Journal of NeuroVirology (1997) 3, 322–330

http://www.jneurovirol.com

Efficient gene transfer into primary and immortalized human fetal glial cells using adeno-associated virus vectors: establishment of a glial cell line with a functional CD4 receptor

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Adeno associated virus (AAV) is a non-pathogenic dependent parvovirus with a broad host range, capable of high levels of transduction and stable integration into the host cell genome. We have investigated the potential for using AAV as a vector for gene transfer into glial cells of the human fetal nervous system. Recombinant AAV vectors expressing either the reporter gene β-galactosidase or a human CD4 receptor were able to transduce both primary glial cells of the human fetal nervous system and an SV40 immortalized human fetal glial cell line (SVG). No difference in transduction efficiency was observed between the primary cells and the cell line which in both cases was as high as 95%. Stable transfectants of the glial cell line expressing the CD4 receptor were selected. An SVG/CD4 expressing line was then established. The presence of the CD4 receptor was confirmed by immunohistochemistry, Westerm immuno-blotting and flow cytometric analysis. The CD4 receptor was shown to be functional by infection of the SVG/CD4 cell line with the human immunodeficiency virus (HIV). Upon infection, the SVG/CD4 cells produced 20-fold higher levels of the HIV intracellular core antigen P24 than the CD4 negative parental cells and in addition formed syncytia. The use of AAV vectors should prove useful in biological investigations of human glial cells and offers promise as a means of *ex vivo* and *in vivo* gene delivery.

Keywords: gene expression; adeno-associated virus; dependovirus; glia

Introduction

The efficient *in vitro* genetic manipulation of central nervous system (CNS) cells is an important goal in neurobiological investigations of the normal and pathological processes affecting the CNS, and in the development of gene therapy strategies for neurological disorders. In an effort to achieve this, much attention has focused on the development of viral vectors.

Integrating retroviral vectors have been used to establish a number of rodent neuronal and glial cell lines which have proved important in developmental studies of the mammalian nervous system and in evaluating the potential of transplanting cell lines for the treatment of neurodegenerative disease (Snyder *et al*, 1992, 1995, Renfranz *et al*, 1991). Despite providing a powerful laboratory tool the use of retroviral vectors has limitations. At this point no human neuronal progenitor cell line has been described and recent attempts to immortalize human oligodendrocyte progenitors with a retrovirus vector have proved unsuccessful, due in part to the poor transduction efficiency of the retrovirus in human cells (Whittemore *et al*, 1994). An adenovirus vector has been used to express the reporter gene β-galactosidase in cells of the human fetal nervous system prior to transplantation in the rat brain (Sabate *et al*, 1995). Such *ex vivo* genetic manipulation of cells may prove important as a means of delivering therapeutic genes for the

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Received 13 January 1997; revised 14 April 1997; accepted 7 July 1997

treatment of neurodegenerative disorders, indeed the transplantation of genetically modified human fetal glial cells has been proposed for the treatment of Parkinson's disease (Tornatore *et al*, 1996). However since adenovirus vectors are not capable of integration any expression in the transduced cells is likely to be transient. Concerns also exist over the inflammatory potential of using adenoviruses in the nervous system (Byrnes *et al*, 1995).

While such studies demonstrate the promise of using viral vectors for the genetic manipulation of CNS cells they also point to the limitations of current vector systems particularly with regards to human tissue.

More recently attention has focused upon the development of adenoassociated virus (AAV) vectors as attractive candidates for gene transfer. Wild type AAV is a non-pathogenic dependent parvovirus which requires co-infection with a helper virus (either Adenovirus or HSV) to provide proteins essential for replication (Carter, 1992). AAV can infect a wide variety of human cells and is capable of stable integration into both dividing and non-dividing cell populations (Samulski *et al*, 1989, Podsakoff *et al*, 1994). Typically, AAV vectors are deleted in 96% of the viral genome, leaving only the inverted terminal repeat sequences which are necessary for packaging, replication and integration. While the cloning capacity of AAV vectors, at approximately 4.5 kb, is limited it nevertheless is sufficient for many potentially therapeutic applications in the nervous system. AAV vectors are produced by cotransfecting plasmids containing vector genome with helper plasmid containing the missing elements of the viral genome without the terminal repeats. Subsequent infection with adenovirus results in production of recombinant AAV and wild type adenovirus (Samulski *et al*, 1987). The contaminating adenovirus can be removed by heat inactivation and cesium banding, leaving a high titer vector, free from helper virus.

The potential for using recombinant AAV vectors for *in vivo* transduction in the CNS has recently been demonstrated. An AAV vector expressing the reporter gene β-galactosidase was shown to promote differential patterns of gene expression in the rodent brain depending on the site of injection (McCown *et al*, 1996). Furthermore an AAV vector expressing the gene for tyrosine hydroxylase was shown to transduce neuronal and glial cells for up to 3 months in the caudate nucleus of 6-OH-dopamine lesioned rats and to promote partial recovery of function (Kaplitt *et al*, 1994a). AAV vectors have also been used to transduce spinal cord neurons of adult rats following injection into the mid-cervical region (Peel *et al*, 1997).

In addition to the *in vivo* studies in rodents, human NT neurons, which are derived from a teratocarcinoma, have been shown to be efficiently transduced by an AAV vector expressing the reporter gene β-galactosidase with subsequent integration of vector DNA (Du *et al*, 1996). There is ongoing controversy however regarding the transduction efficiency of AAV vectors in primary cell populations (Russell *et al*, 1994, Halbert *et al*, 1995). To date no studies have been conducted evaluating the potential for using AAV to transduce primary cells of the human nervous system, or to compare relative transduction rates between primary and immortalized CNS cells.

To address these issues we have investigated whether AAV vectors can be used for gene transfer in primary human fetal glial cells (HFGC's) and in a human fetal glial cell line (SVG) both of which are used extensively in our laboratory for the study of viral pathogenesis of the nervous system (Major *et al*, 1990, Tornatore *et al*, 1994). We report the efficient transduction of HFGC's and SVG's with AAV vectors expressing the reporter gene β-galactosidase and the human CD4 receptor. In addition we report the use of an AAV vector to establish a stable CD4 positive cell line in which the vector DNA is integrated and which can be shown to be functional by subsequent infection with the human immunodeficiency virus.

Results

AAV-LacZ transduction in human fetal glial cells and an immortalized human fetal glial (SVG) cell line

Primary human fetal glial cells (HFGC's) and a human fetal glial cell line (SVG's), previously immortalized with the SV40 large T antigen, were infected with the recombinant AAV vector pdx11 at a multiplicity of infection (MOI) of 5 and the transduction efficiency was calculated by staining for β-galactosidase expression 72 h after infection. In both HFGC's and SVG's the level of transduction at this time point was approximately 95% (Figure 1A and B). Serial dilutions of the virus lowered the transduction rate such that infection at an MOI of 0.1 resulted in a transduction efficiency of approximately 10% (data not shown). At all concentrations of virus used the transduction efficiency in both the primary HFGC's and the immortalized SVG cell line was equivalent. No expression of β-galactosidase was detected in uninfected negative controls (Figure 1C).

AAV-CD4 transduction in human fetal glial cells and the SVG cell line

HFGC's and SVG's were infected with the recombinant AAV vector JM48 at an MOI of 0.5 and the transduction efficiency was subsequently assayed by staining for CD4 receptor expression 72 h later. Between 25–75% of cells expressed the CD4 receptor, as demonstrated by immunofluorescence, with staining seen in both the cell body and in the cytoplasmic processes abutting neighboring nontransduced cells (Figure 2A and B). The presence of CD4 positive perivascular microglia in the primary cultures could have been a potential source of false positives. However no detectable expression of the CD4 receptor was seen on uninfected HFGC's (Figure 2C) or in the SVG's. As with the LacZ expression vector no measurable differences were observed in the transduction efficiencies between the primary cells and the immortalized cell line.

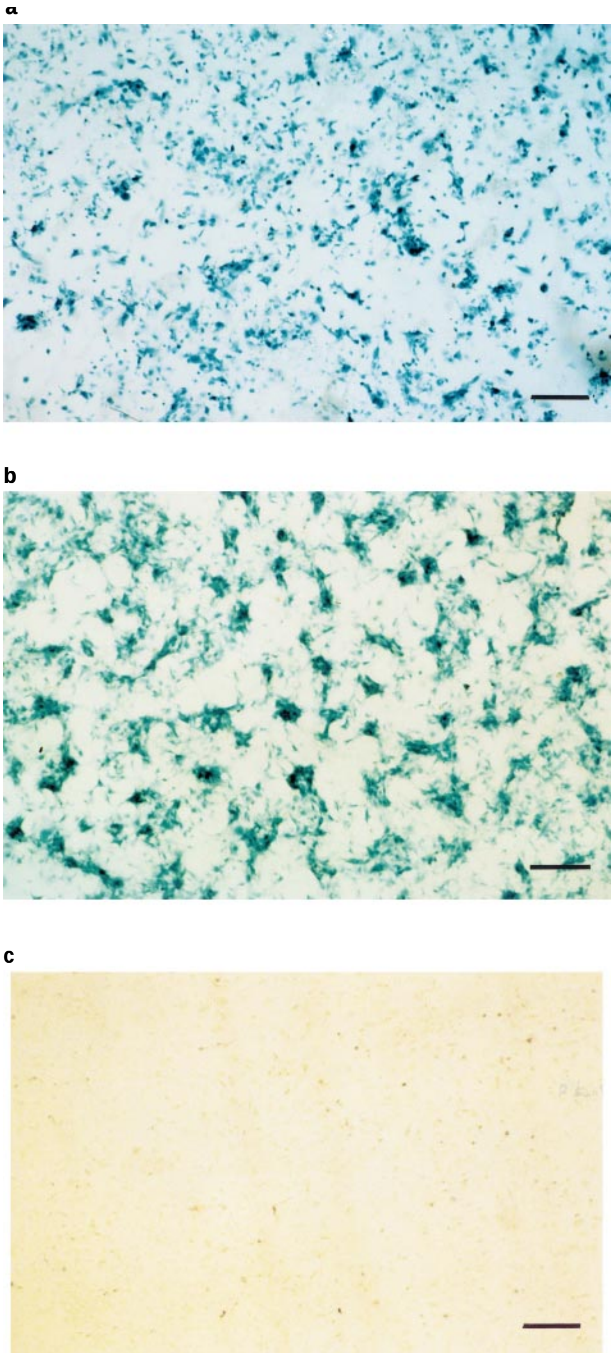

Figure 1 Expression of β-galactosidase in (**A**) primary human fetal glial cells and (**B**) an SV40 immortalized human fetal glial cell line 3 days after infection with the AAV vector pdx11-LacZ at an MOI of 5. The bar corresponds to 333 μm. Uninfected primary human fetal glial cells (**C**) showing no detectable expression of β-galactosidase. The bar corresponds to 500 μm.

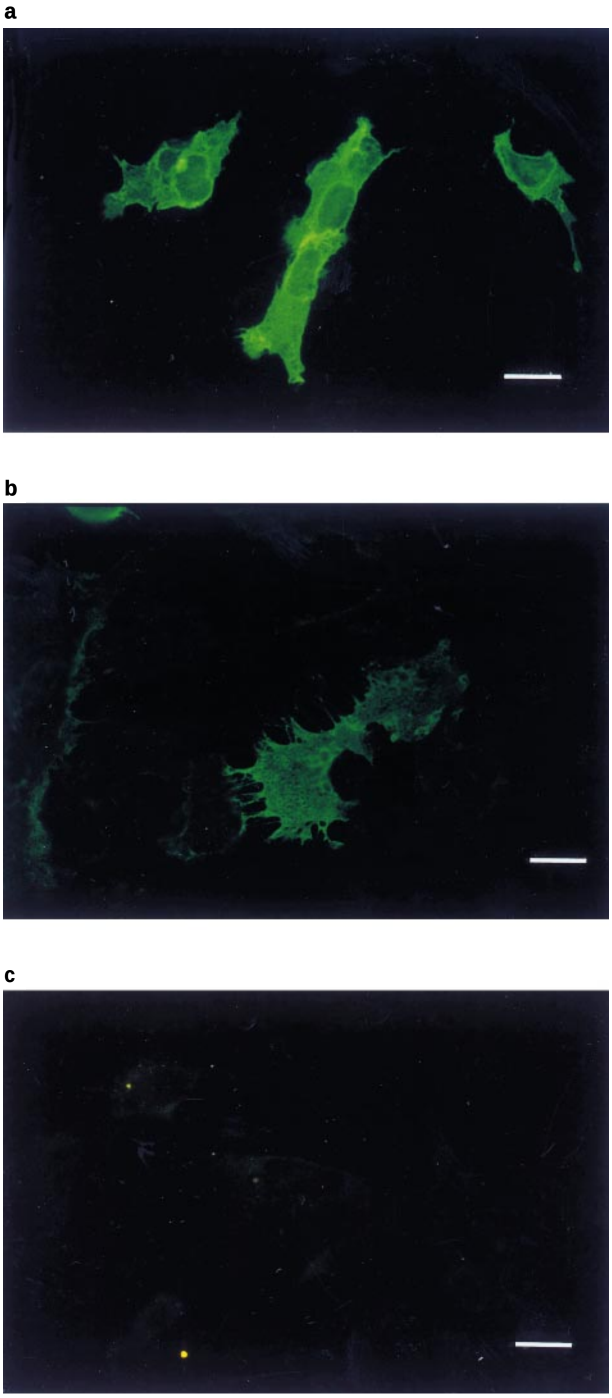

Figure 2 Expression of the CD4 receptor as demonstrated by immunofluorescence in (**A**) human fetal glial cells and (**B**) an SV40 immortalized human fetal glial cell line 3 days after infection with the AAV vector JM48 at an MOI of 0.5. Uninfected primary human fetal glial cells (**C**) showing no detectable expression of the CD4 receptor. The bar corresponds to 15.5 μm.

Geneticin selection of the transduced SVG cells resulted in the establishment of 10 geneticin resistant colonies, suggesting that stable expression occurs at a low frequency in this cell line. These colonies were pooled under continuous geneticin selection to establish an SVG/CD4 cell line which was 100% positive for the CD4 receptor as demonstrated by immunofluorescence and immunoperoxidase (Figure 3A and B).

Expression of the CD4 receptor protein on the SVG/CD4 line was further confirmed by Western transfer and immunoblot analysis. Specific reactivity of the SVG/CD4 lysates to the anti-CD4 was observed, with a band corresponding to the 55 KDa molecular weight of the CD4 receptor protein (Figure 4, lane 3). This band was absent in lysates from the parental SVG cell line (lane 1) and from the supernatant of the SVG/CD4 cells (lane 2). In addition, flow cytometric analysis of the parental SVG cells and the SVG/CD4 cells for CD4 revealed a population shift in fluorescent activity between the two cell populations (Figure 5).

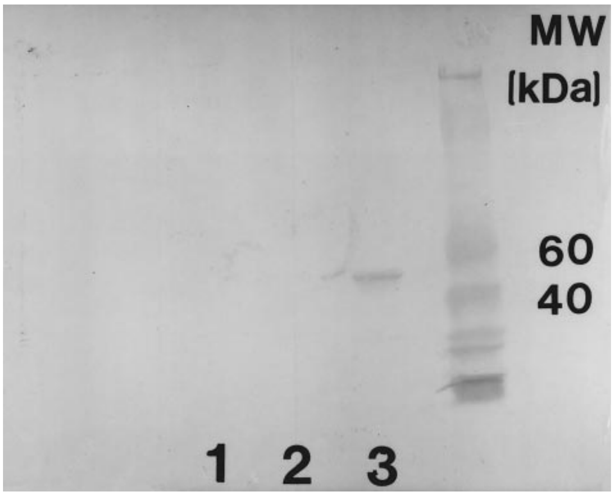

Figure 4 Detection of the CD4 receptor protein in SVG/CD4 cell lysate by Western immunoblot analysis. Lane 1 parental SVG cell lysate. Lane 2 SVG/CD4 supernatant. Lane 3 SVG/CD4 cell lysate showing specific reactivity corresponding to the 55 kDa molecular weight of the CD4 receptor.

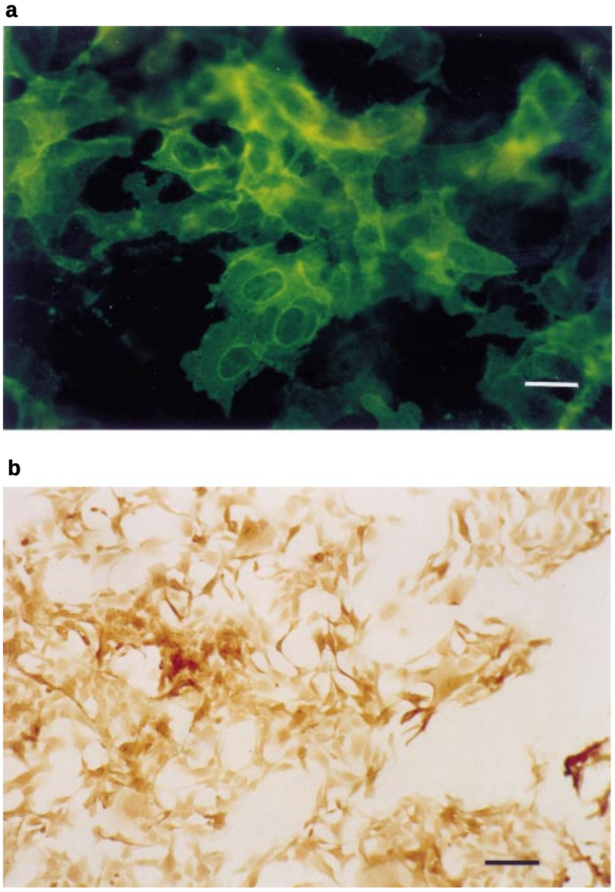

Figure 3 CD4 receptor expression on the SVG/CD4 cell line after continuous geneticin selection for over 1 month as demonstrated by (**A**) immunofluorescence and (**B**) immunoperoxidase. The bar corresponds to 31.25 μm in **A** and 133 μm in **B**.

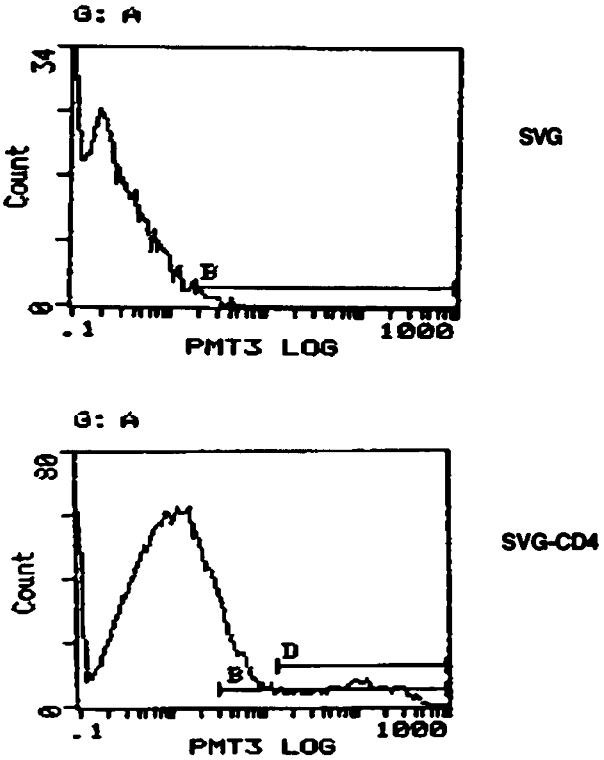

Figure 5 SVG and SVG/CD4 cells were collected for flow cytometric analysis of surface CD4 receptor expression after a brief exposure to trypsin-EDTA. Cells were stained in serum free PBS containing phycoerythrin labeled anti-CD4 antibody and suspended in 4% paraformaldehyde before analysis. Analysis was performed using an Epics Elite instrument with 10 000 events collected. A shift in population is observed in the SVG/CD4 cells as compared to the parental cells.

To investigate whether the viral vector in the stably transduced cells was in an integrated or episomal form a restriction digest with Southern blot analysis was performed on DNA extracted from the SVG/CD4 line and from the parental SVG line. Digestion with the restriction enzyme Bam H1 would result in four bands at 0.2, 0.9, 1.1 and 2.2 kb if the vector DNA was an episomal state. If integrated, the 1.1 and 2.2 kb bands would be conserved while the 0.2 and 0.9 kb bands would be lost since they would lie at the junction of the viral DNA and the host genome. Plasmid DNA from JM48 was used as a control to identify the conserved 1.1 kb fragment and 2.2 kb fragments and a 4.9 kb fragment containing the viral inverted terminal repeats and the plasmid backbone. As seen in Figure 6, the Southern hybridization of the SVG/CD4 genomic DNA revealed the conserved 1.1 and 2.2 kb bands plus an additional three bands at approximately 2.0, 4.6 and 4.8 kb, a finding consistent with integration of vector DNA. A multimeric episome is ruled out by this result since it also would have given the 0.2 and 0.9 kb fragments at the 3 prime and 5 prime portion of the recombinant construct. No hybridization was seen with the parental SVG line which acted as a negative control (data not shown).

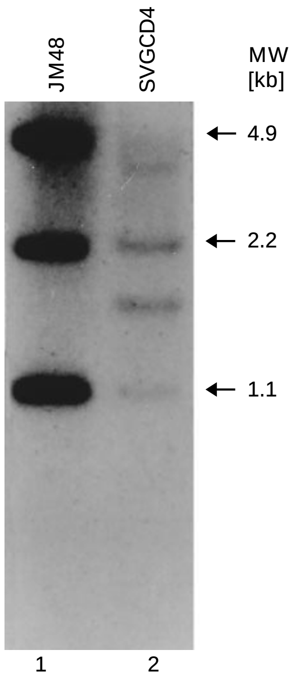

Figure 6 Southern blot analysis of SVG/CD4 cells and plasmid JM48. Lane 1 plasmid JM48 digested with Bam H1 showing the 1.1 kb and 2.2 kb viral DNA fragments and a 4.9 kb fragment containing the viral inverted terminal repeats and the plasmid backbone. Lane 2 SVG/CD4 DNA digested with Bam H1 showing the conserved 1.1 and 2.2 kb fragments and additional junctional fragments.

Infection of the SVG/CD4 line with the human immunodeficiency virus

In order to show that the CD4 receptor was functional, the SVG/CD4 cells and the parental SVG cells were infected with 1×10^5 TCID 50/ml of HIV-1 strain III B. Infectivity was assayed by collecting the supernatants on days 1 – 14 following infection and measuring levels of the HIV intracellular core antigen p24. As expected the parental SVG cells which are CD4 negative produced only low level amounts of newly synthesized p24, peaking at 90 pg/ml. By contrast the CD4 positive cell line produced 20-fold higher levels of p24, peaking at over 1800 pg/ml (Figure 7). The kinetics of the HIV infection as measured by p24 levels was similar for both cell lines, in both cases peak levels of p24 synthesis were observed 24 – 48 h post-infection and declined over subsequent days such that by 14 days post infection p24 protein was not detectable above background levels even in the CD4 positive cells.

In addition to the increased expression of HIV-1 proteins in the SVG/CD4 cells morphological changes including syncytia formation (Figure 8) were detected in the SVG/CD4 cells at 14 days postinfection but not in the CD4 negative SVG cells.

Discussion

Poor transduction efficiency and viral toxicity have limited the application of current viral vectors in the nervous system. We report the successful transduction of cells of the human fetal nervous system with recombinant adeno-associated virus vectors. The efficiency of transduction observed was extremely high (up to 95%) demonstrating that

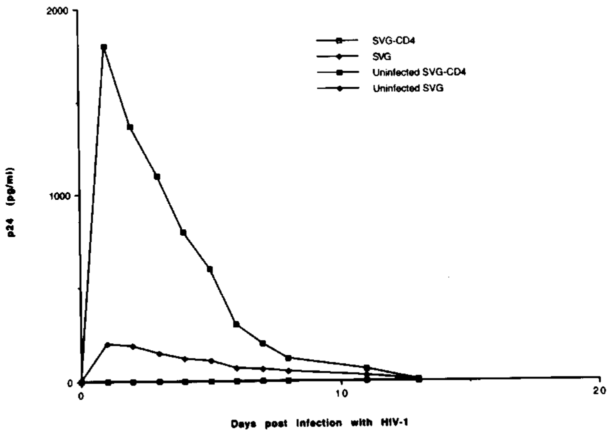

Figure 7 The SVG and SVG/CD4 cells were infected with 1×10^5 TCID 50/ml of HIV-1 strain III B in order to show that the CD4 was functionally active as a receptor for HIV-1. Replication of HIV-1 was assessed by measuring levels of the HIV-1 core antigen P24 secreted in supernatants over a 14 day period and detected by standard enzyme linked immunoabsorbent assay.

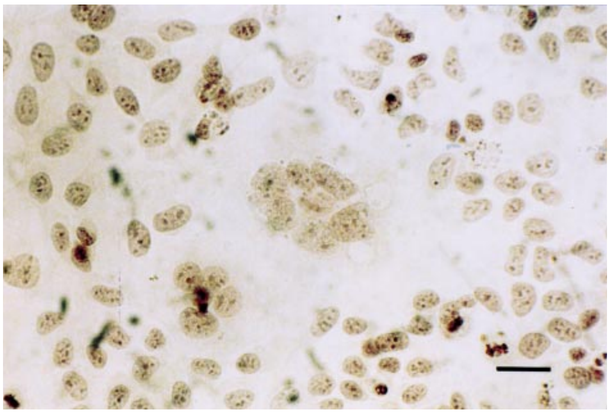

Figure 8 Fourteen days following infection with HIV-1 the SVG and SVG/CD4 cells were fixed and stained with haematoxylin to examine for morphological changes associated with HIV-1 infection. Syncitia formation was observed in the SVG/CD4 cells but not in the CD4 negative parental cells. The bar corresponds to 62.5 μm.

AAV vectors are efficient for gene transfer into both primary and immortalized glial derived cells of the developing human CNS. This finding may appear to contradict an earlier report (Halbert *et al*, 1995) which reported that levels of transduction in primary cells were 10 to 60 times less than in immortalized cells. However the target populations are different as Halbert's investigations were conducted on epithelial cells and fibroblasts. Our results would indicate that in fetal glial cells transduction rates are equivalent between primary and immortalized cells. Whether the high transduction efficiency we observe is due to the target cell population being of fetal or of glial origin remains unclear. It is not surprising however that transduction rates should differ between different cell types and different developmental stages.

While initial transduction levels were high, geneticin selection of SVG cells transduced with JM48 resulted in fewer colonies with stable expression. This supports the findings of others which suggest that AAV vector DNA integration is a relatively rare event and not essential for initial gene expression (Flotte *et al*, 1994). Our data however suggests that integration is required for stable expression at least *in vitro*. The three junctional fragments identified in the SVG/CD4 cells and absence of visible bands at 0.2 or 0.9 kb are consistent with integration of the vector DNA into the host genome, the small size of the 0.2 and 0.9 kb fragments may conceivably render them invisible on the gel however and so the existence of stable episomal DNA cannot be completely ruled out. If integrated the three junctional fragments would suggest that there are two different integration sites in this population of cells. Since the SVG/CD4 cells were derived by pooling 10 separate geneticin resistant colonies, it cannot be determined whether this represents two different clones with a single integrated copy each which developed a growth advantage over the other colonies, or a more homogenous population of cells which has two integrated copies. Attempts to expand cells from a single clone to more fully address this issue have so far proved unsuccessful.

We also demonstrated that a biologically important gene could be introduced into the glial cells and show a measurable effect. Infection of the SVG and SVG/CD4 cell lines with HIV-1 resulted in an initial burst of infection followed by a subsequent decline in both populations. This phenomena was 20-fold higher in the CD4 positive cells. The infection seen in the CD4 negative cells is consistent with an earlier observation from our group that primary human fetal astrocytes can be initially infected with HIV-1 but that the productive infection is followed by a persistent restricted infection (Tornatore *et al*, 1994). During the persistent phase the predominant viral transcript seen is the subgenomic, multiply spliced 2-kb message with a predominance of the viral regulatory Nef transcript. In contrast to mononuclear cells where cytokine stimulation leads to an increase in multiply spliced mRNA and unspliced RNA, similar stimulation of astrocytes results only in an increase in multiply mRNA and not unspliced RNA, suggesting a difference in cellular physiology. Similarly, rapid downregulation of HIV-1 viral replication has been described in a CD4+ glioma cell line (Volsky *et al*, 1992). Recent evidence from an HIV-1 infected astrocytoma cell line suggests that a cellular block in viral Rev function may contribute to the restriction of virus replication (Neumann *et al*, 1995). It has recently been demonstrated (Alkhatib *et al*, 1996) that after HIV-1 binds to CD4 it requires the chemokine receptor CC CKR5 as a fusion cofactor. Whether HIV-1 requires this same cofactor, or any cofactor, for entry into glial cells has not been reported.

The high transduction efficiency of AAV vectors reported here and their ability to promote stable expression of a functional gene indicates that such vectors are likely to prove a powerful tool for the genetic manipulation of cells of the human nervous system and in particular for the targeting of glial derived cells. In addition to *in vitro* investigations, this could have implications for the *ex vivo* manipulation of glial cells prior to transplantation and also for the *in vivo* targeting of glial cells. Since AAV vectors have been shown to transduce neuronal and glial cells it is likely that any *in vivo* cellular targeting strategy will prove challenging. One approach to overcoming this hurdle may be to employ the use of cell type specific promoters, the potential of this approach has recently been demonstrated *in vivo*. The preproenkephalin promoter has for example been shown to promote long term site specific expression in the rodent brain in an HSV amplicon (Kaplitt *et al*, 1994b). In this study expression of β-galactosidase was driven by a 2.7 kb fragment of the rat preproenkephalin promoter. Upon stereotactic inoculation into the rat brain a restricted pattern of β-galactosidase expression was observed in areas of the rat brain that have endogenous expression of preproenkephalin such as the piriform cortex and caudate nucleus. In the piriform nucleus β-galactosidase expression was observed predominantly in cells within the pyramidal cell layer, endogenous preproenkephalin mRNA was also localized to this area, as demonstrated by *in situ* hybridization. In contrast no expression of β-galactosidase was observed in the dorsolateral neocortex which has no endogenous preproenkephalin expression, despite the presence of vector DNA. In addition to the above study, an AAV vector utilizing the neuron-specific enolase promoter has been shown to direct gene expression in neuronal but not glial cells of the rodent spinal cord. It may prove possible to similarly direct gene expression to glial cells with the use of a glial specific promoter, such as the astrocyte specific GFAP promoter. Such an approach coupled with the observed efficiency of transduction of the vector in primary human glial cells may prove important in developing *in vivo* targeting which is an important goal in the development of gene therapy strategies for gliomas.

Materials and methods

Cells and cell line

Preparation of the human fetal glial cell cultures has been described previously (Major *et al*, 1989). Brain tissue was dissected from 8–16 week old fetuses, mechanicaly disrupted by aspiration through a 19 gauge needle, washed in Eagle's minimum essential (E-MEM), medium and plated into poly-D-lysine (0.1 mg/ml in distilled water) treated tissue culture flasks. Cultures were maintained in E-MEM plus 10% fetal bovine serum and fed every 3 to 4 days. The resultant primary cultures are 95% positive for the astrocytic marker glial fibrillary acidic protein (GFAP) but have not been further characterized as type 1 or type 2 astrocytes. The human fetal glial cell line has also been described previously (Major *et al*, 1985). Primary cultures of human fetal glial cells were immortalized with SV40 T protein expression cassette. Cultures are A2B5 negative and GFAP positive and are maintained in Eagle's minimum essential medium (E-MEM) plus 10% fetal bovine serum and fed every 3 to 4 days.

Transduction of cells with recombinant adeno-associated virus

Cesium banded stocks of the recombinant AAV vector pdx11, in which the cytomegalovirus (CMV) promoter drives expression of β-galactosidase, were prepared as described previously (McCown *et al*, 1996). The vector is replication incompetent with 96% of the wild type viral genome including the replication and encapsidation genes having been removed and replaced with a Lac-Z-cytomeg alovirus promoter cassette. AAV-LacZ viral particles were produced by cotransfecting the vector plasmid pdx11 LacZ with the helper plasmid AAV/AD at a ratio of 1:1 into human embryonic kidney 293 cells maintained in Dulbecco's Minimum Essential Medium (DMEM) plus 10% Fetal Bovine Serum (FBS). Adenovirus type 5 was added to the cells at a multiplicity of infection (MOI) of 2. Three days following transfection the cells were harvested and freeze thawed three times. Recombinant AAV was collected by purifying the viral stock through a cesium chloride gradient (1.38 g/ml) formed in a SW41 rotor for 48 h at 40 000 r.p.m. The AAV fraction was collected and dialyzed against DMEM and heated at 56°C for 30 min to inactivate any residual adenovirus. The resultant stock yielded a titer of 1×10^9 transducing units/ml as determined by serial dilutions in 293 cells. No detectable cytopathic effect was seen in recombinant AAV-LacZ infected 293 cells confirming the absence of any active adenovirus. Cell lysate stocks of the recombinant AAV vector JM48, which has a neomycin cassette conferring resistance to geneticin and in which the CMV promoter drives expression of CD4 were prepared as previously described (Anderson *et al*, 1996) with the resultant stock having a titer of 1×10^5 transducing particles/ml. Three days prior to infection, cells were split and plated onto sterile cover slips so as to reach 70% confluency by the time of infection. Cells were infected overnight with virus, either pdx11 or JM48; the following morning the media was changed and cells were incubated at 37°C for a further 48 h before being assayed for expression of reporter genes. Uninfected cells served as negative controls. Geneticin selection on infected SVG cells was carried out with 0.5 mg of geneticin/ml of media.

X-Gal histochemistry and immuno-histochemistry

Cells were washed in PBS, fixed in 100% Ethanol for 5 min and then rehydrated in PBS for 5 min, β-galactosidase activity was detected by incubating cells for 2–3 h at 37°C in substrate solution containing 1 mg/ml 5-bromo-4-chloro-3-indoyl-B-D-galactosidase (X-GAL) as previously described (Keir *et al*, 1995). Immunohistochemical detection of the CD4 receptor was performed at room temperature using standard techniques. The cells were incubated for 1 h with a mouse monoclonal anti-human CD4 (Dako, Carpinteria, CA) diluted 1:10 in 1X phosphate buffered saline (PBS) and then washed in PBS for 10 min. For fluorescence detection the cells were then incubated for 1 h with rabbit anti-mouse directly conjugated to fluorescein isothiocyanate (Dako, Carpinteria, CA) diluted 1:50 in PBS. For peroxidase detection the Histostain SP-kit (Zymed Laboratories Inc., San Francisco, CA) was used. Cells were incubated for 1 h with goat anti-mouse biotinylated antibody, washed in PBS and then incubated in streptavidin-peroxidase for 1 h. The chromagen used was Diaminobenzidine (DAB-Sigma, St. Louis, MO). Controls were uninfected cells, and infected cells stained with secondary antibody alone. Visualization was with a Zeiss epifluorescent microscope.

Preparation of total cell lysates and Western blot
Total cell lysates from the SVG/CD4 and SVG lines were prepared by mixing 1×10^7 cells with 1X sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris, pH 6.8, 0.01% bromphenol blue). The sample was boiled for 5 min and chromosomal DNA was sheared by repeated passage of the sample through a 20 gauge needle, followed by passage through a 26 gauge needle. The sample was then spun at 10 000 g for 10 min and the supernatant collected. Western transfer and immunoblotting were carried out according to the Novex system (Novex, San Diego, CA). Lane 1 was loaded with the SVG parental cell lysate, lane 2 with supernatant from the SVG/CD4 cells and lane 3 with the SVG/CD4 cell lysate. The sample was run at 125 volts for 90 min on a 4–12% Tris-Glycine gel and transferred to a nitrocellulose membrane in a methanol buffer (12 mM Tris, 96 mM Glycine, in 20% methanol) using the Novex Xcell II mini-cell and blot module at 30 volts for 2 h. Detection of Western blots was carried out using the Schleicher & Schuell RAD-free colorimetric detection system (Schleicher & Schuell, Keene, NH). The membrane was incubated at room temperature for 1 h in blocking buffer, and incubated for 24 h at room temperature in anti-human CD4 (Dako, Carpinteria, CA) diluted 1 : 10 in blocking buffer. After washing, the membrane was incubated with goat anti-mouse conjugated to alkaline phosphatase for 30 min at room temperature, washed and incubated with the substrate solution, 5-Bromo-4-Chloro-3-Indolyl Phosphate P-Toluidine (BCIP) until bands appeared.

DNA extraction and Southern hybridization
Total DNA was isolated from cells with standard DNA extraction techniques followed by phenol:-chloroform washes and ethanol precipitation. Two samples each containing 15 μg of DNA were digested overnight at 37°C with the restriction enzymes Bam H1 and then electrophoresed through a 1% agarose gel for 2 h at 140 volts. The agarose gel was rinsed once with distilled water, denatured twice in 1 M NaCl and 0.5 M NaOH for 15 min, then neutralized twice in 0.5 M Tris and 1.5 M NaCl for 15 min. The DNA was then transferred to a nylon filter by capillary action. The DNA was cross-linked to the filter by ultraviolet radiation (Stratalinker, Stratagene) and prehybridized in 50% formamide, $6 \times$ SSPE (0.9 M NaCl, 0.06 M NaH_2PO_4, 0.006 M EDTA-NA_2), $5 \times$ Denhardt's solution, 0.5% SDS and 100 μg/ml calf thymus DNA at 42°C for 1 h. The filter was hybridized in an identical solution, which also contained 1×10^6 d.p.m./ml of nick translated P^{32} labeled JM48. The probe was allowed to hybridize to the filter at 42°C for at least 16 h. The filter was washed twice in $6 \times$ SSPE and 0.1% SDS for 30 min at room temperature, followed by two washes in $1 \times$ SSPE and 0.5% SDS for 30 min at 64°C and a final wash in $0.1 \times$ SSPE and 0.5% SDS for 30 min at 64°C. The filter was dried and used for autoradiography.

Infection of SVG and SVG/CD4 cells with HIV-1
SVG and SVG/CD4 cells at 70% confluency were infected with 1×10^5 TCID 50/ml of the laboratory strain IIIb of HIV-1. From days 1–14 post-infection the supernatants were removed and replaced with whole media. Supernatants were stored at −70°C until assayed for p24 levels by enzyme linked immunoabsorbent assay (Cellular products Inc. Buffalo NY). At 14 days post-infection the cells were fixed in 100% ethanol and stained with hematoxylin to examine for cytopathology.

Acknowledgements

We would like to thank Dr Eugene Major for support and Dr Maneth Gravell for the p24 measurements.

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