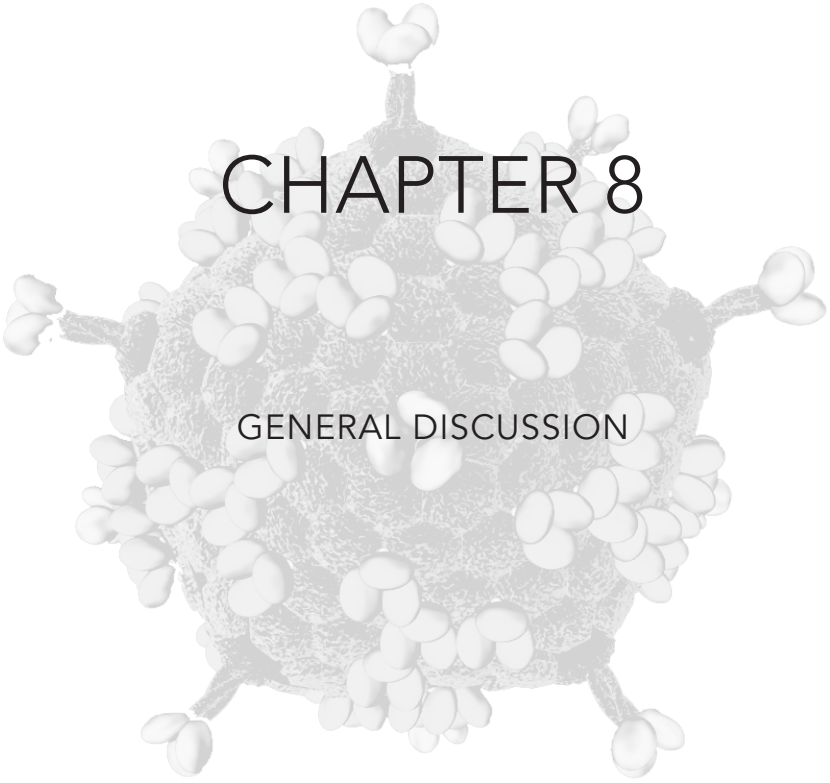
Improvement of oncolytic adenovirus vectors through genetic capsid modifications

Vrij, J. de



CHAPTER 8

GENERAL DISCUSSION

8.1 INTRODUCTION

Human adenovirus type 5 (HAdV-5)-derived vectors are among the most promising viral vectors for cancer gene therapy. Although clinical trials have shown safety for anti-tumor therapy with HAdV-5 vectors, the efficacy in general remains limited. A variety of efficacy-limiting aspects has been identified, as outlined in Chapter 2 of this thesis. A major problem is the poor penetration of the tumor due to the paucity of the coxsackievirus and adenovirus receptor (CAR) on the surface of the tumor cells.[1,2] Genetic modification of HAdV-5 capsid proteins might lead to the development of vectors that are specifically targeted to tumor cells, thereby improving efficacy as well as safety. Development of genetically modified vectors that can infect CAR-negative cells has mainly focused on the incorporation of heterologous ligands in the fiber knob, or on replacement of the entire knob domain by a heterologous ligand.[3] The complexity of incorporating ligands into the adenovirus fiber locale has prompted the identification of other capsid proteins amendable for ligand incorporation.[4] These approaches have the potential to incorporate an increased number of complex ligands per virion.

Major part of this thesis describes proof-of-principle studies on the usability of the minor capsid protein IX for targeting HAdV-5 to tumor cells (Chapters 3, 4, 5). Different ligands (single-chain antibody fragments (scFv's), single-chain T-cell receptors (scTCRs) and Affibody molecules) were efficiently incorporated in the virus capsid after genetic fusion to the carboxyl terminus of protein IX. This leads to enhanced transgene delivery to the targeted tumor cells. Also, a variety of analyses was performed on HAdV-5 vectors lacking the protein IX gene, motivated by the fact that the biological role of protein IX has not been fully elucidated (Chapter 7). This revealed aberrant characteristics for the protein IX-deficient vectors, such as enhanced transgene expression in CAR-negative cell lines, and enhanced activation of immune cells. Besides the assessment of protein IX-based tumor targeting, Chapter 6 of this thesis involves an alternative tumor-targeting methodology, i.e. through the fusion of ligands (scTCRs) to the fiber protein.

In this section, the major conclusions from the work presented in this thesis are summarized and recommendations for further research are provided. Furthermore, a general perspective is given on the future directions of developing oncolytic adenoviruses.

8.2 CONCLUSIONS

From the work presented in this thesis, we can draw the following major conclusions:

1. Large and complex polypeptide moieties with proven potential for tumor targeting, such as single-chain antibody fragments, single-chain T-cell receptors, or Affibody molecules, can be efficiently incorporated in the capsid of HAdV-5.
2. HAdV-5 can be targeted *in vitro* to cancer-testis (CT) antigens through fusing a single-chain T-cell receptor with protein IX or fiber molecules.
3. HAdV-5 can be targeted *in vitro* to Human Epidermal growth factor Receptor-2 (HER2) expressing tumor cells through fusing ZH-Affibody molecules with protein IX. This methodology requires the introduction of a cathepsin-cleavage site in between protein IX and the Affibody molecule.

4. Deleting the protein IX gene from HAdV-5 vectors results in enhanced delivery of transgenes into CAR-negative cells and enhanced activation of immune cells

8.3 PERSPECTIVES ON PROTEIN IX-LIGAND MEDIATED TUMOR TARGETING OF ADENOVIRUSES

Our studies present novel strategies for targeting HAdV-5 to tumor cells, which may be of great value for the future development of oncolytic adenovirus technology with improved efficacy and/or safety. The applicability of protein IX as an anchor for fusing large and complex polypeptides opens new opportunities for tumor cell targeting, as an alternative or in addition to the currently used fiber modification strategies. Of special interest, our studies also demonstrate the feasibility of targeting HAdV-5 to CT antigens through the fusion of scTCRs to protein IX or fiber. This significantly extends the number of tumor cell-specific molecules that can be targeted. Although these results are highly promising, a variety of further studies is needed to delineate the true clinical potential.

One important aspect will be to introduce the protein IX-ligand modifications in the genome of replication competent adenoviruses. Our studies mainly involved assessments of transduction efficiencies using replication deficient vectors. However, it is currently anticipated that the clinical anti-tumor efficacy of replication deficient vectors remains insufficient, irrespective the type of foreign transgene delivery.[5] This has lead to the ongoing development and clinical testing of different variants of Conditionally-Replicating Adenoviruses (CRAds), such as the HAdV-5.RGD.Δ24 virus that is tested for its anti-tumor efficacy and specificity in ovarian carcinoma and glioma patients.[6] Future work may include the incorporation of protein IX-attached targeting ligands in CRAds to further improve their efficacy or specificity.

One aspect probably hampering the efficacy of oncolytic adenoviruses, and possibly all other types of oncolytic viruses, is the heterogeneity of tumor cells within a solid tumor mass. As such, highly differentiated tumor cells exist beside (a minor percentage of) tumor stem cells, which differ in the amount and type of cell surface receptor presentation. As a consequence of the heterogeneities, a significant fraction of a tumor might escape from infection with an oncolytic adenovirus. As a solution, 'mosaic' adenovectors might be developed, that are harnessed with multiple tumor targeting ligands. Protein IX offers new potential for the establishment of mosaic HAdV-5 vectors, for example combining fiber- with protein IX-mediated targeting. Also, vectors might be developed that have protein IX fused to different targeting ligands in a single virus particle. Proof-of-principle of creating protein IX-polypeptide mosaic viruses has already been shown through simultaneously incorporating a targeting, imaging, and therapeutic motif in the capsid of HAdV-5.[7]

Alongside our applied studies on the feasibility of using protein IX as an anchor for the attachment of tumor targeting ligands, aberrant characteristics were found for protein IX-gene deleted vectors (Chapter 7). Most strikingly, protein IX-deficient vectors showed increased transgene expression in a variety of cell types, as compared to the control vectors. The highest enhancements were observed on CAR-low/negative cells. Also, protein IX deletion appeared to lead to increased activation of cellular

subsets of peripheral blood mononuclear cells (PBMCs), as demonstrated by changes in cell surface marker expression. Although our experiments ruled out various aspects as causative mechanism, the exact molecular mechanism could not be elucidated. Recently, Strunze *et al.* reported an essential role for protein IX in facilitating access of viral DNA to the cellular nucleus.[8] Upon infection of a cell, the HAdV-5 particle successively travels towards the nucleus, binds with hexon epitopes to a nuclear pore complex (NPC), uncoats, and inserts its DNA into the nucleus. Protein IX molecules were found to link the virus with microtubule motor kinesin-1 molecules after docking to the NPC. This results in nuclear access of viral genomes through compromising the integrity of the NPC and uncoating of the virus particles. Infection of cells with a protein IX-gene lacking HAdV-5 was shown to be less efficient as compared to wild-type HAdV-5 infection, as demonstrated by the formation of lower levels of progeny virus particles. These findings by Strunze *et al.* do not necessarily contradict our observations. Reduced microtubule motor binding of protein IX-lacking adenoviruses likely causes enhanced innate inflammatory responses, such as the interferon response, as a result of cytoplasmic sequestration and/or altered movement kinetics of the virus particles. In support of this, we observed enhanced activation of PBMCs. The cellular inflammatory responses might have had serious consequences on our protein IX-lacking vector's luciferase and GFP expression levels, since both transgenes were driven by the cytomegalovirus (CMV) promoter. The activity of this promoter is known to be stimulated by the cellular interferon response.[9] Still, it remains to be investigated why CAR-negative cell lines display the strongest increase in transgene expression for protein IX-gene deleted HAdV-5 vectors.

Obviously, translation of protein IX-mediated tumor targeting towards the clinic requires the performance of studies in models that resemble the eventual application in humans, including animal studies. This will reveal the anti-tumor efficacy of protein IX-ligand viruses in the complex environment of a tumor, which consists of a large variety of cell types and extracellular matrix components. These studies should include careful analyses on the interactions of protein IX-liganded viruses with non-tumor cells, such as immune cells or hepatocytes.

Our studies suggest inefficacious protein IX-mediated targeting after the attachment of high-affinity binding ligands. To obtain definite answers, it will be informative to perform a side-by-side comparison between vectors containing protein IX-linked ligands with different affinities for a certain receptor. Also, further studies are necessary to determine the most suitable type of polypeptide ligand to be fused with protein IX, e.g. comparing single-chain antibody fragments with single-chain T cell receptors. Again, it is recommended to use relevant model systems, since the outcome of such experiments probably depends on the tumor (micro)environment.

8.4 GENERAL PERSPECTIVES ON THE FUTURE DEVELOPMENT OF ONCOLYTIC ADENOVIRUSES

Recent years have witnessed the publication of a large variety of preclinical improvements on HAdV-5 vectors. These developments, as summarized in Chapter 2 of this thesis, have been driven by an increased knowledge on oncolytic HAdV-5

performance in humans (through evaluation of clinical trial data), improved insights into tumor biology, and improved vector modification techniques.

Clearly, this plethora of 'next generation oncolytic AdV techniques' needs extensive evaluation in the near future. Performing well-designed studies (for example comparing different vectors) will facilitate translation of the technology to the clinic. Preclinical studies suggest differences in the performance of various oncolytic viruses, with certain viruses outperforming others, depending on the tumor type or the delivery technique.[10] Therefore, it will be important to compare AdV-derived oncolytic viruses with other oncolytic viruses, such as Reovirus,[11] Newcastle Disease Virus vectors,[12] and Herpes Simplex Virus vectors.[13] Also, it will be essential to use testing models that resemble the eventual clinical setting. With this respect, important lessons can be learned from previous studies, which demonstrated remarkable differences in the *in vivo* behavior of HAdV-5 vectors between animals and humans. HAdV-5 binds extensively to human erythrocytes, thereby hampering systemic delivery. This is in contrast to rodents.[14,15] Important differences occur between animal species as well. It has been found that tissues and cells of mice do not support HAdV-5 replication, whilst other species (e.g. cotton rats and Syrian hamsters) are permissive.[16] For this reason, mouse models are less suitable for studying aspects on the specificity of oncolytic HAdV-5 vectors, such as liver toxicity and immune cell activation.

The existence of 'human versus animal' discrepancies makes human models more suitable to answer certain research questions. Various human models have been developed that are of special interest for the evaluation of oncolytic vectors. Vectors might be tested in *ex vivo* blood circulation models, a model that has already been used with success to compare blood circulation times and binding to blood components between AdV vectors.[17] Also, oncolytic AdV testing is anticipated to benefit from the recent developments of *ex vivo* human tumor models.[18] As an example, novel methodologies enable the *ex vivo* culturing of primary tumor material resected from brain tumors, including tumor stem cell cultures in specialized growth media (either as three-dimensional spheres,[19] or as two-dimensional monolayers[20]) and organotypic brain slice cultures.[21] To simulate the heterogeneity of a solid tumor, efforts are undertaken to create spheres that consist of co-cultures of tumor cells with stromal cells, like fibroblasts or endothelial cells.[22] These systems require further optimization, mainly to prevent the stromal cells from rapidly losing their viability or phenotype.

Of additional benefit, *ex vivo* culturing of primary tumor material opens opportunities for the design of 'personalized therapeutics'. In theory, therapeutic vector regimens can be screened on patient-derived tumor material before initiating a therapy. Personalizing viral gene therapy might improve the clinical success rate, taking into account that most tumor types consist of a 'family' of tumor subtypes. It is unlikely that all patients suffering from a single tumor type respond similarly to an oncolytic virus therapy.

Besides the above mentioned requirements for vector testing in appropriate tumor models, it will be important to investigate the effects of combining oncolytic AdV therapy with other therapies. Combining chemotherapy with oncolytic virus treatment can lead to enhanced or even synergistic therapeutic efficacy, as demonstrated in animal models.[23,24] Moreover, oncolytic virus treatment might enable the usage of lower concentrations of certain chemotherapeutics, thereby reducing the toxicity.

Taken together, the availability of improved experimental models and improved viral vectors offers great opportunities for the development of 'next generation oncolytic AdV therapies'. As a response on the previous identification of treatment efficacy-limiting aspects, a large plethora of novel vectors and strategies has been developed. Transductional targeting via attachment of ligands to protein IX offers a methodology for further improvements on oncolytic AdV vectors. Future studies in well-designed preclinical and clinical settings will teach whether the regained expectations for oncolytic adenoviruses as therapeutic regimens for cancer treatment will be fulfilled.

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