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Development of Group B Adenoviruses as Gene Transfer Vectors

DANIEL STONE[1], NELSON C. DI PAOLO[1] AND ANDRÉ LIEBER[1,2]*

[1]*Division of Medical Genetics, Department of Medicine and* [2]*Department of Pathology, University of Washington, Seattle, WA 98195, USA*

Introduction

One of the major limitations to gene therapy is the need for effective gene delivery vehicles and, due to their natural high efficiency of exogenous gene transfer, viral vectors have been widely studied. The tropism of each virus is a major determinant of its therapeutic use and, due to their ability to efficiently infect multiple therapeutic target cell populations, adenovirus (Ad) vectors have shown considerable potential as vectors for delivery of therapeutic genes. To date, 51 human Ad serotypes have been identified, classified into groups A through F (*Table 6.1*), and for almost two decades, vectors derived from group C serotype Ad5 have been extensively used for gene transfer studies. These Ad5-based vectors are able to efficiently infect many mammalian cell types (including both mitotic and post-mitotic cells) through interaction with a primary attachment receptor, the coxsackie and adenovirus receptor (CAR) (Bergelson *et al.*, 1997). Although they can transduce many tissue types, Ad5-based vectors are unable to efficiently transduce several potential disease target cell types, including haematopoietic stem cells (HSCs) (Neering *et al.*, 1996; Watanabe *et al.*, 1996) and dendritic cells (DCs) (Arthur *et al.*, 1997), without using

* To whom correspondence may be addressed (lieber00@u.washington.edu)

Abbreviations: Ad, adenovirus; AIDS, acquired immune deficiency syndrome; BMP, bone morphogenetic protein; CAR, coxsackie and adenovirus receptor; CAT, chloramphenicol acetyl transferase; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; CTL, cytotoxic T lymphocyte; DC, dendritic cell; EBV, Epstein–Barr virus; GFP, green fluorescent protein; hBM-MSC, human bone marrow-derived mesenchymal stem cell; HD, helper dependent; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; HSC, haematopoietic stem cell; LMP, latent membrane antigen; MOI, multiplicity of infection; MSC, mesenchymal stem cell; NK, natural killer; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; PFU, plaque-forming unit; SGPT, serum glutamic pyruvic transaminase; SIV, simian immunodeficiency virus; SMC, smooth muscle cell; SP, side population; TAA, tumour-associated antigen; UCB, umbilical cord blood; YAC, yeast artificial chromosome.

Biotechnology and Genetic Engineering Reviews – Vol. 22, January 2006
0264-8725/06/22/101-123 $20.00 + $0.00 © Lavoisier/Intercept, 14 rue de Provigny, F-94236 Cachan cedex, France

Table 6.1. Adenovirus associated illnesses. B group Ads are underlined in bold. Ad serotypes are grouped into subgroups A (12, 18, 31), B (3, 7, 11, 14, 16, 21, 34, 35, 50), C (1, 2, 5, 6), D (8, 9, 10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51), E (4) and F (40, 41).

Disease	Most affected groups	Principal serotype
Acute febrile pharyngitis	Infants/young children	1, 2, **3**, 5, 6, **7**
Pharyngoconjunctival fever	School children	**3**, **7**, **14**
Acute respiratory disease	Military recruits	**3**, 4, **7**, **14**, **21**
Pneumonia	Infants/young children	1, 2, **3**, **7**
Pneumonia	Military recruits	4, **7**
Epidemic keratoconjunctivitis	Any age group	8, **11**, 19, 37
Pertussis-like syndrome	Infants/young children	5
Acute haemorrhagic cystitis	Infants/young children	**11**, **21**
Gastroenteritis	Infants/young children	40, 41
Hepatitis	Infants/children with liver transplants	1, 2, 5
Persistence of virus in urinary tract	Immunosuppressed patients	**11**, **34**, **35**

high multiplicities of infection (MOI) and causing cytotoxicity or loss of cell function. This is primarily due to low expression of primary attachment (CAR) and secondary internalizing (integrins) (Wickham *et al.*, 1993) receptors on target cells. Additionally, following systemic *in vivo* delivery of Ad5 vectors, acute vector-mediated toxicity is seen (due to uptake by cells of the reticuloendothelial system and subsequent production of pro-inflammatory cytokines/chemokines), and immune responses to adenoviral proteins promote clearance of virus and limit duration of transgene expression. Also, Ad5 is an endemic virus, and pre-existing humoral immunity to Ad5 is widespread among humans (D'Ambrosio *et al.*, 1982; Vogels *et al.*, 2003; Nwanegbo *et al.*, 2004). The presence of anti-Ad5 neutralizing antibodies will inhibit systemic vector application and limits the therapeutic potential of these vectors. Furthermore, upon systemic delivery, Ad5-based vectors are directed to the liver through pathways thought to involve binding to blood factors (Shayakhmetov *et al.*, 2005). This liver sequestration is detrimental to therapeutic strategies that involve *in vivo* targeting of Ad vectors to other organs, or even tumours. While the pros and cons of Ad5-based vectors have been extensively discussed before (for a review see Imperiale and Kochanek, 2004), there has been increasing interest in the development of newer Ad vectors that circumvent the disadvantages of Ad5-based vectors by utilizing other serotypes of Ad.

In this review, we will introduce recent advances in the development of group B adenoviruses as gene transfer vectors. Over the past decade, several groups have attempted to overcome the disadvantages of Ad5-based vectors by developing vectors that utilize elements of group B serotypes. These new Ad vectors show great promise for use in therapies where Ad5-based vectors have been unsuccessful. The currently available Ad vector systems that utilize group B Ads will be discussed, along with experimental data demonstrating their successful use as gene transfer vectors.

Vectors utilizing B group adenoviruses

RATIONALE: PROPERTIES AND DISEASE PATHOGENESIS

Of the currently identified human Ads, 9 serotypes belong to subgroup B, and they have been further divided into subgroups B:1 (Ads 3, 7, 16, 21, and 50) and B:2 (Ads 11, 14, 34, and 35), based on DNA homology. Typically, B:1 serotypes are isolated from patients with respiratory tract infections, whereas the B:2 viruses, with the exception of Ad11a and 14 (Mei *et al.*, 1998), are described as causing kidney and urinary tract infections (Hierholzer *et al.*, 1975; Shields *et al.*, 1985; Shindo *et al.*, 1986), while both B:1 and B:2 Ads have been associated with conjunctivitis (Sawada *et al.*, 1987; Saitoh-Inagawa *et al.*, 2001). B group Ad infections often occur in immunocompromised patients, including AIDS patients, recipients of bone marrow transplants, or chemotherapy patients. In general, B group Ad infections (with the exception of Ad3 and Ad7) are relatively rare, as reflected by the low percentage (<10%) of humans with neutralizing antibodies against B group Ads that have been found in Europe, USA, Asia, and Africa (D'Ambrosio *et al.*, 1982; Vogels *et al.*, 2003; Nwanegbo *et al.*, 2004).

Unlike groups A, C, D, E, and F Ads, the B group Ads do not utilize CAR as a primary attachment receptor (*Figure 6.1*), and this reflects their different tissue tropism and subsequent disease pathogenesis (Roelvink *et al.*, 1998). Recently, Gaggar and colleagues (Gaggar *et al.*, 2003) and others (Segerman *et al.*, 2003b; Sirena *et al.*, 2004) demonstrated that B group Ads are able to use the complement regulatory protein CD46 as a primary attachment receptor. CD46 is a membrane protein that is expressed on all nucleated human cells, and is also used as a receptor by keratoconjunctivitis causing D group Ads (Wu *et al.*, 2004), measles virus laboratory strains (Dorig *et al.*, 1993), human herpes virus 6 (Santoro *et al.*, 1999), pathogenic *Neisseria* (including *Neisseria meningitides, Neisseria gonorrhoeae*), and *Streptococcus pyogenes* (Johansson *et al.*, 2003). In humans, there are four major isoforms of CD46 (BC1, BC2, C1, and C2), depending on the alternative splicing of a region encoding an extracellular domain and the choice between one or two cytoplasmic tails, Cyt1 and Cyt2 (Purcell *et al.*, 1991). CD46 expression is greatly up-regulated in malignant tumour cells (Hara *et al.*, 1992; Thorsteinsson *et al.*, 1998; Kinugasa *et al.*, 1999; Murray *et al.*, 2000) and haematopoietic stem cells (Cho *et al.*, 1991; Manchester *et al.*, 2002). In addition to CD46, it has been suggested that other B group receptors exist (Segerman *et al.*, 2003a). Recently, CD80 and CD86 were identified as candidate receptors for Ad3 (Short *et al.*, 2004), but the existence of additional receptors is likely since CD80 and CD86 are not expressed by several cell lines that have shown differential binding levels for B group Ad serotypes (Mei *et al.*, 1998).

In previous studies, the cell binding characteristics of group B Ads were investigated for several therapeutic target cell populations. Studies have demonstrated efficient binding of Ad35 and Ad11p to human CD34 positive cells (Shayakhmetov *et al.*, 2000; Stecher *et al.*, 2001), Ad11a and Ad11p to various epithelial cell lines (Mei *et al.*, 1998), Ad11p and Ad35 to cells of haematopoietic origin (Segerman *et al.*, 2000), Ad11p to neural cell lines (Skog *et al.*, 2002), Ad11p, Ad16 and Ad21 to primary neural tumour cells (Skog *et al.*, 2004), and Ad11p to endothelial and

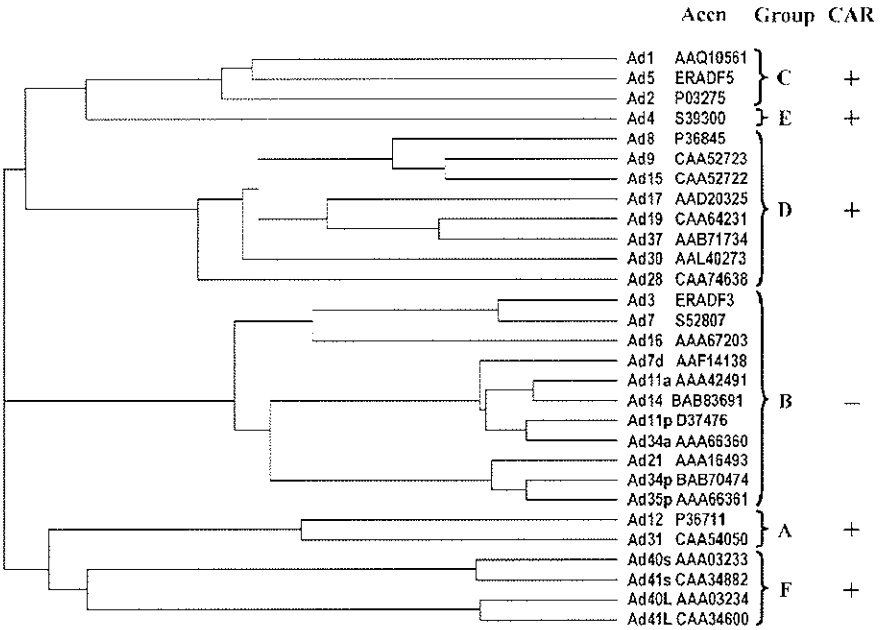

Figure 6.1. Phylogenetic tree of adenovirus fibre sequences available in Genbank. Serotype, Genbank accession number, subgroup, and CAR usage are indicated.

carcinoma cells (Zhang *et al.*, 2003). Overall, the cell binding efficiency of these Ad serotypes demonstrates the potential of B group Ads as gene transfer vectors.

In order to improve the prospects of Ad vectors for use in gene therapy, the limitations of currently used Ad5-based vectors will have to be overcome. One way of potentially doing this may be to utilize group B Ads in the development of new vector systems. As B group Ad infections are relatively rare, the resulting low levels of pre-existing immunity in humans would enable B group vectors to be used more readily than Ad5-based vectors. This is relevant to both *ex vivo* and *in vivo* vector applications, as both humoral and cellular elements of pre-existing immunity can be detrimental to genetic therapies through inhibition of vector transduction and elimination of transgene expression. The CAR-independent tropism of B group Ads also would be beneficial for certain therapeutic applications. By using different receptors, including CD46, for cell entry, B group Ads can infect target cell populations that Ad5-based vectors cannot. Taken together, the lack of pre-existing immunity and enhanced tropism of group B Ads make them ideal candidate viruses for the development of improved gene transfer vectors.

CHIMERIC GROUP B VECTORS

As a means to improve gene transfer vectors, several groups have attempted to harness the unique properties of B group Ads in the context of Ad5-based vectors. The first example of this by Stevenson and co-workers demonstrated that fibre knob domains could be swapped between Ad serotypes (Stevenson *et al.*, 1995). An Ad5

vector possessing the fibre knob domain of Ad7 (Ad5/7) was shown to bind to a different receptor than an Ad5 vector, demonstrating the feasibility of developing fibre chimeric Ad vectors. Around the same time, Krasnykh and colleagues also developed a chimeric Ad5/3 virus possessing the fibre knob domain of Ad3 (Krasnykh *et al.*, 1996). Subsequently, chimeric Ad5 vectors possessing the fibre knob, or fibre knob and shaft domains of the other group B serotypes, including Ad11 (Goossens *et al.*, 2001; Stecher *et al.*, 2001), Ad14 (Havenga *et al.*, 2002), Ad16 (Havenga *et al.*, 2001), Ad21 (Havenga *et al.*, 2002), Ad34 (Havenga *et al.*, 2002), Ad35 (Shayakhmetov *et al.*, 2000), and Ad50 (Knaan-Shanzer *et al.*, 2001), were also developed, and data have been generated suggesting that these vectors are extremely efficient at infecting a variety of human target cell types. Human cells that can be readily transduced by group B fibre chimeric Ads include DCs (Rea *et al.*, 2001), CD34 positive HSCs (Shayakhmetov *et al.*, 2000; Knaan-Shanzer *et al.*, 2001), mesenchymal stem cells (Olmsted-Davis *et al.*, 2002), immortalized and primary tumour cells (Havenga *et al.*, 2002; Kanerva *et al.*, 2002b; Shayakhmetov *et al.*, 2002b), synoviocytes (Goossens *et al.*, 2001), retinal cells (Mallam *et al.*, 2004), endothelial cells (Havenga *et al.*, 2002), cardiovascular cells (Havenga *et al.*, 2001), fibroblasts (Havenga *et al.*, 2002), amniocytes (Havenga *et al.*, 2002), and chondrocytes (Havenga *et al.*, 2002). This is highly encouraging as it suggests B group Ad vectors may be useful for treating a variety of diseases.

Over the course of our experiments, we have recently noticed an unanticipated advantage to using group B fibre chimeric Ads. Upon systemic injection of chimeric Ad vectors possessing short-shafted B group Ad fibres into mice, a significant reduction in virus-induced toxicity was seen when compared to Ad5-based vectors. While this was not initially expected, this welcome observation is likely a reflection of the alternate tropism seen from B group receptor targeted Ads *in vivo*. Initially, we found that a chimeric Ad containing the short-shafted Ad35 fibre produced lower levels of liver serum glutamic pyruvic transaminase (SGPT) enzyme than an Ad5-based vector 4 days after systemic injection, indicating that less liver damage had been caused by vector administration (Bernt *et al.*, 2003). Subsequently, we were able to show that lower levels of pro-inflammatory cytokine and chemokine gene transcription, and plasma pro-inflammatory cytokines and chemokines, accompany this reduced liver damage within the first 24 hours of systemic vector delivery (Shayakhmetov *et al.*, 2004b). The short-shafted Ad5/35 vector was unable to efficiently interact with hepatocellular receptors and was not taken up by Kupffer cells after 30 minutes, consequently greatly reducing cytokine production and hepatocellular damage. This is important since Ad vector induced toxicity within the first 24 hours of administration was implicated in the death of a patient in a clinical trial utilizing an Ad5-based vector (Raper *et al.*, 2003). The development of Ad vectors with reduced toxicity profiles will be extremely useful for future therapeutic applications of Ad vectors.

In an additional approach, aimed at avoiding pre-existing anti-adenovirus immunity, two studies recently demonstrated that the hexon of Ad5 could be substituted with that of another serotype. In the first study, it was demonstrated that hexons from Ad3, Ad4, and Ad9 could be incorporated into infectious Ad5 virions (Ostapchuk and Hearing, 2001). Consequently, this observation led the way to development of a group B chimeric Ad5/H3 vector based on Ad5 but containing the

Ad3 hexon (Wu *et al.*, 2002). Sera from mice pre-immunized with Ad5 could not inhibit *in vitro* infection by Ad5/H3. Furthermore, Ad5/H3 infection was not inhibited *in vivo* by pre-immunization of animals with Ad5. Although it has been suggested that T cells specific to Ad5 may cross-react with B group Ads *in vitro* (Smith *et al.*, 1998; Heemskerk *et al.*, 2003), the data from Wu and colleagues support the theory that B group Ad vectors may avoid pre-existing anti-Ad5 immunity generated *in vivo* as hexon is the major determinant of Ad neutralizing immunity.

In a further approach to Ad vector modification, a recent paper demonstrated that new targeting ligands can be placed at the C-terminus of the Ad3 fibre knob (Uil *et al.*, 2003). The authors were able to insert a 6-his tag at the C-terminus of the Ad3 knob from a fibre chimeric Ad5/3 vector, and used the resultant vector to infect cells through an artificial receptor. This is an interesting observation as a ligand-targeted Ad vector with reduced toxicity would be useful for treatment of many diseases.

Since B group Ads use CAR-independent pathways for cell infection, several studies have analysed the impact of fibre/receptor interactions on intracellular trafficking of Ad vectors. One study investigated the trafficking of Ad5, Ad7, and a fibre chimeric Ad5/7 virus in A549 cells (Miyazawa *et al.*, 1999). Compared to Ad5, both Ad7 and Ad5/7 showed delayed nuclear localization of virions and genomes within the first hour of infection. Ad7 and Ad5/7 virions were distributed in both the nucleus and cytoplasm, unlike Ad5, which was found primarily at the nucleus after 1 hour. In a subsequent study of Ad5, Ad7, and Ad5/7, the same group showed that, while Ad5 was present in the compartments of the lysosomal pathway of neutral pH (pH 7) early after infection, Ad7 occupied acidic compartments (pH 5) over the first 2 hours, with a gradual shift toward neutrality by 8 hours (Miyazawa *et al.*, 2001). Unlike Ad5, Ad7 was seen to localize with late endosomal and lysosomal marker proteins. For membrane lysis, the optimal pH for Ad7 and Ad5/7 was pH 5.5, while for Ad5, it was pH 6.0. In another study, the intracellular trafficking of an Ad5 vector and a fibre knob chimeric Ad5/35 vector were investigated in HeLa cells (Shayakhmetov *et al.*, 2003). This study demonstrated that the selection of intracellular trafficking routes was determined by the fibre knob domain and did not depend on the length of the fibre shaft. Ad5 efficiently escaped from endosomes early after infection, while Ad5/35 remained longer in late endosomal/lysosomal compartments and used them to achieve localization to the nucleus. Recycling of Ad5/35 particles back to the cell surface was seen, and this resulted in significantly less efficient Ad5/35-mediated gene transfer compared to that of Ad5. In a following study, the same authors showed that vectors possessing an Ad35 fibre knob domain migrated to the nucleus through late endosomal and lysosomal cellular compartments in the immortalized human haematopoietic cell line Mo7e (Shayakhmetov *et al.*, 2004a). The authors showed that, unlike E1/E3 deleted and helper dependent (HD) Ad5/35 vectors, ΔAd5/35 vectors with a short 12.6 kb genome were unable to efficiently escape from endosomes and deliver their DNA to the nucleus. Although these studies demonstrate that Ad vectors with B group Ad fibre knobs utilize an alternate route of intracellular trafficking, efficient gene transfer is still achieved in many cell types.

Although no receptor for B group Ads has been found in mouse cells (Havenga *et al.*, 2002; Shayakhmetov *et al.*, 2002b), several studies have investigated the *in vivo* properties of B group chimeric Ads in non-permissive strains of mice. In one study,

Ad5 vectors, or fibre chimeric Ads with the Ad7 fibre or the Ad7 and Ad41(short) [non CAR interacting] fibres, were administered retro-orbitally to B129 mice (Schoggins *et al.*, 2003). When compared to Ad5, significantly lower levels of liver transduction (<1%) were seen 3 days post injection, with Ad7 fibre or Ad7 and Ad41(short) fibre containing vectors. In the spleen, significantly lower levels of transduction were seen, with Ad7 fibre or Ad7 and Ad41(short) fibre containing vectors, but the lowest transduction levels were seen with the vector containing only the Ad7 fibre. In another study, an Ad5 virus ablated for binding to CAR and integrins, and containing the short shaft of the Ad35 fibre (Ad5ΔPΔF-S35), was injected systemically into C57BL/6 mice (Koizumi *et al.*, 2003). When compared to an Ad5 vector, Ad5ΔPΔF-S35 showed lower levels of gene transfer 48 hours after infection in all organs tested. Analysis of viral genomes showed that Ad5ΔPΔF-S35 was cleared from blood more quickly than Ad5, and was completely cleared from the liver between 1 and 48 hours post injection. Further analysis showed that Ad5ΔPΔF-S35 localized with non-parenchymal liver cells, while Ad5 localized with parenchymal and non-parenchymal liver cells. In a more recent study, we analysed C57BL/6 mice after injection with Ad5/9L, Ad5/9S, Ad5/35L, and Ad5/35S vectors (Shayakhmetov *et al.*, 2004b). Ad5/9L and Ad5/9S possess the Ad9 fibre knob domain and the long Ad5 shaft (Ad5/9L), or the short Ad9 fibre shaft (Ad5/9S). Ad5/35L and Ad5/35S possess the Ad35 fibre knob domain and the long Ad5 fibre shaft (Ad5/35L), or the short Ad35 fibre shaft (Ad5/35S). Equal numbers of vector genomes were seen in the liver at 30 minutes post injection, but the number of vector genomes at 6 and 24 hours post injection was dramatically lower for short-shafted Ads. Analysis of hepatocytes isolated 15 and 30 minutes post injection showed that all vectors except Ad5/35S were able to confer gene transduction. Immunohistochemistry revealed that short-shafted Ads were unable to efficiently interact with hepatocellular receptors and were not taken up by Kupffer cells. Furthermore, the lower level of liver cell transduction by short-shafted Ads correlated with a significantly reduced inflammatory anti-Ad response, as well as liver damage induced by the systemic administration of these vectors. Together, these studies demonstrate that, unlike Ad5 vectors, Ad vectors with short fibre shafts, such as those from B group Ad fibres, are cleared from blood more quickly, and are not trapped in the liver upon systemic delivery.

GROUP B AD VECTORS

As a result of previous studies demonstrating that group B fibre/knob containing Ad vectors show improved infection of certain cell types, several groups have attempted to generate Ad vectors derived entirely from a group B Ad serotype. The first such group B Ad vector was based on serotype Ad7, was deleted in E1A, and contained a chloramphenicol acetyltransferase (CAT) expression cassette in the E1A region (Abrahamsen *et al.*, 1997). This vector was constructed with DNA fragments from Ad7a and an Ad7a recombination reporter plasmid in 293 cells. Efficient expression of CAT was seen after *in vitro* infection of A549 cells, while systemic delivery to BALB/C mice resulted in CAT expression in the liver, spleen, kidney, and lung at 3 days post injection. Subsequently, another method of generating E1/E3 deleted Ad7 vectors in cosmids was reported (Nan *et al.*, 2003). An Ad7 human immuno-

deficiency virus (HIV) env expressing vector was generated for vaccination studies and shown to infect cell lines and CD4(+) T lymphocytes.

The second serotype to be used in development of a B group Ad vector was Ad35, with 4 groups recently publishing methods for generating E1/E3 deleted Ad35 vectors. The first group at Crucell in the Netherlands has developed a system for generating Ad35 vectors by homologous recombination of two shuttle plasmids in PER.C6 cells expressing the Ad35 E1B-55K protein (Vogels *et al.*, 2003). They showed that Ad35 vectors are not hampered by pre-existing Ad5 immunity and can efficiently infect DCs, smooth muscle cells (SMCs), and synoviocytes. The second group at Genetic Therapy, Inc. were the first to publish a sequence for the Ad35 genome (Seshidhar Reddy *et al.*, 2003). In the same study, they also introduced a system of making E1A deleted Ad35 vectors in PER.C6 cells or E1A/E1B deleted Ad35 vectors in 293 cells expressing Ad5 E1, E2A, and E4 proteins. Ad35 vectors did not efficiently transduce mouse cells, and biodistribution studies in C57BL/6 mice revealed low levels of Ad35 in all organs evaluated, including liver, lung, spleen, and bone marrow, compared to Ad5. Minimal hepatotoxicity was seen with Ad35, and its half-life in mouse blood was found to be two to three times longer than that of Ad5. The third group developed E1A/E1B deleted Ad35 vectors and was able to grow them in 293 cells expressing Ad5 E1 and E4 (Sakurai *et al.*, 2003a). In experiments with human CD34(+) cells, Ad35, Ad5, and Ad5/35 infected 53%, 5%, and 52% of cells at MOI 300 PFU/ml. The mean of fluorescence intensity in the CD34(+) cells transduced with the Ad35 vectors was 12–76 and 1.4–3 times higher than that in the cells transduced with the Ad5 and Ad5/35 vectors, respectively. In another study, the same group showed that an Ad35 vector efficiently transduced CAR-positive and CAR-negative cells (Sakurai *et al.*, 2003b). Biodistribution studies in C57BL/6 mice showed that Ad5 and Ad35 vectors were rapidly cleared from the bloodstream, with a half-life of approximately 3 minutes. When compared to an Ad5 vector, Ad35 showed lower levels of gene transfer 48 hours after infection in all organs tested, and vector genomes were almost completely cleared from the liver between 1 and 48 hours post injection. PCR analysis showed that more Ad35 localized with non-parenchymal liver cells at 1 and 48 hours, while Ad5 localized with parenchymal and non-parenchymal liver cells at 1 and 48 hours. The fourth group reported both the sequence of Ad35 Holden strain and a plasmid-based system for making E1, E3, or E1/E3 deleted Ad35 vectors (Gao *et al.*, 2003). Production of Ad35 vectors was achieved in 293 cells transiently transfected with a plasmid encoding the Ad35 E1B gene. Testing showed that the Ad35-based vector efficiently infected both human and rhesus macaque DCs.

As an alternative to Ad7 and Ad35, we recently sequenced the group B Ad serotype Ad11p (Stone *et al.*, 2003) and developed an Ad11 vector system based on Ad11p (Stone *et al.*, 2005). With this system, E1-deleted Ad11 vectors can be generated by homologous recombination in *E. coli*, or homologous recombination in a 293-based complementing cell line that expresses Ad11-E1B55K. We found that, like fibre chimeric Ad5/11 vectors, Ad11 vectors infect cells in a CAR-independent/CD46-dependent manner. Also, Ad5/11 and Ad11 vectors can efficiently infect tumour cell lines, human dendritic cells, and human peripheral blood mononuclear cell- (PBMC) derived CD34(+) cells. Compared to Ad5 and Ad5/11 vectors, Ad11 vectors are cleared from blood plasma more rapidly 3 minutes

after systemic administration to CD46 transgenic mice, although clearance over time was comparable. Analysis of blood cell fractions for genomes showed that more Ad11 genomes are associated with blood cells than Ad5 or Ad5/11 genomes. Examination of tissues for Ad genomes revealed that Ad11 vector genomes are cleared from the liver between 30 minutes and 72 hours post administration more rapidly than Ad5 or Ad5/11 vector genomes. Another group has also proposed developing Ad11 vectors and recently sequenced the Ad11 genome to this purpose (Mei *et al.*, 2003). Overall, the development of Ad7-, Ad11-, and Ad35-based vector systems demonstrates that B group Ads are effective as gene transfer vectors.

Vector applications

ANIMAL MODELS

Before CD46 was identified as a receptor for B group Ads, an appropriate small animal model for testing B group fibre chimeric or B group Ad vectors was not available. Previously, it was known that B group Ads could infect human and primate cells, but not rodent cells, although the reason for this was unclear. While all human nucleated cells express CD46, the expression of the murine CD46 homologue is restricted to the testis, which would seem to explain the specificity of infection towards human and primate cells. The identification of CD46 as the B group Ad receptor has enabled the use of existing CD46 transgenic mouse strains with widespread CD46 expression for gene transfer studies. The most representative strain of CD46 transgenic mice was developed using a ~400 kb yeast artificial chromosome (YAC) clone carrying the complete human CD46 gene (Kemper *et al.*, 2001). The CD46 expression profile of these mice closely mimics that observed in humans, including the same pattern of isoform expression as the donor. Tissue-specific isoform expression in the kidney, salivary gland, and brain, parallel to that seen in humans, is also seen. We have utilized this strain for B group fibre chimeric vector transduction studies (*Figure 6.2*) and *in vivo* biodistribution studies of a fibre chimeric Ad5/35 vector (Gaggar *et al.*, 2003). After intravenous injection, viral genomes were found in the liver, spleen, bone marrow, and lung. However, compared to mice injected with Ad5, the levels of Ad5/35 genomes were more than 20-fold lower in the liver (Shayakhmetov *et al.*, 2002b). Ad5/35-mediated transgene expression in livers was seen only in sparse hepatocytes in the periportal region (in cells that are theoretically exposed to the highest dose of incoming virus). We have also found that Ad5/35 uptake into Kupffer cells of CD46 transgenic mice is inefficient, while likely dendritic progenitor cells (CD11c-positive) are transduced in the marginal zone of the red pulpa within the spleen (Gaggar *et al.*, unpublished observations).

As an alternative to CD46 transgenic mice, large animal models are potentially available for group B Ad vector studies. Non-human primates potentially could be used since, unlike mice, they require CD46 expression for complement regulation. In baboons, the CD46 expression profile is similar to that of humans (Hsu *et al.*, 1997), and this would enable their use in gene transfer studies. As macaques have previously been used as host pathogens in measles (which also uses CD46 as a receptor) and vaccine studies (Combredet *et al.*, 2003), they could also be used in gene transfer studies. Although no studies have been published about delivery of

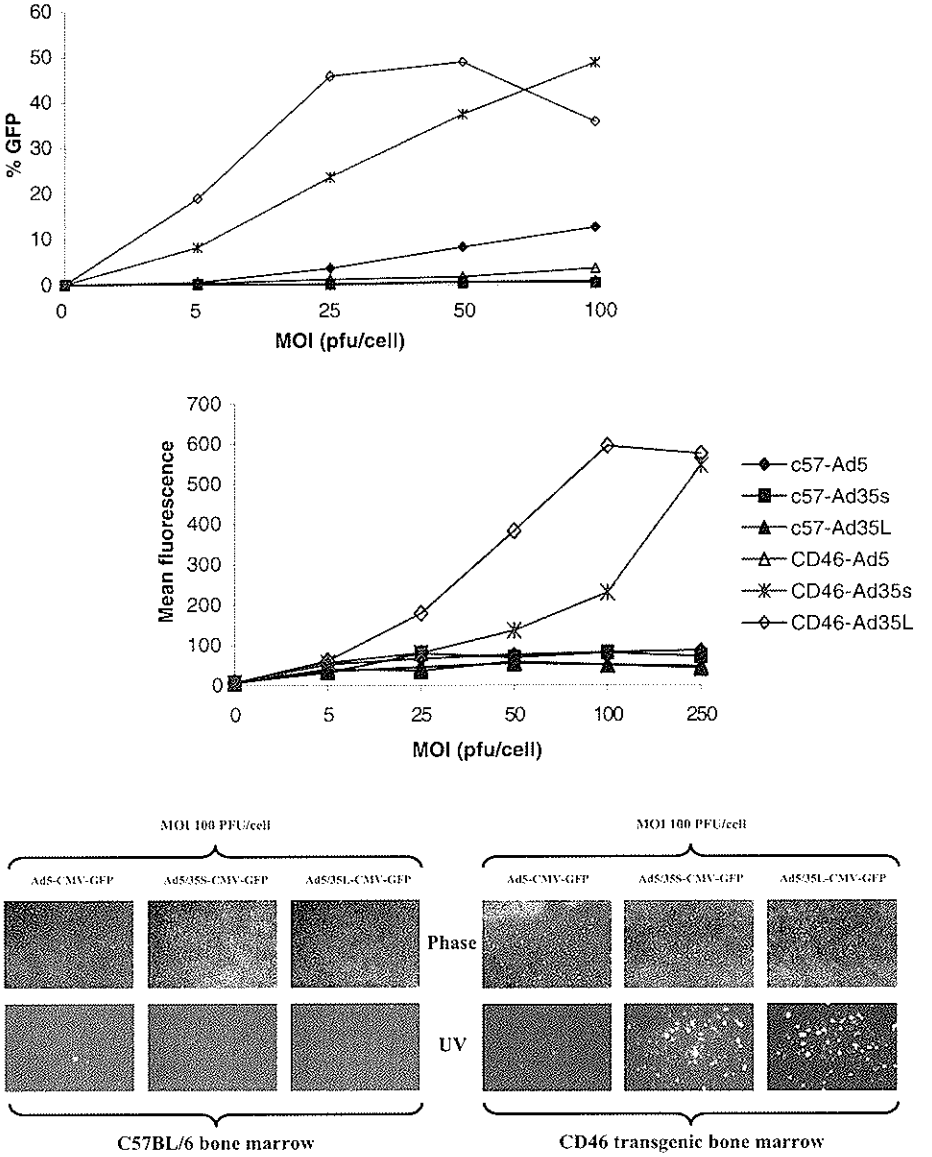

Figure 6.2. Transduction of CD46 transgenic mouse bone marrow. Bone marrow was harvested from normal (C57BL/6) or CD46 transgenic mice and infected with GFP expressing vectors Ad5 (an Ad5-based vector). Ad5/35s (an Ad5-based vector with the Ad35 fibre shaft and knob) and Ad5/35L (an Ad5-based vector with the Ad5 fibre shaft and Ad35 fibre knob). Upper panel shows percentage of cells transduced and mean fluorescence following flow cytometry of transduced cells. Lower panel shows GFP fluorescence of transduced cells.

chimeric B group or B group Ad vectors to non-human primates, we are currently investigating acute vector-mediated toxicity to Ad5, Ad5/11, and Ad5/35 vectors in baboons. Preliminary results indicate that Ad5/11 and Ad5/35 vectors are less toxic than Ad5 vectors following systemic delivery (Ni *et al.*, 2005).

With the identification of CD46 and other potential B group Ad receptors, new animal models for testing chimeric B group or B group Ad vectors will be obtained. Each new model will give a more appropriate representation of likely vector characteristics and host responses in humans. It is important that future experiments use these new models to thoroughly test the safety profiles of these vectors following *in vivo* delivery.

CANCER: TUMOUR TARGETING

One of the major targets for adenovirus-mediated gene therapy has been cancer since Ad5-based vectors are able to infect established tumour cell lines efficiently. One limitation to this is the low levels of CAR expressed or the down-regulation of CAR in primary tumour cells and tumours *in situ*. To circumvent this problem, group B fibre chimeric Ads have been utilized in order to infect cells in a CAR independent manner. As CD46 expression levels are high or up-regulated in many tumours, a higher level of infection in many cancer cell types has been seen from group B fibre chimeric Ads compared to traditional Ad5-based vectors. Studies from the Curiel group have shown that Ad5/3 chimeras demonstrate improved infection in renal cell carcinoma cells (Haviv *et al.*, 2002), ovarian cancer cell lines and purified primary ovarian cancer cells (Kanerva *et al.*, 2002a), and primary melanoma cells (Volk *et al.*, 2003) when compared with Ad5 vectors. Furthermore, in a mouse model of ovarian cancer, an Ad5/3 vector showed higher levels of gene transfer than an Ad5 vector (Kanerva *et al.*, 2002b). Other studies by us (*Figure 6.3*) or others have shown that Ad5/35 vectors demonstrate improved infection of cancer cells. Shayakhmetov and co-workers demonstrated targeting of human MDA-MB435 breast cancer cells *in vitro*. However, the capsid-modified Ad5/35 vector transduced only approximately 8% of metastases in an MDA-MB435 mouse tumour model following systemic Ad delivery, due to anatomical barriers around tumour nests (Shayakhmetov *et al.*, 2002b). A study by Bernt and co-workers using a conditionally replicating oncolytic Ad5/35 vector showed tumour-specific infection *in vitro*, but no enhanced tumour cell transduction, viral replication, or oncolysis was seen upon systemic delivery in a mouse tumour model (Bernt *et al.*, 2003). Factors including the stability of virus in the blood, trapping within the liver sinusoids, transendothelial transfer, and/or vector diffusion of viral particles to tumour cells limited tumour transduction. Studies using a conditionally replicating oncolytic Ad5/35 vector demonstrated efficient infection and killing of human LoVo colon cancer cells, primary human cervical cancer cells, and primary human ovarian cancer cells *in vitro* (Sova *et al.*, 2004). The same virus enabled elimination of pre-established LoVo cell-derived liver metastases in a human metastatic colon cancer model, while showing reduced hepatotoxicity. An Ad5/35 vector expressing GFP was able to transduce Ad5-refractory malignant myeloid and B lymphoid cell lines, as well as primary chronic myeloid leukaemic (CML) cells and chronic lymphocytic leukaemia (CLL) B cells (Nilsson *et al.*, 2004b). Also, a study in which a panel of cancer cell

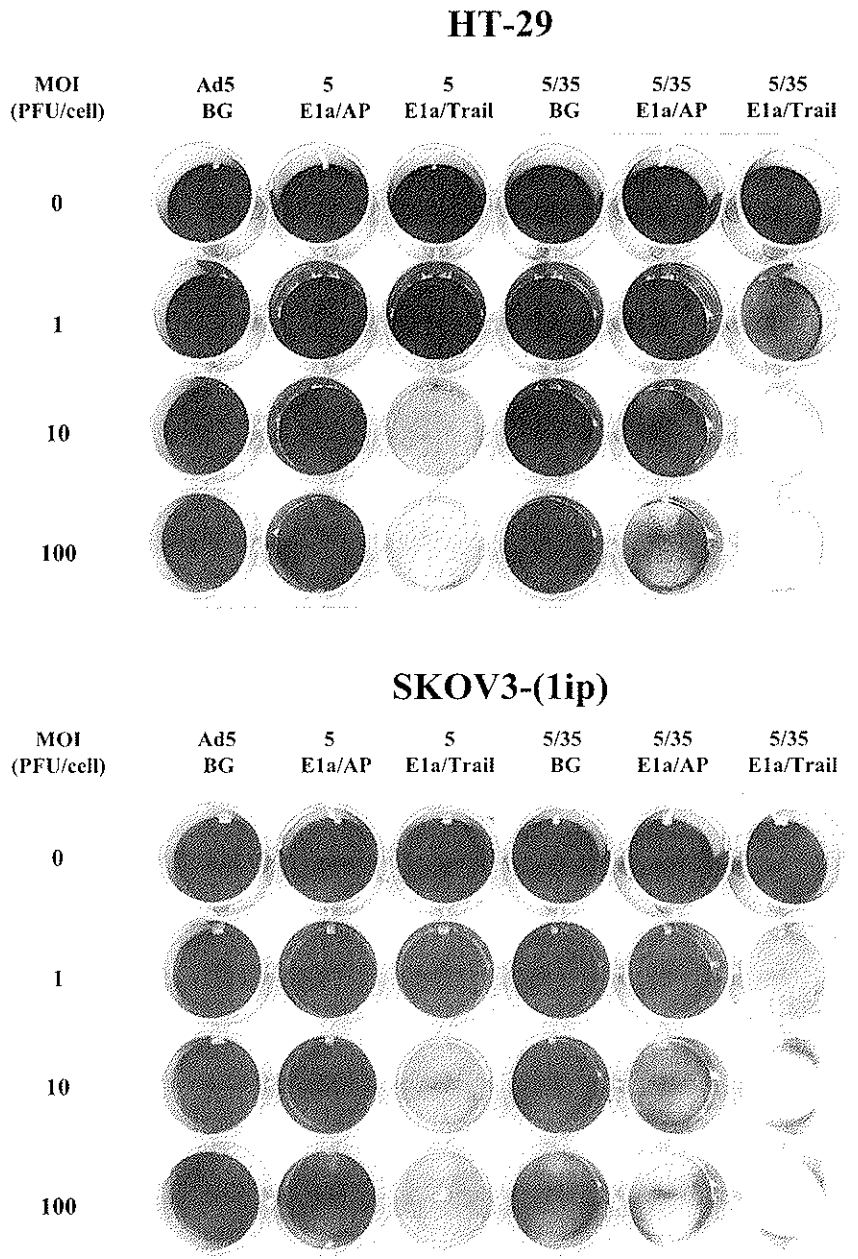

Figure 6.3. Cell killing of human tumour cell lines by tumour-specific Ad vectors. Colon (HT29) or ovarian (SKOV-3-lip) cell lines were infected at indicated MOIs and stained with crystal violet after 4 days. Ad5.BG and Ad5/35.BG are control viruses expressing beta-galactosidase. Ad5.E1A-Trail and Ad5/35.E1A-Trail are conditionally replicating tumour-specific Ads expressing Ad5. E1A and TNF-related, apoptosis-inducing ligand (TRAIL). Ad5.E1A-AP and Ad5/35.E1A-AP are control viruses where TRAIL has been replaced by the reporter gene alkaline phosphatase.

lines was screened for susceptibility to infection by a panel of chimeric Ads with different human Ad fibres demonstrated that many cell lines were most susceptible to infection by group B fibre chimeras Ad5/16, Ad5/35, and Ad5/50 (Havenga *et al.*, 2002). We have also found that Ad5/11 and Ad11 vectors are able to efficiently infect tumour cell lines from multiple organs (Stone *et al.*, 2005). Overall, these studies indicate that Ad vectors possessing a group B fibre will be useful in targeting many types of human cancer.

CANCER: IMMUNOTHERAPY I

In addition to targeting tumour cells directly, an alternative approach to adenovirus-mediated cancer therapy is through transduction of NK or T cells. Lymphocytes are poorly infectable by Ad5 vectors, but recent studies suggest that group B fibre chimeric Ads can infect them efficiently. In one report, an Ad5/35 vector showed enhanced infection of NK, CD3+, CD4+, and CD8+ T lymphocytes when compared to an Ad5 vector, while transduction of lymphocytes did not result in any impairment of proliferative functions (Schroers *et al.*, 2004). In another report, EBV-specific T cells were used as Ad vector producer cells for a cancer immunotherapy approach (Yotnda *et al.*, 2004). EBV cytotoxic T lymphocytes (CTLs) transgenic for Ad5 E1, under the control of the cell activation-dependent CD40L promoter, were transduced with E1 deficient adenoviral vectors. These CTLs produced high levels of infectious virus when exposed to HLA matched EBV-expressing targets, but not on exposure to MHC mismatched or otherwise irrelevant cells. This approach enabled localized delivery of an Ad vector while avoiding the risks associated with systemic administration of large doses of adenoviral vectors. The development of cell/gene therapy treatments for cancer provides a viable alternative to therapies utilizing direct vector-mediated cell tumour killing.

CANCER: IMMUNOTHERAPY II

To induce tumour-specific immune responses in patients, an efficient method for delivery of tumour associated antigens (TAAs) to DCs will be needed. Previously, approaches that deliver TAAs to DCs, either *ex vivo* or *in vivo*, have been utilized but, to date, no efficient TAA delivery system has been developed. Although Ad5-based vectors have been used as TAA delivery vehicles in cancer immunotherapy approaches, the efficiency of gene transfer in human DCs has been poor. Several studies utilizing group B fibre chimeric Ads suggest they may be better vectors for delivery of TAAs in cancer immunotherapy treatments. The first study to investigate this tested a panel of chimeric Ads with alternate serotype fibres for infectivity of human DCs (Rea *et al.*, 2001). Rea and co-workers found that the most efficient vector was the Ad5/35 fibre chimera, which was 100-fold more potent than Ad5 for gene transfer and expression, and that Ad5/35 showed enhanced synergistic effects with other activation signals to trigger DC maturation. They also showed that DCs infected with an Ad5/35 vector expressing the melanoma-associated TAA, gp100, were able to generate gp100-specific CTLs, and that *in vivo* delivery of the Ad5/35 vector enabled *in vivo* targeting of DCs. A subsequent study by the same group demonstrated that Ad5/35 was the best group B fibre chimeric Ad for infecting

human DCs (Havenga *et al.*, 2002). Further experiments from the same group then demonstrated that an Ad35 vector was extremely efficient at infecting DCs without being hampered by pre-existing Ad5 immunity (Vogels *et al.*, 2003). We have also found that human PBMC-derived immature DCs are efficiently infected by Ad5/11 and Ad11 vectors (Stone *et al.*, 2005). In an attempt to utilize Ad5/35 vectors for tumour immunotherapy, vectors expressing the TAAs latent membrane antigens (LMP) 1 and 2 and CAMEL/NY-ESO-ORF2 have also been generated. In one study using LMP-1 or 2 as TAAs, strong LMP-specific CTL responses were generated in a mouse tumour model and human cells with Ad5/35 vectors (Duraiswamy *et al.*, 2004). It is important to note, however, that the mouse studies were carried out in mice negative for human CD46.

In another study, the same group showed that, following infection of peripheral blood with an LMP2 Ad5/35 vector, LMP2-specific cytotoxic lymphocytes are produced that contain both CD4(+) and CD8(+) T cells, and recognize multiple LMP2 epitopes (Bollard *et al.*, 2004). In further studies, efficient generation of CAMEL/NY-ESO-ORF2-specific immune responses were seen following infection of human PBMCs or DCs with an Ad5/35-CAMEL/NY-ESO-ORF2 vector (Slager *et al.*, 2003, 2004).

The preliminary data suggest that group B fibre chimeric and group B Ad vectors will be useful vectors for tumour immunotherapy as TAA-specific immune responses can be generated *in vitro* upon infection of human DCs. Until recently, it was not possible to find out if anti-tumour immune responses *in vitro* translate to anti-tumour immune responses *in vivo* due to lack of a relevant mouse tumour model. Since CD46 transgenic mice can now be used for *in vivo* animal studies of group B fibre chimeric and group B Ad vectors, it should be possible to test the immunotherapy potential of these vectors in a relevant mouse tumour model.

VACCINATION

Ad5-based vectors have been extensively studied for use in vaccination against disease (for review, see Tatsis and Ertl, 2004). Although protective immunity can be conferred by Ad5-based vectors, the ability of group B fibre chimeric Ads to infect DCs more efficiently has led to investigations into their use in vaccine development. Since Ad5/35 vectors showed the most efficient DC transduction, studies have concentrated on their use as vaccines. In one report, a fibre chimeric Ad5/35 vector was shown to infect cultured human DCs and circulating myeloid-derived DCs with greater efficiency than Ad5, which resulted in increased T cell activation *ex vivo* (Ophorst *et al.*, 2004). *In vivo*, however, the authors found that Ad5/35 did not perform as well as Ad5 in mice or primate vaccination studies. In another study, the efficacy of Ad5 and Ad35 vaccines expressing simian immunodeficiency virus (SIV) gag was assessed in mice with pre-existing anti-Ad5 immunity (Barouch *et al.*, 2004). Levels of anti-Ad5 immunity similar to those found in humans inhibited the immunogenicity of an Ad5-gag vector, but not the cellular immune responses elicited by an Ad35-gag vector. Cross-reactive Ad5/Ad35-specific CD4(+) T lymphocytes were found, but they were unable to suppress vaccine immunogenicity. A further study investigated an Ad35 vector for use in HIV vaccination (Kostense *et al.*, 2004). Analysis of serum from normal or HIV positive European or African

patients revealed that seroprevalence to Ad35 was much lower than for Ad5, indicating Ad35 might be used as a vaccine vehicle.

ANTI-ADENOVIRUS THERAPY

Adenovirus infections have been implicated with complications following transplantation (Shields *et al.*, 1985). In an attempt to find a therapy against transplant-related adenovirus infections, Leen and co-workers have investigated adenovirus-specific memory T cells. In an analysis of epitopes from adenovirus-specific CTL responses, five novel CD8+ T cell epitopes were located in conserved regions of the hexon protein (Leen *et al.*, 2004b). These reactive T cells were all able to cross-react between Ad subgroups. In order to harness this observation, the same authors developed a protocol for activating specific CD4(+) and CD8(+) T cells capable of recognizing and lysing target cells infected with wild-type Ads from different Ad subgroups (Leen *et al.*, 2004a). The Ad-specific CD4(+) and CD8(+) T cells are activated from PBMCs infected with a fibre chimeric Ad5/35 vector. The majority of adenoviruses causing disease in immunocompromised patients should be identified by this T cell population. These experiments demonstrate that fibre chimeric Ad5/35 vectors are able to potentiate antigen-specific immune responses in PBMCs.

HAEMATOPOIETIC GENE TRANSFER

Haematopoietic progenitor cells are an important target for gene therapy of diseases, including sickle cell anaemia and thalassaemia. As efficient and stable transduction of HSCs is an important prerequisite for haematopoietic gene therapy, Ad5-based vectors are not considered good vectors for this purpose since they are unable to infect HSCs efficiently, due to low levels of CAR and integrin expression. In order to circumvent this problem, studies have investigated the potential of utilizing B group Ad fibres to target Ad infection of HSCs. Following initial studies showing wild-type B group, Ad11p and Ad35 are able to bind human haematopoietic cells with high efficiency (Segerman *et al.*, 2000; Shayakhmetov *et al.*, 2000; Stecher *et al.*, 2001), the utility of group B fibre chimeric Ads for haematopoietic gene transfer was subsequently tested. In a pilot study, Shayakhmetov and colleagues demonstrated that a fibre chimeric Ad5/35 vector was able to infect umbilical cord blood-(UCB) derived cells with potential stem cell capacity more efficiently than an Ad5 vector (Shayakhmetov *et al.*, 2000). Both CD34(+) and CD34(+) c-Kit(+) cells were infected more readily with the Ad5/35 vector. In a subsequent study, the same authors were able to demonstrate delivery of γ-globin to haematopoietic cells with an integrating Ad5/35 vector deleted of all viral genes (Shayakhmetov *et al.*, 2002a). In another study, the transduction of undifferentiated human haematopoietic cells by group B fibre chimeric Ads was analysed (Knaan-Shanzer *et al.*, 2001). Efficient transduction of UCB-derived monocytes, granulocytes, and undifferentiate CD34(+), CD33(-), CD38(-), CD71(-) cells was seen, with Ad5/35 and Ad5/50 chimeras showing the best levels of infection. In a study by Stecher and colleagues, efficient infection of PBMC-derived CD34(+) cells was seen with an Ad5/11 vector (Stecher *et al.*, 2001). A study by Yotnda and co-workers

demonstrated efficient infection of CD34(+) and CD34(-)lin(-) haematopoietic progenitor cells and Hoechst negative 'side population' (SP) cells of bone marrow with Ad5/35- but not Ad5-based vectors (Yotnda *et al.*, 2001). In an analysis of transplantation efficiency, one study demonstrated that CD34(+) cells infected with an Ad5/35 vector and sorted for GFP expression were able to consistently reconstitute bone marrow in the NOD/SCID mouse with multi-lineage differentiation (Nilsson *et al.*, 2004a). The same group was also able to demonstrate highly efficient infection of primary chronic myeloid leukaemia (CML) cells and chronic lymphocytic leukaemia (CLL) B cells with an Ad5/35 vector (Nilsson *et al.*, 2004b). More recently, a HD fibre chimeric Ad5/35 vector was developed that showed enhanced infection of erythroid K562 cells (Balamotis *et al.*, 2004). Another study utilizing an Ad35 vector was able to demonstrate that CD34(+) cells were as infectable with an Ad35 vector as with an Ad5/35 vector (Sakurai *et al.*, 2003a). We have also found that human PBMC-derived CD34(+) cells are efficiently infected by Ad5/11 and Ad11 vectors (Stone *et al.*, 2005).

OTHER THERAPIES

In addition to the studies mentioned above, group B fibre chimeric Ads have shown the ability to infect other cell types and organs implicated in disease. One such example is synoviocytes, which previously have been utilized for local delivery of anti-inflammatory proteins in therapies for arthritis, and are infected inefficiently by Ad5 vectors. In a study utilizing several fibre chimeric Ads, an Ad5/16 vector was able to transduce synoviocytes more efficiently than any other Ad vector (Goossens *et al.*, 2001). Another area of interest is cardiovascular disease, where two studies have investigated the ability of fibre chimeric Ads to infect cell types targeted in vascular gene therapy. In the first study, an Ad5/16 vector was found to infect primate cardiovascular cells and tissues more efficiently than other Ad vectors tested (Havenga *et al.*, 2001). Similarly, efficient infection was seen in the second study using an Ad5/3 vector to infect human smooth muscle cells from the aorta, coronary, renal, popliteal and pulmonary arteries (Su *et al.*, 2001). Ad5/3 was also able to transduce fresh human arterial tissues. Such a vector might be of use in treating patients with heart problems, such as coronary artery restenosis following angioplasty. A further area of interest is osteogenesis, where studies have investigated the use of B group fibre chimeric Ads to induce bone formation. In one study, an Ad5/35 vector was used to deliver the human bone morphogenetic protein 2 (BMP2) gene to human bone marrow-derived mesenchymal stem cells (hBM-MSCs) (Olmsted-Davis *et al.*, 2002). These transduced cells were then used in an *in vivo* heterotopic bone formation assay, and mineralized bone was only radiologically identified in muscle that received hBM-MSCs infected with the Ad5/35 vector. In a second study, the same group was able to demonstrate heterotopic bone formation in the same assay using hBM-MSCs, primary human skin fibroblasts (SFs), or a human diploid fetal lung cell line (MRC-5) infected with an Ad5/35-BMP2, but not Ad5-BMP2, vector (Gugala *et al.*, 2003). Considering a recent report that MSCs home to tumours upon re-transplantation (Studeny *et al.*, 2002), an application of B group fibre-containing vectors in a combined gene/cell tumour therapy approach could also be envisioned. An additional organ of interest has been the eye, where Ad5/3 and Ad5/35 vectors

have been used for transduction experiments. A study by von Seggern and co-workers showed that an Ad5/3 vector selectively transduced ciliary bodies after intraocular injection of mice, which is of interest for treating diseases like glaucoma (von Seggern *et al.*, 2003). A study by Mallam and co-workers showed that human retinoblastoma cells could be efficiently infected by an Ad5/35 vector *in vitro* (Mallam *et al.*, 2004). The same study showed that mice given a single subretinal injection of Ad5/35-GFP showed transduction of cells in all layers of the retina, especially photoreceptors and occasional neuronal cells and Muller cells, as well as retinal pigment epithelial cells. Mice given Ad5-GFP only showed transduction of retinal pigment epithelial cells and occasional photoreceptors and Muller cells. These studies demonstrate how group B fibre chimeric or group B Ad vectors will be useful tools in developing treatment of many diseases.

Conclusions and future directions

Recent data demonstrate that vectors containing fibres from B group Ads efficiently transduce human cell types that are relatively refractory to Ad5 infection, including malignant tumour cells, bone marrow-derived haematopoietic stem cells and mesenchymal cells, dendritic cells, and endothelial cells. Furthermore, we have recently found that intravenously-injected vectors containing Ad35 or Ad11 fibres (which have short fibre shafts) only inefficiently transduce Kupffer cells (whose transduction requires long-shafted fibres) and therefore elicit significantly reduced innate toxicity as compared to Ad5 vectors. Finally, since B group infections (with the exception of Ad3 and Ad7) are rare in humans, the percentage of humans with neutralizing antibodies is very low. These findings, together with the ability to modify the Ad genome to confer tumour-specific replication, to replace all viral genes with up to 30 kb long transgene cassettes, or to mediate transgene integration into the host cell genome, make B group Ad vectors promising tools for gene therapy. We believe that they will be applicable to *ex vivo* transduction of autologous or allogeneic cells (such as stem cells or dendritic cells) with subsequent retransplantation, or systemic *in vivo* vector administration (for example, for treatment of metastatic cancer or cardiovascular diseases). So far, only a small number of vectors containing B group Ad fibres have been constructed, and little is known about mechanisms and effects of B group Ad vector infection *in vitro* and *in vivo*.

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