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Oncolytic virotherapy: Challenges and solutions

Nasser Hashemi Goradel[a], Alexander T. Baker[b], Arash Arashkia[c], Nasim Ebrahimi[d], Sajjad Ghorghanlu[a], Babak Negahdari[a,*]

[a] Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran
[b] Division of Cancer and Genetics, School of Medicine, Cardiff University, Cardiff, United Kingdom
[c] Department of Molecular Virology, Pasteur Institute of Iran, Tehran, Islamic Republic of Iran
[d] Division of Genetics, Department cell and molecular Biology & microbiology, Faculty of Science and Technology, University of Isfahan, Isfahan, Islamic Republic of Iran

ABSTRACT

Viruses as cancer therapies have attracted attention since the 19th century. Scientists observation that viruses can preferentially lyse cancer cells rather than healthy cells, created the field of oncolytic virology. Like other therapeutic strategies, oncolytic virotherapy has challenges, such as penetration into tumor bulk, anti-viral immune responses, off-target infection, adverse conditions in the tumor microenvironment, and the lack of specific predictive and therapeutic biomarkers. Whilst much progress has been made, as highlighted by the first Food and Drug Administration approval of an oncolytic virus talimogene laherparepvec (T-VEC) in 2015, addressing these issues remains a significant hurdle. Here we discuss different types of oncolytic viruses, their application in clinical trials, and finally challenges faced by the field of oncolytic virotherapy and strategies to overcome them.

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* Correspondence to: Babak Negahdari, Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Islamic Republic of Iran.
E-mail address: negahdari_md@yahoo.com (B. Negahdari).

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Introduction

Despite significant advances in diagnostic and therapeutic choices, including surgery, chemotherapy, and immunotherapy, cancer is still one of the most significant causes of morbidity and mortality globally, with a heavy socio-economic burden.[1] Virotherapy has been studied for the treatment of cancer since the 19th century, but, because of genetic engineering hurdles and safety concerns, it saw little development until the last 2 decades.[2,3] In principle the field of oncolytic virotherapy aims to engineer viral genomes to replicate selectively within cancer cells, thereby lysing them without affecting normal cells.[4] Oncolytic virotherapy is now considered a type of cancer immunotherapy owing to induction of immune responses toward the viral epitopes in infected tumor cells as well as virus-induced tumor cell death.[5] The US Food and Drug Administration approved T-VEC (Imlygic) for the treatment of melanoma as the first oncolytic virus (OV) in 2015.[6] T-VEC is a modified form of herpes simplex type 1 virus (HSV-1) in which deletion of specific genes leads to selective replication within cancer cells and increased presentation of viral and tumor antigens.[7] To promote immune responses, human granulocyte macrophage-colony stimulating factor (GM-CSF) was inserted into the HSV-1 genome.[8] GM-CSF is an immunomodulatory cytokine that promotes the development and prolongation of humoral and cellular immunity.[9] RIGVIR and Oncorine have also been approved in other countries as OVs for cancer therapy. RIGVIR, or enteric cytopathic human orphan type 7, is a no-genetically engineered virus strain from the *Picornaviridae* family used as a treatment for melanoma,[10] approved in Latvia in 2004.[11] State Food and Drug Administration of China approved Oncorine (H101) for head and neck squamous cell carcinoma in 2005. Oncorine is a genetically modified type 5 human adenovirus (HAdV-C5) in which the E1B-55KD and E3 regions were deleted to induce selective replication in p53 defective cells and increase safety.[12] In this paper, we will focus on OVs, their structure, and application in clinical trials, and finally oncolytic virotherapy challenges and solutions to overcome them.

Mechanisms of action of OVs

Direct cell lysis and induction of anti-tumor immunity are 2 primary mechanisms by which OVs destroy cancer.[13] The first mechanism leverages the virus's natural life cycle. Infection and replication of virus in the tumor causes cell lysis or apoptosis. Following replication and lysing the cell, viral particles repeat the lytic cycle by infecting neighboring cells, making the therapy self-amplifying at the point of need.[14] This cycle continues until immune responses attenuate virus replication, or susceptible host cells deplete.[15] These immune responses can also attack the tumor, with the breaking of immunological tolerance of cancer being recognized as a crucial aspect of OVs mode of action.[16,17]

Uninfected cells can also be affected by the OV, for patient benefit. It has been shown that oncolytic vaccinia virus disrupts tumor angiogenesis, reduces blood flow to tumor cells, and finally leads to hypoxia, by affecting vascular cells.[18,19] Angiogenesis is a hallmark of cancer, supplying nutrients and oxygen to tumor cells to increase tumor growth.[20,21]

In addition to the natural ability of OVs in tumor cell lysis, further modifications can increase their lytic ability. For example, herpes simplex virus-1 thymidine kinase (HSV-1 TK) expressing adenovirus (Ad-OC-HSV-TK) in which expression of HSV-1 TK is under the osteocalcin promoter have been developed to target bone tumors.[22] HSV-1 TK is able to activate thymidine analogs such as ganciclovir, a competitive inhibitor of deoxyguanosine, by converting them into monophosphates. Monophosphates terminate DNA synthesis and subsequently cause cell death by incorporation into the DNA of replicating cells.[23] Cytosine deaminase (CD) which transforms 5-fluorocytosine into the highly cytotoxic 5-fluorouracil is another suicide gene that has been used.[24] Insertion of *adp* gene into the adenovirus genome also enhances lytic activity. ADP encodes adenovirus death protein (ADP) which is necessary for late-stage species C adenoviruses infection and releasing viral particles.[25]

The second mechanism of action of OVs is the enhancement of immune responses (Fig. 1). Following infection of tumor cells with OVs, cell death, and releasing tumor-associated antigens, tumor-specific immune responses increase which leads to the elimination of distant and uninfected tumor cells.[26] Tumor cell lysis also leads to release of cytokines (such as type I interferons [IFNs], IFNγ, tumor necrosis factor-α, interleukin-12), viral pathogen-associated molecular patterns, and additional cellular DAMPs danger-associated molecular pattern signals such as heat shock proteins (HSPs), calreticulin, uric acid and ATP which enhance immune responses.[27] OVs have been engineered to further enhance immune responses. In this approach, embedding an immune stimulatory molecule into OV genomes alters the immunosuppressive tumor microenvironment. GM-CSF is the most broadly utilized example and has been inserted into OV genomes as an immune stimulatory molecule with the aim of maturation and recruiting of antigen-presenting cells (APCs), especially dendritic cells, and induction of tumor antigen-specific T cells and natural killer (NK) cells.[13] To increase intracellular antigen delivery to the proteasome, and antigen presentation, Li et al modified the oncolytic adenovirus genome to overexpress the HSP70 protein. They showed that following the modified oncolytic adenovirus administration the numbers of NK cells, CD4+ and CD8+ T cells were elevated.[28] Due to expression of HSP receptor on the APCs, including CD91 and LOX-1, HSP70 enhances tumor antigen delivery to APCs.[29]

Oncolytic viruses

Various viruses have been used as candidates for lysis of cancer cells. Table 1 lists some ongoing clinical trials in the field of oncolytic virotherapy.

Adenovirus

Adenoviruses (Ads) are nonenveloped viruses with double-stranded linear DNA genomes and an icosahedral capsid. Human Ads are classified into 7 species (A to G) based on their DNA homology, oncogenic, hemagglutination, and serum neutralization properties, though there is some controversy surrounding the correct way to classify adenoviruses.[30,31] Depending upon the classification method used there are between 57 and >103 adenovirus types, (http://hadvwg.gmu.edu/), however, HAdV-C5 remains the most commonly studied adenovirus in vaccine and gene therapy.[32] Though the success of the Ad26.ZEBOV vaccine in clinical trials against Ebola virus has created a resurgence in interest in HAdV-D26 as well.[33,34]

Two general approaches have been employed to create cancer selectivity of oncolytic adenoviruses.[35] Small deletions in the essential adenoviral genes[36] is the first approach. Small deletions in E1B-55K and E1A genes lead to selective replication of Ads in p53 and retinoblastoma (pRb) mutated cancer cells, respectively.[35] The pioneer in the field of oncolytic adenovirus was ONYX-015 which is defective for E1B-55K gene as well as *14.7k* genes in the E3B and *rid* genes. Oncorine (H101), the first OV approved by a regulatory agency for clinical use, has a similar structure to ONYX-015.[36] In addition to deletion in the E1B encoding gene, Oncorine also carries a partial deletion in the E3 region.[37] Deletion in the E1A region has also been performed to construct oncolytic Ads. Since E1A binds to pRb, deletion of 24-bp (Delta24) in E1A leads to E2F releasing and finally the viral replication in tumor cells.[38] The rationale behind this mutation was based on the virus replication following induction of S-phase in the host cell cycle due to binding of E1A protein to pRb. Due to an intact G1/S checkpoint in normal cells, replication of E1A-mutated adenoviruses is restricted in normal cells. Since almost all cancer cells carry mutations in the Rb pathway, Delta24 viruses are able to replicate in cancer cells.[39] In the second approach, tumor selectivity of Ads is related to insertion of tissue-specific promoters[40] in which replacement of the E1A promoter by tumor-specific ones, such as the prostate-specific antigen,[41] alpha (α)-fetoprotein,[42] or the human telomerase reverse transcriptase[43,44] have been

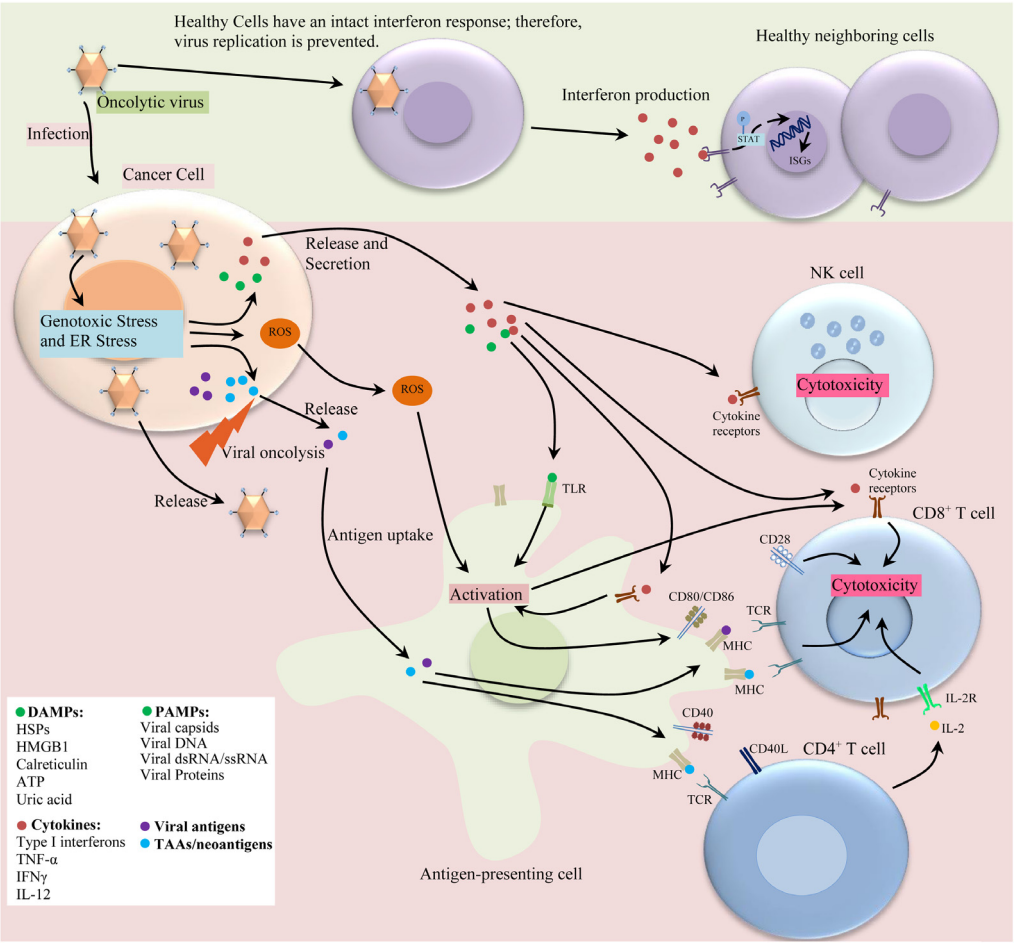

Fig. 1. The stimulation of local and systemic antitumor immunity by oncolytic viruses.

Table 1
Some ongoing clinical trials using oncolytic viruses.

Virus type	Virus name	Cancer type	Clinical phase	Combinational therapy	Identifier
Ad5	OBP-301	Solid tumors	I	Pembrolizumab	NCT03172819
	OBP-301	Esophageal	I	Radiotherapy	NCT03213054
	OBP-301	HCC	I	-	NCT02293850
	DNX-2401	Glioma	I	Surgery	NCT03896568
	DNX-2401	Glioma	I	-	NCT03178032
	AdVince	NETs	I/IIa	-	NCT02749331
	VCN-01	Retinoblastoma	I	-	NCT03284268
	Ad5-yCD/ mutTKSR39rep-hIL12	PaC	I	5-FC; chemotherapy	NCT03281382
Ad5/35	LOAd703	PaC	I/II	Gemcitabine; Nab-paclitaxel	NCT02705196
Ad5/3	ONCOS-102	CRC; OC	I/II	Durvalumab	NCT02963831
	ONCOS-102	Melanoma	I	CP; Pembrolizumab	NCT03003676
Ad3/11p	EnAd	OC	I	-	NCT02028117
HSV-1	T-VEC	Sarcoma	II	Pembrolizumab	NCT03069378
	T-VEC	Breast cancer	I/II	Paclitaxel	NCT02779855
	T-VEC	Melanoma	II	Pembrolizumab	NCT02965716
	OrienX010	Melanoma	Ic	-	NCT03048253
	TBI-1401/HF10	Melanoma	II	Nivolumab	NCT03259425
	TBI-1401/HF10	PaC	I	Gemcitabine; Nab-paclitaxel	NCT03252808
Vaccinia virus	GL-ONC1	OC; FTC; PC	Ib/II	-	NCT02759588
	TG6002	CNS cancers	I/II	5-FC	NCT03294486
	Pexa-VEC/TG6006	HCC	I/IIa	Nivolumab	NCT03071094
	Pexa-VEC/TG6006	Solid tumors	I	Ipilimumab	NCT02977156
Measle virus	MV-NIS	UCC	I	Surgery	NCT03171493
	MV-NIS	Multiple myeloma	II	CP	NCT02192775
CAV21	CAVATAK	NSCLC	I	Pembrolizumab	NCT02824965
PV1	PVSRIPO	Glioma	II	Lomustine	NCT02986178
VSV	VSV-IFNβ-NIS	Solid tumors	I	-	NCT02923466

5-FC, 5-fluorouracil; CP, cyclophosphamide; CRC, colorectal cancer; FTC, fallopian tube cancer; HCC, hepatocellular carcinoma; NETs, neuroendocrine tumors; NSCLC, non–small cell lung cancer; OC, ovarian cancer; PaC, pancreatic cancer; PC, peritoneal carcinomatosis; UCC, urothelial carcinoma.

performed. This renders the OV capable of replication, but only in cells in which these promoters are stimulated: cancer cells.

Herpes simplex virus

Herpes simplex virus (HSV), especially HSV type 1 (HSV-1), is one of the most widely studied DNA viruses, as an OV.[45] HSV has a large genome with parts that are non-essential for replication, leaving space to add engineered transgenes without limiting the packaging efficiency of the virus. Combined with its replication in the nucleus without insertional mutagenesis, HSV-1 makes an attractive candidate for oncolytic virotherapy.[27] T-VEC (Talimogene laherparepvec, or Imlygic) is an oncolytic HSV-1 containing 2 deletions in its genome: ICP34.5 and ICP47.[46] Deletion of ICP34.5, which encodes the neurovirulence factor, prevents the virus replication in neurons without affecting its replication in other cells, especially tumor cells.[47,48] ICP34.5 is essential for blocking the host antiviral innate immunity pathway protein kinase R-Interferon (PKR-IFN). Most tumor cells are deficient in the PKR pathway,[49] making ICP34.5 deleted HSV more selective for cancer cells.[50] In place of ICP34.5, T-VEC contains GM-CSF which promotes dendritic cell maturation and enhances immune responses to tumor cells.[46] Deletion of ICP47, which encodes the inhibitor of antigen presentation, results in tumor-associated

antigens and viral antigen access to major histocompatibility complex (MHC) class I complexes, and promotes immune responses against tumor cells.[51] Early activation of herpes unique short 11 gene is the last alteration in T-VEC which increases virus half-life and cytolytic effects through blocking PKR phosphorylation.[52] Preclinical studies demonstrated tumor lysis potential of T-VEC, especially in melanoma and pancreatic cancer models.[53] Following favorable phase I and II clinical trial endpoints a randomized phase III study was evaluated in 436 patients with late-stage melanoma.[8] Twenty-four doses of intratumoral injection of T-VEC enhanced overall survival and objective response rate compared to subcutaneous administration of GM-CSF.[46]

Vaccinia virus

Vaccinia virus is a double-strand (ds) DNA virus which replicates in the cytoplasm of host cells. It is ability to infect a wide range of cells, strong tropism for tumor cells, and ability to carry large foreign DNA sequences attracts attention as an OV.[54] To increase selective replication and lytic capabilities of the vaccinia virus, some modifications have been employed, including the deletion of viral thymidine kinase (TK), vaccinia type I IFN-binding protein (B18R), or vaccinia growth factor (VGF).[55,56] Vaccinia virus preferentially replicates in metabolically active cells in which nucleotide levels are high, such as dividing tumor cells. The deletion of vaccinia TK gene results in viral replication independent of TK expression of the host cell.[57] It has been shown that the highest level of TK, which is regulated by E2F transcription factor, during the cell cycle of normal cells is in the S-phase, but its expression remains high throughout the cell cycle of cancer cells.[58] Therefore, vaccinia virus dependence on host cell nucleotides and compensation of TK activity, TK-deleted vaccinia predominantly replicates in tumor cells.[57] High avidity of B18R to type I IFNs leads to blockade of type I IFN signaling and subsequent infection of healthy cells with vaccinia virus.[59] So, by deletion of B18R, healthy cells become refractory to vaccinia virus infection due to intact type I IFN responses, while because of disruption of the type I IFN pathways in cancer cells, they are susceptible to infection with vaccinia virus and finally lysis.[27] VGF is an epidermal growth factor (EGF) analog that activates the RAS-MEK-ERK signaling pathway by binding to EGF receptor (EGFR) on the host cells.[60] Therefore, deletion of VGF leads to selective replication of the virus in cells with aberrant FGFR-RAS signaling, such as cancer cells.[27]

Another oncolytic vaccinia virus, GLV-1h68 (also called GL-ONC1 in clinical trials), was constructed by replacing viral TK, hemagglutinin, and F145L genes with 3 expression cassettes encoding β-galactosidase, β-glucuronidase, and Renilla luciferase/green fluorescence (RLuc-GFP) fusion, respectively.[61] A phase I clinical trial of intravenously administered GL-ONC1 in patients with head and neck carcinoma was performed between 2012-2014. Thirty months follow-up showed that administration of GL-ONC1 in combination with standard chemotherapy enhances overall survival and progression-free survival. The study also proved the safety of the virus for further investigations.[62] In addition to the therapeutic application and due to the expression of marker genes including Ruc-GFP, β-galactosidase, and β-glucuronidase, GL-ONC1 could be used for real-time monitoring of cell lysis and tumor treatment. Repetitive biopsies from patients treated with GL-ONC1 opens up the possibility of analyzing efficacy of tumor colonization, track viral replication, and oncolysis thresholds.[63,64]

JX-594 (Pexa-Vec) is another oncolytic vaccinia virus which has entered clinical trials (NCT01469611 and NCT00554372)[65,66] and disrupts tumor angiogenesis.[18] JX-594 carries 3 modifications on genome: insertion of GM-CSF encoding gene to induce systematic immune responses, deletion of TK gene to gain tumor selectivity, and the introduction of lac-Z gene under p7.5 promoter control.[67] Park et al demonstrated that intravenous administration of JX-594 has acceptable safety in patients with treatment-refractory colorectal cancer.[65] Another study proved the safety of intratumoral administration of JX-594.[67] It has been demonstrated that response rate to the OV is dose-dependent. A randomized clinical trial in patients with hepato-

cellular carcinoma showed that median survival in patients treated with the high-dose and the low-dose of JX-594 is 14.1 months and 6.7 months, respectively.[66]

Newcastle disease virus

Newcastle disease virus (NDV), which belongs to the *Paramyxoviridae* family, is an avian enveloped virus with non-segmented negative-stranded RNA.[68] Binding of viral haemagglutinin-neuraminidase (HN) protein to sialic acid-containing receptors on host cells triggers endocytosis of the virus.[69] Replication of NDV occurs in the cytoplasm, there has never been any observed recombination with the host genome.[27] Host defense mechanisms rapidly stop NDV replication, because it is highly sensitive to IFN-α and IFN-β.[69] Therefore, due to the weaker type I IFN responses, cancer cells are sensitive to NDV.[70] Hence, tumor cell deficiencies in anti-viral and apoptosis responses lead to selective replication of NDV in tumor cells, and finally oncolysis.[68]

Oncolytic activities of NDV are related to induction of cancer cell apoptosis and activation of the innate immune system through increased cytokine production (IL-12, GM-CSF, RANTES (regulated on activation, normal T cell expressed and secreted), and type I IFNs) improved antigen presentation.[71] It has been shown that HN protein of NDV acts as a potent antigen which enhances cytotoxic T lymphocytes responses against tumor cells.[72] Due to acceptable antitumor activity of NDV in preclinical studies, there are accumulating positive results in clinical trials.[73]

Coxsackievirus

Coxsackievirus, which belongs to the *Picornaviridae* family, is a nonenveloped virus with a single-stranded RNA genome. Like NDV, coxsackievirus replicates in the cytoplasm of host cells, attenuating the probability of insertional mutagenesis.[27] Another similarity between coxsackievirus and NDV, is that there is no requirement for genetic modification in order to achieve oncolytic activity.

The most widely used coxsackievirus, as an OV is coxsackievirus A21 (CVA21). CVA21 utilizes intracellular adhesion molecule-1 (ICAM-1) as a primary receptor, and decay-accelerating factor (DAF), as a coreceptor, for infecting host cells.[74,75] It has been shown that some solid tumors including melanoma, colon, breast, head and neck, endometrial, lung, and pancreatic cancer highly express the ICAM-1 and DAF receptors.[76-78] So, CVA21 may have a natural tropism toward cancer cells.

Coxsackievirus type B3 (CVB3) is another important oncolytic coxsackievirus. It has been shown that CVB3 also has a natural tropism to cancer cells, especially non–small-cell lung cancer cells due to overexpression of DAF and Coxsackie-Adenovirus Receptor (CAR). Anticancer activity of CVB3 depends on its ability to induce apoptosis.[79] PI3K/AKT signaling pathway accelerates CVB3 replication[79] and since this pathway is activated in most of the cancers,[80] CVB3 selectively replicates in these cancer cells.[79]

Measles virus

Measles virus (MeV), which belongs to the *Paramyxoviridae* family, is an enveloped virus with nonsegmented negative-stranded RNA. The RNA genome contains 6 genes that encode 8 viral proteins.[81] The virus has 3 receptors on host cells: signaling lymphocyte-activation molecule (SLAM/CD150), CD46, and nectin-4 (poliovirus-receptor-like-4, PVRL4).[82] MeV has a natural ability to infect tumor cells due to overexpression of CD46 in many cancer cells, though it is important to note that CD46 is expressed on all nucleated human cells so it is not tumor-selective.[81]

In addition to natural infectious mechanisms, some modifications have been performed to increase the measles oncolytic efficacy, such as generating a more cancer-specific tropism, arming

Table 2
Challenges and solutions of oncolytic viruses' application in cancer therapy.

Challenges	Solutions
1. Spread and penetration	1. Using junction opener 2. Using extracellular matrix (ECM) modulators 3. Induction of apoptosis
2. Passive targeting	1. Capsid modifications 2. Bispecific adapters
3. Immune responses	1. Stealthing 2. Using cellular carriers 3. Epigenetic alterations 4. Using anti-angiogenesis agents 5. Using alternative serotypes
4. Hypoxia	1. Using hypoxia-response element (HRE)-containing promoter
5. Choosing patients	1. Using reliable biomarkers

to improve cancer cell killing, and shielding to avoid antiviral immune responses which retard the therapeutic efficacy.[82,83] Antiviral immunity is an especially difficult hurdle for MeV based oncolytics due to the pervasive use of measles vaccine. Various strategies have been used to avoid off-target side effects and re-direct MeV specifically to cancer cells, including insertion of tumor-specific ligands, insertion of integrin-binding peptides, insertion of single-chain T-cell receptors, and modifications on the envelope fusion properties.[83] Moreover, defects in type 1 interferon responses are another reason for tumor selectivity of MeV.[83]

In Switzerland, a phase I clinical trial using the measles virus-Edmonston Zagreb (MV-EZ) strain was conducted. MV-EZ was administered intratumorally, following IFN-α pretreatment, in 5 patients with cutaneous T-cell lymphomas (CTCLs). MV-EZ treatment led to increased IFNγ production and reduction of the CD4/CD8 ratio, a measure in which a low ratio is associated with a better prognosis.[84] Higher levels of CD4 T lymphocytes are associated with lymph node metastasis and worse outcomes in cancer patients. On the other hand, high levels of CD8 T lymphocytes are associated with better outcomes. Thus, the CD4/CD8 ratio is a valuable indicator for effectiveness of cancer immunotherapy.[85,86] Further studies demonstrated the efficacy and safety of different oncolytic MeV strains.[87-89]

Other viruses

In addition to the aforementioned viruses, there are many others being investigated as oncolytic agents for cancer therapy, including poliovirus,[27] parvovirus,[90] Seneca Valley Virus,[91] vesicular stomatitis virus and the closely related Maraba virus,[92] and retroviruses.[93]

Challenges and solutions

Using OVs, like other strategies, has challenges. To overcome these challenges, scientists have designed numerous solutions (Table 2), discussed below.

Spread and penetration

In carcinomas, intracellular junctions of epithelial cells are barriers to the penetration of therapeutic agents with high molecular weight, leading to resistance.[94-96] Furthermore, phenotype

shifts during metastasis through epithelial-to-mesenchymal transition and then mesenchymal-to-epithelial transition tighten epithelial junctions and makes treatment difficult.[97,98] Epithelial junctions also act as a barrier to intracellular penetration of OVs, especially adenoviruses.[99] Some types of adenovirus including HAdV-B3, B14, and B14p may overcome the junctions by releasing penton-dodecahedra (Pt-Dd) in the early phase of infection and before cell lysis, whilst non-Pt-Dd producing adenoviruses generate an excess of the fiber protein at the same stage.[100,101] However, HAdV-C5 remains the most used serotype in constructing oncolytic adenovirus, and does not not release PtDd. Yumul et al modified Ad5Δ24 to produce epithelial junction opener (JO). They demonstrated that the JO expressing oncolytic Ads compared with unmodified viruses have a significantly stronger anti-tumor effect. They also reported that co-administration of JO with unmodified oncolytic adenoviruses attenuates tumor growth more than virus injection alone.[99] JO is an HAdV-B3 fiber knob-containing self-dimerizing recombinant protein in which C-terminal of fiber knob is engineered to increase its affinity to desmoglein 2 (DSG2).[102] DSG2 is a member of the cadherin protein family involved in cell-cell junctions[103] and is over-expressed in epithelial cancers.[102,104] Following JO binding to DSG2 the signaling pathway activates the matrix metalloproteinase ADAM17, leading to cleavage of the extracellular domain of DSG2 disassociation of epithelial cells.[99]

Extracellular matrix (ECM), which is composed of proteoglycans, hinders the dispersal of anticancer agents within the solid tumors.[105,106] To access cancer cells and lyse them, OVs need to navigate complex ECM[107]. To this end, pretreatment of the tumor with collagenase[108] or co-administration of hyaluronidase with oncolytic adenoviruses[109] led to enhanced spread of the virus. Moreover, engineering OVs to express matrix metalloproteinases-1 and -8 results in degradation of tumor-associated sulfated glycosaminoglycans which increased virus diffusion and therapeutic efficacy.[110]

As mentioned above, ECM and cellular junctions are major obstacles to OVs spread and penetration. In addition to proteases, cancer cell apoptosis also enhances viral spread. Nagano et al reported that induction of apoptosis by cytotoxic agents and caspase-8 activation led to increased intratumoral penetration and therefore the anti-tumor efficacy of oncolytic HSV. They interpreted that shrinkage or removal of apoptotic cancer cells produced channel-like structures and void spaces which facilitate the spread of oncolytic HSV.[111]

Passive targeting

Despite therapeutic benefits after direct administration of T-VEC against melanoma,[112] it has been observed that systematic application of the therapeutic virus is ineffective in the clinic owing to insufficient tumor cell tropism and transduction.[113] Thus, surface modifications of OVs have been applied to achieve improved tumor cell targeting.[114,115]

The initial interaction between HAdV-C5 fiber protein, and many other adenovirus species, and CAR on the target cells leads to interaction between the RGD (arginine-glycine-aspartic acid) motif on viral penton base protein and host cell integrin.[116,117] This interaction trickers clathrin-mediated endocytosis and viral entry into the cell.[118] Since the expression of CAR is down-regulated in many tumor cells,[119,120] modifications have been performed to increase tumor tropism of oncolytic Ads. One of the modifications to increase the infection efficiency of adenovirus is inserting an RGD motif into the HI loop of the adenovirus fiber knob domain.[121] It has been shown that RGD-modified oncolytic adenoviruses treatment in CAR-negative tumor models significantly increase infection efficiency and anti-tumor activity.[121,122] Another strategy for targeting oncolytic Ads is using different serotypes. Lenman et al have discovered that HAdV-G52 is able to bind to polysialic acid on target cells.[123] Due to the high-level expression of polysialic acid on cancer cells including lungs[124,125] and brain,[126,127] using HAdV-G52 as an OV could preferentially infect corresponding cancer types, though would have to be modified to remove any potential neurotropism. Also, antibody-based targeting by antibody single-chain variable fragments (scFvs) fusion with the capsid protein IX (pIX)[128] or generating fiber chimeras[129] could be applied for redirecting adenoviruses.

Another strategy that has been developed to target viruses toward tumor cells is using bispecific adapters. A bispecific adapter consists of 2 arms, virus-binding arm and tumor cell-binding arm.[130] The arms are joined together via chemical linking or with a flexible linker.[131] Since polysialic acid (polySia) is overexpressed on tumor cells including lung cancer, Kloos et al designed a bispecific adapter comprising a polySia-binding scFv domain and the ectodomain of CAR to retargeting of oncolytic Ad toward lung cancer. They reported that pretreatment of adenovirus vectors with adapter effectively infects polySia-expressing tumor cells in tumor-bearing mice and improves survival.[132] Another bispecific adapter containing the ectodomain of CAR fused to CXCL12, as CXCR4 chemokine ligand, shows increased infectivity of the chemokine receptor-positive cancer cells and lower hepatotoxicity.[133] Nakano et al developed an adapter protein composed of EGFR-binding scFv and the N-terminal domain of nectin 1 for redirecting HSV-1 to the EGFR.[134]

Immune responses

Another challenge in using OVs is preexisting immunity due to previous immunization or infection leading to short half-life following intravenous delivery. Coating oncolytic adenoviruses with polymers are known as “stealthing”, can protect adenoviruses during delivery.[135] The most utilized polymers have been *N*-(2-hydroxypropyl) methacrylamide (HPMA),[136] poly ethylene glycol,[137] and polyamidoamine.[138] In addition to extending half-life, another advantage of using polymers is tumor targeting by modification of oncolytic Ad/polymer.[139]

Conjugation of polymer with a specific tumor-targeting moiety such as peptide, antibody, antibody fragment, polysaccharide, or aptamer, can noticeably increase tumor selectivity.[140-143] Some polymers, such as poly ethylene glycol, are permanently coated on oncolytic Ads which interferes with the binding of cellular receptors and adenoviral fiber.[139] To overcome this problem, degradable polymers can be designed which selectively degrade due to specific conditions in the tumor microenvironment, such as hypoxia and low pH.[135] However, this strategy is limited by the fact that the modifications are not genetically integrated, so progeny virions lack these retargeting modifications.

Another strategy in protecting OVs against neutralizing antibodies and reducing toxicity is using cellular carriers as delivery vehicles. There are 3 main types of cells for delivery of OVs: immune, transformed, and progenitor cells.[144] Besides the ability to carry OVs, stem cells have the intrinsic ability to home in on tumor sites which makes them as attractive carrier.[145,146] Expression of various growth factors, cytokines, chemokines, and angiogenesis factors in the tumor microenvironment which is vital for tumor growth, attract stem cells to tumor sites.[147] For example, hypoxia with up-regulation of tumorigenic factors is critical in determining homing of stem cells and progenitor cells to tumor milieu.[148,149] It has been shown that loading of neural stem cells (NSC) with oncolytic adenoviruses increases expression of VEGFR2 and CXCR4, and therefore homing capacity.[150] To avoid allograft reactions in patients, using autologous stem cells is recommended. However, it should be noted that the quality and quantity of the isolated cells from patients who have undergone multiple treatment rounds are variable.[147]

Antiviral cytokines including different types of IFN is an obstacle against effective anti-tumor response to OV as they can retard viral replication.[2] To overcome this problem, several studies have used histone deacetylase (HDAC) inhibitors to induce epigenetic alterations and minimize antiviral cytokine responses in the tumor microenvironment.[151,152] Pretreatment of cancer cells with valproic acid (VPA), an HDAC inhibitor, leads to increased oncolytic HSV replication,[151,152] whereas concomitant treatment with viral infection has very modest outcomes.[151] These results suggest that VPA inhibits viral DNA replication and transcription of late genes in VPA-cotreated cells. On the other hand, VPA limits induction of antiviral responses in VPA-pretreated cells.[151] It has been shown that VPA suppresses infiltration of NK cells and macrophages in the tumor microenvironment following viral infection, which may reduce induction of anti-tumor immunity.[153] Furthermore, HDAC inhibitors shift the gene expression profile toward the induction of cancer cell arrest and apoptosis by epigenetic modifications.[154]

Besides IFN the system, which is a primary immune response against OVs, there are 2 other pathways limiting viral infection: 2′,5′-oligoadenylate synthetase (OAS)-RNase L system and RNA-dependent protein kinase (PKR).[155] Following type I IFN production in response to viral infection, double-stranded RNA activates transcription of OAS-1, -2, -3 genes, resulting in activation of RNase L and finally degradation of viral and cellular single-stranded RNA.[156] As well as this, infection of cells with double-stranded RNA and type I IFN system leads to activation of PKR. PKR is a serine/threonine protein kinase that phosphorylates the α-subunit of eukaryotic initiation factor 2α (eIF2α). Phosphorylation of eIF2α traps it in an inactive state and inhibits synthesis of viral proteins.[155] Jha et al showed pretreatment with sunitinib, an inhibitor of PDGF-R and VEGF enhances the efficacy of oncolytic virotherapy.[157] It has been demonstrated that sunitinib has an inhibitory effect on antiviral enzymes RNase L and PKR both *in vitro* and *in vivo*.[158] Also, it has been shown that bevacizumab, an inhibitor of VEGF, increases oncolytic adenovirus diffusion which can be due to the fact that bevacizumab reduces the interstitial fluid pressure.[159] This combination of anti-angiogenic agents with OVs shows synergic effects; inhibition of angiogenesis and thus the supply of oxygen and nutrients which are essential for tumor growth, and enhanced replication and spread of OVs within tumors.[160,161] Treatment with TGF-β[162] and immunosuppressive chemotherapeutics such as cyclophosphamide[163] have also been used for inhibiting innate immune responses.

Hypoxic effects

Hypoxia is a feature of solid tumors that occurs during tumor development and growth and has been shown to have contradictory effects on OVs.[164] It has been found that hypoxic conditions reduce replication and lytic potential of adenovirus, without affecting the expression of surface receptors.[165,166] Since hypoxia can induce cell cycle arrest, this ability may affect Ads, and any other viruses dependent on cell cycle progression, ability to replicate.[166] To overcome hypoxic inhibition on the replication of adenoviruses and take advantage of hypoxic conditions for targeting, Clarke et al designed an oncolytic adenovirus in which the expression of *E1A* gene is controlled by the hypoxia-response element-containing promoter. The modified oncolytic adenovirus gained the ability to replicate sufficiently under hypoxic conditions.[167]

Two groups in 2009, 1 by Aghi et al and the other by Fasullo et al, demonstrated that a hypoxic environment enhances viral replication of oncolytic HSVs.[168,169] This could be related to the natural tropism of HSV for reduced oxygen cells or oxygen-derived free radical-induced DNA damage which stimulates HSV replication.[168] Furthermore, the transcription of several genes involved in the replication of HSV are activated by hypoxia-inducible factor-1α (HIF-1α).[168] Infection with AF2240, an oncolytic NDV, leads to degradation of HIF-1α under hypoxic conditions and therefore down-regulation of HIF-1α target genes in different cancer cell lines.[170] Others viruses are also able to enhance their replication under hypoxic conditions including vesicular stomatitis virus[171] and vaccinia virus.[172]

Choosing patients

Assuming that all other challenges are solved, the question remains: what patients are suitable for treatment? At time of writing, no robust predictive biomarkers have been identified which can forecast patients who are expected to respond to oncolytic virotherapy. On the other hand, due to the experimental nature of most OVs, patients undergoing oncolytic virotherapy have usually already undergone numerous cycles of conventional cancer therapy, and therefore their immune system is disrupted and the tumors are radically altered compared to their initial form.[173] Zloza et al designed a study in which the changes in the expression of peripheral blood mononuclear cell genes were measured before and after treatment with oncolytic vaccinia virus in melanoma patients. Microarray data revealed that following virus administration, 301 and

960 genes were up-regulated and down-regulated, respectively. Further analysis showed that immunoglobulin-like transcript 2 could be used as a therapeutic biomarker in patients treated with oncolytic vaccinia virus.[174] In another study high-mobility group box 1 was suggested as a predictive and prognostic biomarker for treatment with oncolytic adenoviruses.[175]

Conclusions

OVs are a new branch of cancer therapy that has attracted scientists' attention globally and, in some countries, are now approved clinical therapeutic agents. Despite great progress in overcoming the initial concerns, such as safety and ease of administration, there are several challenges which still need to be addressed.

The field of oncolytic virotherapy has now been vindicated in terms of safety after the concerns following early clinical failures,[176] as indicated the approval of T-VEC and other agents in late stage trials. However, advances are required to properly capitalize upon this new mode of cancer treatment. While OVs are seen to work effectively in synergy with existing therapeutics, efficacy as a monotherapy remains elusive due to the challenges discussed in this review.

Progress in the field is now driven by a shift in focus towards efficacy. Increasingly innovative targeting strategies are under investigation. These utilize a combination of selective replication, and cancer cell targeting, as previously discussed. Many lessons can be learned from other fields which utilize viral vectors, including gene therapy and viral vectored vaccines which are seeing great clinical progress.[177,178]

As ever, there is unlikely to be a single solution to the issues surrounding the use of virotherapeutics for cancer or for the treatment of any given cancer type. As such it is imperative that we maintain a diverse repertoire of both vectors and strategies to enable the field to overcome the myriad obstacles in translating these therapies to the clinic.

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