

Arming viruses in multi-mechanistic oncolytic viral therapy: current research and future developments, with emphasis on poxviruses

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Abstract: The field of oncolytic virology has made great strides in recent years. However, one key finding has been that the use of viral agents that replicate selectively in tumors is usually insufficient to achieve anything beyond small and transient responses. Instead, like most cancer therapies, oncolytic viruses are most effective in combination with other therapies, which is where they have proven therapeutic effects in clinical and preclinical studies. In cases of some of the smaller RNA viruses, effects can only be achieved through combination regimens with chemotherapy, radiotherapy, or targeted conventional therapies. However, larger DNA viruses are able to express one or more transgenes; thus, therapeutic mechanisms can be built into the viral vector itself. The incorporated approaches into arming oncolytic viruses through transgene expression will be the main focus of this review, including use of immune activators, prodrug converting enzymes, anti-angiogenic factors, and targeting of the stroma. This will focus on poxviruses as model systems with large cloning capacities, which have routinely been used as transgene expression vectors in different settings, including vaccine and oncolytic viral therapy.

Keywords: vaccinia, poxvirus, immunotherapy, angiogenesis, prodrug

Introduction

In early results from a recent Phase III trial of over 400 melanoma patients, Andtbacka reported that an engineered virus, talimogene laherparepvec (T-Vec), led to meaningful tumor shrinkage for at least 6 months in 16% of patients, compared against 2% in the control arm (treated with granulocyte-macrophage colony-stimulating factor [GM-CSF] alone): a statistically significant difference.¹ Meanwhile, Heo et al have also recently reported on a 30-patient, randomized dose-comparison Phase II trial in hepatocellular carcinoma of a second, engineered virus, pexastimogene devacirepvec (Pexa-Vec), with statistically significant, dose-dependent, overall survival benefit at the higher dose.² They have also completed enrolment in a Phase IIb TRAVERSE trial,³ treating 120 liver cancer patients who have previously failed sorafenib therapy.

These therapeutics have several factors in common: they are both based on large DNA viruses (T-Vec is a modified *Herpes simplex* virus [HSV],⁴ while Pexa-Vec is a modified vaccinia virus),⁵ both viruses have been attenuated through genetic engineering, such that viral replication is restricted to cells with a malignant phenotype, and both are armed with a therapeutic transgene (GM-CSF, in both cases).

It has been observed for many decades that microbes, especially bacteria, can selectively colonize solid tumors for many decades; the first case reports of viral infections or immunizations leading to tumor responses were recorded more than a century ago.^{6,7} However, better understanding of tumor biology and the advent of genetic engineering

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were required before the first viral therapies designed to selectively replicate in tumor tissues could be produced.^{8,9} These vectors were initially designed to self-amplify selectively in the tumor, leading to viral mediated lysis of infected malignant cells and rapid spread throughout tumor tissue.¹⁰ The first oncolytic viruses to enter clinical testing were based on adenovirus serotype 5.^{11–13} Although the safety of the platform was clearly demonstrated, it became apparent that viral-mediated lysis of the tumor alone was insufficient for complete tumor eradication. As such, the potential for arming these vectors with additional therapeutic power, through expression of selected transgenes, was examined.^{14,15} Because directly-infected tumor cells will inevitably be destroyed by viral replication or through immune targeting, it was necessary to employ transgenes that might induce a bystander effect, such that, surrounding uninfected cancer cells are also destroyed. This was realized through the incorporation of human GM-CSF expression in the second generation of oncolytic vectors, which are currently showing promise in randomized clinical testing.

Current challenges

Despite the clinical promise being shown with these armed oncolytic vectors, there is still significant optimization required and problems that need to be overcome before oncolytic viruses can realize their true potential.

The manufacture of these agents remains complex and costly, with considerable requirements for extensive release testing, while the possibility of acquisition of genetic mutations means that careful monitoring of vector sequence is needed. However, it is likely that, as the platform develops, these procedures will become more streamlined, and so, costs will become manageable, although the biological nature of these agents means that they will never be inexpensive.

A more significant limitation to the effective therapeutic application of oncolytic viruses is in the systemic delivery and intratumoral spread of the agents.^{16,17} The use of viral backbones (such as HSV and vaccinia), which have evolved to spread within the vasculature, has allowed some demonstration of systemic delivery in a clinical setting.^{18–20} However, efficient delivery to all tumors (and to micrometastases that may not be exposed to the vascular system) has not yet been achieved. Targeted delivery approaches involving coated viral particles²¹ or cell-based delivery vehicles^{22,23} have helped to overcome these limitations in preclinical models. However, even with the most efficient viral delivery systems, the raising of antiviral immunity, after an initial round of therapy, means that repeat treatments remains a particular concern.

An alternative approach to achieving successful targeting of residual tumor cells is incorporation of additional tumor-killing mechanisms into oncolytic viral vectors, through transgene expression, or through careful and logical use of therapeutic combinations.

In addition, the use of larger viral vectors typically allows greater potential for engineering of tumor-targeting mutations, and provides greater cloning capacity for transgene expression. However, larger viruses may also have a reduced capacity to spread within the tumor. Viral spread can be severely restricted by a combination of large, inert viral particles, the presence of many non-cancer cell types within the tumor (which may be resistant to oncolytic viral infection), regions of necrosis, and the involvement of extracellular matrix. In addition, it has been demonstrated that the presence of tumor vasculature is necessary for oncolytic vaccinia delivery,²⁴ demonstrating further limitations with trying to deliver large viruses to micrometastases not exposed to the vascular system.

Several approaches that might enhance delivery include the use of smaller viral vectors, which have demonstrated increased capacity to spread within the tumor,^{25,26} as well as through the expression of transgenes, whose products can specifically target the tumor components that restrict viral spread.²⁷

It is noteworthy also that many different oncolytic viruses have demonstrated a capacity to synergize with multiple other therapies, including traditional radiotherapy and chemotherapy, as well as targeted therapies and immunotherapies.^{28–35} A clearer understanding of the mechanisms behind these synergies is needed, to help develop and optimize the clinical application of these vectors.

The need for further optimization applies also to the vectors themselves, where it is likely that increased potency ultimately will be achieved through logical expression of combinations of multiple therapeutic transgenes, ideally with careful regulation of the level and kinetics of transgene expression, and with transgene combinations matched to certain cancers or cancer phenotypes. This will be the primary focus of this review.

Arming oncolytic viruses

The high degree of selectivity demonstrated with many oncolytic viruses means that it is unlikely significant advances will be made through further manipulation of the viral backbones. Instead, the primary areas of focus of future development and enhancement of therapeutic activity are likely to center on 1) enhancing delivery, 2) careful design

of combination therapies, and 3) expression of additional therapeutic transgenes from the viral backbones.

Transgene expression may be used to enhance the natural antitumor mechanisms employed by oncolytic viruses, including improving replication and spread within the tumor, directing the antitumor immunity induced by viral infection, and enhancing the effects of viral-mediated vascular collapse within the tumor microenvironment. Alternatively, transgenes may be incorporated to provide the viral vectors with additional tumor-killing mechanisms, for example, through targeting of nontumor cells within the tumor microenvironment, or the production of anti-angiogenic effects. Finally, transgenes may be incorporated specifically to better allow oncolytic viral therapies to synergize in combination with other therapies. The transgenes chosen to arm oncolytic viruses can therefore be considered to fall into several distinct groups:

Immune activators

Because an infected cell will ultimately be destroyed as a result of viral replication, the most effective therapeutic transgenes must incorporate a bystander effect. This requires either the direct release of the expressed protein, or the release of some other factor from the infected cell, as a result of transgene expression. These secreted factors must be capable of mediating destruction of surrounding tumor cells. For this reason, cytokines have been extensively and successfully incorporated in arming many oncolytic viruses. Ever since Dranoff first reported on the antitumor effect of GM-CSF expression,^{4,36} this cytokine has held particular interest, and the benefits of its expression can be clearly seen with current, clinical oncolytic virus strains.¹⁸ However, many other cytokines have displayed therapeutic benefit, especially in preclinical models, including interleukin 2 (IL2),³⁷ tumor necrosis factor alpha (TNF α),³⁸ type I interferon (IFN),^{39,40} etc. The potential of several of these cytokines has been confirmed through their use in therapeutic treatment of cancer, as recombinant cytokines. However, one of the key advantages of expressing the cytokines from an oncolytic virus, rather than systemically applying recombinant proteins, is that production is primarily from within the tumor; thus, there is a far greater concentration of cytokine within the tumor, and less systemic toxicity.

However, because the immune response is a double-edged sword, acting both as an additional therapeutic mechanism of oncolytic viral activity, and acting to clear the therapeutic,³⁸ there is a fine balance to be met when looking to enhance immune activation. This is seen with the fact that many cytok-

ines appear to enhance overall therapeutic activity, despite reducing oncolytic capacity,⁴¹ with premature clearance of the oncolytic vector and reduced levels of initial tumor colonization and replication being common side effects of cytokine expression. Several approaches have been attempted to overcome this, including regulating transgene expression.⁴¹

Alternatively, more subtle approaches to manipulating viral interaction with the host immune response can result in less profound limitations on viral oncolytic activity, such as through incorporation of CpG dinucleotide regions into viral DNA, to increase Toll-like receptor 9 (TLR9) activation.⁴²

Finally, another approach is to alter the targeting, rather than the activation, of the immune response, such as through expression of chemokines, such as CCL5, CCL19 or CXCL11 from the oncolytic vectors.⁴³ This approach has been reported to enhance therapeutic activity in pre-clinical models, especially when used in combination with other immunotherapies, such as vaccines or adoptive T-cell transfer. However, chemokine production does not appear to deleteriously effect viral replication in most of the models examined.^{43,44}

Prodrug converting enzymes

A prodrug is a nontoxic, small molecule that can be converted to a toxic product through the action of a specific enzyme.⁴⁵⁻⁴⁷ If such prodrug converting enzymes are expressed from an oncolytic virus, their genomic copy number and expression can be selectively amplified from within the tumor. Subsequent systemic addition of the prodrug would then result in production of the toxic drug exclusively within the tumor microenvironment, providing a powerful bystander effect to viral infection, with little systemic toxicity.^{48,49} This approach has been pioneered with the use of HSV thymidine kinase (TK) to convert the prodrug ganciclovir,⁵⁰ and cytosine deaminase to convert 5-fluorocytosine to 5-fluorouracil.⁵¹ Other examples include carboxypeptidase-G2⁵² and carboxylesterases.⁵³ Although they are attractive, there are several drawbacks, which have limited successful clinical translation to date. In particular: 1) the requirement for addition of a second molecule creates a more complex therapy; thus, additional manufacturing is required, and additional toxicity- and dose-finding trials are often needed; 2) the timing of addition of the prodrug is critical, as the concentration of converted drug will be highest in and around infected cells, so that the majority of infected cells are destroyed, and so that oncolytic activity is rapidly curtailed (which may then have additional negative impacts on secondary oncolytic viral mechanisms of action, such as immune activation; for this

reason, prodrug converting enzymes have been proposed to be used as suicide genes, providing a mechanism to shut off viral replication if there are any toxic side effects, or if an uncontrolled infection is produced);⁵⁴ and finally, 3) although preclinical data has demonstrated the potential for prodrug converting enzyme/prodrug combinations to dramatically increase the destruction of large primary tumors, they provide no direct additional benefit in the clearance of micrometastases, which are largely uninfected by the virus after initial delivery (unlike immune-enhancing approaches that can raise antitumor adaptive immunity), which has the potential to assist in the clearance of minimal disease, and provide long-term immune surveillance to prevent relapse.⁵⁵

Targeting of tumor microenvironment

The tumor microenvironment is highly heterogeneous, involving many tumor-associated stromal cells (including tumor fibroblasts, endothelial cells, and lymphocyte populations), as well as extracellular matrix, which can act as barriers to oncolytic virus spread and dissemination, or can act to maintain an environment that encourages tumor regrowth and limits viral replication. Because most oncolytic viruses are primarily designed to selectively replicate in malignant cells, these critical, other components of the tumor are often spared, and may mediate relapse.

One commonly used approach to help overcome these components involves the expression and secretion of selected proteases to help break down the extracellular matrix, allowing the virus to spread and disseminate more effectively within the tumor. This was first demonstrated with pioneering work with expressing relaxin from oncolytic adenovirus.²⁷

Alternatively, some key phenotypic properties of the cancer itself apply also to some tumor stromal cells (such as enhanced proliferation of tumor associated fibroblasts and endothelial cells); thus, some oncolytic agents that target these properties in the cancer will also selectively replicate in tumor-associated stromal cells.⁵⁶ However, these effects might be further enhanced with transgene expression. One of the most common approaches has been the use of anti-angiogenic transgenes to target endothelial cells – an approach that is particularly effective in combination with some viral therapies that are known to induce an early and tumor-specific vascular collapse (such as vesicular stomatitis virus and vaccinia virus).^{56,57} The most commonly used transgenes in this respect involve expression and secretion of the extracellular binding domains of vascular endothelial growth factor (VEGF) receptors, such as Flk1.⁵⁸ These can act as competitive inhibitors of the growth factors, and so

provide a profound anti-angiogenic effect, again amplified by their selective and high-level expression within the tumor.^{59,60} Alternatively, anti-VEGF antibodies have also been expressed directly from an oncolytic vaccinia virus⁶¹ (see below).

Receptor or membrane transporters

Another approach, which was initially pioneered for imaging purposes, but can have additional therapeutic implications, is the expression of cell surface receptors or plasma membrane transporters. These can be targeted with ligands that selectively bind, or are taken up by, cells expressing them. The expression of such receptors/transporters from oncolytic viruses can be utilized in two ways. Firstly, the attachment of imaging contrast agents to the ligands (such as those used in magnetic resonance or radiological imaging) can permit clinical imaging of the location and levels of viral gene expression.^{62,63} This can be of benefit in more rapidly advancing the early clinical development of novel therapies, through identifying the reasons for treatment success or failure, and has also the potential for assisting in early assessment of treatment response, even with an approved therapy. However, as an alternative approach, if a radioisotope-producing, short-range, highly ionizing radiation is attached to the ligand, then a profound and localized destruction of surrounding tissue can be achieved.⁶⁴

Arming oncolytic viruses: the example of poxviruses

Poxviruses, such as vaccinia virus, have been extensively used as oncolytic agents. They are large, double-stranded DNA viruses that show promiscuous infection of mammalian cells.^{65,66} The enveloped viral nuclear capsid measures 140–220 nm in diameter, and 220–450 nm in length. Poxviruses are distinct from other DNA viruses, as they replicate in the cytoplasm. Thus, they do not have the potential to mutate a host cell through integration of viral DNA into the host cell genome. In order to do this, poxviruses carry the enzymes necessary for genome replication, and are able to initiate transcription of viral DNA soon after entry into the cell, leading to rapid viral replication and spread. There are several different forms of the virus, including the intracellular mature virus (IMV), cell-associated enveloped virus (CEV), and extracellular enveloped virus (EEV). The predominant form of the virus is IMV, which is released upon lysis of the cell. However, a fraction of the IMV leaves the cytoplasmic viral factory on microtubules, prior to cell lysis, and becomes wrapped in a double membrane, derived from the trans-Golgi network. This virus moves to the cell surface and either

remains attached to the cell surface (CEV) or is released (EEV). The CEV and EEV forms are relatively resistant to complement- and antibody-mediated targeting (due to the host cell-derived membrane shroud), and so are key factors in the dissemination of the virus.

Poxvirus has been used extensively in humans as a vaccine to eradicate smallpox. Hence, there are plenty of data available to define any possible adverse effects and contraindications to its use. Also, its use in multiple clinical trials, especially for cancers, has not resulted in any serious complications. Poxviruses are particularly promising as oncolytics, as they 1) can be delivered systemically; 2) produce no known disease in humans, and have demonstrated safety profiles in the clinic; 3) have a large cloning capacity for expression of transgenes, with standard protocols for construction of recombinants; 4) can spread rapidly and with a lytic infection; 5) induce a robust immune response; and 6) do not integrate into the host genome and do not display latency.

Several engineered strains of vaccinia have been shown to act as promising oncolytic agents. For example, double-deleted vaccinia virus (vvDD) has been used most extensively in preclinical studies. This strain carries deletions in the viral TK and viral growth factor (VGF) genes.⁶⁷ VGF is a secreted growth factor homolog that binds the endothelial growth factor (EGF) receptor, inducing proliferation in surrounding cells, but is redundant in more than 80 percent of cancers, where mutations in the EGF-R signaling pathway mean that it is unregulated.¹⁸ Since TK is upregulated in cancer cells, the viral TK gene is also redundant, and deletion of this gene results in tumor-selective viral replication.⁶⁸ The vvDD virus has displayed selectivity and antitumor potential in preclinical studies,^{18,67} and is currently undergoing Phase I testing.

However, the vaccinia strain used most extensively in clinical trials has been JX-594, which is based on the Wyeth backbone, with a single deletion in the viral TK gene, and with the expression of GM-CSF.⁶⁹ In Phase I studies of intratumoral delivery in melanoma⁷⁰ and hepatocellular carcinoma,⁷¹ responses were reported at multiple doses. Furthermore, systemic delivery was also demonstrated after intravenous delivery.¹⁹ In a Phase II trial in hepatocellular cancer patients, it was shown that patients receiving the higher dose had significantly improved survival.² In a subgroup of patients who had failed sorafenib therapy (and so, had a survival expectancy of 2–4 months), JX-594 therapy had a median survival of 13.6 months, and two of the subjects have continued living for longer than 2 years.

A further, clinical oncolytic vaccinia virus has also been reported: GLV-1h153 (and its derivatives, such as GLV-1h68). This virus is based on the Lister backbone, with deletions in viral TK and hemagglutinin genes, and with additional intragenic insertions in the F14.5L locus. This virus has also displayed tumor selectivity and decreased virulence to normal host cells, and is capable of providing therapeutic benefits preclinically, in a range of cancer models.^{72–76} GLV-1h68 has entered Phase I clinical testing, although no clinical results have been reported to date.

In addition to these clinical strains, a variety of further oncolytic vaccinia vectors have been described in preclinical studies. These include a strain with deletion of the viral type I IFN binding protein (B18R), which selectively replicates in cells with loss of an IFN response phenotype,³⁹ viruses with enhanced spread, to treat metastatic tumors,⁷⁷ and a virus with deletions in the viral serpins, which targets cells with a loss of apoptotic potential.⁷⁸ Thus, different vaccinia strains have the potential to target different cancers through different phenotypic properties.

In addition to the growing panel of tumor-targeting mutations that have been reported in vaccinia virus backgrounds and that target different phenotypic properties of cancer, a large number of therapeutic transgenes have been expressed from vaccinia. One advantage of this virus as an oncolytic backbone is its ability to integrate more than 25 kilobases of foreign DNA into the viral genome, without affecting viral packaging capacity.⁷⁹ As such, a wide variety of transgenes and combinations of transgenes have been reported. These include cytokines (such as type I IFN,³⁹ TNF α ,³⁸ IL2,⁴¹ and GM-CSF) and chemokines (such as CCL19⁴⁴ and CCR5),⁴³ together demonstrating the potential for using vaccinia strains as immunotherapeutic agents, in addition to their oncolytic power.

It is also known that vaccinia can induce an antivascular effect in the tumor, after initial delivery.⁵⁶ Therefore, transgenes that target the process of angiogenesis are attractive options. As such, oncolytic vaccinia has been cloned to express VEGF-targeting transgenes (including a solubilized Flk1,⁶⁰ an endostatin-angiostatin fusion,⁸⁰ a CXCR4 antagonist,⁸¹ and an anti-VEGF antibody).⁶¹ The use of imaging (or theranostic) reporters has also been reported, including hSSTR2⁸² and the sodium iodine symporter.⁸³ Finally, other novel transgenes expressed from oncolytic vaccinia strains have included erythropoietin, to help alleviate cancer-related anemia⁸⁴ (and so permit higher doses of radiotherapy and chemotherapy), melanin, to assist in laser-induced thermotherapy,⁸⁵ and BMP-4, to target cancer stem cells in glioblastoma.⁸⁶

Future considerations for arming viruses

Enhancing the action of other therapeutics

It has been reported in many preclinical and clinical studies that different oncolytic viral therapies have the potential to combine synergistically with traditional radiotherapies and chemotherapies, as well as targeted therapies (such as TK inhibitors and monoclonal antibodies). One reason for this is that oncolytic viruses target and destroy tumor cells through different mechanisms than these other therapies, leading to robust therapeutic combinations.³² Furthermore, it is apparent that oncolytic viruses have the potential to sensitize previously-resistant tumors to some therapeutics. Although the mechanisms mediating these effects are often unknown or poorly defined, it is evident that they might be further enhanced through transgene expression from the oncolytic agent. This is currently being investigated by various groups, and represents a research area of great promise.

Expression of antibodies

An area that holds great promise for future development is that of expression of specific antibodies from oncolytic agents. As approaches have become available to express entire and functional antibodies in a single peptide, the use of antibody transgenes has become more widespread. This has the potential to allow more direct and specific targeting of both tumor and nontumor cells within the tumor.

Regulating transgene expression

Consideration must also be given to the promoters used to drive transgene expression, including the strength and timing of expression, with stronger late promoters (usually tied to successful viral replication in susceptible cells) most often incorporated. In some cases, the use of tissue- or tumor-specific promoters has also been reported. However, this is typically redundant, if the viral vector itself is highly selective. An ideal scenario would allow for exogenous and specific control of transgene function. A variety of approaches have been reported in preclinical models.⁴¹ Although these approaches typically require the addition of an exogenous small molecule (which acts as a regulator of protein expression, mRNA stability, protein stability or function, etc), thus adding an additional level of complexity, the resultant therapeutic benefits can be profound. This has been demonstrated in the scenario of cytokine transgene expression, which typically acts to limit oncolytic activity through

early immune activation. The ability to delay the functional production of cytokine, until such time as the early oncolytic phase of therapeutic activity has neared completion and the vector is beginning to switch to an immunotherapeutic stage of activity, can provide significant therapeutic benefits. Such approaches might ultimately be extended to involve independent regulation of multiple transgenes, whose mechanisms of action would be most beneficial at different phases of oncolytic virus activity. This would allow greater synergy between transgene and viral activity, and potentially allow activation of a transgene's function to coincide with addition of a secondary therapeutic.

Multiple transgenes

Indeed, as the benefits of different individual transgenes are identified and defined in isolation, it is likely that the next logical step will be to express multiple transgenes from a single oncolytic vector, thus allowing additional therapeutic benefit. Obviously, such combinations will have to be carefully monitored, to prevent antagonistic activity or increased toxicity. But, ultimately, a "Swiss army knife" approach, with multimechanistic viral therapies expressing additional transgene combinations in an ordered and regulated fashion, is likely to provide the power needed to overcome tumor heterogeneity, and so prevent relapse. This may allow us finally to begin to produce oncolytic viral therapies that are capable of inducing regular complete responses in a variety of different cancer types.

Conclusion

It has become apparent that oncolytic viruses, such as strains of vaccinia virus, have the potential to be delivered safely and systemically to cancers in the clinic, leading to tumor-selective replication and antitumor effects. However, it has also become clear that the viruses alone are usually insufficient to eradicate entire tumors, including metastases; thus, additional boosting of existing antitumor effects (ie, replication and spread, immune activation, and antivascular activity) or the addition of further mechanisms of action is needed. Although this can be achieved in some situations through combinations with other therapeutics, a simpler approach has been to express therapeutic transgenes from the virus directly. This has distinct advantages (such as, increased concentration of the therapeutic transgene product within the tumor, the potential for controlling transgene product levels, and the capacity for expressing multiple transgenes) and, if harnessed correctly, will be expected to result in the production of powerful novel therapies.

Disclosure

Dr Thorne is a founder of and shareholder in Jennerex Biotherapeutics Inc., (San Francisco, CA, USA). Dr Thorne holds several patents relating to oncolytic viral therapy. The authors report no other conflicts of interest in this work.

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