CIRCUMVENTING ANTI-VECTOR IMMUNITY: POTENTIAL USE OF NON-HUMAN ADENOVIRAL VECTORS

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ABSTRACT

Adenoviruses are efficient gene delivery vectors based on their ability to transduce a wide variety of cell types and drive high-level transient transgene expression. Whilst there have been advances in modifying human adenoviral (HAdV) vectors to increase their safety profile, there are still pitfalls that need to be further addressed. Pre-existing humoral and cellular immunity against common HAdV serotypes limits the efficacy of gene transfer and duration of transgene expression. As an alternative, non-human adenoviral (NHAdV) vectors can circumvent neutralizing antibodies against HAdVs in immunised mice and monkeys and in human sera suggesting NHAdV vectors could circumvent pre-existing humoral immunity against HAdVs in a clinical setting. Consequently, there has been an increased interest in developing NHAdV vectors for gene delivery in humans. In this review, we outline the recent advances and limitations of HAdV vectors for gene therapy and describe examples of NHAdV vectors focusing on their immunogenicity, tropism, and potential as effective gene therapy vehicles.

KEYWORDS: Adenovirus; serotype; tropism; pre-existing immunity; toxicity; gene therapy.

1. INTRODUCTION

Adenoviruses (AdVs) are non-enveloped viruses with an icosahedral capsid containing a double stranded linear DNA genome of 26 to 46 kb (Davison *et al.*, 2003). Since their discovery in 1950s in human cell cultures (Rowe *et al.*, 1953), AdVs have been identified in many species, ranging from fish to humans. Today AdVs form five accepted genuses (*Mastadenovirus, Aviadenovirus, Siadenovirus, Atadenovirus, and Ichtadenovirus*) within the family *Adenoviridae.* Recently, one more genus was proposed, named *Testadenovirus* (Doszpoly *et al.*, 2013). So far 57 different AdV serotypes (Lukashev *et al.*, 2008; Walsh *et al.*, 2011) have been isolated from humans (HAdVs) and are classified within the genus *Mastadenovirus* as 7 species, HAdV A through HAdV G (Jones *et al.*, 2007). Whilst classification was previously based on biological characteristics (erythrocyte agglutination, oncogenicity, and by specific anti-sera neutralisation), phylogenetic relationships have been revised and clarified on the basis of genotype similarity (Bailey & Mautner, 1994; Davison *et al.*, 2003). Accordingly, individual AdV serotypes frequently share characteristics such as tropism and associated host pathologies with other serotypes within their species. The tropism is in large part determined by the capsid proteins which interact with the host cell surface receptors to mediate cell entry. The capsid consists primarily of hexon and penton proteins which form pentameric structures at each of the 12 vertices, securing a trimeric fibre that projects outwards. Other viral proteins include the cement proteins (IIIa, VI, VIII, and IX) and the core proteins (TP, V, VII, and mu) that associate with the AdV genome (Vellinga *et al.*, 2005). Whilst the basic structure of these proteins is maintained, variations in the length and composition of the amino acid sequences of these proteins across species and genera cause them to bind to a number of receptors and host factors with variable affinity. Thus, the coxsackie and adenovirus receptor (CAR), CD46, desmoglein-2 (DSG2), CD80, CD86, vascular cell adhesion molecule-1 (VCAM-1), heparan sulfate proteoglycans (HSPGs), major histocompatibility complex class I-α2 (MHC-I-α2), sialic acid (SA), dipalmitoylphosphatidylcholine (DPPC), lactoferrin and others can be utilised by one or more HAdV serotypes to transduce cells (Sharma *et al.*, 2009b; Arnberg, 2012).

Viral vectors remain to be the most promising gene transfer vehicles for gene therapy due to their higher gene-transfer efficiencies when compared with other approaches.

Currently AdV vectors have been used in 23.5% of all clinical trials (http://www.wiley.com//legacy/wileychi/genmed/clinical/) as they can efficiently transduce a wide variety of dividing and quiescent cells, they very rarely integrate into the host genome minimising the risk of insertional mutagenesis (Stephen *et al.*, 2010), they have a large packaging capacity up to 36 kb and they can routinely be produced at high titres. Of the 57 HAdV serotypes, HAdV-5 of species C has been the best characterised and the most widely used in clinical trials so far, mainly in the treatment of cancer (http://www.wiley.com//legacy/wileychi/genmed/clinical/) but also as a vaccine (Sullivan *et al.*, 2003; Shiver & Emini, 2004).

It has, however, been estimated that over 80% of the adult population have been naturally exposed to the common HAdV serotypes (Garnett *et al.*, 2002). As a result, pre-existing humoral and cellular immunity may preclude efficient gene transfer (Kuriyama *et al.*, 1998) and local systemic immune responses against the vector and transgene product may result in a number of clinically undesirable side-effects. Severe immune responses against AdV vectors can have serious consequences in humans, which have been exemplified by a fatal case of systemic inflammatory response syndrome that was directly attributed to the systemically administered AdV gene transfer vector (Raper *et al*., 2003). Moreover, upon systemic administration of recombinant HAdV-5, transduction of immune cells in the liver and spleen leads to immune responses against the AdV vector that can limit its therapeutic efficacy. Eliminating liver tropism and the epitopes involved in viral proteins recognition by neutralizing antibodies (NAbs) has been proposed to reduce these immune responses. Taking advantage of the diversity of AdVs capsid protein isoforms and their variety of ligand-receptor interactions, vectors based on low seroprevalent AdVs could potentially enable targeting to different cell types as well as overcoming pre-existing immunity. Naturally, non-human AdV (NHAdV) species have a low seroprevalence in all human populations and therefore represent a source of potential vectors that could fit this niche. Since the beginning of their development in 1990s (Mittal *et al.*, 1995), recombinant NHAdV vectors have been derived from many different AdV species: canine AdV-2 (CAdV-2), bovine AdV-3 (BAdV-3), porcine AdV-3 (PAdV-3), ovine AdV-7 (OAdV-7), murine AdV-1 (MAdV-1), several simian (SAdVs) and fowl (FAdVs) AdVs. Here we outline the current status of HAdV vectors, summarise the development of NHAdV vectors and discuss the advantages and shortfalls of each.

2. INNATE AND ADAPTIVE IMMUNE RESPONSES AGAINST ADENOVIRUSES

Innate and adaptive immune responses play a critical role in resolving AdV infections. The presence of specific anti-viral antibodies significantly increases antibody constant fraction receptors (FcR)-dependent viral internalization in macrophages, triggering an innate immune response to AdVs which leads to type I Interferon (IFN) production and caspase-dependent interleukin (IL)-1β maturation (Zaiss *et al.*, 2009). Internalized AdV DNA induces maturation of pro-IL-1β in macrophages dependent on NALP3 and ASC, components of the innate cytosolic molecular complex called the inflammasome, but independent of Toll-like receptors (TLRs) and IFN regulatory factors (Muruve *et al.*, 2008). Later studies reported that this response requires the binding of the arginine-glycine-aspartic acid (RGD) motif in the AdV penton base with macrophage β3 integrins (Di Paolo *et al.*, 2009). Barlan and colleagues also demonstrated that the specific AdV penetration mechanism of the endosomal membranes is required for IL-1β release (Barlan *et al.*, 2011a,b). Macrophages and conventional dendritic cells (cDCs) can detect cytosolic AdV DNA in a TLR9 independent manner (Basner-Tschakarjan *et al.*, 2006; Zhu *et al*., 2007) whilst plasmacytoid DCs (pDCs) detect non-methylated AdV CpG-containing DNA in the endosomes through TLR9. Also, internalised HAdV-5 and HAdV-6 immune complexes have been reported to induce DC maturation via TLR9 agonist motifs present in the AdV genome, whereas HAdV-35, -36 and -26 have a limited potency to induce DC maturation due to having a smaller number of motifs (Perreau *et al.*, 2012). TLR9 signalling pathway is dependent on myeloid differentiation primary response 88 (MyD88) (Yamaguchi *et al.*, 2007; Zhu *et al.*, 2007) and induces the secretion of IL-6 and IL-12 (Yamaguchi *et al.*, 2007). Heparin-sensitive receptors on DCs bind to the AdV shaft fiber region and trigger T cell immune responses (Cheng *et al.*, 2007). HAdV-5-induced Natural Killer (NK) cell activation relies on T cell contribution *in vitro* (Pahl *et al.,* 2012), and also appears to be related to presence of type I IFN *in vivo* (Zhu *et al.*, 2007, 2008). In contrast to HAdV-5, in response to HAdV-35 pDCs alone are sufficient for NK cell activation in a TLR9-dependent manner, after which NK cells enhance pDCs IFN-α secretion (Pahl *et al.*, 2012). This indicates that both pDCs and T cells may be necessary for NK cells activation. Therefore, it is clear that different AdV serotypes can trigger immune responses in

different ways, thus it is essential to study immune responses in detail to ensure that efficient strategies to circumvent immune responses are developed. The complement system also plays a critical role in AdV immune responses with effects mediated through the classical and alternative complement pathway. The complement system appears to be related to humoral immune responses to the vector capsid or the encoded transgene (Appledorn *et al.*, 2008). IgM isotype neutralization of virions activates the classical complement pathway hampering receptor-ligand interactions with the target cell (Xu *et al.*, 2013). On the other side, AdV-induced thrombocytopenia has been demonstrated to be dependent on factor B and C3 of the alternative complement pathway, and AdVs mediate induction of the nuclear factor-kappaB (NF-κB) upon binding to C3 (Appledorn *et al.*, 2008). In addition to macrophages, DCs and NK cells, binding of CAR protein and αv-integrins on non-immune cells also activates immune responses (Liu *et al.*, 2005; Schoggins *et al.*, 2005). In particular, CAR-mediated responses have been reported in epithelial cells (Tamanini *et al.*, 2006) and integrin-mediated responses in kidney epithelium-derived (REC) cells, inducing expression of CXCL10 (IP-10) through NF-κB in a RGD motif-dependent manner (Liu *et al.*, 2005).

These anti-AdV innate immune responses are linked to adaptive immune responses through direct interactions and production of cytokines (Ginsberg, 1996; Appledorn *et al.*, 2008). When a cell is infected by AdVs, it releases type I (α and β) IFN. Plasmacytoid DCs, cDCs, and macrophages produce IFN-α upon AdV recognition. The surrounding uninfected cells recognize IFN by IFN receptors and they induce, via the Jak-STAT signalling pathway, the expression of anti-viral enzymes such as MxA, 2',5'-oligoadenylate synthase and protein kinase R (PKR) (Katze *et al.*, 2002). Also, Type I IFN induces up-regulation of co-stimulatory molecules in DCs such as CD80, CD86 and CD40, leading to DCs maturation (Vujanovic *et al.*, 2009). Moreover, IFN takes part in B cells activation for antibody production. Type II IFN (IFN-γ) is an immunoregulatory cytokine secreted by Th1-type T CD4+ cells, T CD8+ cells and NK cells. IFN-γ induces the expression of major histocompatibility complex class I (MHC-I) on nearly all cells and major histocompatibility complex class II (MHC-II) on professional antigen presenting cells (APCs) promoting antigen presentation to helper T CD4+ cells, and it also activates macrophages (Gattoni *et al.*, 2006).

Dendritic cells have been shown to engulf infected cells and present antigens to

naive T CD8+ cells through MHC-I (Albert *et al.*, 1998). At the same time, APCs present antigens to Th1-type T CD4+ cells through MHC-II and upon activation they produce IL-2. If the T CD8+ cell receives both antigen presence and IL-2 signal it gets activated (Jankovic *et al.*, 2001). Once the T CD8+ cell interacts with an infected cell, mainly recognizing conserved epitopes in hexon protein (Olive *et al.*, 2002; Leen *et al.*, 2004; Tang *et al.*, 2006), it begins a cytotoxic response leading to apoptosis of the infected cell (Schumacher *et al.*, 2004). On the other hand, Th2-type T CD4+ cells induce B cell maturation and isotype switching (Jankovic *et al.*, 2001).

Neutralizing antibodies (NAbs), mostly IgG isotype, interact with AdV capsid proteins (Gall *et al.*, 1996; Sumida *et al.*, 2005). It was reported that NAbs recognize mainly the hypervariable regions (HVRs) of the hexon protein (Roberts *et al.*, 2006; Abe *et al.*, 2009; Shiratsuchi *et al.*, 2010; Bradley *et al.*, 2012b) and to a lesser extent the fiber knob domain (Myhre *et al.*, 2007; Bradley *et al.*, 2012a; Yu *et al.*, 2013) and penton base proteins (Yu *et al.*, 2013). AdV neutralization prevents cell attachment and facilitates aggregation and phagocytosis mediated by opsonisation (Wohlfart, 1988). FcRs in macrophages and NK cells recognize NAbs bound to AdV antigens, facilitating their phagocytic and cytolytic action, respectively (Gattoni *et al.*, 2006).

In summary, AdV gene transfer vectors are subjected to anti-viral immune responses (Figure 1) once administered to the patient. This process highly limits their efficiency for gene therapy applications. Therefore it is critical to understand the immune response to AdVs and have strategies to circumvent anti-viral immune responses. These will be discussed in the following sections, focusing in the main limiting factors: NAbs against AdV proteins and CD8+ cytotoxic T lymphocytes (CTLs) against infected cells.

3. IMPLICATIONS OF ANTI-VIRAL IMMUNE RESPONSES AGAINST HUMAN ADENOVIRAL VECTORS AND STRATEGIES TO CIRCUMVENT IT

Since the tragic death of a patient participating in a clinical gene therapy trial involving administration of a replication-incompetent HAdV-5 vector (Raper *et al.*, 2003), many efforts have been made to avoid anti-viral immune responses against AdV vectors to improve their safety and efficacy. Since the AdV vectors recognition by the immune system depends on the existence of antigenic epitopes in the AdV proteins (i.e. NAbs recognize AdV proteins, and CD8+ CTLs recognize infected

cells), most of the strategies to circumvent the immunogenicity of HAdV vectors are based on protein modifications.

Functionally significant HAdV-5-specific NAbs are directed primarily against the HAdV-5 hexon protein and to a lesser extent to fiber and penton base proteins (Gall *et al.*, 1996; Sumida *et al.*, 2005; Tian *et al*., 2011). Despite several groups successfully generated hexon-chimeric HAdV-5 vectors that partially evaded anti-HAdV-5 immune responses (Gall *et al.*, 1998; Roy *et al.*, 1998; Ostapchuk & Hearing, 2001; Wu *et al.*, 2002; Roberts *et al.*, 2006; Bruder *et al.*, 2012), hexon pseudotyping appears to be a limited strategy as it has been reported the formation of many non-viable virions (Youil *et al.*, 2002).

The hexon protein (Rux & Burnett, 2000) contains 7 HVRs (Crawford-Miksza & Schnurr, 1996). Many groups have exchanged HAdV-5 HVRs with those from low prevalent serotypes and demonstrated that the resultant AdV vector can evade HAdV-5 immunity (Roberts *et al.*, 2006; Abe *et al.*, 2009; Shiratsuchi *et al.*, 2010). Roberts *et al* showed that HAdV-5 with all seven HVRs replaced with the corresponding ones from the rare serotype HAdV-48 could circumvent HAdV-5 NAbs in both mice and rhesus monkeys (Roberts *et al.*, 2006). Further studies (Bradley *et al.*, 2012b) exchanged only some HVRs and demonstrated that HAdV-5-specific NAbs target multiple HVRs, suggesting that mutation or replacement of all 7 HVRs would probably be necessary to evade anti-HAdV-5 immunity. However, in 2012, Coughlan *et al* demonstrated that, despite HAdV-5 containing all seven HVRs from HAdV-48, it displayed a decreased hepatocyte transduction and accumulation in Kupffer cells, and it triggered a robust pro-inflammatory response not present with the wild-type HAdV-48 (Coughlan *et al.*, 2012). In order to generate novel viable HVR-chimeric vectors able to evade immune responses, it would be necessary to use HVRs from other serotypes, and for that it is essential to determine the viability of HVR replacements in AdV vectors based on the structural and biochemical constraints of the different HVRs that limit their manipulation.

Regarding the immunogenicity of the fiber protein, little is known about the epitopes involved in fiber recognition by NAbs, although it is thought that anti-knob NAbs represent the major population of anti-fiber NAbs (Myhre *et al.*, 2007; Bradley *et al.*, 2012a; Yu *et al.*, 2013). It has been reported that fiber pseudotyping of AdV vectors can lead to reduced AdV-associated innate (Schoggins *et al.*, 2005) and adaptive immune responses (Parker *et al*., 2009; Rogée *et al*., 2010). Despite the C-terminal

and shaft region heterogeneity, the N-terminal region (fiber tail) that is bound to penton base protein presents high similarity amongst diverse AdV species (Tarassishin *et al.*, 2000). Thus, designing chimeric AdV vectors containing all proteins from HAdV-5 but fibers from other serotypes (mainly species B and D) is a very interesting approach to retarget HAdV-5 to different tissues while circumventing anti-viral pre-existing immunity. Thus, several chimeric AdV vectors with tropism for different cell types such as HAdV-5/3 (Haviv *et al.*, 2002; Kanerva *et al.*, 2002; Volk *et al.*, 2003 ; Ulasov *et al.*, 2007), HAdV-5/11 (Havenga *et al.*, 2001*;* Stone *et al.*, 2005; Wang *et al.*, 2011b), HAdV-5/16 (Havenga *et al.*, 2001) and HAdV-5/35 (Havenga *et al.*, 2001) were generated. Interestingly, HAdV-5/35 and -5/11 were assessed *in vivo* in murine and non-human primate animal models*,* showing reduced toxicity and limited induction of inflammatory cytokines (Ni *et al.*, 2005). However, other studies showed that HAdV-5/35 presented tropism for CD34+ human hematopoietic stem cells *in vitro* (Shayakhmetov *et al.*, 2000), monocytes, granulocytes and blast cells of human bone marrow (Rogozhin *et al*., 2011), and CD4+ and CD8+ T lymphocytes *in vitro* (Zhang *et al*., 2013b). Since transduction of immune cells can lead to immune responses, extensive characterization of chimeric AdV vectors is essential.

Other retargeting strategies such as the truncation or removal of the fiber knob domain and its replacement with foreign trimerization motifs and heterologous peptides fused to the fiber shaft are also recently being assessed (fiber de-knobbing) (Coughlan *et al.*, 2010). Despite the removal of the fiber knob domain results in lower number of fiber copies per virion leading to a less efficient production of the AdV vector, this approach has been shown that may contribute to evade NAbs (Myhre *et al.*, 2007).

Regarding the adaptive immunity mediated by CD8+ CTLs, the response depends on viral antigenic epitopes loaded in the MHC of the infected cell. High-capacity, helper-dependent (HD) or also called gutless vectors bypass this problem (Kochanek *et al.*, 1996) as they are devoid of all viral genes and, therefore, they are less immunogenic and allow long-term transgene expression (Maione *et al.*, 2001; Ehrhardt & Kay, 2002; Dudley *et al.*, 2004). Furthermore, as in these vectors DNA is constituted of stuffer DNA as part of the vector backbone to maintain optimum vector size, non-methylated viral CpG-containing DNA (recognized by DCs through TLR9) can be eliminated. However, one study showed that HD AdVs also trigger an innate immune

response dependent on TLR9 (Cerullo *et al.*, 2007). Despite the advantages they offer, HD vectors are equally neutralized by pre-existing antibodies against capsid proteins and also may present transgene-encoded proteins immunogenicity (Tripathy *et al.*, 1996).

On the other hand, the activation of immune responses due to interactions between AdV capsid proteins and receptors in the target cell can limit AdV vector efficacy in gene therapy applications. HAdV-5 binds to the CAR protein on human erythrocytes in the bloodstream, limiting its delivery to the *in vivo* target tissue and contributing to toxicity (Seiradake *et al.*, 2009). To avoid this interaction, Nicol *et al.* described two CAR-binding mutations that abolished the previously described agglutination in human and rat erythrocytes (Nicol *et al.*, 2004). Moreover, HAdV-5 binding to CAR activates immune responses that can limit its potential as a gene transfer vector (Schoggins *et al.*, 2005; Tamanini *et al.*, 2006). Other interactions such as RGD with macrophage β3 integrins (Cheng *et al.*, 2007; Di Paolo *et al.*, 2009) or the one between the shaft fiber region and the heparin-sensitive receptor from DCs (Cheng *et al.*, 2007) have also been associated with immune responses and, in the case of RGD motif, through IL-1α production (Di Paolo *et al.*, 2009). Interestingly, the mutation of the RGD motif to arginine-glycine-glutamic acid (RGE) resulted in a significant 5-fold reduction in spleen uptake diminishing the anti-viral inflammatory response after intravascular administration (Bradshaw *et al.*, 2012). These studies indicate that de-targeting from native receptors could be a useful approach to avoid immune responses against the AdV vector. Nevertheless, further research needs to be done to describe the pathways through which the AdV vectors trigger immune responses. There are more factors involved in cellular recognition that could also be implicated in promotion of immune responses. These include alternative receptors and co-receptors such as FcR (Xu *et al.*, 2008), complement-3 receptor (CR-3) (Xu *et al.*, 2008), HSPGs and low-density lipoprotein receptor-related protein (LRP) (Shayakhmetov *et al.*, 2005), complement receptor-1 (CR1) (Carlisle *et al.*, 2009), scavenging receptor-A (SR-A) (Khare *et al*., 2012), bridging molecules such as the coagulation factor IX, X, VII and protein C (Parker *et al.*, 2006), complement component C4-binding protein (C4BP) (Shayakhmetov *et al.*, 2005) and IgM antibodies (Xu *et al.*, 2008). Further studies will have to be done in order to describe their influence in the use of HAdV vectors for gene therapy applications.

Apart from genetic engineering of HAdV vectors, other strategies such as chemical

304 modifications by addition of cationic polymers or lipid molecules to shield AdV vectors
305 and prevent them from binding undesired proteins have been widely studied. Several
306 variants of polyethylene glycol (PEG) have been used but better shielding was found
307 with multivalent copolymers of poly[N-(2-hydroxypropyl)methacrylamide] (pHPMA).
308 Shielding of HAdV vectors with pHPMA showed an increased biological stability of
309 the vector and a longer persistence in blood, decreased liver transduction and
310 therefore a lower immune response (Green *et al.*, 2004). Furthermore, coated HAdV
311 vectors have been shown to be less vulnerable to recognition by pre-existing NAbs *in*
312 *vitro* (Romanczuk *et al.*, 1999; Wang *et al.*, 2011a) and by Kupffer cells in the liver
313 (Mok *et al.*, 2005), likely due to the hampered interaction of PEGylated AdVs and
314 Kupffer cells SR-A (Haisma *et al.*, 2009). However, other factors could be affecting
315 liver transduction of the coated HAdV such as the limited size of the sinusoidal
316 endothelial cells fenestrae (Hofherr *et al.*, 2008). The use of PEGylated HAdV vectors
317 resulted in decreased levels of IL-6 production *in vitro* (Mok *et al.*, 2005) and of IL-6
318 and IL-12 *in vivo* (Croyle *et al.*, 2000). Moreover, coated HAdV vectors induce lower
319 IL-6 levels in serum due to decreased levels of spleen AdV uptake (De Geest *et al.*,
320 2005). Furthermore, several groups successfully retargeted coated AdV vectors
321 (Parker *et al.*, 2005; Stevenson *et al.*, 2007; Morrison *et al.*, 2008, 2009; Wang *et al.*,
322 2010). Although in some studies they presented similar levels of anti-tumoral efficacy
323 (Morrison *et al.*, 2009) or transgene expression (Parker *et al.*, 2005; Stevenson *et al.*,
324 2007; Morrison *et al.*, 2008; Wang *et al.*, 2010) to unmodified wild-type HAdV-5,
325 others achieved a greater therapeutic effect with negligible side effects (Eto *et al*.,
326 2010; Yao *et al*., 2010). Despite the limited retargeting efficiency in comparison with
327 unmodified HAdV-5, the use of PEGylated AdV vectors achieved lower hepatic
328 tropism and reduction in liver and spleen toxicity *in vivo*. Moreover, evidence
329 highlights the potential of AdV polymer coating strategies to circumvent pre-existing
330 immunity to all viral capsid proteins (Romanczuk *et al.*, 1999; Croyle *et al.*, 2000; De
331 Geest *et al.*, 2005; Mok *et al.*, 2005; Haisma *et al.*, 2009; Zeng *et al*., 2012).
332 Nevertheless, the reported PEG immunogenicity could hamper PEGylated AdVs
333 efficacy (Shimizu *et al.*, 2012).
334 Despite the advances made in HAdV vectors to evade anti-AdV immune responses,
335 the current strategies are limited by the formation of non-viable virions following
336 molecular engineering and by the limited knowledge and characterization of capsid
337 epitopes motifs responsible for cross-reactivity. Therefore, the use of low

seroprevalent HAdV serotypes has been proposed as an alternative to hexon or fiber-chimeric AdV vectors in order to circumvent pre-existing NAbs. More than 15 years ago, several studies showed that the alternate use of AdV vectors from different serotypes, even within the same AdV species, can circumvent anti-AdV humoral immunity (Kass-Eisler *et al.*, 1996; Mastrangeli *et al.*, 1996; Mack *et al.*, 1997; Parks *et al.*, 1999). A study on the presence of NAbs against different HAdV serotypes in the Belgium population reported high seroprevalence towards species A, C, and E, while low seroprevalence towards species B and D, among which, serotypes 11, 34, 35, 50 (species B), 43 and 48 (species D) showed the lowest values (Vogels *et al.*, 2003). This indicates that these serotypes could be potentially used to evade pre-existing immunity. Vectors have been developed from multiple HAdV serotypes with lower seroprevalence than HAdV-5: HAdV-11 (Holterman *et al.*, 2004; Stone *et al.*, 2005; Abbink *et al.*, 2007), HAdV-35 (Gao *et al.*, 2003; Sakurai *et al.*, 2003; Seshidhar Reddy *et al.*, 2003; Vogels *et al.*, 2003; Barouch *et al.*, 2004; Wu & Tikoo, 2004; Abbink *et al.*, 2007; Brouwer *et al.*, 2007; Sakurai, 2008; Sakurai *et al.*, 2009; McVey *et al.*, 2010; Geisbert *et al.*, 2011), HAdV-49 (Lemckert *et al.*, 2006; Abbink *et al.*, 2007), HAdV-26, -48 and -50 (Abbink *et al.*, 2007; Geisbert *et al.*, 2011), HAdV-7 (Nan *et al.*, 2003) and HAdV-6 (Capone *et al.*, 2006). However, since the presence of NAbs depends on AdV exposure which is closely related to geographical location, seroprevalence studies in different specific populations are very important. Moreover, better characterization of such serotypes in terms of tropism and specificity, biodistribution and toxicity is yet required to design safe HAdV vectors.

4. USE OF NON-HUMAN ADENOVIRAL VECTORS

The idea about NHAdVs as vectors appeared in 1990s, with the assumption they would be more useful for vaccines and gene therapeutic approaches in human medicine than HAdV-based vectors. Design and characterization of NHAdV vectors was reviewed earlier (Bangari & Mittal, 2006), with a focus on vaccine vectors. In this review we focus on the progress in development of NHAdV vectors (Table 1) for gene therapy.

4.1 CANINE AdV VECTORS

Canine AdV (genus *Mastadenovirus*, species CAdV A) derived vectors are currently the best described NHAdV vectors. Up to 90% of the residues involved in CAdV-2 fiber tail-penton base interactions are conserved compared to HAdV-5 (Schoehn *et al.*, 2008). CAdV-2 binds to CAR (Tomko *et al.*, 1997; Soudais *et al.*, 2000) but it does not contain an integrin-interacting RGD motif in the penton base (Chillon & Kremer, 2001) in contrast with HAdV-5 (Greber, 2002). CAdV-2 penton base appears to be ~20% shorter than that of HAdV-5 (Schoehn *et al.*, 2008). In addition, CAdV-2 has shorter protuberances on the top of the penton base, mainly where RGD motif is contained in HAdVs, and a shorter N-terminal region. The hypervariable loops in the hexon protein are shorter in CAdV-2 and the protruding structures containing the epitopes recognised by anti-hexon HAdV-5 antibodies are absent, whilst the fiber is more complex since the shaft region contains two bends (Schoehn *et al.*, 2008). Also, protein IX and IIIa are shorter in CAdV-2 (Schoehn *et al.*, 2008). Production of CAdV-2 vectors started more than 15 years ago (Klonjkowski *et al.*, 1997) in an attempt to avoid the inhibitory effect of the pre-existing humoral and cellular immunity against HAdVs when used in the clinics. Later, it was shown that CAdV-2 vectors rarely cause neither the activation and proliferation of T cells nor the maturation of DCs (Perreau & Kremer, 2006). The advantage of these vectors over HAdV vectors is that CAdV-2 mainly interacts with CAR (Soudais *et al.*, 2000) and they present specific tropism for neurons in the central nervous system (Soudais *et al.*, 2001) with great capacity for axonal transport *in vivo*. Taking into account that CAdV-2 vectors development was recently reviewed (Bru *et al.*, 2010), the following data will focus on the more recent trials performed with the mentioned vector. Until very recently, the production of CAdV-2 vectors was based on dog kidney (DK) cell lines (Kremer *et al.*, 2000), which is not accepted by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA). To circumvent this problem, CAdV-2 E1-transcomplementing cell lines, based on Madin-Darby canine kidney (MDCK) cells accepted by the FDA and EMA for the production of vaccines, have been developed (Fernandes *et al.*, 2013a). CAdV-2 vector DNA replication in the MDCK-E1 cell line was shown to correlate with the expression levels of the transcomplementing E1A and E1B. Whilst Cre recombinase expression in MDCK-E1-Cre cell lines (for HD CAdV-2 vectors production) can impair cell growth, controlled expression of Cre was determined not to result in negative effects on viral production (Fernandes *et al.*, 2013b). CAdV-2 based vectors are ideally suited for treating neurological disorders,

but due to vector titer limitations, large volumes are necessary for applicability in the clinics. Therefore, better purification and scalable production according to good manufacturing practice (GMP) are being currently developed (Segura *et al.*, 2012; Fernandes *et al.*, 2013a) and the improvement of producer cell lines have increased vector titers (Ibanes & Kremer, 2013). In a comparative study on HD HAdV, HD CAdV-2 and a VSV-G lentiviral vector, the transduction efficiency and transgene expression in human midbrain neuroprogenitor cells was assessed. HD CAdV-2 was determined to have the most promising vector profile: equal transduction efficacy, no negative effect to neuronal development and milder induced immune response (Piersanti *et al.*, 2013). HD CAdV-2 was also used as a vector in order to evaluate a gene therapy approach to correct the neurodegenerative lysosomal storage disorder mucopolysaccharidosis type IIIa (Lau *et al.*, 2012). It was shown to effectively transduce neurons and provide long term transgene expression, however its use *in vivo* was limited to discrete areas of the brain, indicating a need to find a way to increase transgene expression throughout the whole brain. Furthermore, in a clinically relevant stroke model in rats, cortical CAdV-2 vector injection transduced neurons at a greater level and covered a larger region than lentiviral vectors (Ord *et al.*, 2013), reducing infarct volume and improving neurologic recovery. For all the potential of CAdV-2 vectors, there ultimately remains the need for further understanding of the effects of CAdV-2 vectors on human cells before they are able to enter human clinical trials.

4.2 BOVINE AdV VECTORS

Bovine AdVs (genus *Mastadenovirus*, species BAdV A, B, C; genus *Atadenovirus*, species BAdV D) were the first NHAdVs used for vectoring purposes. Mittal and colleagues substituted the non-essential E3 region of BAdV-3 (BAdV B species) with the firefly luciferase reporter gene and luciferase expression lasted at least 6 days post-infection in human embryonic kidney (HEK)293 cells (Mittal *et al.*, 1995). BAdV-3 might be a better option to target a specific tissue or organ, since its cell entry is independent of CAR (Bangari *et al.*, 2005b) and other primary receptors of HAdV-5 (Bangari *et al.*, 2005a). BAdV-3 uses sialic acid molecules as a primary receptor to enter into host cells (Li *et al.*, 2009) and it can efficiently transduce different cell types of various species. However, it shows limitations in transducing human cell lines (Rasmussen *et al.*, 1999; Wu & Tikoo, 2004; Bangari *et al.*, 2005b). To circumvent

this problem, capsid proteins (fiber and pIX) were genetically modified to increase the ability of BAdV-3 to transduce non-bovine cells (Zakhartchouk *et al.*, 2007). Studies on BAdV-3 vectors *in vivo* biodistribution in a mouse model showed efficient transduction of the heart, kidney, and lung in addition to liver and spleen with a longer duration and higher levels of transgene expression than HAdV-5 vectors (Sharma *et al.*, 2009a). In a mouse model of breast cancer, the biodistribution of BAdV-3 vector was comparable to that of HAdV-5 vectors, the vector had the ability to efficiently transduce tumor and systemic tissues in the presence of HAdV-5 immunity, and it evaded sequestration by Kupffer cells (Tandon *et al.*, 2012). Also, it has been shown that there is minimal cross-neutralization by pre-existing anti-HAdV immunity in humans (Bangari *et al.*, 2005b). These results show the suitability of BAdV-3 vectors as a delivery system for cancer gene therapy while circumventing pre-existing anti-vector immunity. Moreover, BAdV-3 and HAdV-5 vectors have a comparable biological behaviour in human, bovine, porcine and mouse cell lines and both genomes persist at high levels in vector-permissive cell lines in the absence of immune pressure, proving BAdV-3 vectors safety for their use as gene delivery vehicles (Sharma *et al.*, 2011).

4.3 PORCINE AdV VECTORS

The idea of using PAdV (genus *Mastadenovirus*, species Porcine A, B, C) vectors was published over 20 years ago (Tuboly *et al.*, 1993). A few years later it was discovered that the virus can enter but is unable to replicate in dog, sheep, bovine and human cells (Reddy *et al.*, 1999a). Soon after, PAdV-3 (PAdV A species) vectors were constructed with the long-term objective to develop replication-competent PAdV-3 as a live gene transfer vector to induce a mucosal immune response in pigs (Reddy *et al.*, 1999b). Recombinant PAdV-3 is an effective delivery system in pigs (Hammond & Johnson, 2005) and it is often studied together with BAdV-3 vectors, showing similar properties: lack of cross-neutralization with pre-existing anti-HAdV immunity in humans (Bangari & Mittal, 2004; Bangari *et al.*, 2005b), CAR and integrin independent cell entry (Bangari & Mittal, 2005; Bangari *et al.*, 2005b), similar safety profile and biological characteristics (Sharma *et al.*, 2011), efficient transduction of several cell types of various species with PAdV-3 having an advantage in specific targeting of the breast tissue (Bangari *et al.*, 2005b), and the vector genomes remain as linear episomes (Sharma *et al.*, 2009a). In contrast with BAdV-3 vectors, the

genome levels of PAdV-3 vector were shown to be comparable or lower than those of HAdV-5 vectors *in vivo* in a mouse model, except in the case of the heart where the levels were comparable or higher (Sharma *et al.*, 2009a). The specificity of PAdV vectors for distinct tissues is very interesting for the design of vectors for targeted gene delivery.

4.4 SIMIAN AdV VECTORS

Simian AdVs are grouped into several human (certain HAdV species contain simian or ape AdVs, based on species-demarcation criteria like phylogenetic distance, nucleotide composition etc.) and simian AdV species within the genus *Mastadenovirus*. The first gene transfer vector derived from chimpanzee AdV-68 (ChAdV-68/Pan 9/SAdV-25; species HAdV E) was able to grow in HEK293 complementing cells without being neutralized by antibodies against HAdV serotypes (Farina *et al.*, 2001). Since neutralizing seroprevalence to ChAdV-68 in different regions of the world is significantly lower than that to HAdV-5 (Xiang *et al.*, 2006; Ersching *et al.*, 2010; Zhang *et al.*, 2013a), that makes it a good candidate for application in humans. Interestingly a single surface loop defines a major neutralization site for the ChAdV-68 hexon (Pichla-Gollon *et al.*, 2007), which when mutated, permitted the virus to escape from neutralization by polyclonal antisera obtained from animals *in vitro*. This was actually the first neutralizing site identified for any AdV, which was later very important for the successful application of the vectors in the clinics. Gene delivery by ChAdV-68 is CAR-dependent (Cohen *et al.*, 2002), and unlike the E1 of HAdV-5, the flanking sequences of ChAdV-68 are non-homologous with cell-derived E1, preventing the formation of replication-competent viruses (Xiang *et al.*, 2002). Development of vectors derived from chimpanzee AdVs Pan 5 (ChAdV-5/SAdV-22), Pan 6 (ChAdV-6/SAdV-23), and Pan 7 (ChAdV-7/SAdV-24), members of HAdV E species, started in 2004 (Roy *et al.*, 2004). The vectors transduced skeletal muscle with the same efficiency as HAdV-5 vectors, but without being neutralized by human sera, and the neutralization domains showed to be different in Pan 6 and Pan 7 both *in vitro* and *in vivo.* One study investigated the importance of neutralizing determinants on capsid proteins by using chimeric vectors with different combinations of Pan 7 and Pan 6 penton base, hexon and fiber proteins (Roy *et al.*, 2005), reporting that both hexon and fiber have neutralization epitopes and that *in vivo* transduction was more affected by anti-hexon antibodies, with no

effect of anti-penton base NAbs. Other studies on seroprevalence rates of various ChAdVs were carried out, with Pan 6 and Pan 7 seeming promising as gene therapy vectors although depending on the region where the potential patients live (Jian *et al.*, 2013). A tropism modified derivative of Pan 7 showed several advantages over HAdV-5 for the use in gene therapy: lower degree of inactivation, diminished liver transduction caused by poor stability of FX-Pan 7 complexes, greater gene delivery efficiency than the wild-type control Pan 7 vector *in vitro,* and higher transduction *in vivo* of the Her2 (human epidermal growth factor receptor type 2)-expressing human tumor cells injected intravenously in mice but not statistically significant compared to the control (Belousova *et al.*, 2010). The last finding indicates that there is a need to improve the vector to achieve the desired levels of target specific transduction. Pan 6 vector demonstrated a better capacity than HAdV-5 vector in transduction of brain tumor cells and glioma cells, indicating that it is a promising vector candidate for the brain tumor therapy (Skog *et al.*, 2007). Twenty E1 deleted vectors were generated from different AdVs isolated from chimpanzees, bonobos and gorillas, of which 12 could be propagated, rescued and expanded in HEK293 cells (Roy *et al.*, 2011b). SAdV-7 (HAdV G species) was proposed for vectoring a few years earlier (Purkayastha *et al.*, 2005b), and was subsequently constructed as an E1-deleted vector which could be complemented by HAdV-5 E1 genes in HEK293 cells (Roy *et al.*, 2011a). The latter observation was surprising since SAdVs from HAdV G species are phylogenetically quite distant from HAdV C members. Recently it was shown that humans have low seroreactivity against simian (SAdV-11, -16) or chimpanzee-derived (ChAdV-3, -63) AdV vectors compared with HAdV vectors (rHAdV-5, -28, -35) across multiple geographic regions (Quinn *et al.*, 2013). Chimpanzees and macaques elicited a systemic humoral but not systemic cellular immune response to endogenous AdVs. The structure of the E3 locus, involved in modulating the host's response to infection, is smaller in HAdVs than in ape AdVs, which may impact the ability of the ape AdVs to evade host immune detection and elimination (Calcedo *et al.*, 2009). ChAdV-68 showed the ability to transduce immature and mature human DCs, followed by secretion of IFN-α and IL-6 but not IL-12 or tumor necrosis factor (TNF)-α, and transduced immature DCs could stimulate proliferation of autologous T lymphocytes (Varnavski *et al.*, 2003). Therefore, ChAdV-68 could be used as a vector for transduction of human DCs.

4.5 FOWL AdV VECTORS

Construction of FAdV (genus *Aviadenovirus*, species FAdV A to FAdV E) vectors started with the demonstration that recombinant FAdV-10 (FAdV C species) could be used to express an antigen to induce a protective immune response in pathogen-free chickens (Sheppard *et al.*, 1998). After the identification of FAdV-9 (species FAdV D) regions that are non-essential for the virus (Ojkic & Nagy, 2001, 2003; Corredor & Nagy, 2010a), FAdV-9 recombinants demonstrated wild-type growth kinetics, virus titers, cytopathic effect and plaque morphology (Corredor & Nagy, 2010a), expression in avian and mammalian cells, and lack of replication in mammalian cells, indicating their applicability as gene delivery vehicles for mammalian systems (Corredor & Nagy, 2010b). FAdV-8 (species FAdV E) vectors were also constructed and showed efficacious *in vivo* delivery of antibody fragments against pathogenic infectious bursal disease virus (IBDV) (Greenall *et al.*, 2010). FAdV-1 (species FAdV A) or CELO (Chicken Embryo Lethal Orphan) vectors have been widely used. The genomic regions that could be deleted were evaluated more than 10 years ago and a cosmid-based strategy was developed for generating CELO vectors (Michou *et al.*, 1999; François *et al.*, 2001). FAdV-1 has several interesting properties: it interacts with CAR and its capsid architecture allows changes in its tropism (Tan *et al.*, 2001), it transduces mammalian cells as efficiently as HAdV-5 vector and has a potential for mammalian gene transfer applications, especially because it has larger DNA packaging capacity and greater physical stability (Michou *et al.*, 1999). It was shown that FAdV-1 is able to deliver p53 transgene into various tissues *in vivo* restoring p53 function in human tumor cells xenografts in mice and leading therefore to the inhibition of tumor growth (Logunov *et al.*, 2004). Moreover, FAdV-1 encoding human IL-2 gene can successfully produce biologically active recombinant IL-2 *in vitro*, *in ovo*, and *in vivo*, and it is capable of increasing the median survival time of mice carrying melanoma tumors (Shmarov *et al.*, 2002; Cherenova *et al.*, 2004). Also, recombinant FAdV-1 encoding the HSV-1 thymidine kinase showed cytotoxicity in carcinoma cell lines and suppressed tumor growth and increase median of survival of mice with melanoma tumors (Shashkova *et al.*, 2005), demonstrating its efficacy for the gene delivery to tumor cells *in vitro* and *in vivo*. Furthermore, one study showed that FAdV-1 successfully enhanced expression levels of secreted alkaline phosphatase reporter gene in transduced mammalian cells *in vitro* and *in vivo*

(Tutykhina *et al.*, 2008). Finally, PEGylated and retargeted with fibroblast growth factor FAdV-1 showed increased levels of binding and internalization in a variety of human cell lines (Stevenson *et al.*, 2006), with transgene expression being greater than the unmodified version in PC-3 human prostate cells, and being fully resistant to inhibition by human serum *in vitro* (Stevenson *et al.*, 2006). All together indicates that retargeting of CELO virus to human cells via disease-specific receptors is possible, while avoiding pre-existing humoral immunity.

4.6 OVINE AdV VECTORS

The production of OAdV (genus *Mastadenovirus*, species OAdV A, B; genus *Atadenovirus*, species OAdV D) vectors started more than 15 years ago (Xu *et al.*, 1997) after determining the non-essential regions within the genome (Vrati *et al.*, 1996). Surprisingly, much larger insertions than expected are tolerated, and portions of sequences can be deleted without affecting virus viability (Xu *et al.*, 1997). The primary receptor for OAdV-7 (OAdV D species) is not CAR, as it was shown that OAdV-7 does not compete with HAdV-5 for the entry into cells (Xu & Both, 1998; Voeks *et al.*, 2002). Recombinant OAdVs can infect a variety of non-ovine cells such as rabbit, human and murine cells but cannot replicate within them (Khatri *et al.*, 1997; Hofmann *et al.*, 1999; Löser *et al.*, 2000; Xu *et al.*, 2000; Kümin *et al.*, 2002; Lockett & Both, 2002). They are not neutralized by polyclonal serum against HAdV-5 (Xu & Both, 1998) and they can overcome pre-existing humoral immunity against HAdVs *in vivo* (Hofmann *et al.*, 1999). A cosmid-based system proved useful for efficient generation of recombinant OAdVs and vectors could be rescued in an ovine fetal skin fibroblastic producer cell line (Löser *et al.*, 2003). OAdV-7 carrying purine nucleoside phosphorylase (PNP), which converts the prodrug fludarabine into its activated form 2-fluoroadenine, was effective *in vivo* against prostate cancer progression upon prodrug addition (Voeks *et al.*, 2002; Martiniello-Wilks *et al.*, 2004). Moreover, recombinant OAdV-7 expressing ovalbumin showed efficacy in inducing antitumor response in a mouse model (Tang *et al.*, 2012). OAdVs biosafety profile and their application as a gene delivery vectors were reviewed in the last decade (Both, 2004).

4.7 MURINE AdV VECTORS

The production of MAdV (genus *Mastadenovirus*, species Murine A, B, C) mutants started with the MAdV-1 (MAdV A species) E3 deleted recombinant (Beard & Spindler, 1996; Cauthen *et al.*, 1999) and E1 deleted recombinant (Ying *et al.*, 1998). MAdV-1 presents mouse endothelial cells tropism (Charles *et al.*, 1998; Kajon *et al.*, 1998; Lenaerts *et al.*, 2005) but it can also infect human endothelial cells (Nguyen *et al.*, 1999) and it displays higher affinity for primary human smooth muscle cells than recombinant HAdV-5 (Lenaerts *et al.*, 2009). MAdV-1 lacks the RGD motif in the penton base but it contains it in the fiber knob domain (Raman *et al.*, 2009). The RGD motif was reported to play a role in MAdV-1 infection through α_v integrins, which act as a receptor for the virus, and it was shown that cell surface heparan sulfate glycosaminoglycans (HSGAGs) are involved in MAdV-1 infection (Raman *et al.*, 2009). Conversely, the primary attachment of MAdV-1 is independent of CAR (Lenaerts *et al.*, 2006). Distribution of MAdV-1 to the liver is markedly lower in intravenously injected immunodeficient mice than that observed with recombinant HAdV-5 (Lenaerts *et al*., 2009). Moreover, MAdV-1 has been used as an oncolytic vector proving that it is a suitable murine homolog model to test the mechanism of action of murine oncolytic AdV vectors in an immunocompetent, tumor-bearing host (Robinson *et al.*, 2009). Furthermore, MAdV-1 has preference for ovarian carcinoma cell lines, probably because of their increased expression of the enzyme involved in HSPGs biosynthesis, therefore having the potential to be used in the oncolytic treatment of ovarian cancer (Lenaerts *et al.*, 2012).

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The main limitation of clinically applied HAdV based vectors today is their failure in achieving high levels of efficient transduction in their target tissues. This is primarily due to the pre-existing NAbs leading to the rapid clearance of circulating vector and cellular immune responses resulting in the elimination of transduced cells, greatly limiting the expression of the transgene. These immunological responses most likely stem from the high frequency of HAdV exposure in multiple human populations across the globe, which results in immunological memory against HAdV serotypes that most of today's vectors are based on. In order to circumvent this, several strategies have been developed for HAdVs, including molecular engineering of chimeric vectors or the use of low seroprevalent HAdV vectors. As an alternative,

NHAdV vectors have been developed and are being tested in various non-human and human cell lines. Most of the NHAdV vectors have two important features which make them more appropriate than HAdV vectors: there is no or very low pathogenicity for their natural host, and there is no presence of pre-existing NAbs, CD4+ T cells and CD8+ CTLs against them. However, it should be noted that caution must be applied when using non-human primate AdVs, since their ability to infect human cells and their structural similarity could potentially result in anti-HAdV T-cell and/or NAb cross-reactivity with non-human primate AdV antigens. Different NHAdVs have specific tropism for different cell types, which makes them applicable in treating different diseases especially when the targeting of specific cells is required, and therefore they could extend the range of potential target tissues. Furthermore, the inability of NHAdVs to co-replicate with wt HAdVs, as well as the fact that they are replication defective in human cells, makes their vectors much safer than HAdV vectors. Many of the NHAdVs are able to tolerate longer sequences of exogenous DNA than HAdVs. For some of the NHAdVs there are low-cost propagation systems available, for example, chicken embryos for CELO virus (Laver *et al.*, 1971; Michou *et al.*, 1999). Despite these advantages, there are still many steps necessary before using the NHAdV vectors in the clinics. First challenge is to find the appropriate cell lines for efficient production. There are also safety issues compared to HAdVs such as the possible occurrance of interspecies adaptation. For example HAdV-4 is the only HAdV member of HAdV E species which is very similar to SAdVs from that group, indicating its zoonotic origin and molecular adaptation to its new host (Purkayastha *et al.*, 2005a; Dehghan *et al.*, 2013). As AdVs have co-evolved with their hosts (Benkö & Harrach, 2003; Davison *et al.*, 2003), there is a possibility that humans and monkeys could be infected with the same AdVs, a concern when considering chimpanzee or simian AdV vectors for use in humans. Nevertheless, NHAdV vectors present great potential as effective gene therapy vehicles and will hopefully be part of the successful AdV vectors used in the clinics in the coming years.

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