Ovine adenovirus vectors promote efficient gene delivery in vivo

Research Article

Peter Löser¹, Günter Cichon², Gary S. Jennings¹, Gerald W. Both³, and Christian Hofmann¹

¹HepaVec AG für Gentherapie, Berlin, Germany, ²Max Delbrück Center for Molecular Medicine, Berlin, Germany, ³Division of Molecular Science, CSIRO, North Ryde, New South Wales 2113, Australia

Correspondence: Dr. Christian Hofmann, Vice President R & D, HepaVec AG für Gentherapie, Robert-Rössle-Str. 10, 13125 Berlin-Buch, Germany, Tel: ++49-30-94892283, Fax: ++49-30-94892913, E-mail: chofmann@hepavec.com

Key words: ovine adenovirus vectors, pre-existing antibodies, human α₁-antitrypsin, viral tropism

Received: 8 October 1999; accepted 25 October 1999

Summary

The use of vectors derived from human adenoviruses in gene therapy is limited by pre-existing humoral immunity against these vectors in many individuals. We have recently reported the use of a vector derived from ovine adenovirus (OAV) isolate 287 that can transduce cells in vivo (Hofmann et al., 1999). In this report we present data regarding the physical stability of the OAV particles and demonstrate their ability to infect mice pre-immunized with a human adenoviral (hAd) vector. The tissue distribution of the vector delivered by several routes of administration is also examined and shown to be different from that observed for vectors derived from human adenoviruses. The construction and rescue of a recombinant virus that expresses the green fluorescent protein gene will further facilitate studies on the tropism of OAV in vivo.

I. Introduction

A. Rationale for using non-human adenovirus vectors in gene therapy

Vectors derived from human adenoviruses (hAd) have been widely used for transfer of potentially therapeutic genes into animals, but successful use of these vectors in humans has been limited by immunological barriers. Transgene expression is often transient due to both a CD8⁺-dependent T cell response to infected cells which leads to clearance of virus from target tissues (Yang et al., 1994 a, b and 1995), as well as by an immune response against the transgene product (Tripathy et al., 1996; Morrall et al., 1997). Moreover, formation of neutralizing antibodies to the vector prevents effective re-application of adenoviral vectors (Smith et al., 1993; Dai et al., 1995). This problem has been overcome in animals by strategies involving immunosuppression (Kay et al., 1995; Vilquin et al., 1995; Yang et al., 1996; Kolls et al., 1996; Kay et al., 1997), induction of immune tolerance (Kass-Eisler et al., 1996; Ilan et al., 1998) or co-expression of immuno-modulating proteins (Ilan et al., 1997). However, these strategies cannot be applied to humans due to the fact that most adenovirus vectors are derived from group C subtype 2 and 5 adenoviruses that have infected the vast majority of the population (Horwitz, 1996) and neutralizing antibodies would cause immediate vector inactivation making even a single unimpeded application impossible. For hAd vectors, serotype switching has been proposed as one possible solution to pre-existing immunity (Mastrangeli et al., 1996; Mack et al., 1997). This strategy might indeed allow the application of adenovirus vectors to humans if a rare serotype is used. However, safety concerns regarding trans-complementation of hAd vectors by wild-type hAd infection cannot be overcome with this approach.

The problem of pre-existing humoral immunity against the vector also affects other potential gene therapy vectors of human origin such as AAV, which is endemic in 85% of the population (Mayor et al., 1976), or herpes simplex virus, which is endemic in 50 to 95% of the population (Whitley et al., 1996). Therefore, to approach the problem of pre-existing humoral immunity to vectors derived from human viruses in a more general way, we and others have suggested the use of recombinant adenoviruses of non-human origin as...
gene therapy vectors. Adenoviruses from at least five non-human species, which might have some potential for gene therapy applications, have been used for generation of recombinant vectors (Mittal et al., 1995; Klonjkowski et al., 1997; Zakhartchouk et al., 1998; Reddy et al., 1999; Michou et al., 1999; see also Table 2). Among these, the ovine adenovirus (OAV) isolate 287 (Boyle et al., 1994) has been studied extensively (Vrati et al., 1995, 1996a,b; Khatri et al., 1998; Venktesh et al., 1998) and developed as a vector with the capability to infect mammalian cells in vitro and in vivo (Vrati et al., 1996c; Khatri et al., 1997; Xu et al., 1997; Xu & Both, 1998; Hofmann et al., 1999). In this report we describe recent progress with this novel vector system.

B. OAV287 derived vectors

On the basis of phylogenetic analyses of protease and hexon genes OAV287 has been grouped together with several BAV subtypes and Egg Drop Syndrome virus in the proposed genus Atadenovirus (Harrach et al., 1997; Harrach and Benkö, 1998) which is distinguished from the other adenovirus genera by base composition and genome arrangement. The organization of the OAV287 genome is shown in Figure 1. As in all adenoviruses the genome is organized into early and late transcription units and homologies to most structural and E2 genes of the genus Mastadenovirus are evident (Vrati et al., 1995; Vrati et al., 1996a). However, unlike the Mastadenoviruses there is no typical E1A/B region, no obvious E3 region and the genomic location of the putative E4 region also differs (Vrati et al., 1995 and 1996b). Homologous sequences to genes coding for structural protein IX and core protein V of human adenoviruses as well as a virus-associated RNA gene are absent in the OAV genome (Vrati et al., 1996b; Venktesh et al., 1998). In addition, OAV uses a primary receptor that is distinct from the Ad5 receptor (Xu and Both, 1998) and lacks an identifiable integrin-binding domain (Vrati et al., 1996a).

Recombinant OAVs were initially constructed by insertion of DNA cassettes in either orientation (Xu et al., 1997; Hofmann et al., 1999) into a region of the genome between the pVIII and fiber protein (designated as site I in Figure 1), or into a unique SalI site located near the right hand end (designated as site II in Figure 1). Recently, viruses carrying an expression cassette in site III (base 26575, plasmid OAV600, Xu et al., 1997), which comprises an ApaI/NotI polylinker inserted between the RHE and E4 transcription units (Khatri and Both, 1998), have also been rescued (G. W. Both, unpublished results). For sites I and II, up to 4.3 kb of foreign DNA could be inserted without a compensating deletion and foreign gene insertion did not reduce virus growth. Thus, DNA comprising at least 114% of the wild type viral genome can be packaged in the OAV capsid (Vrati et al., 1996c; Xu et al., 1997). Moreover, deletion of a 2kb sequence between sites II and III that contains apparently redundant ORFs did not effect virus growth, suggesting that OAV may package at least 6.3 kb foreign DNA without further deletions (Xu et al. 1997).

Figure 1. Organisation of the OAV287 genome. The complete sequence of OAV287 is available (Genbank Accession Number U40839). The locations of the early regions LHE, RHE, E2 and putative E4 are shown in bold type and arrows. Reading frames with homologues in other adenoviruses are named except for the p32 which is unique to Atadenoviruses. Promoter regions (Khatri and Both, 1998) are identified by filled circles. Non-essential sites I-III for insertion of gene cassettes are indicated.
Plasmids to generate OAV recombinants are produced either by direct cloning of the sequence of interest into the OAV genome (Vrati et al., 1996c; Xu et al., 1997 and 1998; Khatri et al., 1998) or by homologous recombination in E.coli BJ5183 (Hofmann et al., 1999). Recombinant viruses are generated by releasing the infectious, linear genome from such plasmids with KpnI digestion, followed by transfection of DNA into the ovine foetal lung cell line, CSL503, which is permissive for OAV replication (Pye, 1989). Lipofectamine was initially used for transfection but more recently cationic lipids related to the series described by Cameron et al., (1999) have produced increased transfection of CSL503 cells (G. W. Both, unpublished results), with a corresponding increase in the ability to rescue recombinant OAVs. OAV grows to useful titers in CSL503 cells although, in contrast to Ad5 growth in 293 cells, about 50% of OAV is released into the medium prior to harvest. Yields of >10,000 opu/cell can routinely be obtained from the cell pellet after CsCl-gradient purification.

II. Results

A. Heat stability of OAV vectors

Human adenoviruses become inactivated at temperatures higher than 48°C. In contrast, CELO and related viruses from the avian adenovirus group were shown to be stable at temperatures of up to 56°C (Michou et al., 1999). To check if OAV-derived vectors are more stable than hAd vectors OAVhaat and Ad5haat were incubated for 30 minutes at several temperatures and the remaining virus titer was determined on permissive CSL-503 or HEK293 cells, respectively. As shown in Figure 2, incubation of Ad5haat at 51°C diminished virus titer by more than one order of magnitude, and incubation at 54°C completely abolished its ability to produce CPE on 293 cells. In contrast, OAVhaat remained stable at up to 54°C and retained some ability to infect CSL503 cells even after incubation at 57°C. Complete inactivation was observed at 60°C. Thus, with regard to heat stability, OAV holds an intermediate position among adenoviruses in that it is more heat stable than hAd vectors but marginally less stable than CELO. This is interesting because protein IX, which is of major importance for Ad5 capsid stability (Ghosh-Choundhury et al., 1987), is absent from both OAV287 and CELO. Thus, other protein-protein interactions or new capsid protein(s) might substitute for pIX in the OAV capsid leading to a more pronounced heat stability. In addition, the heat stability of OAV could be of importance for vector preparation protocols since it may facilitate the purification of OAV-derived vectors from heat-sensitive contaminants.

B. Inactivation of hAd vector by human serum

Pre-existing humoral immunity against hAd vectors might be critical for the use of these vectors in humans. Figure 3 shows the results of an in vitro experiment to examine this. Ad5luc, a hAd5-derived vector expressing the luciferase reporter gene under control of the RSV 3’LTR, was incubated with a random human serum sample and subsequently used for infection of human liver-derived HuH7 cells at a moi of 100pfu/cell. Pretreatment of the hAd vector with heat-inactivated (30 min at 56°C) serum resulted in a dramatic decrease in luciferase activity (0.8% of control) in these cells. This decrease reflects the influence of pre-existing neutralizing antibodies against hAd5 in the
serum, the titer of which (1:320) was determined to be the mean of that within the human population (Hofmann et al., 1999). In addition to neutralizing antibodies, the immune system produces an excess of antibodies after a natural infection that bind to the virus, but do not neutralize it directly. However, these antibodies consequently activate the complement system. Several antibody- and complement-dependent mechanisms generally mediating virus inactivation have been described (Cooper, 1998): i) opsonization and rapid clearance by the reticuloendothelial system, ii) envelopment with antibody and/or complement, thereby masking (hAd)-receptor ligands on the viral surface, iii) antibody and/or complement-dependent viral aggregation. To investigate these possibilities in vitro, we explored the fate of hAd5luc vector in the presence of either untreated serum (measuring the effect of neutralising antibodies + intact complement), or sera, depleted in distinct activation routes (alternative and classical pathway of the complement system). The classical complement pathway can be blocked by EGTA/Mg²⁺-treatment. Proteins of the alternative pathway can selectively be blocked by treatment of sera at 48°C. We observed that blocking of the classical complement pathway by EGTA-Mg²⁺-treatment did not significantly increase the inhibitory effect of heat-inactivated serum at 56°C (due only to neutralising antibodies), whereas blocking of the alternative pathway by heating at 48°C or untreated serum (intact complement system) caused a nearly complete inactivation of the hAd vector (Figure 3).

Thus, inactivation of the hAd vector in vitro is predominantly caused by neutralizing antibodies, but the data imply that the complement system increases the inactivation rate of human adenovirus vectors through deposition of C-proteins of the classical pathway on the viral surface. Moreover, in vivo (clinical situation), a rapid clearance of opsonized hAd vectors via the reticuloendothelial system and viral aggregation might be expected in patients with pre-existing anti-hAd antibodies.

C. OAV overcomes pre-existing immunity to hAd vectors

To mimic the situation of pre-existing immunity in humans, Balb/C mice were immunized with 5x10⁹ pfu of Ad5lacZ or Ad5haat, respectively. At day 31 p.i., a second infection at the same dose was performed: one group of mice received Ad5haat while the second group were given OAVhaat. When immunization at day 0 was performed with Ad5lacZ, injection of OAVhaat, but not Ad5haat, resulted in strong expression of the reporter gene at day 3 after the second injection (Table 1). This confirms, that preexisting immunity to the hAd vector prevented in vivo transduction with the same hAd vector but not with the OAV vector. However, when the vector first injected was Ad5haat, no haat protein was detectable by ELISA after the second injection of either vector. This phenomenon was clearly due to antibodies against haat in Balb/C mice at the time of the second injection (1:10.000 to 1:100.000 as determined by anti-haat ELISA).

**Figure 3:** Ad5 vector is inactivated by human serum. Ad5luc (5x10⁶ pfu) was incubated at 37°C for 30 min with 50µl of various human sera pre-treated for 30 minutes as indicated and then used to infect of 5x10⁴ HuH7 cells in 24 well plates. At 48 hours post-infection, cells were lysed and luciferase activity was determined. The graph represents the average of three independent experiments.

36
Analysis of RNA in liver at day 4 after second vector application showed clear expression of haat specific transcripts in liver and heart when the second injection was performed with OAVhaat (Figure 4), but not when the second vector was Ad5haat (not shown). Thus, in addition to the pre-existing immunity to the vector, humoral immunity against the transgene product might prevent its function in the organism and must be taken into consideration.

D. Infection and tissue distribution of OAV in vivo

To test the ability of OAVhaat to infect cells in vivo, mice were injected by several routes, and haat expression was analysed. As shown in Figure 5, intravenous as well as intraperitoneal application of the vector to Balb/C mice resulted in high levels of reporter gene expression at day 3 post-infection, although individual differences in haat expression were observed after intraperitoneal injection. In addition, intraportal application of 1x10⁹ infectious particles of OAVhaat to C57bl-6 mice produced hAAT values ranging from 10.3 to 16.1 µg/ml in serum at day 3 post-infection. However, as observed previously (Hofmann et al., 1999), expression was transient, peaking at day 3 to 4 post-infection.

To analyse the tissue distribution of the vector, Southern blot analysis was performed with DNA harvested at day 3 from several organs of infected mice. Intravenous injection (Hofmann et al., 1999) resulted in comparable infection of the liver, spleen, heart and kidney and, to a lesser extent, lung tissue. Similarly, intraperitoneal application of OAVhaat resulted in the infection of all tissues investigated (Figure 6a) with no particular preference for any organ. After intraportal application of the vector, the level of OAV DNA in liver was significantly elevated relative to other organs (Figure 6b) but the liver was still not the overwhelming site of accumulation as seen for hAd5 in rodents (Smith et al., 1993; Fang et al., 1994; Kay et al., 1994; Huard et al., 1995).

Table 1: OAV overcomes pre-existing immunity to a hAd vector. Balb/C mice were infected with 5x10⁹ pfu of Ad5lacZ at day 0 and reinjected 31 days later with 1x10⁹ infectious particles of OAVhaat or 5x10⁹ pfu of Ad5haat, respectively. haat expression was determined in mouse sera 3 days after the second injection. bg, background (<20 ng/ml).

<table>
<thead>
<tr>
<th>1. Ad5lacZ (day 0)</th>
<th>1. Ad5lacZ (day 0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 µg/ml</td>
<td>&lt;bg</td>
</tr>
<tr>
<td>2.8 µg/ml</td>
<td>&lt;bg</td>
</tr>
<tr>
<td>1.9 µg/ml</td>
<td>&lt;bg</td>
</tr>
<tr>
<td>2.8 µg/ml</td>
<td>&lt;bg</td>
</tr>
<tr>
<td>2. Ad5haat (day 31)</td>
<td>2. Ad5haat (day 31)</td>
</tr>
</tbody>
</table>

E. Construction and rescue of an OAV/GFP recombinant

The problem with a reporter such as haat is that it does not identify the types of cells infected in vivo. Therefore to extend these studies on the cell tropism of OAV we have constructed a virus (OAV217A) in which the human CMV immediate early promotor/enhancer was used to drive expression of the green fluorescent protein (GFP) gene. The cassette was inserted in the left-to-right orientation in site I (Figure 1). Attempts were made to rescue viruses carrying one of two expression cassettes, one that had an intron and one that did not. Only the virus that lacked the intron was rescued, albeit after several attempts. Using this virus it was confirmed that OAV infects a range of cell types (Khatri et al., 1997), including human prostate (PC3) and cervical carcinoma (HeLa) as well as monkey kidney (COS-7) and mouse prostate (RM-1, Hall et al., 1997) cells (Figure 7). However, subsequent passage of this virus in CSL503 cells and BamHI analysis of its DNA showed that, unlike many other viruses that we have rescued, the foreign gene cassette was unstable. This may explain why the virus was difficult to rescue initially. Nevertheless, useful stocks of passage 3 virus have been purified and will facilitate in vivo studies to determine precisely which cells are infected in particular mouse organs. The HCMV/GFP cassette is being reconstructed in site III such that it will be located between transcription units (Khatri and Both, 1998) rather than interrupting the transcript for the fiber protein.
Figure 5: In vivo expression of haat gene after transduction of mice with OAVhaat. Balb/C or C57/bl-6 mice were infected with 1x10^9 infectious particles of OAVhaat via the route indicated and haat levels in serum samples were determined three days after infection. Each bar represents an individual animal.

Figure 6: Tissue distribution of OAVhaat in mice. Animals were injected with 1x10^9 infectious particles of OAVhaat or PBS (n.i.) via the (A) intraperitoneal or (B) intraportal routes. DNA was harvested at day 3 post-infection from the tissues indicated and analyzed by Southern blotting using an OAV-specific radiolabelled probe. DNA equivalent to 1 or 5 copies of the virus genome per cell (2.5 or 12.5 pg) was used as a standard. The position of the OAV-specific 2399 bp fragment is shown. Numbers refer to tissues from the same animals.
Figure 7: Infection of murine RM-1 cells by OAV as monitored by the expression of green fluorescent protein gene. Cells were infected at the multiplicity of infection indicated and examined by fluorescence (left and middle panels) or light microscopy (right panel) at 48hr post-infection.

III. Discussion

Pre-existing neutralizing antibodies against adenoviruses in the vast majority of the human population represent a major hurdle to the use of hAd derived vectors for gene delivery. We have shown here and elsewhere (Hofmann et al., 1999) that pre-incubation of hAd vectors with human serum results in complete inactivation of the vector. In addition, the complement system accelerates hAd vector inactivation, most likely by masking the viral surface with C-proteins. Thus, in vivo, it can be expected that the clearance of opsonized vectors by the reticuloendothelial system will dramatically reduce the efficacy of hAd-vectors in patients with pre-existing immunity. The antibody titer of the serum used was similar to that induced in mice after infection with hAd vectors. The unsuccessful readministration of hAd vectors to mice immunised with a first injection of the same hAd vector very likely predicts the outcome in humans.

Since other gene therapy vectors such as AAV and HSV-1 are also derived from viruses which commonly infect humans, we and others favour the use of non-mammalian (Hofmann et al., 1995) or non-human viral vectors for use in human gene therapy approaches (Mittal et al., 1995; Klonjkowski et al., 1997; Zakhartchouk et al., 1998; Reddy et al., 1999; Hofmann et al., 1999; Michou et al., 1999). Adenoviral vectors of non-human origin developed so far are summarised in Table 2. Although most of these vectors were created for vaccination purposes they might also be useful as gene therapy vectors. However, these vectors are all derived from viruses belonging to the Mastadenoviruses and some cross-reactivity of antibodies in human sera was observed with canine adenovirus-derived vectors (Klonjkowski et al., 1997). No such cross-reactivity was observed with OAV287 (Hofmann et al., 1999) which is phylogenetically distant from human adenoviruses (Harrach et al., 1997).

In this paper we have shown that OAV vectors are valuable tools for achieving high-level transgene expression in vivo under conditions that are unfavourable for hAd vectors. We observed that both the i.p. and intraportal routes of vector administration led to infection of several organs in mice and to secretion of significant amounts of the transgene product, human α1-antitrypsin, into the serum.

The problem of transience of gene expression after OAV mediated gene transfer needs further investigation. We were unable to detect residual expression of OAV early and late genes in mouse tissues after local injection of the vector by RT-PCR (P. Löser, unpublished results), and the major late promoter of OAV was shown to be only weakly active in semi-permissive BNT cells and silent in non-permissive cells after infection with OAV (Khatri et al., 1997). However, de-novo synthesis of viral gene products was shown to be dispensable for an immune response to hAd vectors (Kafri et al., 1998). Thus, closer inspection of immune infiltrates present in tissues infected by OAV will help to reveal the reason for vector clearance in vivo. On the other hand, gutless adenoviral vectors were reported to remain stable in mouse liver allowing for long-term transgene expression (Morsy et al., 1998). Therefore, construction of analogous OAV vectors similar to those developed for hAd (Kochanek et al, 1996) might help to overcome the problem of short-term expression after OAV mediated gene transfer. Alternatively, OAV vectors may be better suited to problems where short-term gene delivery is sufficient.
### Table 2: Data on non-human adenovirus recombinants published to date.

<table>
<thead>
<tr>
<th>Adenovirus</th>
<th>bovine adenovirus type 3</th>
<th>ovine adenovirus 287</th>
<th>canine adenovirus type 2</th>
<th>porcine adenovirus type 3</th>
<th>chicken embryo lethal orphan adenovirus type 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>abbreviation</td>
<td>BAV-3</td>
<td>OAV-287</td>
<td>CAV-2</td>
<td>PAV-3</td>
<td>CELO</td>
</tr>
<tr>
<td>genus</td>
<td>mastadenovirus</td>
<td>atadenovirus</td>
<td>mastadenovirus</td>
<td>mastadenovirus</td>
<td>aviaadenovirus</td>
</tr>
<tr>
<td>number of publications</td>
<td>4</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>phylogenetic relationship to Ad5</td>
<td>close</td>
<td>distant</td>
<td>close</td>
<td>close</td>
<td>distant</td>
</tr>
<tr>
<td>insertion of transgene in:</td>
<td>E3</td>
<td>site I, II or III</td>
<td>E1</td>
<td>E3</td>
<td>right end</td>
</tr>
<tr>
<td>non-essential or deleted regions</td>
<td>E3 (1249 bp)</td>
<td>RHE (2000 bp)</td>
<td>E1</td>
<td>E3 (600 bp)</td>
<td>right end (3600 bp)</td>
</tr>
<tr>
<td>human cell lines successfully infected (published to date)</td>
<td>non</td>
<td>HepG2, MRC-5, MCF-7, T47D-2, HAT-29, PC-3, HeLa, COS-7</td>
<td>293, HeLa, HIB, myocytes</td>
<td>293, A549</td>
<td>HepG2, A549, HeLa, primary fibroblasts</td>
</tr>
<tr>
<td>animal models</td>
<td>cotton rat</td>
<td>mouse</td>
<td>chicken embryo</td>
<td>none</td>
<td>chicken embryo</td>
</tr>
</tbody>
</table>

The tissue distribution of OAV is significantly different from that observed for hAd vectors which mainly infect the liver after systemic application in rodents (Smith et al., 1993, Fang et al., 1994, Kay et al., 1994, Huard et al., 1995). We found nearly equal amounts of vector DNA after i.p. (this paper) or intravenous (Hofmann et al., 1999) vector delivery. Moreover, even after local injection into the portal vein of C57/bl-6 mice the vector is only moderately enriched in the liver and is still found in all tissues examined. This is consistent with evidence (Xu and Both, 1998) that OAV vectors use a primary receptor that is distinct from CAR, the Ad5 receptor (Bergelson et al., 1997, Tomko et al. 1997). The full spectrum of cells that are infected by OAV remains to be determined but it is likely that there will be some cells that are better infected by OAV compared with hAd vectors and vice versa. The availability of an OAV/GFP recombinant will greatly facilitate these studies.

In summary, we have shown that OAV vectors are valuable tools for achieving high-level gene expression in animals. Further studies are in progress to extend the investigations of this novel vector system to other animal models. The prospect that OAV-derived vectors may replace or supplement their hAd counterparts warrants further development of this vector system to broaden its potential application in the field of gene delivery.

### IV. Experimental Procedures

#### A. Cells and viruses

Human embryonic kidney 293 cells, permissive for E1-deleted human adenoviruses and HuH7 (human hepatoma) cells were cultured in Dulbecco’s modified Eagles medium (GibcoBRL) with 2mM glutamine (Sigma, Deisenhofen, Germany) and 10% foetal calf serum (Roche Diagnostics, Mannheim, Germany) at 5% CO₂. CSL503 cells (foetal ovine lung, permissive for OAV) were grown under the same conditions but in 15% foetal calf serum. RM1 cells were grown in DMEM with additives (Cat # 12100-103; Life Technologies) plus 10% foetal bovine serum (Hall et al, 1997). Ad5Luc (a generous gift of M. Hillenberg, Berlin) contains a hCMV IE promoter-driven luciferase gene. The generation of OAVhaat in which expression of the human α₁-antitrypsin (haat) cDNA is driven by the RSV 3’LTR has been described (Hofmann et
al., 1999). Ad5haat, which contains the identical haat gene expression cassette, was a generous gift of Mark Kay, Stanford. To construct OAV217A containing the HCMV/GFP cassette we used a GFP gene that was modified by Dr. Shinichi Aota (Biomolecular Engineering Research Insitute, Japan) to optimise expression in mammalian cells. The gene was blunt-cloned into the XhoI/Smal sites of plasmid pCI (Promega Corp, Madison WI) and the promoter/gene cassette was excised by BglII/BamHI digestion and blunt-cloned into the XbaI site of pGem11zf (Promega Corp, Madison WI). A clone with a 5' Apal and 3' NotI site was selected and the insert was cloned into these sites in pOAV200 for virus rescue (Vrati et al, 1996b).

Subsequently, the cassette was further subcloned and modified by AflII digestion and blunt end ligation to remove the intron provided in pCI. The virus was rescued after transfection of C5L503 cells as described previously (Vrati et al, 1996b) except that cationic lipids were used (Cameron et al, 1999) in place of lipofectamine.

Viruses were grown on permissive cell lines and purified as described (Sandig et al, 1996). Virus titers were determined by an end point dilution assay on permissive cell lines. Particle/infectious unit ratios for Ad5 recombinants and OAV/haat were <40:1.

B. Treatment of vectors by human serum and heat

Ad5luc was incubated with 50 µl of human serum for 30 minutes at 37°C. Serum was either untreated, treated to remove complement at 56°C for 30 min, or heated at 48°C for 30 min. EGTA/Mg-treated serum contained 10mM EGTA and 7mM MgCl₂. Serum-treated virus was then used to infect HuH7 cells at a moi of 100. At 48hr post infection, cells were harvested and luciferase activity was determined as described previously (Löser et al, 1996). HuH7 cells infected with Ad5luc incubated for 30 minutes with PBS served as positive control. For heat treatment, 1x10⁵ pfu of either OAV/haat or Ad5haat were incubated for 30 minutes at 4, 42, 45, 48, 51, 54, 57 and 60°C, respectively, and virus titer was subsequently determined on permissive cells using an end point dilution assay.

C. Animal procedures, antibodies and detection of adenovirus-mediated gene transfer

Female Balb/C or C57/bl-6 mice aged 6 to 8 weeks (Charles River, Germany) received intravenous or intraperitoneal injections of adenoviral vectors as indicated. For portal vein injection a mid-line incision of 2 cm was made below the region of the liver. The intestines were carefully displaced to the right and the ileum extended to display the portal vein. A capillary tube connected to a syringe was inserted about 1 cm into the portal vein and vector suspension (a maximum of 150µl) was injected at a rate of 100µl per minute. After removal of the tube the portal vein was clamped for one minute to allow closure.

For determination of haat gene expression, blood samples were collected from the external jugular vein of mice and used in an enzyme-linked immunosorbent assay as described (Cichon and Strauss, 1998). Antibody titers to haat were determined according to Morral et al. (1997). Detection of hAd-specific antibodies was performed as described (Hofmann et al., 1999). For Southern blotting and RNase protection assay animals were sacrificed, organs of interest were frozen immediately and homogenized in liquid nitrogen and DNA and RNA were isolated separately from the same tissue piece using standard methods. For Southern blotting, genomic DNA (20µg) was digested with EcoRI, which releases a 2399 bp fragment from the OAV genome. After separation on a 1% agarose gel and transfer to a nylon membrane, hybridisation was performed using a probe spanning bp 1968 to 3408 of the OAV genome. A specific OAV EcoRI fragment (2.5 or 12.5 pg, equivalent to 1 or 5 copies per cell, respectively) was used as a standard. RNase protection assays were carried out with total RNA (20µg) following standard procedures. A radiolabelled RNA fragment of 362 bases comprising the EcoRI fragment of the hAAAT gene was used as a probe. In vitro transcribed haat RNA (10 or 25 pg, respectively) served as a standard.

Acknowledgement

We thank V. Sladek, E. Bennett and K. Smith for excellent technical assistance and Dr. Z. Xu for providing the image in Figure 7.

References


Vrati S, Brookes DE, Boyle DB, and Both GW (1996a) Nucleotide sequence of ovine adenovirus tripartite leader sequence and homologous of Iva2, DNA polymerase and terminal proteins. *Gene* 177, 35-41.


Yang Y, Ertl HCJ, and Wilson JM (1994b) MHC class I restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *Immunity* 1, 433-442.

