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Oncolytic Virotherapy: Molecular Targets in Tumor-Selective Replication and Carrier Cell-Mediated Delivery of Oncolytic Viruses

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Abstract

Tremendous advances have been made in developing oncolytic viruses (OVs) in the last few years. By taking advantage of current knowledge in cancer biology and virology, specific OVs have been genetically engineered to target specific molecules or signal transduction pathways in cancer cells in order to achieve efficient and selective replication. The viral infection and amplification eventually induces cancer cells into cell death pathways and elicits host anti-tumor immune responses to further help eliminate cancer cells. Specifically targeted molecules or signaling pathways (such as RB/E2F/p16, p53, IFN, PKR, EGFR, Ras, Wnt, anti-apoptosis or hypoxia) in cancer cells or tumor microenvironment have been studied and dissected with a variety of OVs such as adenovirus, herpes simplex virus, poxvirus, vesicular stomatitis virus, measles virus, Newcastle disease virus, influenza virus and reovirus, setting the molecular basis for further improvements in the near future. Another exciting new area of research has been the harnessing of naturally tumor-homing cells as carrier cells (or cellular vehicles) to deliver OVs to tumors. The trafficking of these tumor-homing cells (stem cells, immune cells and cancer cells), which support proliferation of the viruses, is mediated by specific chemokines and cell adhesion molecules and we are just beginning to understand the roles of these molecules. Finally, we will highlight some avenues deserving further study in order to achieve the ultimate goals of utilizing various OVs for effective cancer treatment.

Keywords

cancer; oncolytic virus; oncolysis; selective replication; target molecule; signaling pathway; chemokine; drug

1. Introduction

An oncolytic virus (OV) is a virus used to treat cancer due to its ability to specifically infect and lyse cancer cells, while ideally leaving normal cells unharmed. A number of viruses, either naturally occurring or developed through genetic engineering, are being investigated as oncolytic agents for cancer treatment. Significant progress in the field has been made as showcased by the recent approval of the first OV (H101) for the treatment of cancer patients

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in China. The field has come a long way from the original pioneering research with herpes simplex virus type-1 (HSV-1) [1], and the first multiple clinical trials with ONYX-015 in cancer patients [2,3]. The early clinical trials with ONYX-015 (*dl*1520) demonstrated safety, but single agent efficacy was relatively limited. However, when combined with cisplatin and 5-fluorouracil chemotherapy, effective antitumoral activity has been demonstrated [4]. A phase III study of H101, which is very similar to ONYX-015, combined with cisplatin-based chemotherapy for head and neck squamous cell carcinoma and esophageal cancer showed significant improvements in clinical outcome [5]. This paved the way for China to approve H101 as the world's first OV for clinical use [6].

Probably the most serious limitation to the successful application of oncolytic virotherapy has been the limited ability to deliver viruses specifically and efficiently to the tumor, while perhaps its greatest strength has been the ability to achieve selective and efficient amplification of the virus and lysis of the cancer cells. Through extensive investigations in the last few years, a number of novel strategies to achieve tumor-selective replication and potent oncolysis have been developed. In addition, the issue of tumor-selective systemic delivery of OVs via the use of chemokine-guided cellular vehicles has been explored.

Although a number of review articles have been written on the topic recently, most have focused on only one virus - adenovirus [7–15]. Our aim is to to provide an overview of molecular targeting mechanisms and to discuss the recent application of trafficking cells as viral carriers to the tumor microenvironment. We will primarily focus on adenovirus (Ad), HSV, and poxviruses, although other OVs including vesicular stomatitis virus (VSV), measles virus (MV), Newcastle disease virus (NDV), influenza virus A (IFA), parvovirus, and reovirus are also discussed. Finally, we will provide some perspectives on new developments in the field and new avenues to be investigated.

The reason we are primarily focusing on three DNA OVs is that the ease of genetic manipulation of these viruses makes them more amenable to the rapid study of multiple gene deletions or modifications. Therefore, a great deal of information about the mechanisms of tumor targeting of replication-selective viruses has been achieved in these backgrounds. A variety of RNA viruses have been studied, with several displaying promising early clinical data (including reovirus, VSV, NDV, measles and coxsackie viruses). Because it is technically more challenging to make recombinant OVs from these RNA viruses many are used without genetic modification, usually relying on the limited ability of these small viruses to respond to IFN-mediated anti-viral responses to achieve tumor-selectivity. However, several recombinant OVs have been made and shown to possess enhanced tumor selectivity. Some recent reviews have extensively discussed the progresses made on reovirus, measles virus, VSV, MV and NDV [16–20].

2. Three representative oncolytic viruses

Because Ad and HSV have been the most extensively studied OVs, and because poxviruses represent relatively new family of OVs that have shown promising results in preclinical models, we will discuss the biological properties of these viruses related to their applications as OVs. The molecular mechanisms for viral DNA replication have been well characterized for Ad and HSV [21,22], and are still under intensive investigation for poxviruses [23,24].

2.1. Adenovirus

2.1A. Tumor retargeting—Ad enters a cell via receptor-mediated endocytosis involving two separate receptors. This begins with the binding of the fiber knob of viral capsid proteins to the primary receptor, the coxsackie-adenovirus receptor (CAR), and is then followed by the internalization of the virus, which is mediated by the interaction of a penton-base Arg-Gly-

Asp (RGD) motif and cellular αvβ integrins. This leads to endocytosis of the virion via clathrin-coated pits. In the endosome, the virus is disassembled followed by endosomal lysis [25]. A number of research teams have developed strategies for selective retargeting of oncolytic Ad. These strategies exert restrictions on the virus at three different levels: cell entry, viral gene transcription and viral protein translation. In the first level retargeting strategies are employed whereby adenoviruses can be designed to selectively infect tumor cells [9]. This may be achieved by the use of bispecific binding ligands (that bind both the virus and a cell surface receptor); by the exchange of virus capsid proteins between different serotypes; or by the creation of genetically modified vectors incorporating a cellular retargeting ligand. The second level involves transcriptional retargeting whereby certain tumor or tissue-specific promoters are used to control viral genes essential for viral replication [26–28], while in the third level, translational control of viral protein expression in tumor cells is used for tumor selectivity [29].

2.1B. Selective replication in tumor cells or other types of cells in cancer—A variety of conditionally replicating Ads (CRAds) have been created through extensive genetic engineering. For example, some have been designed to target cancer cells mutated for the tumor suppressor proteins p53 or pRB. Inhibition of p53 protein activity must be blocked in normal cells in order to allow efficient viral replication. The viral 55-kDa protein encoded by the *E1B* region of wild type adenovirus binds and inactivates *p53*, allowing viral replication in normal cells. ONYX-015 contains an 827-bp DNA deletion in the *E1B* region of the viral genome and thus produces a mutant E1B-p55 protein. It was hypothesized that normal cells, upon infection with ONYX-015, should generate a *p53* response that leads to apoptosis, preventing ONYX-015 virus replication [Fig. 1]. In contrast, tumor cells lacking a functional *p53* gene should be unable to suppress viral replication. In this way, replication of ONYX-015 should be restricted to *p53*-deficient tumor cells, resulting in their selective destruction. However, while the original idea was brilliant, the mechanism, as originally proposed, was incomplete and exact role of p53 may not be as pivotal as originally ascribed. Recent studies indicated that ONYX-015 could replicate in a variety of tumor cell lines with wild-type *p53* possibly because other defects within the *p53* pathway other than mutations of *p53* itself can render cells permissive for ONYX-015 [30]. A more likely explanation is that late viral RNA export, rather than *p53* inactivation, determines the tumor selectivity of the virus ONYX-015 [31].

Other CRAd have been generated by mutating the *E1A* region of the adenoviral genome. A virus with a mutation in the RB family-binding region of *E1A* (*dl922–947*), which targets defects in the RB pathway commonly found in cancer cells, has perhaps demonstrated the most promising antitumor efficacy in animal models [32]. *Dl*922-947 demonstrated greater efficacy than a similar virus Ad-Δ24, which contains 24-bp deletion in E1A from nucleotide #919 to 943 [33]. Although the viruses were administered via tail vein injection, no viral biodistribution studies were performed making it impossible to evaluate the tumor targeting potential of these viruses.

Oncolytic Ad can also be engineered to target other types of cells in the tumor rather than the cancer cells. Stromal cells constitute a major portion of the tumor mass and so an oncolytic Ad was constructed with *E1A* and *E1B* expression under the control of endothelial cell-specific promoters. This oncolytic Ad selectively replicates in dividing endothelial cells that are essential for solid tumor development [34]. Using two replicating Ads, one targeting cancer cells and the other stromal cells, Jin et al have shown that cotargeting tumor and tumor endothelium effectively inhibits the growth of human prostate cancer in mouse models [35]. A similar approach has been to design oncolytic Ads that target tumor cells for viral replication but that secrete products from infected cells (protein or siRNA) that act on endothelial cells and so inhibit tumor angiogenesis. This can be achieved in either one virus or two-virus

systems. Single oncolytic Ads expressing a gene encoding an antiangoiogeneic protein (such as endostatin or Flt-1) or a VEGF-specific short hairpin RNA were shown to elicit potent inhibition of both angiogenesis and tumor growth [36–38]. Using two Ads together, one virus (Ad Flk1-Fc) a non-replicating vector that expresses a soluble VEGF receptor capable of inhibiting tumor angiogenesis and growth, and the second an OV, *dl*922-947, it was demonstrated that the second virus with oncolytic activity could provide *in trans* functions needed for the replication of the first virus. This enhanced the expression of intratumoral levels of Flk1-Fc protein with increased antiangiogenic activity [39]. These studies illustrate that OVs capable of targeting multiple cell types in the tumor may be a highly effective approach for cancer therapy.

2.2. Herpes simplex virus

HSV is an enveloped double-stranded DNA virus. The genome consists of 152-kb of linear double-stranded DNA arranged as long and short components that are covalently linked. The transcription, replication and packaging of HSV take place in the nuclei of infected cells. In permissive cells, the entire replication cycle is usually completed within 20 hours, releasing thousands of progeny virions upon cell lysis. A variety of engineered HSV have been explored as OVs in different laboratories [8,40,41].

2.2A. Cell entry and cell retargeting—Entry of HSV into a cell depends upon multiple cell surface receptors and multiple proteins on the surface of the virion. The cell surface receptors include heparan sulphate chains on cell surface proteoglycans, a member of the tumor necrosis factor receptor family (HVEM; LIGHT) and two widely distributed cell adhesion molecules of the Ig superfamily, nectin-1 and nectin-2. The HSV ligands for these receptors are the envelope glycoproteins gB and gC for heparan sulphate and gD for the protein receptors and for specific sites in heparan sulphate generated by certain 3-O-sulfotransferases [42]. HSV enters cells either by fusion with the plasma membrane or via an endocytic route. The same receptor may initiate different routes of infection, depending on the cell in which it is expressed. Hence, the cell is a key determinant that controls whether a given receptor initiates a plasma membrane or an endocytic route of entry [43]. Recent studies also indicate that human defensins play multiple roles in multiple steps in the life cycle of HSV [44].

As with oncolytic Ads, HSV can be retargeted for cell type selectivity. For example, it has been retargeted to the EGF receptor through a gD-specific soluble bridging molecule [45]. By genetic engineering, it is further possible to separate receptor-binding and profusogenic domains of glycoprotein gD. This finding opens the way for developing recombinant HSV for cell retargeting in which the profusion domain of gD and independently furnished, interacting receptor-binding domains effect entry of the virus via a range of receptors [46].

2.2B. Genetic engineering for tumor-selective replication—One way to target HSV to cycling tumor cells and to prevent replication in normal quiescent tissue rests on the fact that nucleotide precursors are required for HSV to replicate. Wild-type HSV-1 can replicate in both cycling and noncycling cells, in part because it encodes multiple enzymes involved in DNA synthesis. Many of these viral proteins have cellular homologs that could provide the viral protein function *in trans* in the infected cells. Deletion of these viral genes therefore restricts replication of the mutant virus to tumor cells where the gene function is provided by the cell.

G207, NV1020 and G47Delta are examples of well-studied oncolytic HSV. G207 contains deletions of both copies of ICP34.5 and a *lacZ* gene insertion inactivating the ICP6 gene (U_L39) [1,47]. NV1020 (R7020) contains a deletion of one copy of U_L39, U_L24, U_L55 and U_L56 while viral *tk* gene is under the control of the viral α4 promoter [43]. G47Delta is a third-

generation oncolytic replication-competent HSV, containing deletions of the gamma34.5 and alpha47 genes and an inactivating *LacZ* insertion in ICP6 (U_L39) [49].

ICP34.5 is a neurovirulence factor. It forms a complex between proliferating cell nuclear antigen (PCNA) and DNA-binding HSV proteins [50]. In general, dividing cells support replication of ICP34.5-null HSV while nondividing cells cannot. Harland et al. proposed that in nondividing cells, ICP34.5 is required to switch PCNA from repair to replication mode, a prerequisite for the initiation of HSV replication [45]. Early studies with single deletion mutants confirmed that they are significantly attenuated in normal cells but replicate efficiently in malignant cells [40,41]. The molecular basis of this tumor selectivity has been controversial however. Some studies indicate that it lies in the activation of the protein kinase R (PKR)-mediated host cellular defense pathway, which inhibits protein synthesis in infected normal cells. Tumor cells overexpressing MAPK kinase (MEK) activity support robust replication of $\Delta\gamma_1$34.5 mutants via MEK-mediated inhibition of PKR, resulting in tumor oncolysis [51,52]. However, another study showed that permissiveness to viral replication of a gamma34.5-deleted HSV-1 (R3616) is neither associated with enhanced Ras signaling nor with defective PKR activity. Instead, disregulation of the PI3K signaling pathway allowed the R3616 virus to overcome the cellular antiviral activity in cancer cells [53].

In another example of tumor-selectivity produced by viral gene deletion, a U_S3 locus-deficient HSV, L1BR1, was investigated as an OV. L1BR1 was more than 10,000-fold less virulent than the parental virus in mice, and showed very low replication capacity in normal human hepatocytes, but impressive anti-tumor effects *in vivo* [54]. Another Us3 mutant virus, R7041, demonstrated up to 3-log replication inhibition in normal cells due to enhanced apoptosis. In contrast, R7041 replicated very well in tumor cells. *In vivo*, R7041 showed no signs of toxicity after systemic delivery in both immunocompetent and immunodeficient mice and showed preferential and prolonged replication in tumors compared with normal tissues. R7041 also displayed significant antitumoral efficacy after intratumoral or intravenous administration [55].

HSV-2 has only recently been examined as a potential OV backbone. The N-terminus of the ICP10 gene in HSV-2 encodes a serine/threonine protein kinase (PK) domain that facilitates HSV-2 replication by activating the Ras/MEK/MAPK mitogenic pathway and suppressing apoptosis. Mutants carrying deletions in this region preferentially replicate in and lyse tumor cells in which the Ras signaling pathway is constitutively activated [56].

As was seen with Ad, another strategy for the creation of tumor-selective, replicating HSV strains has been to utilize a tumor-specific promoter to drive an essential viral gene [28,40].

2.3. Vaccinia and other poxviruses

In one of the most successful stories in medical history, vaccinia virus (VV) was utilized as the vaccine for worldwide smallpox eradication. Recombinant VV and other related poxviruses have served as vectors for gene delivery and as vaccines for infectious disease and cancer [57]. Despite the fact that it has been used as a vaccine and a gene expression vector for many years, it is only in the last decade that VV has been specifically genetically engineered and explored as an OV [58–61]. In addition, other poxviruses have also been investigated as oncolytic agents [62].

Poxviruses are a family of large DNA genome viruses. VV has served as the prototype virus for research on poxviruses. VV viral genome is composed of ~190 Kb dsDNA encoding about 200 viral genes. VV relies on both its own encoded proteins and host cell proteins and functions for its life cycle, allowing for rapid, efficient transcription, translation and replication in poxvirus factories located in the cytoplasm of the host cell [23,24].

2.3.A. Cell entry and signal transduction pathways—In tissue culture, VV can infect and replicate in many types of cell lines derived from mouse to man, but it exhibits a more restricted tropism for primary human cells [58]. Moss and associates have recently shown that entry of VV into cells occurs by an endosomal route as well as through the plasma membrane. Two distinct low-pH steps promote entry of VV through endosomes into the cells [64]. It has been suggested that different forms of vaccinia (IV: intracellular virus and EV: extracellular virus forms) enter cells by different mechanisms, perhaps reflecting the use of different cellular receptors [65]. IV entry requires actin dynamics, while the EV does not. Even more interestingly, the IV, but not the EV form of the virus, induces a signaling cascade [66], indicating that EV seems to have evolved the capacity to enter cells silently, without a need for signaling. Bonjardim and associates have extensively investigated the signaling events in cells upon VV infection. They showed that MAPK and PKA pathways are required for efficient virus multiplication [67,68]. VV and other poxviruses carry out DNA replication in the cytoplasm. They create cytoplasmic 'mini-nuclei', called poxvirus factories, distinct sites that are surrounded by membranes derived from the rough endoplasmic reticulum that support viral replication [23]. Colocalization of transcription and translation within these poxvirus factories coordinates viral expression and subjugates host functions [24].

A specific cell receptor for VV has not been identified, and the broad tropism of the virus implies that many receptors, or non-specific cell-association events may occur. Human chemokine receptors (CCR1, CCR5 and CXCR4) have been identified as important for some poxviruses [69]. In addition, recent work has suggested that VV activation of CCR5 renders cells permissive for viral replication in otherwise non-permissive cells [70].

2.3B. Genetic engineering for tumor-selective replication—VV strains may possess a natural tumor tropism after systemic delivery in mouse models [71–73] **[**Fig. 2]. Part of the reason for this may lie in the fact that VV activates cell-signaling pathways and alters the progression of the cell cycle upon infection by inactivation of the tumor suppressors p53 and pRb. This may contribute to more efficient replication of the virus in rapidly growing cells such as many cancer cells [74]. As a large virus, 250 × 350 nm in size, it may also require leaky vasculature for extravasations from circulation and into tissues. Tumor tissues are well documented for their vascular endothelial growth factor (VEGF) production and leaky vasculature. A number of strategies have been designed to further enhance viral replication in cancer cells and reduce replication in normal cells and thus maximize its safety.

Aberrant epidermal growth factor receptor (EGFR) signaling is a major characteristic of a number of human malignancies including breast cancer, lung cancer, colon cancer, and head and neck cancer. Alterations in the functions of the EGFR signaling pathway are associated with oncogenic transformation and are associated with essentially all of the key features of cancer, such as autonomous cell growth, invasion, angiogenic potential, and development of distant metastases [75]. Vaccinia growth factor (VGF), a 19-kD secreted glycoprotein encoded by the virus, shares sequence homology and functional properties with cellular growth factors, EGF and TGF-α. Like EGF and TGF-α, VGF binds and induces tyrosine phosphorylation of the EGF receptor.

Early work by Moss and associates demonstrated that viral genes encoding both thymidine kinase (*tk*) and vaccinia growth factor (*vgf*) are virulence genes and are needed for efficient viral replication in normal cells *in vivo* [76,77]. When the *tk* gene was deleted from the viral genome of the WR strain of VV, the resulting virus displayed reduced replication in normal cells and yet retained full capacity to replicate in cancer cells [73,78]. When both viral genes for VGF and TK were deleted, the resulting virus (vvDD) retained its ability to target and replicate in cancer cells both *in vitro* and *in vivo*, and displayed significant oncolytic potency in multiple tumor models [73,79]. This virus was found to be dependent on cellular TK for

viral replication, which is expressed to high levels in cancer cells as a result of aberrant EGF-R signaling leading to E2F-mediated TK expression. The additional deletion of the *vgf* gene prevented activation of EGFR signaling in non-cancer cells, further restricting viral replication to cancer cells [80]. The addition of GM-CSF gene to this virus was further found to be very useful for combination oncolytic immmunotherapy. Systemic administration of this virus (JX-963) led to systemic efficacy against both primary carcinomas and widespread metastases in immunocompetent hosts [80] (Fig. 2).

In addition, by taking advantage of cancer cells' ability to evade apoptosis, the deletion of two viral genes (B22R and B13R) (Fig. 1) encoding anti-apoptotic proteins and serpins SPI-1 and SPI-2 was examined. The resulting virus, named vSP, displayed enhanced tumor targeting, yet significant oncolytic potency in a tumor model [81]. The tumor selectivity could be explained at least partly by the fact that normal cells infected with vSP die with faster kinetics and thus viral replication is limited, while viral replication in cancer cells is sustained. However, there is a limitation to the strategy of deleting non-essential viral genes as illustrated in a new VV (vSPT) with a triple deletion of viral genes, *spi-1*, *spi-2* and *tk*. This virus showed remarkable tumor selectivity, yet lost significant oncolytic potency in tumor models *in vivo* [82].

Other strains of VV have also been explored as OVs. Szalay and colleagues have described an oncolytic VV based on insertion of reporter genes into three viral sites (J2R [TK], A56R [HA] and F14.5L) in the LIVP strain [83]. This virus, GLV-1h68, replicated poorly in normal mouse cells yet replicates well in cancer cells. It was also found to cause tumor regression in a human breast cancer xenograft in nude mice. Gene expression profiling of regressing tumors revealed gene expression signatures consistent with immune defense activation.

Other poxviruses have also been explored as oncolytic agents. Yaba-like virus does replicate in human cancer cells, but the cytopathic effect of the virus was rather weak [84]. Much more exciting results have been recently obtained from myxoma virus, a rabbit specific poxvirus. It was demonstrated that myxoma virus of Lausanne strain can infect and replicate in the majority of human glioma cell lines [85]. It functions as an OV in a human glioma model in nude mice and in primary and metastatic B16F10 melanoma in syngeneic C57BL6 mice after intratumoral injection or systemic administration [85,86]. Infection of human cancer cells with this virus requires Akt activation via physical interaction with the viral M-T5 protein, a viral host range ankyrin-repeat protein [87]. Furthermore, rapamycin treatment was found to increase myxoma virus tropism for human cancer cells [88]. Genetic engineering may further enhance the oncolytic potency and safety of this virus.

3. Selective replication of oncolytic viruses in cancer cells

Cancer cells possess at least 6 hallmarks to distinguish themselves from their normal counterparts [89]. A manifestation of six essential alterations in cell physiology collectively dictate malignant growth of these cells: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Interestingly, there is a profound functional overlap between biological programs of viruses and those of tumor cells [90,91]. By taking advantage of special biological features of cancer cells and viruses, investigators have come up with a number of approaches to create unique OVs. These strategies can be grouped into two general approaches. One is to delete viral genes that are critical for viral replication in normal cells but are dispensable in tumor cells. The second approach is to limit the expression of a critical viral gene essential for viral replication to tumor tissue through the use of tumor or tissue-specific promoters.

3.1. Targeting defective pRB or p53 pathways

Ad encodes a number of early viral proteins to drive infected quiescent primary cells into unscheduled replication, while simultaneously preventing premature apoptosis. A number of oncolytic Ads have been generated by manipulating the functions of these early proteins carefully. Mutations in specific regions of the Ad *E1A* gene have resulted in tumor-specificity. The retinoblastoma tumor suppressor protein (pRb) pathway is dysregulated in a majority of human cancers [92] and is targeted by the Conserved Region 2 (CR2) of the E1A protein. The mutant Ad (dl922-947) replicates in and lyses a broad range of cancer cells with abnormalities in cell-cycle checkpoints [32], while a similar *E1A* modified virus (Ad-Δ24) targets the Rb pathway [33]. The human E2F-1 promoter is selectively activated in tumor cells with a defect in the Rb pathway. Therefore, an alternative means to achieve tumor-selectivity in Ad is to control an essential viral gene with an E2F-1 promoter to target Rb pathway-defective tumors [93]. The development of second and third generation viruses with multiple modifications may result in further enhancement of tumor selectivity. For example, ICOVIR-5 is an Ad that encompasses an *E1A* deletion in the Rb-binding region, substitution of the E1A promoter for E2F-responsive elements, and an RGD-4C peptide motif inserted into the adenoviral fiber to enhance adenoviral tropism [94].

Cancer stem cells are believed to be critical for initiation and progression of both leukemias and solid tumors. Those cancer stem cells with the Rb/p16 pathway deficiency could be treated by an Rb/p16-targeted oncolytic Ad. Indeed, a recent report showed that breast cancer stem cells isolated from patients can be infected and treated with E1A-CR2 gene region deleted Ad with modified capsid [95].

As discussed previously, ONYX-015 and H101 are viruses with a deletion in the *E1B* gene, which target cells with an ability to support RNA export [31].

E1A-deleted Ad could be utilized to target other unique features of cancer cells. If some types of cancer cells express cellular E1A-like activity, they may support the replication of *E1A* gene-deleted Ad. Indeed, an *E1A*-deleted Ad can efficiently replicate and produce high titers of progeny in HepG2 and Hep3B human liver cancer cells that produce an E1A-like activity. Replication of the *E1A*-deleted virus inhibited tumor formation and destroyed this type of tumors *in vivo* [96].

Targeting defective IFN/dsRNA-dependent protein kinase (PKR) pathways— Another frequent genetic defect in transformed cells is in the innate immune response, mediated primarily by the interferon (IFN) system [97,98] (Fig. 3). Type I IFNs are antiviral cytokines that are induced and secreted from cells exposed to virus and a number of other stimuli such as double-stranded RNA and lipopolysaccharides. VSV is a negative–stranded RNA virus normally sensitive to IFN-α/β. The attenuated VSV strains, AV1 and AV2, replicate in cancer cells and have the ability to treat metastases systemically before ultimately being cleared by the host in mouse models [97,99]. Another group made similar discoveries and showed that defects in translational regulation can cooperate with impaired IFN signaling to facilitate VSV replication, and may represent a common hallmark of tumorigenesis [98,100]. A more recent study indicated that an activated RAS/Raf1/MEK/ERK pathway plays a critical role in the defects in IFN-α/β signaling seen in cancer cells. The activated pathway negatively regulates the alpha interferon-induced antiviral response, thus allowing propagation of VSV or an IFN-sensitive VV (delE3L) in the presence of alpha interferon [101]. Similarly, targeting of IFN-β by deleting the B18R gene that produces a protein neutralizing the secreted type-I IFNs and then overexpresses IFN-β from VV has generated a specific, multi-mechanistic oncolytic VV [102]. About 30% of all cancers have constitutive activation of the RAS/Raf1/MEK/ERK pathway, and these engineered viruses would be an ideal OV for targeting such cancers [103].

HSV-1 mutants lacking the $\gamma_1$34.5 gene have long been studied as OVs as they are severely attenuated in normal tissues but not cancer cells. The molecular basis for this specificity probably lies in the activation of the protein kinase R (PKR)-mediated host cellular defense pathway, which inhibits protein synthesis in infected normal cells [Fig. 3]. Tumor cells overexpressing MAPK kinase (MEK) activity support robust replication of $\Delta\gamma_1$34.5 mutants via MEK-mediated inhibition of PKR, resulting in tumor oncolysis [51,52].

Newcastle disease virus (NDV), an avian paramyxovirus, is tumor selective and intrinsically oncolytic [104,105]. Again, the inherent oncolytic properties of this virus are believed to derive, at least in part, from defective IFN signaling pathways in tumor cells. Further enhancement of the oncolytic properties of NDV may be harnessed with novel reverse genetic approaches [106].

Influenza virus A (IFV) has also been explored as an OV. The NS1 protein is a virulence factor that counteracts the PKR-mediated antiviral response by the host. As a consequence, an IFV lacking the NS 1 open reading frame (delNS1) fails to replicate in normal cells but produces infectious particles in PKR-deficient cells. The oncolytic properties of this mutant virus are dependent on activated Ras [107], because oncogenic Ras induces an inhibitor of PKR. Further studies indicated that IFN-resistant tumors are also suitable targets for oncolysis induced by NS1-modified IFA mutants [108].

Targeting activated EGFR or Ras pathways—EGFR is overexpressed in various tumor types, and its expression correlates with metastatic behavior and poor prognosis. Growth factor binding to EGFR induces receptor oligomerization and activation of tyrosine kinase signaling. In tumor cells, high levels of EGFR lead to hyperactivation of the signaling pathways and activation of Ras signaling downstream. The Ras family of proteins consists of three isoforms, H-, K-, and N-Ras, which play critical roles in control of normal and transformed cell growth. Mutated K-*ras* is one of the most frequent genetic events in human cancer, with the highest incidences in pancreatic carcinomas (90%), colorectal tumors (50%), lung carcinomas (30%) and myeloid leukemia (25%) [109,110]. Activated K-Ras may not only promote tumor initiation, but also tumor progression and metastasis formation [111,112].

The human reovirus is a naturally occuring OV that specifically targets cancer cells with an activated Ras pathway [113,114]. A single intratumoral injection of virus resulted in regression of tumors in 65 to 80 percent of mice in pre-clinical studies [113]. Systemic reovirus therapy of metastatic cancer has also been studied in immune-competent mice [115]. Initially it was thought that activated Ras signaling was important for efficient viral protein synthesis [116]. However it has more recently been proposed that Ras transformation mediates reovirus oncolysis by enhancing virus uncoating, particle infectivity, and apoptosis-dependent release [117].

The need for VA RNA genes in Ad should also be bypassed in cells with an active Ras pathway. Therefore, Ad mutants deleted in virus-associated I (VAI) or/and VAII RNAs have oncotropism characterized by up to 100-fold replication deficiency compared with wild-type Ad in normal cells and an unaffected ability to replicate in and kill Ras-activated tumor cells [118,119]. However, in another study, it was shown that activating Ras mutations fail to ensure efficient replication of adenovirus mutants lacking VA-RNA. Thus the authors proposed that Ras mutations predispose tumor cells to undergo secondary changes that sometimes enable the replication of interferon-sensitive viruses [120].

Another oncolytic Ad has been designed to target tumor cells through activated RAS/MAPK-selective mRNA stabilization [121]. It was previously found that the *PTGS* (also known as *COX2*) 3′-UTR acts in normal, quiescent cells to destabilize the messenger mRNA to levels

where translation of the corresponding protein is very low or negligible. In cells with constant proliferative signals such as activated RAS/MAPK, the *PTGS2* 3′ UTR would act to restabilize the mRNA to levels at which expression of the corresponding protein is detectable and biologically relevant. Based on this hypothesis, the authors constructed a CRAd by ligating the essential gene *E1A* to the *PTGS2* 3′ UTR. Expression of E1A in normal cells is abrogated, however, E1A is increased in cells receiving proliferation signals through expression of the activated RAS oncoprotein, as a result of restabilization of the E1A mRNA in tumor cells.

Other genetically engineered OVs, such as HSV-2 (*ICP10* mutation) targeting the activated Ras pathway, and VV targeting the activated EGFR pathway (*vgf* deletion), have been discussed in detail earlier [56,73,80].

3.4. Targeting Wnt signaling pathway

Aberrant activation of the Wnt pathway is implicated in driving the formation of various human cancers, particularly those of the digestive tract [122]. Activation of the pathway results mainly from mutations in the adenomatous polyposis coli (APC) and β-catenin genes, although mutations have also been described in the axin gene in hepatocellular carcinoma. In response to wnt signals, GSK3β is inhibited and β-catenin is stabilized. β-catenin then enters the nucleus, binds to Tcf/Lef family transcription factors, and activates transcription of wnt target genes, such as cyclin D, myc, and $PPAR_{\delta}$.

By taking advantage of unique features in these cancer cells, Iggo and associates have developed CRAds that express the viral *E1B* and *E2* genes from promoters controlled by the Tcf4 transcription factor [123]. Additional improved versions of oncolytic Ads targeting the wnt pathway have been made and tested in tumor models with defective wnt signaling [124]. Based on the same principles, a replicating parvovirus was also developed, however, the virus replicated poorly in wnt activated cancer cells and further improvements remain challenging [125]. In contrast, an oncolytic HSV (bM24-TE) that was constructed with the ICP4 gene and thus viral replication driven by a synthetic promoter containing the tandem repeats of a Tcf responsive element was efficacious against colorectal cancers carrying an APC gene mutation between the first and second 20-amino-acid repeats [126].

3.5. Targeting the hypoxic tumor environment

Hypoxia is a hallmark of solid cancer that is associated with metastases, therapeutic resistance and poor patient survival [127]. Tumor hypoxia presents an obstacle to the effectiveness of many antitumor therapies. However, a number of strategies, including OVs targeting hypoxia and/or hypoxia inducible factors (HIF), have been developed by investigators [128].

The first study harnessing the hypoxic environment for tumor-selectivity of OV incorporated an oncolytic Ad with the *E1A* gene controlled by a minimal dual-specificity promoter that responds to hypoxia as well as estrogens [129]. Subsequent studies made specific modifications such as total dependence on HIF for viral replication and those geared toward specific types of cancer [130–132]. A dual-regulated oncolytic Ad CNHK500, in which the *E1b* gene is controlled by a hypoxia-responsive promoter and the *E1a* gene is controlled by an hTERT promoter, showed increased safety and should be useful for a number of solid tumors [133]. Recent mechanistic studies have revealed some negative effects of hypoxia on Ad viral infection, gene expression and replication [134,135]. These results could guide further improvements of oncolytic Ads targeting the hypoxic tumor environment.

Connor and colleagues have shown that VSV is capable of replication under hypoxic conditions [136], demonstrating that VSV has an inherent capacity for infecting and killing hypoxic cancer cells.

3.6. Targeting anti-apoptosis pathways in cancer cells

Many cancer cells have evolved to evade apoptosis [89,91,137,138]. During evolution of host-pathogen interactions, viruses have also evolved mechanisms to evade apoptosis in order to assist their replication. The viral genes encoding anti-apoptosis functions are therefore theoretically expendable in cancer cells but essential for viral replication in normal cells [91, 138]. Figure 1 illustrates the interactions of cellular apoptosis signaling pathways with a number of viral proteins derived from Ad, HSV and VV [89,138–141]. Work with Ad, HSV and VV have demonstrated that deletion of a variety of these viral genes have generated tumor-selective and potent OVs.

For Ad, the viral protein E1B-19 kDa is a Bcl-2 homologue that blocks apoptosis induction via the intrinsic and extrinsic pathways. Kirn and associates have demonstrated that an E1B-19 kDa gene deletion mutant had TNF-enhanced cancer selectivity, due to genetic blocks in apoptosis pathways in cancer cells [142]. In addition, this mutant demonstrated significantly enhanced viral spread and antitumoral potency relative to ONYX-015 and wild-type adenovirus *in vitro*. Significant antitumoral efficacy was also demonstrated *in vivo*. The same team of investigators hypothesized that the deletions of two functionally redundant genes, *E1B* and *E3B*, that both independently antagonize TNF-α, could lead to enhanced oncolytic potency while maintaining selectivity. Studies determined that E3B deletion alone resulted in a potent OV while deletion of both viral genes was relatively deleterious for viral replication in cancer cells [143].

In VV, a virus (vSP) with deletion of two viral genes encoding antiapoptotic proteins and host range factors (SPI-1 and SPI-2) resulted in enhanced tumor-selective replication. Viral replication was reduced in normal cells due to enhanced apoptosis, thus this virus displayed enhanced tumor selectivity [81].

A number of viral genes in HSV encode anti-apoptotic functions which may not be essential for viral replication and survival in cancer cells. Us3 locus-deficient HSV have been investigated as OVs. One of such viruses, L1BR1, has been shown to possess the greatest reduction in replication capacity in normal human hepatocytes, but the highest tumor-reducing effect *in vivo* among the oncolytic HSV tested. In addition, L1BR1 significantly increased the induction of apoptosis in cancer cells when administered in combination with anticancer drugs [54]. Another Us3 mutant virus, R7041, exhibits a 3-log replication reduction in normal cells due to enhanced apoptosis while replicating very well in tumor cells. In vivo, R7041 shows preferential and prolonged replication in tumors and displays significant antitumoral efficacy [55]. HSV-2 ICP10 protein has anti-apoptotic activity in virus-infected hippocampal cultures through activation of the Ras/Raf-1/MEK/ERK pathway. As has been discussed, an HSV-2 with a mutation in ICP10 gene also functions well as an OV [56].

3.7. Targeting tumor-associated viruses in tumor cells

Infectious viruses are among the few known causes of cancer and contribute to a variety of malignancies [144]. The viruses include human papillomaviruses (cervical carcinoma); human polyomaviruses (mesotheliomas, brain tumors); Epstein-Barr virus (B-cell lymphoproliferative diseases and nasopharyngeal carcinoma); Kaposi's sarcoma herpesvirus (Kaposi's sarcoma and primary effusion lymphomas); hepatitis B and hepatitis C viruses (hepatocellular carcinoma) and human T-cell leukemia virus-1 (T-cell leukemias). These virus-associated cancers account for 10–20% of malignancies worldwide. One of these viruses, EBV, is a ubiquitous human herpesvirus found latently expressed in 90% of the human adult population [145]. This virus is closely associated with the development of a variety of malignant diseases, including endemic Burkitt's lymphoma, B-cell lymphoma in immunocompromised patients, nasopharyngeal carcinoma, Hodgkin's disease, T-cell

lymphoma, natural killer cell leukemia/lymphoma, smooth-muscle tumors, and gastric cancer [146].

Early work in the 1980s has demonstrated that the two Epstein-Barr virus-encoded RNAs can efficiently complement for the Ad VAI. EBV-encoded small RNA1 is uniformly expressed in most EBV-associated human tumors and can functionally substitute for the Ad VAI RNAs [147]. This enables replication of a VAI-deletion mutant Ad to proceed through complementation. Indeed, VAI mutant virus replicated efficiently in EBV-positive tumor cells compared to EBV-negative and normal human primary cells. *In vivo*, VAI-deleted Ad showed superior antitumoral efficacy to wild-type Ad in EBV-positive tumor xenografts, with lower hepatotoxicity than wild-type Ad [148]. These results suggest that VAI-deleted Ad is a promising CRAd with targeting specificity for EBV-associated tumors.

High-risk strains of human papillomavirus (HPV) are associated with several human epithelial neoplasms such as cervical, anorectal, and other carcinomas [149]. These viruses are thus highly significant as biological carcinogens and have major impacts on human health. It has been shown that an oncolytic Ad expressing a p53 variant resistant to degradation by HPV E6 protein exhibits potent and selective replication in cervical cancer [150]. Another group has shown that a CRAd termed Ad-URR/E1AΔ24, in which a mutated E1A gene is driven by the promoter from HPV-16, is highly selective for HPV-associated cancers [151].

3.8. Restricting viral replication utilizing tumor-specific gene promoters

An alternative means to create tumor-selective OV is through the use of tumor-specific gene promoters to drive viral gene(s) essential to viral replication. A number of oncolytic Ads have been designed using such promoters to control one or more essential genes [26–28].

There are two types of tumor-specific gene promoters. The first type is a promoter that is very active in many different tumor types with little or no activity in normal cells, such as developmentally regulated genes. For example, the promoter of human telomerase reverse transcriptase (hTERT) is highly active in most tumor and immortal cell lines but inactive in normal somatic cell types. The native or modified hTERT promoter has been utilized to control E1A expression in oncolytic Ad [152–155]. Alternatively, the human survivin gene promoter was used to control the expression of E1A and the resulting Ads displayed promising therapeutic potential in pre-clinical models [156–158]. The second type of promoter used to drive essential viral gene function represent tumor type/tissue type-specific. Examples are gene promoters for prostate specific antigen, α-fetoprotein and tyrosinase, specifically for cancers of prostate, liver and skin, respectively [8]. Sometimes, tumor-specific promoters display very weak activity. In this regard, it is interesting to note that a binary regulatory system with recombinant GAL4 fusion protein have been utilized to augment the activity of a weak tissue/tumor-specific promoter and could be further incorporated into oncolytic Ads [159]. For this purpose, an efficient construction method will expedite the generation of CRAds that target tumor cells with multiple factors [160].

The strategy of transcriptional targeting of OVs has been most widely applied to oncolytic Ad. However, some investigators have extended the application to other OVs such as HSV. As mentioned previously, a transcriptionally targeted oncolytic HSV (bM24-TE) in which replication is driven by the TE promoter was constructed. This virus efficiently and specifically replicated in and killed tumor cells with strong beta-catenin/Tcf signaling [126]. In order to retarget lytic virulence in addition to viral replication to tumors, the expression of virulence gene gamma34.5 in oncolytic HSV was engineered to be under the control of a tumor-specific promoter, either the E2F-responsive, B-myb gene promoter [161] or a glioma-specific musashi1 gene promoter [162]. In both cases, the authors showed that the gamma34.5-induced

virulence has been retargeted and displayed an enhanced viral cytotoxicity to tumors. These two HSV could be promising therapeutic agents for certain types of cancer.

It should also be pointed out that not all viruses can be controlled in this way. For example, VV completes its entire life cycle in the cytoplasm of the host cell and has its own RNA polymerase and promoter sequences and this approach is unlikely to work for VV. Neither this approach would work for a number of oncolytic RNA viruses. In addition, the fact that most cellular promoters display only weak transcription relative to viral promoters means that careful selection of transcriptional control elements and gene to be controlled is needed.

4. Targeting tumor endothelium and microenvironment

OVs can selectively replicate in and lyse certain types of cells in the tumor, thus inhibiting tumor growth. Some OVs were designed for this purpose while others were later found to possess such property. Using dividing endothelial cell-specific promoters to control *E1A* and *E1B* expression, an oncolytic Ad was generated to selectively replicate in proliferating endothelial cells in cancer tissue [34]. Using human osteocalcin gene promoter, another OV was generated to target both prostate cancer epithelial and stromal cells [35]. In addition, ONYX-015 has also been found to actively replicate in human umbilical vein endothelial cells [163]. Oncolytic HSVs such as G207 and HSV-1716 can replicate in proliferating endothelial cells and purified tumor endothelial cells, and thus may exert direct antiangiogenesis and suppress tumor growth [164,165]. The molecular mechanisms by which these oncolytic HSVs target endothelial cells are yet to be understood.

Two recent studies revealed a previously unappreciated and unexpected interplay between OVs, tumor and inflammatory responses [166,167]. In one study, the authors discovered that infection of a small proportion of tumor cells by oncolytic VSV or VV triggered a significant portion of cancer cells in the tumor to undergo apoptosis. The major reason was that viral infection triggered infiltration of neutrophils with corresponding chemokine induction and inflammation. This resulted in a loss of blood flow to the interior of the tumor, causing induction of massive cellular apoptosis in uninfected tumor cells [166]. In the other study, the authors revealed that treatment with an oncolytic HSV increased tumor vascular permeability, host leukocyte infiltration into tumor, and expression of inflammatory cytokines in the tumor [167]. Therefore, targeting OVs to endothelial cells as well as tumor cells may not only inhibit tumor angiogenesis, but may also play a major role in inducing uninfected tumor cells into cell death pathways, and inducing inflammatory responses that have significant consequences in antitumor immune responses, leading to enhanced therapeutic effects by OVs.

5. Tumor-specific delivery of oncolytic viruses via carrier cells

Because viruses outside of a host cell are essentially inert particles, they rely on passive targeting to reach a tumor systemically. Vehicles that have endogenous tumor-targeting activity could therefore be usefully incorporated to deliver OVs to the tumor tissues in vivo, resulting in greater delivery to the tumor and reduced off-target toxicities. In this regard, specific autologous cells have been investigated as potential carriers for viral delivery to the tumor where replication and amplification of the virus should increase the effective local viral dose in the tumor and thus enhance the oncolytic effects. Recent studies have shown that immune cells can be good carriers for OVs. Other types of cells, such as stem cells and tumor cells can also be utilized as carriers [168,169]. Carrier cells (or cellular vehicles) have been utilized successfully to deliver HSV-1 [170], Ad [171–174], parvovirus [175], VV [176], measles virus [177–179], and VSV [180,181]. Two great advantages for this approach are the specific delivery to the tumor and its great potential to bypass the pre-existing antiviral immunity (173,179,180). An understanding of the molecular events underlying homing of carrier cells to tumors may hold the key to further improvements of this technology.

5.1. Chemokines and integrins in cell homing to cancer

In order to develop efficient cellular vehicles for OVs as well as effective cancer immunotherapy, it is extremely important to develop a systematic understanding of the molecular basis of cell trafficking and biodistribution of candidate carrier cells. Two classes of molecules, chemokines and adhesion molecules such as integrins, have been shown to be important for cell trafficking within immune systems and into tumors [182–184]. Chemokines are a large family of small, generally secreted polypeptides which guide lymphocyte movement throughout the body by controlling integrin avidity and inducing migration. Cancer cells express a number of chemokines which may attract certain types of cells to tumor [185]. Manipulation of the chemokine-chemokine receptor systems may be very useful for efficient trafficking of carrier cells as well as for cancer immunotherapy [186].

The CXC chemokine stromal cell-derived factor-1alpha (SDF-1; official name CXCL12) binds to CXCR4, a seven-transmembrane G protein-coupled receptor that plays a critical role in many physiological processes that involve cell migration and cell fate decisions, ranging from stem cell homing, angiogenesis, and neuronal development to immune cell trafficking. Trafficking of normal stem cells and metastasis of cancer stem cells have both been shown to involve similar mechanisms with the SDF-1-CXCR4 interaction playing a pivotal role [187].

The tumor-derived chemokine RANTES (CCL5) may play dual roles in tumor growth. On one hand, it increases the anti-tumor response by recruiting NK, CD4 and CD8 T cells into the tumor site [188,189]. On the other hand, it promotes the expression of matrix metalloproteinase 9 (MMP9) in certain carcinomas that may contribute to angiogenesis and foster tumor growth [190,191].

Efficient trafficking of adoptively transferred tumor antigen-specific T cells to tumor is crucial to both adoptive immunotherapy and oncolytic virotherapy using activated T cells as cellular vehicles. It has been demonstrated that intratumoral expression of CCL3, CCL21 and CXCL10 (IP-10) enhanced trafficking of adoptive T cell transfer and thus immunotherapy in mouse tumor models [192–194]. On the other hand, T cells can be redirected to tumors by genetic engineering of the T cells to express appropriate chemokine receptors such as CXCR2 [195, 196].

Other types of immune cells are also found to localize to tumors. Monocytes and macrophages extensively colonize solid tumors, where they are thought to promote tumor angiogenesis [197]. It has been shown that integrin alpha4beta1 (VLA4) promotes the invasion of tumors by myeloid cells and subsequent neovascularization [198]. Natural killer (NK) cells, large granular lymphocytes of the innate immune system, are widely distributed in lymphoid and non-lymphoid organs and participate in the early control of microbial infection and cancer. NK cells can also be recruited in various tissues upon inflammation via the functions of integrins and chemokines. However, the mechanisms governing NK cell trafficking remain poorly dissected (199). A recent study indicted that mobilization of NK cells into inflamed organs requires the expression of sphingosine 1-phosphate receptor S1P(5) (200). As the most important class of professional antigen presenting cells, dendritic cells (DC) respond to the environment. Once activated, they migrate to the lymphoid tissues where they interact with T cells and B cells to initiate and shape the adaptive immune response. Kalinski and associates have generated a new type of DC *in vitro*, alpha-type-1-polarized DC, which are highly effective for cancer vaccines [201]. These alpha-DC1 cells show high migratory responses to the CCR7 ligand, CCL21. In summary, intensive research on cell trafficking will provide us a much better picture of how these cells are guided to the sites of cancer and other diseases. This information will be very useful to improving the efficacy of carrier cells to deliver OV to tumor.

5.2. Stem cells and progenitor cells as carriers

Mesenchymal progenitor cells (MPCs) possess properties such as simple isolation and propagation procedures, making these cells attractive candidates as cellular vehicles. Some earlier studies established that MPCs possess key functional vector attributes for cancer gene therapy [202]. The tumor-homing properties of MPCs add further weight to the value of this cell type as potential vehicles [203,204]. Recent studies have shown that MPCs can be infected with oncolytic Ads, and they can carry the viruses to the tumors [171,174]. It is interesting to note that human mesenchymal stem cells lack tumor tropism, but enhance the Ad viral antitumoral activity in orthotopic lung and breast tumors in mice [172].

The recent demonstration that cancer stem cells derived from breast cancer patients can be infected by oncolytic Ad [95], raised the possibility that cancer stem cells could be used as carriers for delivery of OVs to cancer. In addition, investigators have used an *in vivo* screen to identify populations of bone-marrow-derived donor cells that accumulate within tumors [205]. This type of study may direct rational selection of cell types as carrier cells in the future.

5.3. Endothelial progenitors and endothelial cells as carriers

Recent studies have also demonstrated that when transplanted, endothelial progenitor cells can migrate via peripheral blood and home exclusively to the site of tumor neovasculature [206]. Therefore, it has been proposed that endogenitor cells may also act as a “Trojan horse” for systemically delivering OV to metastases [207]. In fact, blood outgrowth endothelial cells have been tested to systemically deliver an oncolytic measles virus to a human glioma model in mice [178].

5.4. Immune cells as carriers

A variety of immune cells can respond to “danger signals” released from cancer tissue by trafficking to the cancer sites [208]. These immune cells may be used as cellular vehicles for delivery of OVs. The types of cells investigated so far have included T cells [177,181], NKT cells [176] and monocytes [179]. In addition, dendritic cells and NK cells may also be useful for this purpose [199–201]. A potential advantage of using immune cells as cellular vehicles is that it may produce a combined immune virotherapy. This has been excellently demonstrated with cytokine-induced killer (CIK) cells and oncolytic VV in mouse tumor models [176]. This strategy could be combined with other types of therapies such as T cell immunotherapy and epigenetic therapy [209–211].

In fact, Vile and associates have recently demonstrated that, using a combination of antigen-nonspecific adoptive T cell therapy, oncolytic virotherapy and immunotherapy, metastases in lymphoid organs could be purged [181]. They showed that metastatic cancer cells in lymphoid organs was initially reduced by viral oncolysis, and was then eradicated by the protective immunity generated. As pointed out by the authors, this combination approach could have a great therapeutic impact and be utilized on many types of cancers in the adjuvant setting.

5.5. Cancer cells as carriers

The idea of using tumor cells as carriers to deliver an OV was first demonstrated in a regional delivery of replication-selective HSV-1 for the intraperitoneal therapy of epithelial ovarian cancer [170]. In later studies, the tumor carrier cells have been inactivated by gamma-irradiation after infection, which did not affect the production and release of oncolytic parvovirus [175]. Using a model of spontaneous metastases of human breast cancer, it was shown that i.v. injected tumor cells localized to metastatic lesions, and were able to carry an oncolytic Ad to the metastases thereby delivering the therapy in a localized and less toxic fashion [212].

The repeated administration of VSV in carrier cells to animals bearing metastatic tumors greatly improved therapeutic efficacy when compared with naked virion injection. It was revealed that carrier cells derived from solid tumors accumulate primarily in the lungs following systemic injection, whereas leukemia carriers disseminate extensively throughout the body [180]. Another study has shown that Ad-GM-CSF augmented the anti-tumor effect of carrier cells by increasing anti-adenoviral and anti-tumoral CTL responses and decreased the number of injections of carrier cells required to induce complete tumor regression [173]. In both studies, carrier cell-based delivery of OVs had circumvented pre-existing antiviral immunity [173,180].

6. Conclusions and prospectives

Over the past few years, a great deal of effort has led to a much greater understanding, at the molecular level, of how viruses and their mammalian host cells interact with each other, allowing the design of genetically engineered viruses that target selected molecules or signaling pathways in cancer cells. In order to potentiate their oncolytic potency, many OVs are armed with genes such as those encoding apoptosis-inducing proteins, prodrug converting enzymes or immunostimulatory molecules. The induction of host anti-tumor response has also been recognized as an important long-term therapeutic benefit. These OVs may further be used in conjunction with other therapeutic modalities such as chemotherapy and radiation therapy leading to highly effective and safe cancer therapies. However, there are still a number hurdles to overcome before oncolytic virotherapy reaches its full potential. In particular, efforts need to be executed in the following areas.

(1). Regulatable gene expression in the viral vectors

Some therapeutic genes need to be expressed at the right place and the right time in order to achieve maximal therapeutic effects. This is especially important for molecules that stimulate host immune systems to generate immunity against tumor antigens and viral antigens and for molecules that induce host cells to enter cell death pathways. One possibility is to express genes from extrinsically regulated promoters [213]. This regulated expression coupled with non-invasive and quantitative imaging of gene expression *in vivo* should greatly facilitate the detection of endogenous disease-specific biological alterations (e.g., signal transduction) and may be used for monitoring the induction and regulation of therapeutic genes [214].

(2). Overcoming the preexisting host immunity

The majority of the human population has previously been exposed to a number of viruses, thus pre-existing immunity against these viruses in cancer patients is a major obstacle to the success of OV therapy. Alternatively, the initial use of any viral therapy will induce a robust anti-viral immune status in the patient that will limit repeat dosing. Effective strategies to safely circumvent pre-existing immunity against these viruses need to be established in order for such viruses to be effectively utilized as OVs. Two types of strategies are under investigation. One has been using single or multiple immune suppressants to inhibit both innate and adaptive immunity of the host. The second approach has been, as described earlier, to use carrier cells to deliver OVs to the tumor sites, bypassing the host immune system (173,179,180).

(3). Enhancing trafficking of carrier cells

Initial studies have shown this approach to be very promising. However, in many cases only small fractions of the carrier cells, and thus the OV they have carried, reached the target tumor tissues after systemic delivery. In order to achieve the ultimate goal of delivering OVs efficiently, we need to better understand the factors affecting the trafficking of these carrier cells.

(4). Enhancing intratumoral spread and oncolytic potency

It is very important for the OVs to distribute throughout the tumor tissue and display significant oncolytic potency in order for this mode of cancer therapy to work effectively. Investigators have documented a number of strategies to enhance the spread and the oncolytic potency of the viruses.

In summary, we have examined several aspects of oncolytic virotherapy and have provided some perspectives on the further development of other areas. There are many additional OVs currently under development, and many hurdles that will need to be addressed, including the emergence of virus-resistant tumor cells [7,10,215]. The discovery of efficient and safe anticancer drugs remains a highly challenging endeavour [216]. OVs, as a special class of cancer drugs, are no exceptions. Considering the heterogeneity of tumors in single patients and among patients, a multi-targeted OV or combinations of OVs may ultimately lead to the most effective results. Even though further modification of OVs might result in increased targeting and oncolytic potency, it is still unlikely that oncolytic virotherapy alone will be able to fully eradicate tumors in human cancer patients. This has been the case for the first clinically used OV H101 and may remain so for other upcoming candidates in the near future. As a plateform gaining momentum, inclusion of OVs into a multimodal cancer treatment protocol combining standard radiation, gene therapy, chemotherapy, and/or immunotherapy, will have the greatest potential of destroying tumors and improving the survival of cancer patients.

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* Papers #74, 207, and 210 are epub ahead of print, page numbers of which will be provided a bit later.

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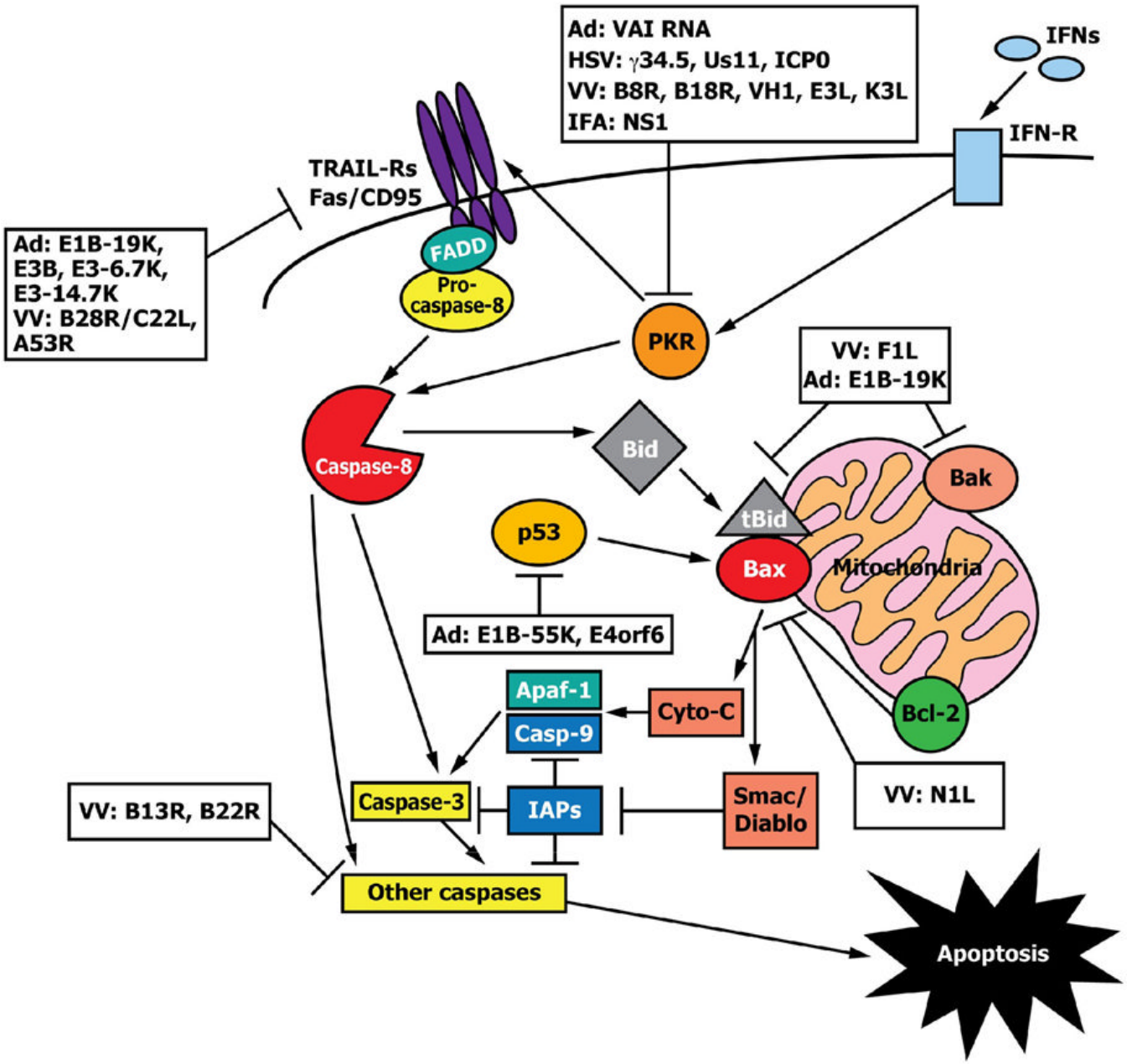

Fig. 1.

Cellular apoptosis pathways and intervention by both cellular anti-apoptotic proteins and viral proteins encoded by oncolytic Ad, HSV and VV. A simplified description of intrinsic, extrinsic apoptosis pathways as well as key players (e.g. interferon pathway and p53) and their interactions are included. Also illustrated are mechanisms that viruses employed to block apoptosis, and some cellular anti-apoptotic proteins which are often over-expressed in cancer cells. Two recent dissected anti-apoptotic proteins encoded by VV, F1L [139] and N1L [140], are illustrated. This figure is modified from a figure by Liu and Kirn, 2005 [138].

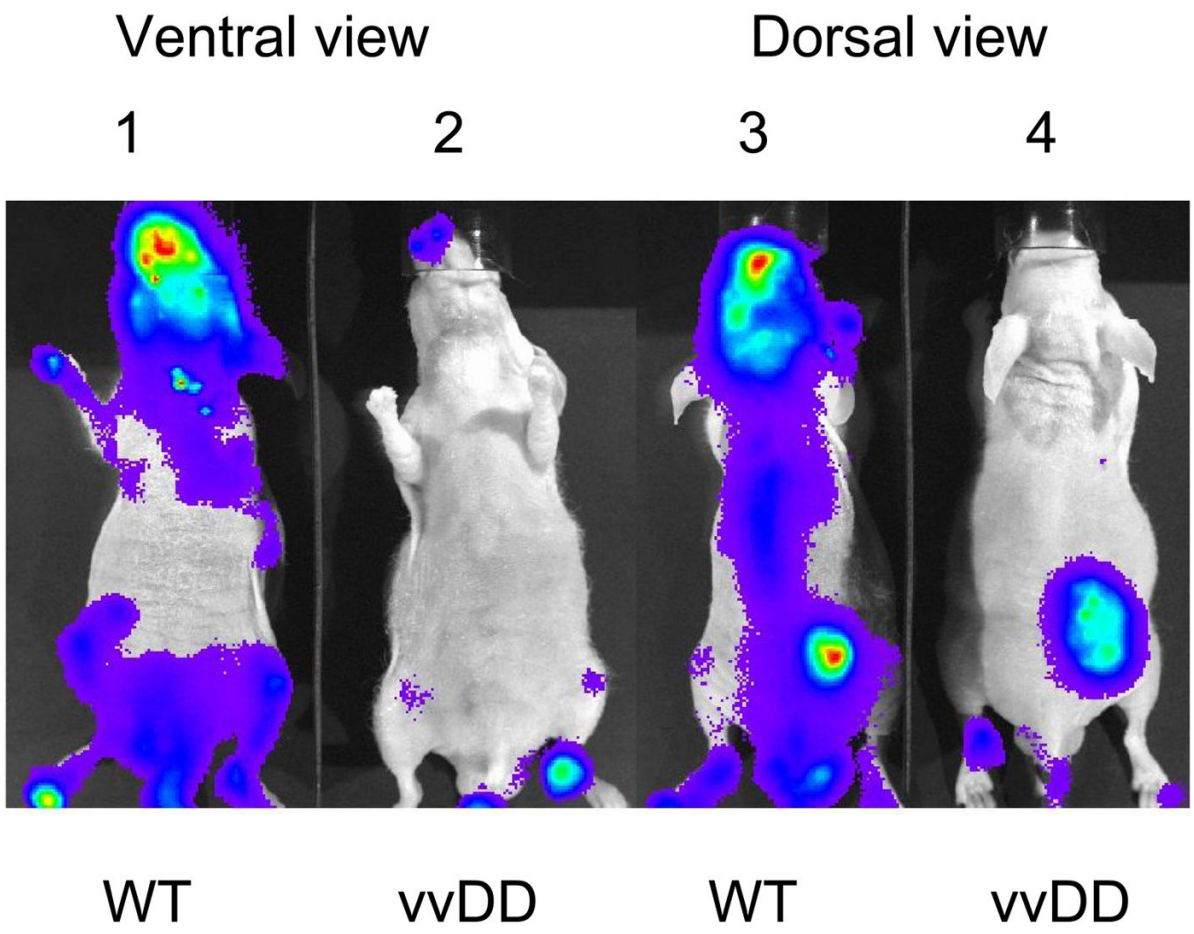

Fig. 2.

Imaging of tumor-selective amplification of an oncolytic VV in tumor-bearing mice after systemic delivery. Subcutaneous tumor (in the back)-bearing nude mice were treated with intravenous injections of wild type WR strain VV (panels 1 and 3), or a tumor-selective oncolytic VV (vvDD; panels 2 and 4) both expressing luciferase. Bioluminescence images are taken 96 hours after treatment. Both ventral (panel 1, 2) and dorsal (panel 3, 4) views are presented. The wild type virus has infected multiple organs and tissues, while vvDD has been removed from most tissues other than the tumor, where it continues to express luciferase. Data are from Thorne et al., 2007 [80].

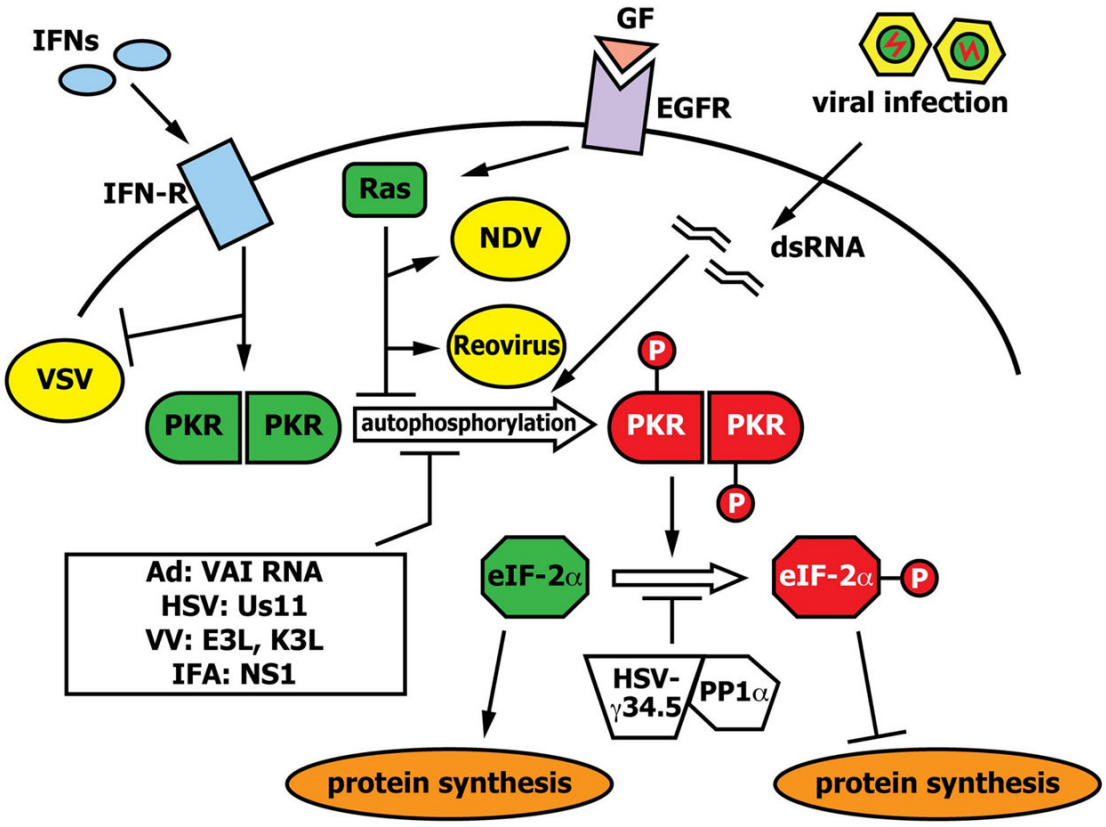

Fig. 3.

The IFN, EGFR/Ras signaling pathways and their interactions with virus-encoded proteins. The kinase PKR is an important downstream effector molecule of the IFN signaling pathway. When a cell is infected by a virus, it is stimulated to produce IFNs that will activate an antiviral defense response of nearby cells. Upon binding of IFNs to cell surface receptors (IFN-R) on neighboring cells, an intercellular signal transduction cascade is engaged, which induces PKR expression. The dsRNA produced by viruses will bind to PKR, and lead to autophosphorylation of the PKR homodimer and activation of its kinase activity. The activated PKR phosphorylates eIF-2α, which in turn inhibits protein synthesis and thereby viral replication in the cells. Ad, HSV and VV encode a number of viral proteins to inhibit the antiviral response. For examples, virus-associated (VA) RNAs produced by Ad; E3L and K3L proteins by VV and NS1 protein by IFA, all can inactivate PKR. Cancer cells have accrued many mutations in these signaling pathways. Inactivating mutations of the IFN signaling pathway can be found in many cancer cells. Oncolytic VSV targets this characteristic for its natural oncotropism. A constitutively activated Ras signaling pathway, another common feature for tumor cells, leads to inhibition of PKR autophosphorylation. Oncolytic NDV and reovirus depend on this characteristic for their tumor cell selectivity. This figure is modified from Everts and van der Poel, 2005 [8].

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Table 1

Examples of tumor cell-selective replicating oncoloytic viruses

Virus	Viral Gene Defect	Cellular Target(s)	Tumor Models	References
Ad: ONYX-015	E1B-55kD	Deficient p53 pathway	brain, cervical, larynx, liver, ovarian, thyroid	2,31
Ad: *dl* 922-947 and Δ24	E1A CR1 & CR2 domains	pRB in p16 pathway	brain, breast, cervical, colorectal, larynx	32,33
Ad: CNHK500	E1a gene controlled by the hTERT promoter while E1b gene by a hypoxia- responsive promoter	Cancer cells in a hypoxic environment	liver	133
HSV: G207, R3616, R1716	ICP34.5	Protein phosphatase 1α, and defective IFN pathway	brain, breast, coloretal, lung, ovarian, prostate	1,47,164
HSV: bM24-TE	ICP4 is controlled by TE promoter	Activated Tcf/Lef due to APC gene mutation	colorectal	126
NDV	none	Activated Ras pathway	fibrosarcoma, neuroblastoma	104,105
Reovirus	Wild type and mutation in S1	Activated Ras pathway	brain, breast, colorectal, ovarian	17,113,114
VSV	wildtype and mutations in M protein (AV1, AV2)	Defective IFN pathway	colorectal, lung, skin	97,98
IFA	NS1	PKR	melanoma	107,108
VV: vvDD	TK and VGF	Elevated dTTP pool and activated EGFR pathway	colorectal, liver, ovarian, skin	73,79,80
VV: vSP	SPI-1, SPI-2	Elevated anti-apoptotic proteins	colorectal, ovarian	81,82