Human adenovirus serotypes 4p and 11p are efficiently expressed in cell lines of neural tumour origin

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Most currently used adenovirus vectors are based upon adenovirus serotypes 2 and 5 (Ad2 and Ad5), which have limited efficiencies for gene transfer to human neural cells. Both serotypes bind to the known adenovirus receptor, CAR (coxsackievirus and adenovirus receptor), and have restricted cell tropism. The purpose of this study was to find vector candidates that are superior to Ad5 in infecting human neural tumours. Using flow cytometry, the vector candidates Ad4p, Ad11p and Ad17p were compared to the commonly used adenovirus vector Ad5v for their binding capacity to neural cell lines derived from glioblastoma, medulloblastoma and neuroblastoma cell lines. The production of viral structural proteins and the CAR-binding properties of the different serotypes were also assessed in these cells. Computer-based models of the fibre knobs of Ad4p and Ad17 were created based upon the crystallized fibre knob structure of adenoviruses and analysed for putative receptor-interacting regions that differed from the fibre knob of Ad5. The non-CAR-binding vector candidate Ad11p showed clearly the best binding capacity to all of the neural cell lines, binding more than 90% of cells of all of the neural cell lines tested, in contrast to 20% or less for the commonly used vector Ad5v. Ad4p and Ad11p were also internalized and produced viral proteins more successfully than Ad5. Ad17p virions neither bound or efficiently infected any of the neural cell lines studied.

Introduction

Adenoviruses are non-enveloped viruses with a linear double-stranded DNA genome. They are commonly used as vectors for in vitro as well as in vivo gene transfer to a number of different tissues (Ebihara et al., 2000; Habib et al., 1999; Musgrave et al., 2000; Reynolds et al., 2000; Trask et al., 2000). One of the benefits of using adenoviruses as gene transfer vectors is the ability to efficiently transduce both dividing and non-dividing cells and induce high expression of the desired gene. There are 51 identified serotypes of human adenovirus, divided into six species (A–F), each with a different tropism. The most commonly used adenovirus vector systems are based on adenovirus serotypes 2 and 5 (Ad2 and Ad5), both belonging to species C. Ad2 and Ad5 have been shown to transduce some cell types efficiently but do not transduce neural cells effectively (Chillon et al., 1999). Adenoviruses of different species have different tropisms, indicating that serotypes other than Ad2 or Ad5 could be evaluated for their potential as a vector (Wadell, 1999). It is likely that other serotypes can alleviate the limited tissue tropism and immunity problems of Ad2 and Ad5.

An adenovirus fibre protrudes from each of the 12 vertices of the icosahedral adenovirus particle. Gene delivery to cells by adenovirus is initiated by the binding of the adenovirus fibre knob to a cellular receptor, followed by an interaction between the penton base RGD motif and αv-integrins in the cell membrane (Wickham et al., 1993). The coxsackievirus and adenovirus receptor (CAR) can act as a primary receptor for adenoviruses in species A and C–F (Roelvink et al., 1998), although some serotypes in these species seem to use receptors other than CAR (Arnberg et al., 2000a). Furthermore, adenoviruses from species B use a receptor that is different from CAR (Roelvink et al., 1998). The α2 domain of major histocompatibility complex class 1 (MHC-1) molecules and the vascular cell adhesion molecule-1 have been described, in addition to CAR, to be receptors for Ad5 (Hong et al., 1997). The main CAR-
binding epitopes of the fibre knob are located in the AB-loop (comprising over 50% of the interfacial protein–protein interactions) but also additional residues in the B β-sheet, the DE-loop and the FG-loop are also important. Some of these residues are conserved among the CAR-binding adenoviruses (Bewley et al., 1998; Roelvink et al., 1999).

Gene transfer to neural cells has gained considerable interest in the field of neuroscience (Hermens & Verhaagen, 1998; Kaplitt & Makimura, 1997). Applications for gene transfer range from treatment of genetic diseases, tumours and acquired degenerative encephalopathies, such as Alzheimer’s disease and Parkinson’s disease, to being a powerful tool in the study of biological mechanisms (Alton & Kitson, 2000; Choi-Lundberg & Rosenthal, 1999; Lang et al., 1999; Miller et al., 1998; Russell & Hirata, 1998; Wood et al., 1996). The in vivo delivery of adenovirus into the brain can induce an immune response regardless of the more protective environment in the CNS. Adenovirus vectors with a deleted early E1 region can be maintained for months (Thomas et al., 1998). A new trend in adenovirus vector construction is always desirable to use a virus with a high affinity for the target tissue. A new trend in adenovirus vector construction is to delete all virus-related genes in the vector to avoid the efficiency of transduction in order to minimize the dose of virus required. Another strategy to avoid the immune response is to delete all virus-related genes in the vector to avoid ‘leakage’ of viral proteins from the infected cells (Fisher et al., 1996; Kochanek et al., 1996). These high-capacity adenovirus vectors can give long-term transduction in the rat brain without extensive inflammation, even after a subsequent peripheral immunization against the adenovirus (Schiedner et al., 1998; Thomas et al., 2000). Whatever strategy is chosen, it is always desirable to use a virus with a high affinity for the target tissue. A new trend in adenovirus vector construction has been to modify the tropism of the vector by swapping fibres or by introducing a new ligand in the fibre (Russell, 2000).

The purpose of this study was to find an adenovirus serotype with tropism for human neural tumour cells. The binding and infectivity properties of Ad4p (prototype), Ad5v (vector strain), Ad11p and Ad17p (species E, C, B and D, respectively) were assessed. Ad17p was clearly an interesting candidate, since it was shown previously that recombinant Ad2 with an Ad17 fibre could infect rat neural cells more efficiently than wild-type Ad2 (Chillon et al., 1999). Binding properties were evaluated by flow cytometry and the ability to express viral proteins was assessed by immunostaining and 35S-labelling of proteins after infection. Binding was then analysed on the basis of CAR recognition. Computer-based models of the Ad4p and Ad17p fibre knob monomers were created from the coordinates of crystallized adenovirus fibre knobs.

Methods

■ Cell lines and culture conditions. A549 cells (human oat cell carcinoma) are permissive for most adenovirus serotypes and were used as a positive control in all experiments. Three different neural cell lines, Mg251 (human glioblastoma), PFSK-1 (human medulloblastoma) and SH-SYS5Y (human neuroblastoma), were chosen to represent different types of neural tumours. Chinese hamster ovary cells (CHO) expressing either human CAR (CHO-CAR) or human x2-integrins (CHO-x2) were grown as described previously (Bergelson et al., 1993, 1997). A549, Mg251 and SH-SYS5 cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) containing 0.75% NaCO3 (w/v), 10% foetal bovine serum (FBS), 20 mM hepes (pH 7.4) and penicillin-streptomycin (10 IU/ml and 10 µg/ml, respectively) (PEST) at 37 °C. PFSK-1 cells were grown in RPMI 1640 (Sigma) containing 10% FBS, 20 mM hepes (pH 7.4), 0.75% NaCO3 (w/v) and PEST at 37 °C. Upon virus infection, the FBS concentration was lowered to 0.5%.

■ Virus strains and purification. Adenoviruses used in this study were Ad4p (prototype, strain Rl67), Ad5v (vector, strain pFG140) (Graham, 1984; Graham et al., 1989), Ad11p (prototype, strain Slobitski) and Ad17p (prototype, strain Ch22). Virus serotypes were all checked according to their DNA restriction patterns (Adrian et al., 1986). Viruses were propagated in A549 cells and purified on a discontinuous CsCl gradient, as described previously (Mei et al., 1998), to purify complete virus particles. The virion band was collected and the density was measured on a refractometer. Virions were assessed under an electron microscope. Virions were then purified from the CsCl by desalting on a NAP-10 column (Pharmacia) and eluted with 1.5 ml 10 mM PIBS. Virus concentration was determined using a spectrophotometer: an OD of 1 unit (OD260 – OD230) corresponds to 280 µg virions or 1014 virus particles/ml.

■ Virus labelling. Virions purified from the CsCl gradient were loaded onto a NAP-10 column and eluted with 1.5 ml labelling buffer (50 mM NaHCO3 and 135 mM NaCl, pH 8.8). The virion concentration was between 1 and 4 mg/ml MgCl2 (3 µl of a 1 M solution) was added to a final concentration of 2 mM. Then, 165 µl N-hydroxysuccinimido-biotin (1 mg/ml) (Sigma) dissolved in DMSO (Sigma) was added and the virions were mixed with biotin in the dark at 4 °C overnight. Free biotin was removed on a NAP-10 column eluted with 10 mM PBS. Glycerol was added to a 10% concentration and the virions were aliquoted and stored at −70 °C until use.

■ Binding experiments using a FACScan flow cytometer. For each binding experiment, 5 × 10⁶ cells were used. Cells were detached from the growth surface using PBS containing 0.05% EDTA, diluted in their respective growth medium and counted. Cells were incubated with three different concentrations of biotinylated virions: 1, 3 and 6 pg per cell. Upon virus infection, the FBS concentration was lowered to 0.5%.

■ Adenovirus binding capacity to non-human cells expressing human CAR. The ability of the viruses to bind to CAR was analysed for each of the different adenovirus serotypes. CHO-CAR cells expressing
CAR and CHO-α2 cells expressing α2-integrins (3 and 6 pg virions per cell) of the different serotypes, as described above. Results are presented as the mean of at least three independent experiments, with a confidence interval of 95%.

**Labelling of proteins after virus infection.** Pulse–chase experiments were performed as described previously (Segerman et al., 2000). To distinguish viral proteins from cellular proteins, a mock-infected control was used for each cell line. Three independent labelling experiments were carried out and the data were consistent.

**Immunostaining.** The cell lines Mg251, PFSK-1, SH-SY5Y and A549 were grown on coverslips in a 24-well microtitre plate and infected with 2 pg virions per cell (the same amount of cells were used with the different serotypes) in serum-free medium for 1 h at 37 °C. At 1 h post-infection (p.i.), medium containing 2% FBS was added and the cells were incubated for a further 24, 48 or 72 h. Cells were fixed in 2% paraformaldehyde for 30 min. A solution containing 2% BSA and 0.1% saponin in PBS (BS buffer) was added for 30 min at room temperature. Cells were then incubated for 1 h at room temperature with a mouse monoclonal antibody (mAb 8052), which recognized the hexon protein of all adenovirus serotypes used in this study (Chemicon International), diluted to 5 μg/ml in BS buffer. Cells were washed in BS buffer and then incubated for 1 h at room temperature with FITC-conjugated rabbit anti-mouse immunoglobulin G (Dakopatts), diluted 1:30 in BS buffer. The cells were washed again in PBS and mounted onto a slide. Micrographs were taken using a fluorescence microscope (Leitz Dialux 20) equipped with a Hamamatsu colour 3CCD (C5610) at a magnification of 130 X.

**Fibre knob alignment.** Sequence alignments were made with the MEGALIGN program (DNASTAR) using the CLUSTAL algorithm with a PAM (point-accepted mutations per 200 aligned positions) 250 residue weight table. Alignments were performed using the sequences from Protein Database (PDBnr): accession numbers s39300 for the Ad4p fibre, ERAD5 for the Ad5 fibre, D37476 for the Ad11p fibre and UNK_52178293 for the Ad17 fibre.

**Ad4p and Ad17p fibre knob modelling.** The Ad4p knob monomer model was created with the swiss-model ProMod II system (Guex & Peitsch, 1997; Guex et al., 1999; Peitsch, 1995) using the X-ray crystallographic coordinates of the Ad5 fibre knob (Xia et al., 1994) and the corresponding alignment of Ad4p (accession number s39300). The Ad17p fibre knob monomer model was modelled based upon the crystallographic coordinates for the Ad2, Ad5 and Ad12 fibre knobs (accession numbers 1QHVA, 1KNB and 1N0BD, respectively) and the corresponding alignment of Ad17p (accession number UNK_52178293). Structures were refined using Swiss-PdbViewer with respect to energy minimization and the molecular surface was computed. The root–mean–square (RMS) value of the Ad4p fibre knob monomer model versus the crystallized Ad5 was 0.55 Å. The RMS value for the Ad17p fibre knob monomer was 1.17 Å. Surface residues with no similarity to Ad5 were coloured blue and the positions of conserved CAR-interacting residues were coloured green. White regions represent amino acid residues also present in Ad5 (see Fig. 4) (Bewley et al., 1999; Roelvink et al., 1999).

**Results**

**Ad11p manifests a superior binding capacity to human neural cell lines**

Binding to the neural tumour cell lines Mg251 (human glioblastoma), PFSK-1 (human medulloblastoma), SH-SY5Y (human neuroblastoma) and the permissive control cell line A549 (human oat cell lung carcinoma) was analysed (Fig. 1). The different adenovirus serotypes showed various binding characteristics. Ad11p, species B, was binding with an exceptionally high capacity, creating between 28- and 130-fold more positive cells in the neural cell lines compared to the commonly used vector Ad5v (binding to PFSK-1 cells and SH-SY5Y cells, respectively, at 1 pg virions per cell). All four adenovirus types were binding better to the A549 cells than to the cells of neural origin, with the exception of Ad17p, which bound equally well to PFSK-1 cells. Ad11p manifested an unsurpassed binding capacity to all of the cell lines tested. Ad5v, species C, showed poor affinity for all of the neural cell lines tested. The vector candidates Ad4p and Ad17p also showed lower binding capacities to the neural cell lines. Both serotypes seemed to have a binding capacity that was as low as that of Ad5v towards the neural cells, even though Ad4p and Ad17p seemed to have a preference for the medulloblastoma cells (PFSK-1).

**Ad4p bound to CAR with a higher capacity than Ad5v, while Ad11p and Ad17p did not bind to CAR**

None of the serotypes could bind to the CAR-deficient CHO-α2 cells (Fig. 2). Using flow cytometry, Ad4p showed a 100-fold higher binding capacity to CHO-CAR cells than to CHO-α2 cells at a concentration of 3 pg virions per cell (virus saturation point). Ad5v bound to CHO-CAR cells eight times better than the CHO-α2 cells, which was indicative of an affinity for CAR, although this efficiency was markedly lower than the binding obtained with Ad4p virions. Ad11p and Ad17p showed no binding to either the CHO-α2 or the CHO-CAR cells, pointing to a very low or no binding capacity to CAR. CAR expression in the neural cell lines was approximately one- to two-fifths of the levels of CAR in CHO-CAR cells (SH-SY5Y and PFSK-1, respectively), as evaluated by flow cytometry (data not shown).

There are a few amino acid residues of the fibre knob that are critical for CAR-binding (Bewley et al., 1999; Roelvink et al., 1999). A protein alignment of the critical CAR-binding amino acids in the AB-, DE- and FG-loops showed conservation of these residues in serotypes Ad4p, Ad5v and Ad17p but not in the species B virus Ad11p (Fig. 3). To further evaluate the differences between the fibre knobs of the serotypes containing CAR-binding residues, two computer-based models of the Ad4p and Ad17p fibre knob monomer were created using the crystallized adenovirus fibre knobs as templates (Fig. 4). Surface amino acid residues in Ad4p and Ad17p with no similarity to Ad5 were distinguished as well as the residues that have been demonstrated to be important for CAR binding. It was revealed that Ad4p has a relatively large accessible area next to the conserved CAR-binding residues, with no similarity to Ad5. Ad4p shared 66% identity to Ad5 in the fibre knob. Ad17p manifested only 48, 46 and 37% identity to the fibre knobs of Ad5, Ad2 and Ad12, respectively, which were used
Fig. 1. Flow cytometry analysis of the binding of biotin–streptavidin FITC-labelled Ad4p, Ad5v, Ad11p and Ad17p virions to the positive control cell line A549 (a) and the human neural glioblastoma cell line Mg251 (b), medulloblastoma cell line PFSK-1 (c) and neuroblastoma cell line SH-SY5Y (d). Results are presented as the mean ± 95% confidence interval of at least three independent experiments.

Fig. 2. Binding capacities toward CHO-α2 cells (a, do not express CAR) and CHO-CAR cells (b, express human CAR). Results are presented as the mean ± 95% confidence interval of at least three independent experiments.

in the modelling of the knob. This indicated that the fibre knob of Ad17p was very different from the other CAR-binding serotypes, despite the conserved CAR-binding residues. Ad17p contained a unique accessible region (the blue region to the left in Fig. 4b) involving the regions of the CD-, GH- and IJ-loops. Ad11p of species B shared only 31% identity with the species C Ad5 fibre knob and the CAR-binding motif was absent, pointing toward a very different receptor-binding motif than the CAR-binding fibre knobs. **Ad4p and Ad11p were expressed early in the neural cell lines**

Binding does not necessarily lead to internalization and expression of viral proteins. An immunostaining protocol was
Adenovirus expression in neural tumour cells

Fig. 3. Protein sequence alignment of the fibre knob region with conserved CAR-binding residues. The fibre knob trimerization motif is shown in green and conserved CAR-binding residues are shown in red. The alignment was made with the MEGALIGN program using the CLUSTAL algorithm and a PAM (point-accepted mutations per 200 aligned positions) 250 residue weight table.

performed to assess whether the different serotypes could also become internalized and start transcription and translation of their genes in these cell lines (Fig. 5). Cultures were analysed for hexon expression at three time-points p.i., showing that the time of virion expression in the cells was dependent both on the cell line and on the serotype of the virus. The glioblastoma cell line Mg251 was permissive for Ad4p, Ad5v and Ad11p infection but refractory to Ad17p, where, only occasionally, a cell could be scored positive 72 h p.i. Viral hexon proteins of Ad5v, Ad11p and Ad17p could not be detected in the glioblastoma cell line 24 h p.i., when Ad4p hexon was produced in a low percentage of the cells (Fig. 5a). After 48 h, the proportion of Ad4p-, Ad5v- and Ad11p-infected cells had increased to a substantial majority of the cells. Ad17p did not show any hexon production 48 h p.i. in this cell line. In the medulloblastoma cell line PFSK-1, a few cells scored positive 24 h after infection with both Ad4p and Ad11p, indicating an efficient internalization and expression in these cells. At 72 h p.i., both Ad4p and Ad11p manifested a high proportion of positive cells. Ad5v was expressed but to a lesser extent than Ad4p and Ad11p in PFSK-1 cells. Ad17p showed a refractory pattern with only a few positive cells at 72 h p.i. (Fig. 5b). The proportion of positive cells was fewer than after infection of the other two cell lines. Ad4p was expressed but to a lesser extent than Ad4p and Ad11p in PFSK-1 cells. Ad17p showed a refractory pattern with only a few positive cells at 72 h p.i. (Fig. 5b). The neuroblastoma cell line SH-SY5Y was only semipermissive to infection by the different serotypes. Ad4p was the only serotype expressed 24 h p.i. but the number of positive cells was fewer than after infection of the other two cell lines. At 48 h p.i., only Ad4p and Ad11p manifested infection in SH-SY5Y cells, Ad17p did not infect the cells at all, while Ad5v showed expression 72 h p.i. Ad4p infected the highest proportion of cells in the neuroblastoma cell line; moreover, Ad4p and Ad11p displayed the most rapid expression (Fig. 5c).

Fig. 4. A molecular surface representation of the Ad4p (a) and Ad17p (b) fibre knob monomer viewed at the CAR-interacting interface. White, amino acid residues shared by the Ad4, Ad5 and Ad17 fibre knobs; green, conserved CAR-interacting residues; blue, the unique amino acid residues in the Ad4 (a) and Ad17 (b) fibre knobs. The analysis is based on an alignment of the adenovirus fibres. The monomer models were created by Swiss-model ProMod II system and then refined using Swiss-PdbViewer.

Ad4p and Ad11p produced the highest concentration of viral structural proteins in infected neural cells

An $^{35}$S-labelling experiment was performed in order to further evaluate the production of viral proteins in the different cell lines. Both quantitative and differential expression of the viral proteins were evaluated (Fig. 6). The hexon was the most abundant structural protein of the virion and the apparent sizes were about 110 kDa for Ad5v and roughly 120 kDa for Ad4p, Ad11p and Ad17p. The intensities of the hexon bands reflect the capacity of virions from the different serotypes to enter the nucleus and express their genes. The $^{35}$S-methionine and $^{35}$S-cysteine labelling experiment showed, following SDS-PAGE, detectable levels of viral proteins of Ad4p in all of the neural cell lines as well as in the positive control cell line A549.
Fig. 5. Immunofluorescence detection after infection of Ad4p, Ad5v, Ad11p and Ad17p in the neural cell lines Mg251 (a), PFSK-1 (b), SH-SY5Y (c) and the permissive cell line A549 (d). NC is a mock-infected negative control.
Ad11p induced detectable levels of viral structural proteins in all cells except the neuroblastoma cells. Ad5v was being adequately expressed only in the glioblastoma and A549 cells, while Ad17p showed expression in the epithelial control cell line but not in the neural cell lines. Downregulation of cellular proteins could be seen in A549 cells infected by Ad11p. Ad17p did not give a very strong hexon band in the A549 cells, indicating that 2 pg virion per cell corresponded to a lower m.o.i. for Ad17p than for Ad4p, Ad5v and Ad11p in A549 cells. The species E serotype Ad4p was the only serotype that produced sufficient amount of viral proteins to be detected in the neuroblastoma cells, even if the level was significantly lower than in the other cell lines. Ad4p also produced several other viral structural proteins in the cell lines; however, this was not seen for Ad11p, which produced predominantly hexons in the neural cell lines.

**Discussion**

Ad5 of species C is not optimal for transfecting cells of neural origin (Chillon *et al.*, 1999; Ehrengruber *et al.*, 2001; Miller *et al.*, 1998) and the present strategy to only use adenoviruses from species C as vectors is probably not ideal because of the restricted tropism of these viruses. Different tissues may require different adenovirus serotypes to be efficiently transduced. To ascertain whether a virus can be used as a vector for gene delivery to a certain cell type, it is vital to determine the binding capacity of the vector candidate for the given cell. The CAR-binding serotype Ad5v of species C showed only a very low capacity to bind the neural cells in the affinity assay, whereas Ad11p of the non-CAR-binding species B manifested a very high affinity for all of the neural cell lines tested. The binding experiment was carried out on ice to avoid virus internalization. The binding capacities for serotypes Ad4p, Ad5v and Ad17p to neural cells were lower, suggesting either a lower number of receptors, a low affinity for the cell receptors or masked receptors. Even if the receptor was expressed on the cell surface, proteins such as CAR can be masked by the glycocalyx on cells (Pickles *et al.*, 2000). Ad17p was shown to have better infectivity in rat neural cells than Ad5 (Chillon *et al.*, 1999), but did not show efficient infectivity in the human neural cell lines in this study.

The data presented in this report support the notion that Ad4p of species E has affinity for CAR, as has been described previously (Roelvink *et al.*, 1998). However, Ad4p was, as shown here, more efficient in infecting neural tumour cells than Ad5v. Furthermore, Ad4p bound with a higher efficiency to CAR on CHO-CAR cells than Ad5v. A difference in the binding characteristics between these two CAR-binding viruses was not surprising. There are some major differences in the fibre knob between Ad4p and Ad5v. They are both binding to the same receptor, but the amino acid sequence reveals a 33% dissimilarity in the fibre knob between these serotypes (Chroboczek *et al.*, 1995), which may have affected the docking on CAR. A structure model of the fibre knob of these viruses showed that a large part of this dissimilarity was located in an area bordering the conserved CAR-binding region. Possibly, this region was responsible for the difference in binding properties seen between Ad4p and Ad5v. Other aspects, such as differences in surface-exposed charges, could increase electrostatic repulsion by the negatively charged glycocalyx. Also, the length of the fibre has been shown to influence binding. A short fibre will facilitate direct binding between the penton base and αv-integrins on the cell but could also make the virion sensitive to electrostatic interactions between the hexon and cell surface (Shayakhmetov & Lieber, 2000). Ad4p belongs to species E but has characteristics from both species B and C. The ‘early’ gene sequences of Ad4p show a large resemblance with species B adenoviruses, while the fibre knob gene is similar to species C adenoviruses. The length of the fibre is an intermediate between species B and C (Gruber *et al.*, 1993).
The fibre knob of Ad4p, species E, showed a higher similarity to Ad5 of species C than the fibre knob of Ad17p of species D did. There was only 48, 46 and 37% identity between the Ad17p fibre knob and the CAR-binding fibre knobs of Ad5, Ad2 and Ad12, respectively, which were used in the modelling of the Ad17p knob. In the model structure of Ad17, there were dissimilarities adjacent to the CAR-binding residues and also a large area of divergence in the regions of the CD-, GH- and JJ-loops (Fig. 4b, blue region on the left) (van Raaij et al., 1999). By modelling, we showed that this region may be easily accessible on the fibre knob and the observed structural difference could account for the differences in the binding characteristics between these serotypes. This question can be further investigated by point mutation analysis of this region in the fibre knob. The presence of the conserved CAR-binding residues in the AB-loop in Ad17p does not necessarily provide a good affinity for CAR. It has been shown that other species D adenoviruses, like Ad37, contain these residues but use sialic acid as a primary receptor instead of CAR (Arnberg et al., 2000a, b). It has also been suggested that Ad17p has receptors other than CAR or MHC-1 for attachment to cells (Zabner et al., 1999). The fibre knob of Ad11p shared only 31% identity with the species C Ad5 fibre knob and could not be properly modelled based on the crystal structure of Ad5.

Ad11p is a very interesting vector candidate because of its high binding capacity towards neural cell lines. The ability to infect a cell population is initially dependent on affinity for the particular cell type. Actions that enhance virus binding will increase adenovirus-mediated gene transfer (Fasbender et al., 1997, 1998; Kaplan et al., 1998; Toyoda et al., 1998). However, the affinity required for efficient transfection of a cell population may be different for different serotypes and cell lines. Also, the stages between internalization and expression of the viral proteins are important. Different serotypes have different strategies before entering the nucleus, which may influence the success rate of infecting different cell types. Species B and C viruses have an equally rapid internalization (t1/2 = 2–3 min) but intracellular trafficking is different and seems to be dependent on the adenovirus fibre (Miyazawa et al., 1999). Adenoviruses of species C are quickly released to the cytoplasm from the early endosome and translocated to the nucleus along microtubules. Members of species B have chosen another route and remain encapsidated until they reach the late endosome or lysosome (pH 5.5 and 5.0, respectively) before transport to the nucleus (Miyazawa et al., 2001).

The biodistribution of CAR in the body seems to be dependent on the stage of cell differentiation. In the CNS, CAR is suggested to be implicated as a cell adhesion molecule in neural network formation of the developing brain (Honda et al., 2000). In the adult brain, expression is more restricted and limited to the ependymal cells lining the ventricular system (Tomko et al., 2000). Other areas of the body also show a developmental restriction of CAR expression. CAR is not expressed on primitive CD34+ and CD38– haematopoietic progenitor cells but expression is acquired during erythroid and myeloid differentiation (Rebel et al., 2000).

CAR expression in different glioma cell lines is highly variable (Asaoka et al., 2000; Miller et al., 1998) and the transfection efficiency with Ad5 vectors is correlated to the CAR content on the cell (Asaoka et al., 2000; Mori et al., 1999). Because of this, alternative non-Ad5 serotypes should be developed for gene therapy of neural tumours. The non CAR-binding serotype Ad11p showed a much higher affinity for all of the tested neural cell lines than Ad5v and is therefore a very interesting candidate vector for tumour cells of neural origin.

High expression of Ad4p, Ad5v and Ad11p viral proteins in the glioblastoma cell line was noted in the 35S-labelling experiment. In the medulloblastoma cell line there was only efficient expression of Ad4p and Ad11p, which was supported by immunostaining experiments in which Ad4p and Ad11p showed the largest and quickest response. The lower Ad5v expression, as visualized by immunofluorescence, was not sufficient to generate visible bands during SDS–PAGE. Only Ad4p produced sufficient amounts of viral proteins in the neuroblastoma cell line. It was interesting to see that Ad4p, despite a low binding capacity for the neural cell lines, could infect a relatively large proportion of cells. Under these experimental conditions, a relatively low binding capacity was demonstrated for Ad4p. However, an efficient expression was detected using both SDS–PAGE and immunofluorescence, making Ad4p an important vector candidate. Virus expression of all the serotypes tested in the neuroblastoma cell line (SH-SYSY) was very slow. The immunostaining procedure showed that the virus types could infect the neural cell lines but only to a varying degree and with a different time span from infection to virus expression. The efficiency of Ad17p was very poor in all of the neural cell lines tested. The two vector candidates Ad4p and Ad11p seem to be more efficient than the currently used Ad5. A slow response in the production of structural viral proteins normally results from a low m.o.i. for the virus serotype on that particular cell line (Fenner et al., 1974). It has also been shown that the length of virus incubation is often more important than the m.o.i. for the infection success rate. A longer incubation time with virions results in a higher transduction efficiency (Marini et al., 2000). Ad4p showed the most rapid response in all of the cell lines and Ad11p also manifested a rapid and potent expression capacity in all of the cell lines.

The large number of adenovirus serotypes and the fact that different adenoviruses have distinctive tropism (Wadell, 1999) give an indication that different adenovirus candidate vectors could be evaluated for different tissues. Another important issue when choosing a suitable vector is the prevalence of immunity against the adenovirus vector candidate and, in this respect, the choice of the endemic Ad2 and Ad5 is not optimal. The percentage of adults with neutralizing antibodies against Ad2 and Ad5 is very high (88 and 68%, respectively) and the fact that the viruses are endemic gives ample possibilities for
boosting the titre (Fox & Hall, 1980; Kremer et al., 2000; Piedra et al., 1998; Wadell, 1999). About 34% of adults have neutralizing antibodies against the epidemic Ad4 (Ludwig et al., 1998). The seroprevalence in adults for Ad11 and Ad17 is not found in the literature. The high prevalence of immunity against Ad2 and Ad5 will impair long-term expression of most vectors based on these serotypes. This also applies to virus expression in the CNS when the recipient can be challenged with the common Ad2 or Ad5 infection (Thomas et al., 2000).

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