Long Term Genetic Modification of Neurons, Astrocytes and Ependymocytes *In Vivo* using a High Capacity Adenovirus Vector

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Abstract: For the evaluation of a possible adenovirus-mediated gene transfer into cells of the CNS a replication deficient 3rd generation gutless adenovirus vector was used.

For cell transduction, vectors with different reporter sequences were applied, resulting in the possibility of measuring the transduction success after infection by the transgene expression. The reporter genes include the green fluorescent protein (GFP), the LacZ (ß-galactosidase) as well as the human secreted placenta alkaline phosphatase (SEAP). Applying various infectious particles (10, 50 and 100 MIO) a concentration-dependent transduction rate can be ascertained. The vector was injected into different brain areas including the ventricles. Applying immunohistochemical techniques using antibodies against NeuN (neuronal specific nuclear protein) and the gilal fibrillary acidic protein (GFAP) it can be shown that after vector administration into the striatum a preferential transduction of astrocytes is achieved. Within this brain compartment a diffusion of the vector up to a distance of 1000 µm from the injection site can be determined. Following injection of vector particles into the corpus callosum, a vector distribution within the entire fibre tract of the white substance including the contralateral hemisphere is observed.

With these results it can be shown that the allocation of the vector is dependent on the structure and features of the receiving tissue. Injecting the adenovirus vector into the cerebrospinal fluid results in transgene expression (GFP, LacZ and SEAP) in the ependymal cells surrounding the entire ventricle.

After installing a permanent catheter in the lateral ventricle cerebrospinal fluid may be removed in a continuous manner in order to analyse/evaluate the marker enzyme SEAP. Using a luminometric screening procedure, the enzyme SEAP can be demonstrated up to a period of 42 days following vector injection into the cerebrospinal fluid. By these data it can be shown that the use of an adenovirus vector at least under experimental circumstances might be a pioneer therapeutical option for diseases of the central nervous system.

Key Words: Adenovirus vector, astrocytes, ependymocytes, gene transfer, cerebrospinal fluid.

INTRODUCTION

As the blood brain barrier represents a problem for the delivery of many therapeutic agents and proteins, in recent years gene therapeutical approaches are increasingly discussed also for the treatment of neurodegenerative diseases or injuries of the CNS as well as for the treatment of glioblastomas. Former studies have used herpes simplex virus (HSV)-based vectors or anti-sense oligonucleotides to dissect the role of anti-apoptotic proteins, receptors and neurotrophic factors in protecting or augmenting injury following ischemic events in adult rodent CNS [1, 2]. However, direct gene transfer for the treatment of chronic neurodegenerative diseases has taken several approaches mainly using adenovirus (Ad) vectors. Such approaches are based on the concept to augment levels of neurotransmitter receptors for example in diseases such as Parkinson`s disease [3]. Significant improvements in animal behavior and dopamine cell survival have been reported following ex vivo and *in vivo* gene transfer of neurotrophic factor genes in rat models of Parkinson`s disease by means of recombinant adenovirus vectors [4]. Further studies have shown that NGF expressed from adenoviral vectors can protect against neuronal atrophy in a rat model of Alzheimer`s disease [5].

For the treatment of CNS malignancies direct intratumoral Ad injection and delivery of Ad after disruption of the blood-brain barrier has resulted in decreases in tumor volume [6-8].

One approach to deliver proteins or peptides to the CNS through gene therapy is either by cells manipulated *in vivo*,

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which are then implanted into the CNS, or by direct transfer of the gene to the CNS using an appropriate vector system [9-12]. The *in vivo* gene transfer as a drug supplying system seems to be rather tantalizing as it excludes any side effects that might occur by using non-autologous carrier cells. Furthermore, an ex-vivo gene transfer is not necessary.

In this study we investigated the suitability of a high capacity adenovirus (HC-Ad) vector to genetically modify neural cells after transplantation into different compartments of the adult rat brain for the transfer of different reporter genes. HC-Ad vectors have several advantages compared with first- or second-generation adenovirus vectors. Their lack of viral coding sequences results in an expanded capacity of up to 36 kb for the uptake of foreign DNA and a reduced toxicity and immunogenicity [13-18].

The use of the reporter genes such as the GFP gene and the ß-galoctosidase (ß-gal)-gene allowed tracking of cells preferentially transduced by the adenovirus vector. Using a vector with the gene for the human placenta alkaline phosphatase (PLAP) allowed the detection of this enzyme in the cerebral spinal fluid after transduction of ependymocytes.

MATERIAL AND METHODS

Construction of the High-Capacity Adenoviral Vector

The high-capacity adenovirus vectors used in this study were generated as previously described [19]. For the production of the high capacity vectors the cre-lox recombinase system of bacteriophage P1 was used (Parks *et al*., 1996). In this process a helper virus with two lox recognition sequences were used which flanked the packaging signal. The vectors were produced in HEK 293 cells, which expressed the cre recombinase of the bacteriophage P1 (Chen *et al*., 1996). After infection of cells with the helper virus as well as with the vector, the packaging signal of the helper virus was eliminated with high efficiency, which resulted in the preferential packaging of the vector. The yield of the adenoviral vector after CsCl equilibrium density centrifugation was 2×10^{12} particles as determined by OD_{260}. The infectious titre (1×10^{7} green-forming units per µl) was determined in triplicate experiments by infecting HeLa cells with different numbers of particles and counting the resulting green cells using a fluorescence microscope.

The vector termed as HC-Ad CV 32 used in this study was carrying the sequence for the reporter gene lacZ encoding the enzyme ß-galactosidase (ß-gal) as well as the sequence for the secreted human placental alkaline phosphatase (SEAP), which were introduced into the E1 region of the vector genome. The reporter genes were under the control of different promoter sequences. The lacZ sequence of the HC-Ad CV 32 vector was under the control of the cytomegalie (CMV) promoter, while the sequence for SEAP was under the control of the semian virus 40 (SV40) promoter.

The second vector used in this study, the HC-Ad CV39 vector was carrying the sequence for the green fluorescent protein (GFP). The GFP sequence of was under the control of the elongation factor 1α-promoter (EF-1 α).

Astrocyte Precursor Cell Line for the Exploration of the Adenovirally Induced Transduction Efficiency *In Vitro*

Spontaneously immortalised astrocytic cell clones derived from neural precursor cells of embryonic day E16 rat striatum were propagated for 4 weeks in DMEM/F12 medium supplements with N2 (Invitrogen, Germany) and containing 20 ng/ml EGF/bFGF. Glial differentiation was induced by plating the undifferentiated cells in DMEM/F12 containing 10% FCS and several continuously proliferating cells lines were isolated after repetitive passaging, as described previously [20]. The cell line of striatal origin was termed S16E referring to the region and embryonic stage of tissue isolation.

Transfection of the S16E Astrocyte Cell Line

For the transfection of the S16 E cell line 200.000 cells were plated into each well of a 24 well plate (Nunc, Germany) in a medium (DMEM, Sigma, Deisenhofen, Germany) supplemented with 1% FCS Gold (PAA Laboratories; Cölbe, Germany), non essential amino acids (100x, Invitrogen), 2mM L-Glutamine (Invitrogen), 100 mM ß-Mercaptoethanol, 50 U/ml penicillin/streptomycin (Invitrogen). Two days after plating the cells were treated for one hour with the proliferation inhibitor Mitomycin C (1.25 ml/500 ml medium). After a washing step the cells were transfected with 50 MOI of the AdCV32 vector. Afterwards the ß-Gal and SEAP expression were analysed histo- and immunocytochemically. Additionally SEAP synthesis was analysed in the culture medium using the Phospha-Light™ System.

Intracerebral Injection of Adenovirus Vectors

For intracerebral injection, adult Wistar rats (250–300 g) were used. Animals were kept in accordance with Federal Government guidelines. Animals were anesthetized by intramuscular injection of a mixture of 6 mg/kg xylazine and 60 mg/kg ketamine. The animals were transferred to a stereotaxic apparatus in a clean field. A 2-5 mm incision was made in the scalp above bregma. For intrastriatal injection a burr hole was made in the bone 0.3 mm rostral and 3.4 mm lateral to the bregma with a dental drill, and the vector was slowly injected over 5 min into the striatum at a depth of 5.4 mm from the surface of the brain. For injection both the Ad Cv 32 and the Ad CV 39 were used. For vector-injection into the fibre tracts of corpus callosum a burr hole was made in the bone 1.5 mm lateral to bregma and the vector suspension was injected at a depth of 2.8 mm. For the intraventricular injection the burr hole was made 0.8 mm caudal and 2.5 mm lateral of bregma and the cannula was introduced to a depth of 3.6 mm.

10 µl of the different vectors (1.3×10^{7} infectious units) were injected using a sterile 0.9% NaCl vehicle solution with a 25 µl N-702-N Hamilton syringe (Bonaduz, Switzerland).

After vector injection, the wound was closed with interrupted surgical sutures. After 7, 14 and 21 days, rats were sacrificed by intracardiac perfusion under deep anaesthesia with xylazine and ketamine in PBS supplemented with heparin and procainhydrochloride, followed by 4% formaldehyde. The brains were removed, the forebrains trimmed, and the samples were frozen after cryoprotection in 18% sucrose.

Installation of a Permanent Catheter in the Lateral Ventricle of the Rat

The catheter was prepared from a 16 G cannula (0.6 x 25 mm). For this procedure the cannula was cut into pieces of 6 mm length and one end of these was grinded conically. The application cannula was also prepared from a 16 G injection cannula. From this a 10 mm piece was cut and used for the stabilisation of the Hamilton cannula, which was either used for the intracerebroventricular injection of the vector or for obtaining cerebrospinal fluid for analysis.

For the introduction of the catheter male Wistar rats were anaesthetised using of a mixture of 6 mg/kg xylazine and 60 mg/kg ketamine as described above and transferred to a stereotactic frame. After creating a 2-5 mm incision in the scalp above bregma a burr hole was made at 0.8 caudal and 1.6 mm lateral to bregma. Two other holes were made caudally to the first one. The catheter was introduced into the first hole and two screws were introduced into the other two for fixation of the catheter together with carboxylate dental cement (Heraeus Kulzer, Hanau, Germany). The correct location of the catheter was tested by an intracerebroventricular injection of Angiotensin II, which causes a temporary anadipsia.

Detection of the Reporter Gene SEAP in Cell Supernatants and Liquor Samples

The Tropix® Phospha-Light™ system is a chemiluminiscent reporter gene assay system designed for the rapid, sensitive and non-isotopic detection of screted human placental alkaline phosphatase in cell culture media or body fluids. SEAP was detected in the supernatants of transfected astrocyte precursor cells as well as in samples of the cerebrospinal fluid according to the manufacturer`s recommendations (Applied Biosystems).

Enzymatic Detection of ß-Gal in Astrocyte Precursor Cells and Tissue Sections

For the detection of the β-gal enzymatic activity in the precursor cells, the medium was aspirated, cells were washed in 0.1 M PBS ad afterwards the cells were fixed in a 25% glutaraldehyde solution (Merck, Darmstadt, Germany) for 15 minutes. After washing off the glutaraldehyde cells were incubated in stain buffer (5 mM $MgCl_2$, 5mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 0.4 mg/ml 5-Bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal) (Sigma) for 24 hr at room temperature (RT). After staining, the cells were rinsed in PBS and after that the cells were dehydrated in a graded series of alcohol. After treatment in xylol they were coverslipped with Entellan (Merck). Cells were then analyzed microscopically. β-gal positive cells could be identified by their dark blue staining.

For detection of β-gal enzymatic activity in brain sections, PBS was aspirated and sections were also incubated in stain buffer as mentioned above for 24 hr at room temperature (RT). After staining, sections were rinsed in PBS and stored in 4% PFA until mounting by placing into 0.5% gelatine in H_20 before transferring to slides coated with 0.5% gelatine, 10% potassium chromium alum. Sections were allowed to dry, dehydrated through graded alcohols to histolene, rehydrated through graded alcohols, rinsed in water and counterstained in 0.05% Nuclear Fast Red in 5% aluminium sulphate for 15 min. Sections were rinsed in H_2O, dehydrated again and mounted in Entellan. Sections were analyzed microscopically and areas which were βgal positive were identified according to the rat brain atlas in stereotactic coordinates by Paxinos and Watson (1986) [21].

Histological Staining of Cells and Tissue Sections

For immunohistochemical studies and detection of GFP expression animals were killed prior to perfusion fixation with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS). After cryoprotection in 18% sucrose, 20 µm cryostat sections of the appropriate brain areas were attached to poly-L-lysine-coated glass slides and processed for immunocytochemistry.

Cells were fixed in the culture dish for 20 min. also using 4% PFA in 0.1 M PBS. After rinsing off the fixative they were further processed for the immunohistochemical investigation.

Immunohistochemistry was performed by incubation with the primary antibodies overnight at 4°C. Cells and tissue sections were treated with blocking antibodies of 2% goat serum and 5% FBS for 30 min. Then, the cells were further treated with 0.025% Triton X-100 for 30 min and rinsed in PBS. The following primary antibodies were used:

Anti-Alkaline Phosphatase (mouse monoclonal antibody, 1:1000,Sigma)

X-gal-40 (mouse monoclonal antibody, 1:1000, Sigma)

GFAP (guinea pig polyclonal, 1:1000, Progen)

NeuN (mouse monoclonal antibody, 1:50, Chemicon)

Anti-Human-Placental alkaline Phosphatase (polyclonal rat, 1:50, Biomeda)

Vimentin (mouse monoclonal, 1:40, Sigma)

IBA-1 (rabbit polyclonal, 1:100, gift of Yoshinori Imai)

ED-1 (mouse monoclonal, 1:2000, BMA)

O4 (mouse monoclonal, 1:20, Chemicon)

Galctocerebroside (mouse monoclonal, 1:20, Roche)

Secondary antibodies were used at the following dilutions:

Cy3-conjugated, or Cy2-conjugated affinity-purified goat anti-mouse or anti-rabbit IgG, 1:1000, Rockland, Gilbertsville, PA, USA.

Alternatively, Biotin conjugated secondary antibodies were used (goat anti-mouse biotin, 1: 400, DakoCytomation, Glostrup, Denmark or goat anti-guinea pig, 1:500, Sigma). Immunoreactivity was visualized with StreptABComplex/ horseradish peroxidase (DAKO), and Nickel chloride enhanced DAB (Pierce, Rockford, USA).

Nuclei of cultured cells and in brain sections were in some experiments stained using the Hoechst 33342 (1 mg/ml, Sigma) for 5 min at room temperature. To exclude the possibility of non-specific staining, secondary antibodies were used at the same concentrations as the primary antibodies. Cells or tissue sections incubated without the primary antibodies served as controls. As a positive control for testing the SEAP antibody human placenta was used. After the staining procedure the sections were cover slipped with Entellan (Merck). Specimens were analysed with a Zeiss

Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany).

Evaluation of the Transduction Efficiency

The total cell population as stained with the Hoechst dye nuclear stain and positively stained cells were counted in 10 random identical visual fields (20x magnification) on four cover slips for each marker in three different experiments. Data were expressed as means ± S.E.M of immunoreactive or GFP expressing cells and total cells per field for each condition. The semiquantitative analysis of the transduction efficiency of cells *in vivo* after vector injection into the brain was performed by counting GFP positive cells in 5 consecutive brain sections, comparing the GFP expression with that of the immunohistochemical detection of GFAP or NeuN positive cells, respectively.

RESULTS

Transduction Efficiency of Astrocyte Precursor Cells *In Vitro*

In preparation for an efficient vector transduction *in vivo*, the determination of the minimal vector dose to be sufficient for the transduction of neural cells was tested with an astrocyte precursor cell line *in vitro*. This cell line was previously generated from the striatum of embryonic rats (embryonic day 16, E16).

For the transfection of cells (5 x 10^4 cells), different concentrations of the Ad CV 32 and the Ad CV 39 vector (10, 50, 100 MOI) were used. After 48 hours, incubation in the presence of the different vectors, in case of cells transfected with the Ad CV 39 vector, the transduction efficiency could be directly evaluated by determining the percentage of GFP expressing cells within the total cell population as marked with the nuclear marker Hoechst dye using fluorescence microscopy. In the presence of 10 MOI the transduction efficiency was determined as 34.1 ± 6.8 %, for 50 MOI 56.3 ± 8.1 and for 100 MOI 74 ± 8.5 % (Fig. **1a-d**).

Similar percentages of astrocyte precursor cells were also transfected using the Ad CV 32 vector, which could be demonstrated immunocytochemically by the expression of the marker enzyme SEAP (Fig. **1e**).

Luminometric Determination of SEAP in the Supernatant of Astrocyte Precursor Cells

After inhibition of the proliferative activity using Mitomycin and transfection with the adenovirus vector Ad CV 32

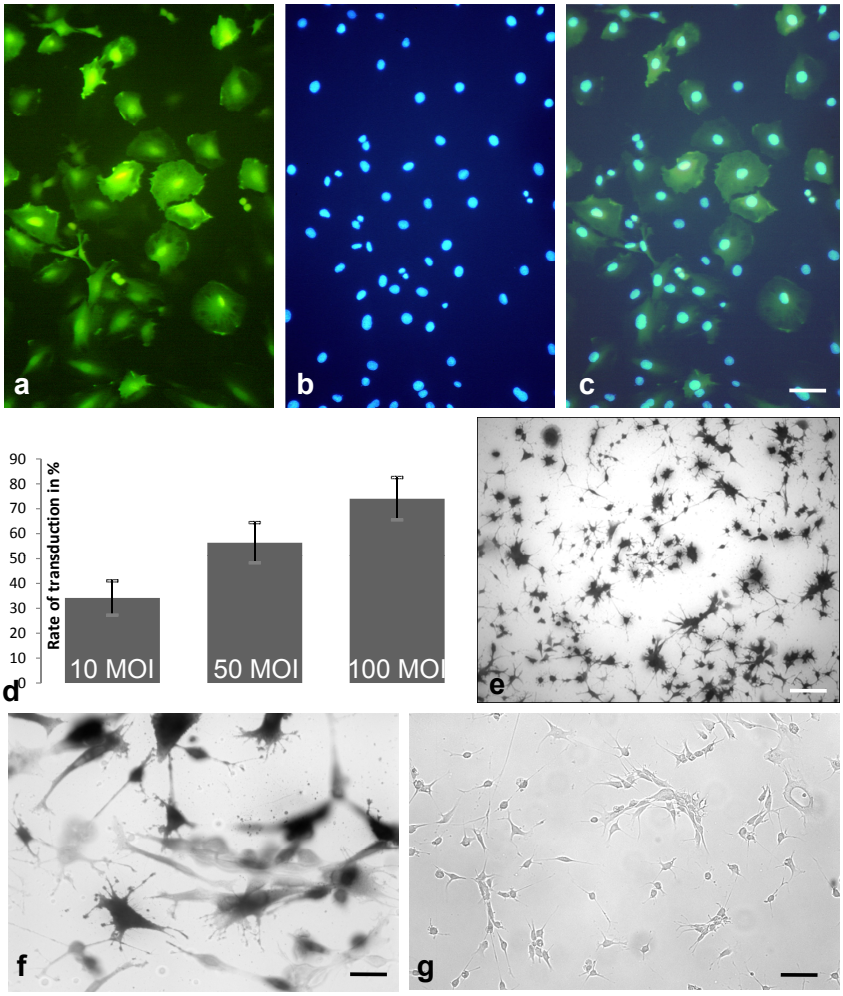

Fig. (1). Investigation of the transduction efficiency using different adenoviral vectors in the S16 astocyte cell line. **a**) GFP expression in transduced astrocytes **b**) total cell population as marked with the nuclear marker Hochst dye, **c**) merge. **d**) Diagram of the quantification of transduction efficiency after transfection using 10, 50 and 100 MOI of the Ad CV39 vector. **e**) Visualisation of cellular transduction using the SEAP reporter gene and an anti-SEAP antibody **f**) higher magnification of transduced astrocytes. **g**) control without SEAP antibody. Scale bar in **a-c** = 15 µm, in **e** and **g** = 25 µm and in **f** = 10 µm.

(50 MOI), astrocyte precursor cells were kept in culture for 24 days. From the cell culture supernatants, which were collected every second day, synthesized SEAP concentrations could be detected using a luminometric assay. There was a strong increase of the SEAP concentrations until day 6 with a peak value of 92 ± 6.8 ng/ml. After day 6 there was a sharp decline to 6.4 ± 1.0 ng/ml on day 8 and to basal values of 3.2 ± 0.8 ng/ml until day 20 after transduction. In contrast, in the control cell population, without vector incubation, no SEAP concentrations could be detected (Fig. **2**). The sharp decline is the result of the reuptake of the proliferative activity of astrocyte precursor cells.

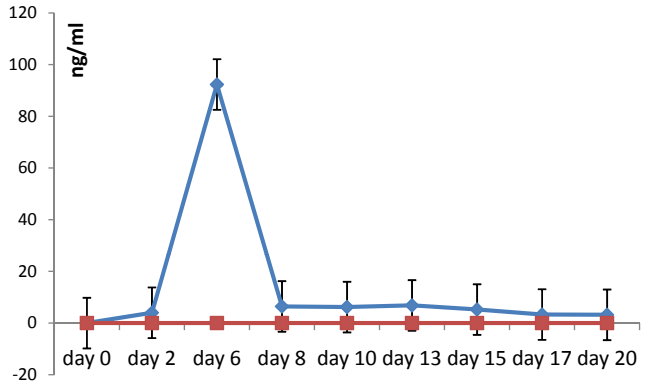

Fig. (2). Representative graphical display of the luminometric SEAP detection in the astrocyte precursor cell line S16. The luminometric assay for the detection of SEAP in cell culture supernatants was performed in three independent experiments (n = 3) Red line = non-transfected control cells, blue line = vector transfected astrocyte precursor cells.

Direct Intracerebral Injection of Adenovirus Vectors

Direct stereotactic injection of the Ad CV 32 vector into the fibre tracts of the Corpus callosum was carried out in order to gain information about the diffusion of the vector within the white matter fibre tracts. Vector transduced cells (SEAP/ LacZ sequence) could be detected within the appropriate brain slices by the enzymatic ß-glactosidase assay. Using this assay it could be shown that the vector, after a survival time of 21 days, had distributed from the injection site, which was situated 1.5 mm lateral to bregma, within the fibre tracts 1 mm lateral into the fibre tracts of the ipsilateral hemisphere as well as 7 mm towards the contralateral hemisphere (Fig. **3a**), resulting in a complete staining of the corpus callosum and adjacent fibre tracts. At the margins of the fully stained fibre tracts single processes and cells preferentially exhibiting a glial morphology were stained (Fig. **3b-e**).

As the striatum is one of the major targets for a possible gene therapeutical intervention in neurodegenerative diseases, in another set of experiments the Ad CV 39 vector, carrying the sequence for the GFP reporter gene, was additionally administered into the striatum. Vector transduced cells could easily be detected by the induced GFP expression (Fig. **4a**, **b**). Furthermore, using a vector carrying both reporter sequences for GFP as well as for Lac Z the expression of both transgenes could be detected side by side in the same cell as shown with light and fluorescence microscopy (Fig. **4c** and **d**). In co-immunostainings using the glial marker GFAP and the neuronal marker NeuN, it could be demonstrated that both cell populations were transduced using this vector. A semiquantitative analysis revealed that 90% of the transduced cells showed a co-immunostaining with the glial marker GFAP, while only 10% were immunopositive for NeuN (Fig. **4e**, **f**). In contrast, a transduction of oligodendrocytes, as investigated by an immunostaining using the oligodendroglial markers O4 and galactocerebrosid, was not detectable (data not shown).

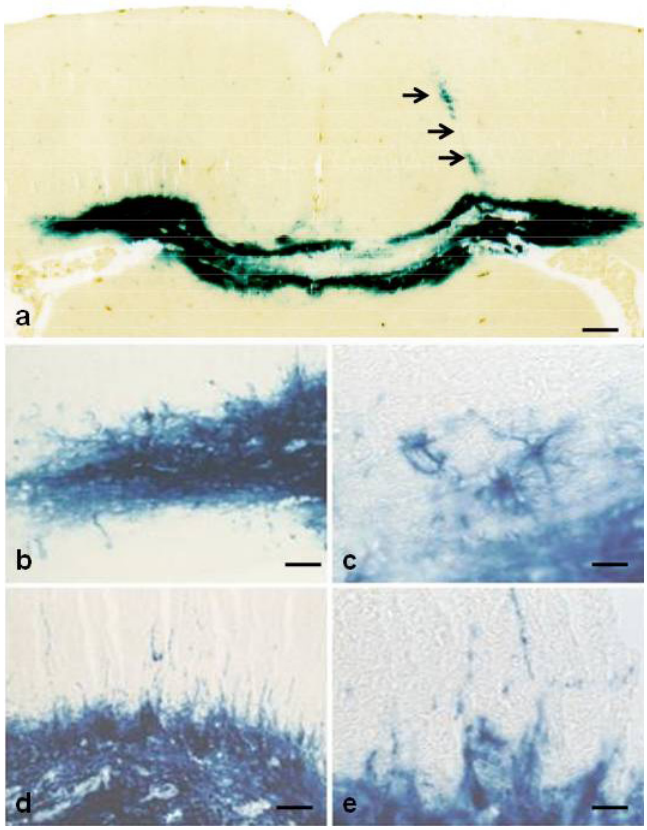

Fig. (3). Distribution of the adenoviral vector after direct injection into the fibre tract of the Corpus callosum as visualised by the ß-galactosidase assay 2 weeks after injection. **a**) distribution of the vector along the white matter fibre tracts of the Corpus callosum. Arrows = branch canal **b**, **c**) in a higher magnification transduction of glial cells in close vicinity to the Corpus callosum are visible, **d**, **e**) ß-galactosidase stain in glial processes in the neighbourhood of the white matter fibre tracts. Vector injection was carried out in 5 animals (n = 5). Scale bar in **a** = 3mm, in **b** = 50 µm, in **c-e** = 25 µm.

Surprisingly, a GFP expression could be detected in cells within the striatum in a distance as far as 1000µm away from the injection site.

Intraventricular Administration of Adenovirus Vectors

After direct stereotactic injection of both adenovirus vectors a transduction of the ependyma throughout the ipsilateral ventricle could be detected in brain cryostat sections of animals that were sacrificed 1, 2 , 4 and 10 weeks after vector injection. Transduction could either be identified after

Ad CV 39 vector transduction by the GFP expression of ependymal cells. These could be clearly identified by their immunopositivity for the marker of the intermediary filament vimentin, which is typically expressed in the ependyma (Fig. **5a**, **b**). Furthermore, a positive transduction after injection of the Ad CV 32 vector, could also be deduced from the positive histochemical staining for ß-Galaktosidase throughout the ventricular lining (Fig. **5d**). The ß-Gal staining is stable for up to 10 weeks after vector injection. Using this staining procedure also a positive staining of the epithelial cells demarcating the choroid plexus could be detected (Fig. **5e**).

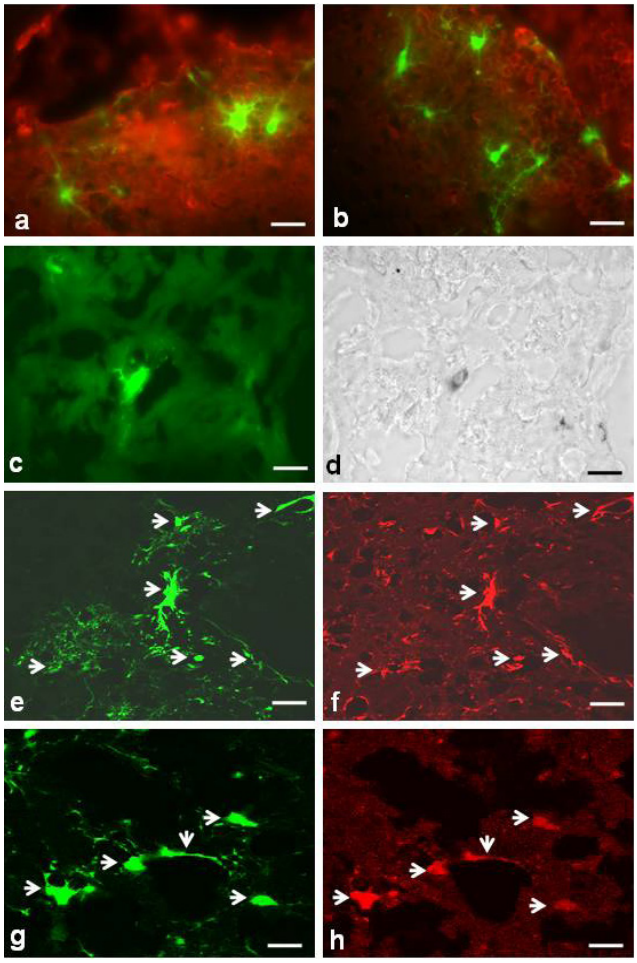

Fig. (4). GFP expression in neural cells of the striatum 2 weeks after direct vector injection. **a**, **b**) Transduced cells show typical neuronal or glial morphologies. **c**) Transduced neural cell after transduction with a vector carrying the sequence for GFP as well as **d**) the reporter sequence for LacZ. **e**) Comparison of the vector induced GFP expression with **f**) the expression of the astroglial marker GFAP. **g**) Comparison with the vector induced GFP expression with **h**) the expression of the neuronal marker NeuN. Vector injection into the striatum was carried out in 5 animals (n = 5). Scale bar in **a** and **b** = 20 µm, in **c** and **d** = 15 µm, in **e**-**h** = 20 µm.

Additionally, after vector injection as can be demonstrated by the ß-Gal staining also cells within the subventricular zone are positive (Fig. **5f**).

The findings of an ependymal and subependymal cell transduction can also be confirmed by our immonocytochemical data generated with an antibody against SEAP (Fig. **6**).

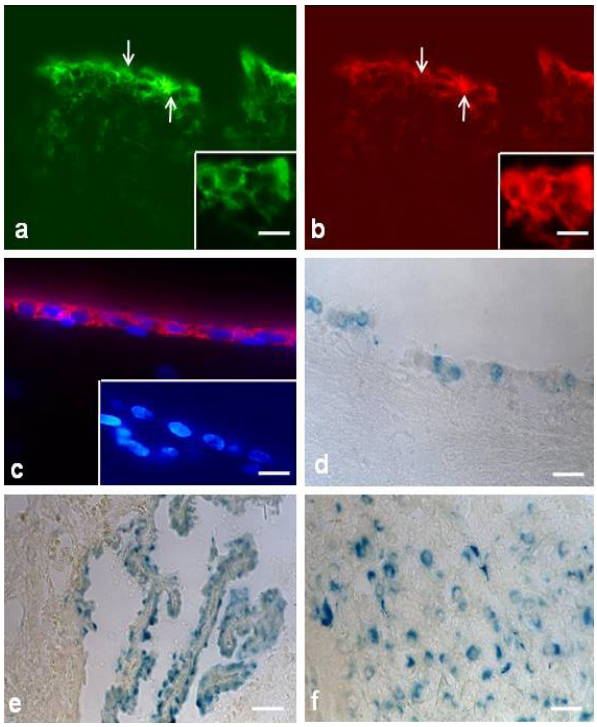

Fig. (5). Ependymal cells after transduction with the high capacity adenoviral vector two weeks after vector injection into the lateral ventricle. **a**) GFP expression, **b**) Expression of the intermediary filament vimentin, a marker for ependymal cells. arrows =labelling of single ependymal cells. Inset in a and b: higher magnification of GFP and vimentin expressing cells **c**) vimentin immunohistochemical staining of the ependymal lining in a counterstain with the Hoechst dye nuclear stain, Inset in c: negative control without primary vimentin antibody **d**) labelling of the ependymal lining after injection of the vector carrying the LacZ construct, **e**) labelling of the epithelial cells of the chorioid plexus, **f**) using the ß-galactosidase stain also transduced cells of the subependymal layer can be detected Vector injection into the lateral ventricle was carried out in 5 animals (n = 5). Scale bar in a, b = 25 µm, in the insets in **a** and **b** = 12 µm, in **c** = 20 µm in the inset in **c** = 15 µm, in **d**-**f** = 25 µm.

Direct Intraventricular Injection of the Adenovirus Vector *Via* a Catheter

A different experimental set up was carried out in order to obtain information whether a successful transduction of ependymal cells could be performed and for what period of time a transgene expression could be achieved. For these experiments a permanent catheter was introduced into the left cerebral ventricle of adult rats. This experimental set up offers the opportunity to perform repetitive vector injections as well as to collect samples of the cerebrospinal fluid for the detection of the marker enzyme.

In correspondence to the conventional vector injection using a Hamilton syringe also introduction of the intraventricular catheter resulted in profound transgene expression, which could be detected histologically throughout the ventricular lining already one week after vector injection. Furthermore, the marker protein SEAP could additionally be detected in the subventricular zone as well as in the choroid plexus. These finding indicate that the vector was distributed *via* the liquor throughout the left lateral ventricle. Furthermore, the histological findings indicate that the vector

was additionally able to cross the ependymal barrier into transducer cells within the subventricular space. Transduced cells exhibited neuronal as well as glial morphologies (data not shown).

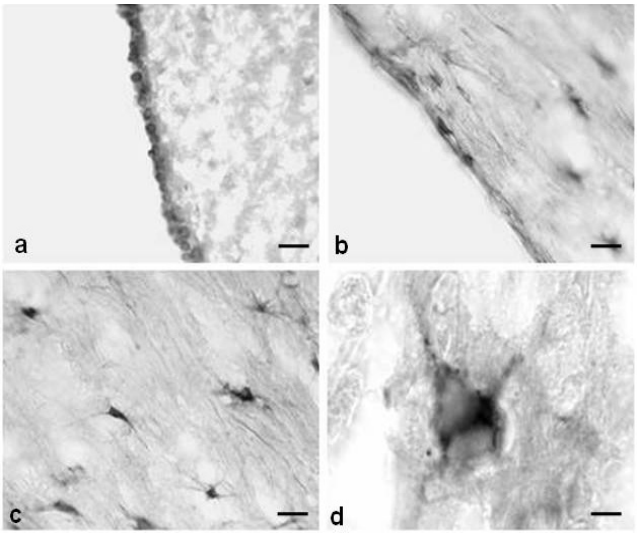

Fig. (6). Imunnohistochemical detection of the SEAP expression 4 weeks after vector injection into the right lateral ventricle *via* a permanent catheter. **a**) SEAP expression throughout the ependyma of the right ventricle, **b** and **c**) there is also an expression of subependymal cells detectable, **d**) higher magnifications of subependymal cells. Vector injection into the lateral ventricle *via* a permanent catheter was carried out in 3 animals (n =3). Scale bar in **a-c** = 30 µm, in **d** = 10 µm.

For the analysis of secreted SEAP within the cerebrospinal fluid after vector injection *via* the permanent catheter into the ventricular sytem the cerebrospinal fluid of 4 rats was collected. 5 µl of the cerebrospinal fluid of each animal were analysed daily for up to 8 weeks using the luminometric SEAP assay. Data from these 4 animals and of one control animal reveal that there was an increase in SEAP concentrations within the fluid between day 0 and up to day 21. Between day 42 and day 56 after vector injection there were no residual SEAP concentrations detectable within the cerebrospinal fluid. In the control animal no SEAP concentrations could be detected at any time (Fig. **7**).

For the detection of a possible immunological reaction after the intraventricular vector injection an immunhistochemical detection was carried out using the microglial markers ED-1 and IBA. The analysis of immunohistochemical stainings for both microglial markers revealed that there was no enhanced immunoreactivity detectable compared to a sham injection using a physiological salt solution (data not shown).

DISCUSSION

Our data describe the possibility to directly transduce cells of the central nervous system by direct injection of a helper virus dependent high capacity adenoviral vector into the brain of adult rats. The specific novelty of our study is, that various reporter sequences can be successfully introduced into glial restricted neural precursor cells *in vitro* as well as into different cell types within the brain by means of the third generation adenoviral vector with a long term expression.

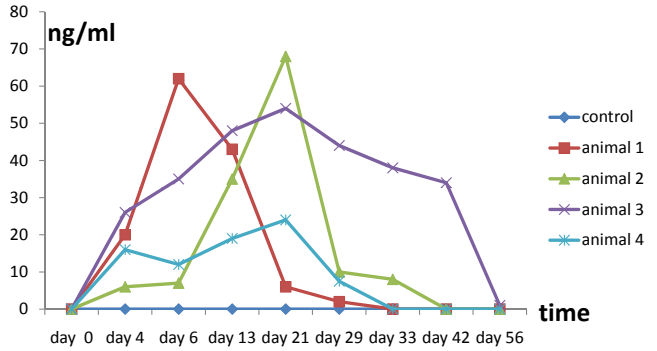

Fig. (7). Time course of SEAP concentrations in the cerebrospinal fluid after vector injection of four animals (n = 4) and one control animal (n = 1).

Our *in vitro* transduction experiments using glial restricted precursor cells reveal that the best transduction rates can be achieved using vector concentrations of 100 MOI, which is in line with transduction rates in earlier experiments using an adeno-associated virus for the modification of human neural progenitor cells [22].

In contrast, genetic modification of human neural progenitor cells using a bacculovirus derived vector and a vector concentration of around 80 MOI only yielded in a transduction efficiency of 25% [23].

For terminally differentiated astrocytes using a first generation adenovirus vector it has been reported that only moderate transduction rates could be attained [24, 25].

The peak value of 92 ng/ml marker enzyme, which could be detected in the cell culture supernatants of astrocyte precursor cells is highly significant provided a similar amount could also be synthesized using a vector encoding for neurotrophic factors such as BDNF. However, in similar transfection experiments also using astrocytes as target cells only neurotrophin concentrations in the picogram level could be achieved [26].

As an alternative to the ex-vivo gene transfer, in the presented study we investigated the possibility of a direct *in-vivo* gene transfer into different brain compartments.

For these experiments we selected regions, which are interesting from the therapeutic point of view in degenerative diseases such as the striatum an important target structure for the treatment of Parkinson`s disease. Our data from co-labelling experiments using the glial marker GFAP reveal that there is preferential transduction of cells of the glial lineage, which are in line with data from our *in vitro* experiments with the immortalised glial restricted precursor cells. They are however in contrast to a similar *in-vivo* study also using a helper dependent adenoviral vector in the hippocampus of rats. In these experiments vector injection yielded in a uniform transduction of neurons and astrocytes [27]. However, in the latter study also a long term transgene expression could be observed and this is one of the major prerequisite for a therapeutical implementation of gene therapy strategies in CNS diseases.

The distribution of the vector after injection within the recipient tissue is of course dependent on the structure of the target tissue. As it can be deduced from our experiments

after adenoviral injection into the corpus callosum, there is an extremely favourable distribution of vector particles within the fiber tracts of this compartment, even towards the fibre tracts of the contralateral hemisphere. This is of course favoured by the anatomical situation in this brain compartment, which mainly consists of nerve fibres as well as of oligodendrocytes [28]. The preferential transduction of oligodendrocytes within this compartment has already been demonstrated using a first generation adenovirus vector [29]. Thus, direct vector injection into this fibre tract may be useful for demyelinating diseases [29] but also for the treatment of glioblastomas and especially those, with a localisation close to the corpus callosum.

The other extremely interesting target structure we have investigated concerning its suitability for an effective transduction is the ependyma of the intracerebral ventricular system. Our data reveal that after vector injection the whole ventricular lining including the choroid plexus can be efficiently transduced. Similar findings have already been documented in a study using a first generation adenoviral vector [30]. However, while we are able to show a transgene expression for up to 10 weeks, in the report by [30] the expression of the reporter gene LacZ could only be detected for up to 7 days.

Besides using a helper dependent high capacity adenovirus vector, the advantage of our methodological approach is the introduction of a permanent catheter into the lateral ventricle so that the reporter enzyme SEAP can be continuously analysed within the cerebrospinal fluid. Thus, the permanent access to the cerebrospinal fluid on one hand guarantees the continuous follow up of transgene expression but could also be exploited for a repetitive therapeutic vector application according to an Ommaya reservoir which is commonly used in brain tumor therapy.

In conclusion, the opportunity to effectively transduce various target structures within the brain by direct injection of a third generation adenoviral vector with preferential transduction of glial cells will become increasingly important for the quest of new therapies for either neurodegenerative diseases or intracranial tumor therapy.

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