Enhancing reovirus for use in oncolytic virotherapy

Kemp, V.

7

General discussion

EXPLORING REOVIRUS AS AN ONCOLYTIC VIRUS

Oncolytic viruses represent promising tools in anti-cancer therapy. The recent FDA approval of T-VEC, a herpes simplex virus expressing copies of the GM-CSF gene, gives an encouraging outlook on approval of other oncolytic virotherapies by the regulatory bodies [1]. Oncolytic reovirus has been studied in a variety of clinical trials, but its efficacy in stand-alone treatments remains moderate at best [2]. Therefore, we sought to explore ways in which we can improve the therapeutic potency of reovirus. Before effective application, we need to have a good understanding of the prerequisites for efficient viral replication, cell death stimulation, and induction of a potent long-lasting anti-tumor immune response.

ROLE OF AUTOPHAGY DURING REOVIRUS REPLICATION

We discovered that specific members of the autophagy machinery are important for reovirus replication, as shown by reduced titers upon knock-out of the corresponding Atg genes [3]. Interestingly, not all autophagy-related proteins influenced reovirus replication, indicating that there may be a non-canonical use of the proteins, independent of their role in autophagy. We proposed several explanations, including a function in endosome maturation, mitochondrial function or inhibition of type I interferon (IFN) production, which are important for viral entry and replication, respectively. Importantly, the effects on viral replication could only be detected at relatively late time points during infection (48-72 hours post infection), suggesting that a late event during viral replication is influenced. Therefore, it seems unlikely that an effect of Atg knock-out on viral replication is caused by an effect on viral entry. Furthermore, we observed only minor effects of Atg knock-outs on the induction of cell death by reovirus [4]. An effect on mitochondrial homeostasis or an anti-viral type I IFN response seem more plausible explanations for the changes in viral replication. Interestingly, the expression of autophagy-related proteins in cancer is very diverse, with several cases of cancers that lack the expression of some autophagy-related protein [5]. It would be interesting to see if there is a correlation between expression of autophagy-related proteins such as Atg3 or Atg5 and cancer susceptibility to oncolytic viruses.

GENETIC MODIFICATION OF REOVIRUS TO ENCODE TRANSGENES

E4orf4

In order to enhance oncolytic reovirus as a monotherapy, we sought ways to genetically modify the virus to encode a potentially therapeutic transgene. We did so by exploiting a plasmid-based reverse genetics system, in which plasmids are used that encode the 10 different reovirus genome segments, driven by a T7 polymerase promoter. Conceptually, upon transfection of cells expressing the T7 polymerase,

these plasmids are transcribed and viral proteins are generated. These will assemble in viral particles which can be propagated on a producer cell line. We replaced the S1-encoding plasmid for a plasmid encoding S1 and a heterologous transgene. In order to not increase the genome size of reovirus, we replaced the S1 region encoding the σ1 head domain by the transgene. The σ1 head domain is responsible for binding to the canonical reovirus receptor JAM-A. Based on the previously isolated *jin-3* mutant reovirus, that is able to infect JAM-A negative cells, we incorporated a G196R mutation in the S1 segment that allows for enhanced sialic acid binding dependent entry. We used the reverse genetics system to encode a cell death inducing protein called E4orf4 in the reovirus S1 segment [6]. This adenovirus-derived protein is known to induce a non-classical form of apoptosis. Interestingly, we found no additional effect of encoding E4orf4 on cell death induction by reovirus. Rather, the truncation of σ1 seems to result in potent cell death induction, presumably making the expression of an additional cell death inducer redundant.

GM-CSF

The mechanism of action by oncolytic viruses has been extended beyond the simple induction of cancer cell death. It is now widely accepted that the efficacy of oncolytic viruses not only depends on replicating capacity but also on the induction of potent and long-lasting anti-cancer immune responses. Therefore, we hypothesized that it may be of more value to genetically modify reovirus to encode an immunostimulatory transgene that can trigger anti-tumor immune responses. We generated recombinant reoviruses expressing GM-CSF [7]. GM-CSF is known to stimulate the generation and activation of dendritic cells (DCs) [8, 9]. The GM-CSF expressing reoviruses triggered the secretion of GM-CSF from infected cells [7]. Furthermore, the secreted murine GM-CSF was further tested and found to be functional in generating and activating dendritic cells *in vitro*. Importantly, we also found systemic effects on the immune composition in mice bearing pancreatic tumors. Therefore, we proposed that this virus may be of value to further test for clinical application. Future studies are needed to determine to what extent the GM-CSF expressing reoviruses improve tumor reduction and/or survival.

PRODUCTION AND STABILITY OF RECOMBINANT REOVIRUSES

To make clinical application feasible, large batches of (genetically) stable reoviruses must be generated with ease. During the generation of our recombinant viruses, we noticed that the infectious titers of these reoviruses were relatively low compared to wild-type reovirus or bioselected mutants such as *jin-3*. Further experiments revealed that σ1 may be incorporated to a lower level into the viral particles, and we propose that this affects viral stability and infectivity. Furthermore, we

occasionally detected deletion mutants in which (parts of) the transgene and sometimes reovirus-derived sequences were removed. Further examination of the deletions revealed that the most plausible explanation for this lies in complexities of the RNA structure. Altogether, we demonstrated that medium-scale batches of recombinant reoviruses can be generated with relative ease. However, before large-scale batches can be generated that are suitable for clinical application, the production to high titers and the (genetic) stability of batches have to be optimized. Therefore, it may be easier to move wild-type reovirus or bioselected mutants into the clinic, as these do not give the infectivity and stability issues that the recombinant reoviruses do. In addition, it may be less complex and more effective to let the virus come up with a solution for eliminating cancer cells than to develop a tumor-specific cell death trigger ourselves. The mutation rate of reovirus makes it possible to generate pools of mutants during virus propagation. Perhaps in this way viruses can be generated that potently combat cancers that resist current therapies.

FUTURE DIRECTIONS

For moving forward, and for getting the right treatment for cancer patients, we need to focus on markers that predict the susceptibility of a cancer to the oncolytic virus treatment. With each cancer having different susceptibility profiles to different oncolytic viruses, it would be of great value to consider therapeutic approaches that combine different oncolytic viruses. Alternatively, many promising combinations of oncolytic viruses with other anti-cancer agents are studied and often work synergistically [10, 11]. The heterogeneity in tumor composition within patients and also between patients continue to form a hurdle. However, with the viruses' intrinsic tumor tropism as a starting point and with the aid of directed virus evolution methodology we have the means to overcome this hurdle and to improve the therapeutic efficacy of oncolytic virus treatment. With new and improved viruses, and in combination with new immunomodulatory approaches and potentially synergizing cytostatic drugs, we may one day reach the point where each patient receives treatment involving the personalized administration of dedicated, efficient, and safe oncolytic viruses.

REFERENCES

1. Pol, J.; Kroemer, G.; Galluzzi, L. First oncolytic virus approved for melanoma immunotherapy. Oncoimmunology 2016, 5, e1115641.
2. Zhao, X.; Chester, C.; Rajasekaran, N.; He, Z.; Kohrt, H.E. Strategic Combinations: The Future of Oncolytic Virotherapy with Reovirus. *Mol Cancer Ther* **2016**, 15, 767-73.
3. Kemp, V.; Dautzenberg, I.J.C.; Limpens, R.W.; van den Wollenberg, D.J.M.; Hoeben, R.C. Oncolytic Reovirus Infection Is Facilitated by the Autophagic Machinery. *Viruses* **2017**, 9, 266.
4. Kemp, V.; Dautzenberg, I.J.; van den Wollenberg, D.J.; Hoeben, R.C. Autophagy has minor effects on reovirus-induced cytolysis. *Unpublished data*. **2017**.
5. Uhlen, M.; Zhang, C.; Lee, S.; Sjostedt, E.; Fagerberg, L.; Bidkhori, G.; Benfeitas, R.; Arif, M.; Liu, Z.; Edfors, F. *et al.* A pathology atlas of the human cancer transcriptome. *Science* **2017**, 357.
6. Kemp, V.; Dautzenberg, I.J.C.; Cramer, S.J.; Hoeben, R.C.; van den Wollenberg, D.J.M. Characterization of a replicating expanded tropism oncolytic reovirus carrying the adenovirus E4orf4 gene. *Gene Therapy* **2018**, 25, 331–34.
7. Kemp, V.; van den Wollenberg, D.J.M.; Camps, M.G.M.; van Hall, T.; Kinderman, P.; Pronk-van Montfoort, N.; Hoeben, R.C. Arming oncolytic reovirus with GM-CSF gene to enhance immunity. *Cancer Gene Ther* **2019**, doi: 10.1038/s41417-018-0063-9.
8. Xu, Y.; Zhan, Y.; Lew, A.M.; Naik, S.H.; Kershaw, M.H. Differential Development of Murine Dendritic Cells by GM-CSF versus Flt3 Ligand Has Implications for Inflammation and Trafficking. *The Journal of Immunology* **2007**, 179, 7577-7584.
9. Zhan, Y.; Carrington, E.M.; van Nieuwenhuijze, A.; Bedoui, S.; Seah, S.; Xu, Y.; Wang, N.; Mintern, J.D.; Villadangos, J.A.; Wicks, I.P.; Lew, A.M. GM-CSF increases cross-presentation and CD103 expression by mouse CD8(+) spleen dendritic cells. *Eur J Immunol* **2011**, 41, 2585-95.
10. Bommareddy, P.K.; Shettigar, M.; Kaufman, H.L. Integrating oncolytic viruses in combination cancer immunotherapy. *Nat Rev Immunol* **2018**, 18, 498-513.
11. Lal, G.; Rajala, M.S. Recombinant viruses with other anti-cancer therapeutics: a step towards advancement of oncolytic virotherapy. *Cancer Gene Ther* **2018**.

ADDENDUM

Nederlandse samenvatting
Curriculum Vitae
List of publications
Acknowledgements (Dankwoord)