Forward and reverse genetics strategies for improving oncolytic reoviruses

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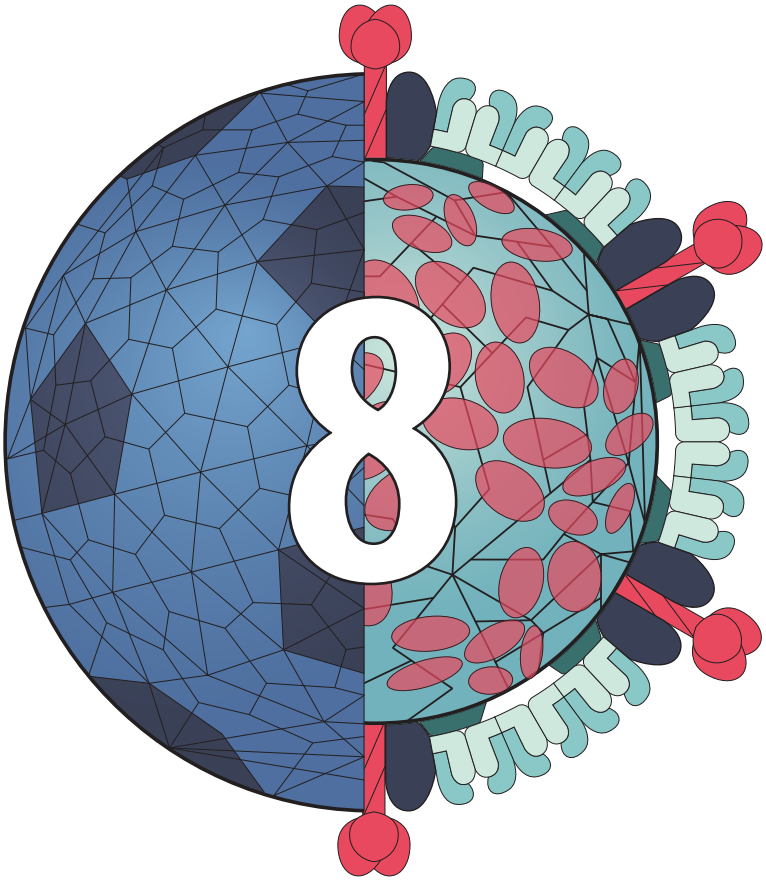
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GENERAL DISCUSSION

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Oncolytic viruses are among the most promising therapeutic agents in the field of cancer therapy. Reovirus T3D and adenovirus type 5 are potent members of this new treatment category. Clinical trials have demonstrated the safety of this new approach, however pre-clinical and clinical research also illuminated several obstacles that thwart the efficacy of oncolytic virotherapy (reviewed in[1-3]). Nonetheless, the feasibility of the oncolytic virus concept has been demonstrated by the recent regulatory approval of the first oncolytic virus in the US, Europe and Australia. This genetically modified herpesvirus, Imlygic™ (talimogene laherparepvec (T-VEC)), is used for treatment of advanced stage melanoma[4].

This chapter discusses the results reported in this thesis and describes the current status of the research involving reovirus as an immune stimulatory agent. Also, future perspectives on the use of oncolytic reovirus are discussed.

8.1 BIOSELECTION AND RATIONAL DESIGN CAN IMPROVE THE EFFICACY OF ONCOLYTIC VIRUSES

Poor penetration of oncolytic viruses into tumours and inadequate transduction of tumour cells due to the absence or inaccessibility of the virus receptor on the tumour cell surface are considered as major barriers upon intra-tumoural injection. The research described in this thesis mainly focussed on the amelioration of these problems by genetically modifying reovirus (and adenovirus) by various strategies (chapter 2, 3, 5 and 6).

Chapter 2 described the improved transduction of tumour cells by human adenovirus type 5 which has a tumour-targeting ligand (HER2/neu-binding ZH *affibody* molecule) fused to the minor capsid protein IX. Retargeting ligands are commonly used in gene therapy to create viral vectors that bind more specific to the target-cell type. Cancer-targeting ligands have been genetically fused to adenovirus capsid proteins (fiber, hexon or protein IX) to create viruses with a higher affinity for tumour cells[5]. The minor capsid protein IX has several characteristics that make the protein an interesting candidate for fusing targeting ligands, i.e. the high abundance in the capsid (240 coiled-coil domains at the surface versus 12 domains for fiber) and the ability to modify the coiled-coin domain without compromising the stability or infectivity of the virus[6]. Nonetheless, early attempts on protein IX-mediated adenovirus targeting appeared inefficient, which was attributed to an inability of the virions to release from their (high affinity-bound) ligand after uptake in the cellular endosome[7]. This could result in viral entrapment in

the endosome. The solution to this problem was inspired by the natural disassembly process of reoviruses inside endosomes. Here, cathepsin proteases prepare the virion for endosomal escape. Indeed, the insertion of a (reovirus-derived) cathepsin-cleavable site between protein IX and the targeting ligand resulted in significantly enhanced transduction of tumour cells in two- and three-dimensional cell cultures, as well as in the chicken chorioallantoic membrane tumour model. It would be worthwhile to investigate the efficacy of this cathepsin-cleavage site between the protein IX anchor and the targeting ligand in *in vivo* tumour models.

From our results (chapter 4) and results obtained by others it became clear that the availability of the cellular receptor for reovirus, JAM-A, may not be as important in tumours as it is for reovirus infection *in vitro* on monolayer cell cultures[8,9]. In chapter 4 it was shown that, whereas monolayer cell cultures of a JAM-A negative glioblastoma cell line resist reovirus infection, three-dimensional tumour spheroid cultures of these cells could be adequately infected. This could be attributed to cathepsin proteases, of which the secretion by spheroids is enhanced as compared to the secretion levels in two-dimensional cell cultures. In monolayers, these cathepsin proteases (predominantly cathepsin L and B) are present in the endosomes where they partially uncoat reovirus, enabling the resulting intermediate subviral particles (ISVP) and ISVP* virus particles to escape the endosome and continue the virus replication cycle in the cytosol[10]. Similar to our spheroid model, *in vivo* this step can occur extracellularly, as many tumours express and secrete high levels of cathepsins[11-16]. Therefore, the absence of JAM-A on a tumour cell may be less of a hurdle for the clinical anti-tumour efficacy of reovirus.

Nevertheless, the generation of the reovirus *jin*-mutants, which are able to infect tumour cells independent of JAM-A expression, is of great value (chapter 3). Upon intratumoural injection of reovirus, the local extracellular virus concentration will be considerable. It could be anticipated that part of the virions will be digested by extracellular proteases to ISVP and ISVP* particles, able to enter the tumour cells receptor-independent, while other virions will enter the cells in a receptor-dependent manner. The presumed enhanced binding of the *jin*-mutants to sialic acids on the cell surface could be advantageous in this respect, as the sialic acids are enriched on tumour cells as the result of overexpression of sialyltransferases[17,18] and the sialylation pattern is altered compared to non-transformed cells[19].

Moreover, the enhanced binding affinity of the virus for sialic acids allows the replacement of the codons for the JAM-A-binding domain in the reoviral spike protein, e.g. for those encoding a fluorescence reporter protein (chapter 5). This replacement strategy allows for the inclusion of a transgene without exceeding the length of the wild-type S1 segment. Also other functional transgenes could theoretically be

incorporated, e.g. encoding cytotoxic products or modulators of the immune system. This strategy is currently under investigation in our group. In addition, replacement of the JAM-A binding head domain of σ1 may result in a further attenuated virus. This was observed in a reovirus isolated from persistently infected human fibrosarcoma HT1080 cells. The reovirus that was isolated contains a S1 segment harbouring a stop codon in the codons for the shaft region (at amino acid 251) of the σ1 protein, resulting in an attachment protein that lacks the JAM-A-binding head domain. These viruses are less pathogenic in mice, while retaining their oncolytic capacity[20].

The *jin*-mutants have proven to be genetically stable at repeated passages. However, the recombinant reoviruses, obtained by reverse-genetics, occasionally acquire deletions upon serial passaging (chapter 5). Although the stability of the S1 gene segment may be transgene-dependent, genetically stable recombinant reoviruses are essential to produce virus batches for clinical use. As the helper-virus free genetic modification technology of reoviruses has been developed recently, new methods to optimize this technique can be foreseen (e.g.[21]). Additionally, it would be valuable to study alternative loci in the reovirus genome to insert a transgene. So far, foreign genes have been inserted in the S1, S2, S3, S4, M1 and L1 gene segments[22-25], but the number of studies addressing transgene stability is still small. Besides genetic stability, also size constraints on the length of dsRNA that can be included inside the virus capsid and the protein amounts that are translated from a certain segment are aspects to consider while designing recombinant reoviruses[22,26]. Moreover, as extracellular proteases in the microenvironment of a tumour may be able to convert reovirus virions into ISVPs and ISVP*s, the loss of capsid proteins σ1, σ3 and part of μ1 outside the cell and, as a consequence, the loss of the tumour-targeting ligand incorporated into these capsid proteins, has to be regarded.

The poor penetration capacity of oncolytic viruses into tumours is an additional hurdle that has to be overcome for clinical applications[27-29]. In chapter 6 we demonstrate that baculovirus can be used as a tool to improve the spread of reovirus into a tumour spheroid. For future studies, it would be highly relevant to determine the mechanism by which baculovirus is able to enhance the reovirus spread within the tumours[30]. This may yield novel strategies to enhance the intratumoural distribution of the therapeutic virus. Currently, baculovirus itself is not approved for clinical use, however regarding the considerable pre-clinical efforts that are being made, clinical admission of baculovirus may be a matter of time.

In chapter 7 fundamental research on the reovirus-host interaction is described and this shows that reovirus infection induces autophagy in several cell lines. Although the role of autophagy in cancer is complex and can be context-dependent, it is noteworthy

that autophagy processes that are induced by viral infection may enhance tumour-associated antigen presentation by DCs to subsequently activate T cells[31-33]. This shows that deeper knowledge of the cellular pathways involved in reovirus replication and cancer cell death could be of great value to ameliorate therapeutic approaches and thereby reovirus' achievements in the clinic.

8.2 REOVIRUS THERAPY TO BOOST ANTI-TUMOUR IMMUNE RESPONSES

The role of the human immune system in oncolytic virotherapy is ambiguous and a paradigm shift on the influence of innate and adaptive immune responses on the efficacy of oncolytic viruses in eradicating cancer has taken place in recent years[34]. Initially, the immune system was perceived merely as a negative determinant in oncolytic virotherapy as pre-existing circulating antibodies and innate immune responses can limit the effectiveness of the oncolytic virus, especially after intravenous administration. Nowadays, pre-clinical and clinical evidence also points to a synergistic role of oncolytic viruses and the immune system[34,35]. These findings have caused major shifts in research on oncolytic viruses. The precise mechanisms underlying the cooperation between oncolytic viruses and the immune system are under extensive investigation. It is evident that infected tumour cells produce cytokines and chemokines which elicit innate and adaptive anti-tumour immunity. Furthermore, upon virus-mediated lysis, the cell releases many tumour-derived (neo-) antigens. These antigens can be ingested by antigen presenting cells, subsequently leading to immune reactions against the tumour[3,36]. Ultimately, a systemic immune response against tumour-derived antigens may remove distant metastases[37].

The number of studies addressing the effect of reovirus-induced anti-tumour immune responses is limited, although results are encouraging. For virus-infected melanoma cells it was demonstrated that they secrete immune stimulatory factors (interleukin-8, interferon-β, macrophage inflammatory protein-1α and 1β and others) which induce a chemotactic response in dendritic cells (DC), natural killer cells (NK) and cytotoxic T cells. This response is dependent on nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and protein kinase RNA-activated (PKR) and it supports, in the absence of live reovirus, lysis of melanoma cells *in-vitro*[38]. Analysis of immunocompetent mice bearing a prostate tumour demonstrated that reovirus infection led to homing of NK cells, cytotoxic $CD8^+$ T cells and DCs to the tumour environment and enhanced tumour-derived antigen presentation by DC and NK[39]. A clinical study also showed

the activation of NK cells upon intravenous administration of reovirus in patients with colorectal cancer and liver metastases, although the effect of this NK stimulation on the tumours was not determined[40].

Also the potential synergistic effect of reovirus in combination with other targeted therapies is investigated in several studies. Reovirus has been combined with several anticancer monoclonal antibody (mAb) therapies, e.g. reovirus with trastuzumab (Herceptin®) showed increased anti-tumour immunity and inhibition of tumour growth in a murine xenograft model of HER-2 positive gastric cancer[41]. Administration of the checkpoint blockade inhibitor anti-programmed cell death protein 1 (anti-PD-1) antibody together with reovirus in a murine melanoma model resulted in prolonged survival compared to reovirus alone[42]. In a recent study three treatment modalities were combined. Reovirus, VSV expressing a cDNA library of melanoma antigens and anti-PD-1 antibody were consecutively systemically delivered in a murine melanoma model resulting in enhanced survival, with long-term cures, compared to the individual or double combination therapies[43]. Clinically, the PD-1 receptor inhibitor Pembrolizumab is tested in combination with reovirus in pancreatic cancer patients. The primary endpoint of this phase I study was safety, but preliminary results showed stable disease and partial response in several patients[44].

Another approach is the loading of reovirus onto DCs[45] or lymphokine-activated killer cells (LAK; a heterogeneous population of activated T, NK and NK-T cells) in combination with DCs, called LAKDCs[46]. These cells act as virus carriers and have been shown to protect the virus from neutralising antibodies and handed-off the virus to tumour cells *in-vitro*[45,46]. Moreover, reovirus-loaded LAKDCs secreted proinflammatory cytokines (Tumor Necrosis Factor-α, Interferon-α, Interferon-γ), which have the potential to override the immuno-suppressive state of the tumour microenvironment[46]. Also adaptive anti-tumour immune responses were initiated by reovirus-loaded LAKDC in an *in-vitro* priming experiment as demonstrated by the induction of cytotoxic T-cell response against co-cultured tumour cells[46]. Together these developments show the therapeutic potential of the alliance between reovirus and the immune system and increasing the insight in the molecular basis of this synergy will likely lead to improved anti-cancer therapies.

8.3 IN CONCLUSION: THE FUTURE OF REOVIRUS AS AN ONCOLYTIC AGENT

Oncolytic viruses constitute a new treatment regimen for cancer. Most oncolytic viruses, and reovirus in particular, have been shown to exhibit an excellent safety profile in contrast to several conventional cancer therapies. Reovirus has been demonstrated in preclinical experiments to be efficacious against a plethora of cancer types, leading to 30+ human clinical trials involving reovirus as single agent or in combination with radiation-, chemo- and immunotherapy[3]. Phase II and III studies have shown promising results of clinical efficacy, especially those analysing reovirus in combination therapies[3]. Clinical trials involving reovirus in combination with various forms of immunotherapy are limited in number, nonetheless the first outcomes of pre-clinical and phase I research using this combination of therapies are encouraging[42,43,47]. It is therefore conceivable that these joined agents become the major focus of upcoming clinical trials[3,34,43,47,48], with the ultimate goal of developing safe and powerful anti-cancer therapies. This approach, however, necessitates close collaboration of researchers in multidisciplinary teams that bring together the expertise of clinicians, immunologists, and virologists.

In this respect, also genetically modified reoviruses may be of great importance. Modifications on the virus capsid can not only improve the virus-host cell interaction but may also alter immune responses against the virus[49]. Furthermore, immune modulatory genes may be included in the virus genome and their product can contribute to the killing of tumour cells. Particularly for the substantial genome changes that foreign gene inclusion requires, several hurdles have to be overcome before clinical grade virus batches will become available. Nevertheless, regarding the advances in reovirus modification techniques that we witnessed in recent years, it is reasonable to assume that also these issues become manageable in the near future.

In addition, it is worthwhile to mention that oncolytic virotherapy is not a 'one virus fits all' cancer treatment. Existential heterogeneity in tumours among different cancer types, as well as inter- and intra-tumour variability in molecular-genetic features in tumours of the same cancer type result in a distinct susceptibility of the tumour for none, one or more oncolytic viruses[48,50,51]. Personalized oncolytic virotherapy will therefore be the future for this new category of cancer treatment.

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