Improvements in adenovirus technology : aiming at replication specificity and vector integration

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Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op donderdag 26 april 2007

klokke 16.15 uur

door

Hendrik Jan Rademaker
geboren te Almelo

in 1976

Promotiecommissie

Promotor:	Prof. dr. R.C. Hoeben
Referent:	Dr. V.W. van Beusechem (VU medisch centrum - Amsterdam)
Overige leden:	Prof. dr. J. Verhaagen (Nederlands Instituut voor Neurowetenschappen - Amsterdam)
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The studies presented in this thesis were performed at the Molecular Cell Biology (MCB) department, Virus and Stem Cell Biology lab, of the Leiden University Medical Center (LUMC).

The studies described in this thesis were supported in part by the Dutch Technology Foundation STW.

The printing of this thesis was financially supported by MCB.

Printing: Printpartners Ipskamp - Enschede

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Proefschrift Leiden - Met lit. opgave - Met samenvatting in het Nederlands

Contents

Thesis outline

Current generation adenoviral vectors (Ads) are not suitable for those gene therapy approaches that require long-term gene expression. This is due to their high immunogenicity and transient gene expression in fast dividing tissue. The development of gutless Ads, also known as helper-dependent Ads, is a major improvement in reducing the immunogenicity of the vector system. Gutless Ads lack virtually all viral protein-coding sequences, thus severely limiting the viral-antigen evoked cellular immune responses that may result in the elimination of the transduced cells. Safety wise, recombinant Ads are considered safe due to their inability to replicate autonomously. However, we show in this thesis that replication of recombinant Ads can be rescued by the co-infection of wild type (*wt)* Ads. In this thesis studies are described that aim at the development of a new system to prevent vector mobilization. Though at its present state not directly applicable, this system could also potentially be used for the production of gutless Ads devoid of helper Ad contamination.
To improve efficacy of the Ad vector in dividing tissue we also studied two integration systems for their applicability in Ads. Overall, the experiments described in this thesis aim at generating safer vectors that should result in prolonged transgene expression due to lower immunogenicity and genomic integration.

In the first part of this thesis (Chapters **2**, **3** and **4**) we explored a new strategy to produce Ads that are replication-deficient and cannot be mobilized by *wt* Ads. Our strategy is based on the sequence specificity of replication initiation of human Ads. All human Ads start their genomes with 5'-cytidine residue. The Ads replication machinery is only able to start replication *in vitro* with dCTP as substrate. This is the result of an intrinsic specificity of pTP/Pol complex for binding dCMP as the first nucleotide. By changing the sequence at the replication-initiation sites in the HAdV5 ITRs (i.e. changing guanine residues at nt 1, 4 and 7 of the template strand to cytidine), replication in the presence of HAdV-5 pTP could be blocked.
The rHAdV-5 that have so far been used in clinical studies are replication deficient due to deletion of their *E1* region. When rHAdV-5 is co-infected with *wt* HAdV5, the *wt* HAdV can complement the *E1* deletion and thus can mobilize these rAd vectors. We show in chapter **2** that rHAdV-5-vector mobilization is not restricted to *wt* HAdV-5, but that *wt* HAdVs of other species (formerly subgroups), too, can mobilize this vector. Vector mobilization is undesirable since it can result in unpredictable side effects, especially when transgenes are used with a small therapeutic window. Use of mobilization-resistant Ads (mrAds) could potentially prevent this problem.
For the generation of these mobilization-resistant Ads a suitable production system must be made available. We searched for Ads that have ITRs that differ from the consensus HAdV ITR and looked for changes in the replication-initiation proteins that could explain this altered specificity for the ITR sequences. In chapter **3** we analyzed a series of Ad ITR sequences and confirmed that most Ad species initiated replication by coupling dCMP to pTP. The only exception is the Fowl Adenovirus 1 (FAdV-1). Some FAdV-1 isolates, i.e. PHELPS and KUR, contain guanine residues at the 5' end of the ITR. Clearly, PHELPS pTP/Pol should be able to initiate replication with dGTP residues as substrate, a feature not present in HAdV-5 pTP/Pol complex as evident from in vitro studies. The highly similar FAdV-1 isolate OTE, resembles the conventional ITR and initiates replication with dCTP. We

studied whether PHELPS had a changed specificity (i.e. can only initiate with dGTP), or a relaxed specificity (i.e. can initiate with both dGTP and dCTP) by comparing pTP and Pol sequences of PHELPS with OTE. We determined the coding sequences of OTE *pTP* and *Pol*, and performed *in vitro* replication assays. From our data we concluded that the FAdV-1 members have relaxed, rather than changed its nucleotide specificity for replication initiation.

In chapter **4** we describe the generation of mdHAdV-5 vectors with mutated ITRs (i.e. guanine instead of cytidine residues at nt 1, 4 and 7 of the top-strand). Transfection of plasmids encoding the mrAd genome on 911 producer cells did not result in the production of virus. Transfection of mrAds where the ITRs were changed back to *wt* ITR did result in virus production. This confirms that HAdV-5 pTP is not able to replicate on ITRs containing cytidine residues at the initiations sites of the template-strand. To propagate these mdHAdV-5 vectors, changes in the HAdV-5 pTP are required. We generated a FAdV-1/HAdV-5 hybrid pTP (H2pTP) by replacing a region surrounding the nucleotide-binding site of the HAdV-5 pTP gene with the corresponding region of the PHELPS pTP gene and generated a 911 cell line stably expressing this H2pTP (911-*H2pTP*). Transfection of mrAdV5-GFP plasmid on 911-*H2pTP* did result in the generation of virus indicating that over-expression of H2pTP could rescue the mutations in the ITR of mrAds.

The second part of this thesis is focused on the generation of integrating adenoviral vectors to prolong transgene expression in dividing cells. Chapter **5** describes the exploration of a plasmid-based minimized bacteriophage Mu integration system for transgene integration. Transfection of the Mu genes with the integration cassette did result in an increase of integration over background, albeit with low efficiency.

In chapter **6** we generated rAdV expressing *Agrobacterium tumefaciens* VirD1 and VirD2 genes to study 'agrolystic' integration in human cells. In this preliminary study we found a 60 fold increase in integration efficiency of Ads containing the integration cassette when both VirD1 and VirD2 were expressed in the same cell. When studying the integration sites, we found that integration did occur via an alternative VirD1/VirD2 enhanced mechanism.

In chapter **7**, we summarize the pros and cons of integrating vectors and discuss their safety.