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Design of virotherapy for effective tumor treatment
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The use of viruses as therapeutic agents against cancer is an old concept that has had a significant revival in the past two decades, in parallel with advances in methods to modify viral genomes genetically. From the initial stage of proof of concept, the field of virotherapy quickly progressed to the clinical setting, where serious limitations, yet promising opportunities, were identified. After demonstrating good safety profiles in humans, the objective in virotherapy has become to improve the efficacy of oncolytic viruses. Virotherapy approaches include incorporating therapeutic genes, evaluating alternative viruses with stronger oncolytic potential, employing new methods to improve biodistribution, and establishing greater insight into the influence of the immune system on both the success and failure of therapies. This review summarizes the most significant advances in recent years in the design of virotherapy for effective tumor treatment.

Keywords Cancer, gene therapy, oncolytic virus, therapeutic gene, tumor treatment, virotherapy

Introduction

Malignant transformation renders cells more susceptible to the lytic effect of certain viruses. The alterations responsible for this phenomenon are still under investigation, and are known to vary depending on the type of tumor and the particular virus. Wild-type viruses, such as measles virus (MV), vesicular stomatitis virus (VSV), Newcastle disease virus (NDV) and reovirus, have long been recognized to cause the preferential destruction of cancer cells. In addition, other viruses, such as adenovirus (Ad), HSV-1 or poxviruses (Poxs, particularly vaccinia virus [VV]), can be genetically modified to gain tumor specificity. This specificity can be obtained by abrogating the viral functions required for replication in normal cells, but that are redundant in cancer cells. An alternative, complementary approach is the transcriptional control of essential viral genes using tissue- or tumor-specific promoters. Modifying viral tropism to restrict infection to cancer cells constitutes an additional level of control. Recent methods to modulate gene expression, such as RNAi, are rapidly being incorporated into the design of oncolytic viruses (OVs). In the past 15 years, virotherapy has undergone notable expansion that is based, in part, on the construction of recombinant viruses. During this period, the acquisition of highly specific replication and cytopathic effects in cancer cells had been the primary objective. Clinical experience with OVs has increased, and at least 800 patients have been treated worldwide in phase I to III clinical trials, indicating that OVs are relatively safe. Tumor responses to OVs have also been reported (reviewed in reference [1]). Significant research efforts in the field of virotherapy are now focused on the search for new approaches to increase efficacy. Thus, fundamental aspects that limit the antitumor effect of OVs are now being

addressed, such as the role of the immune system and the physical barriers that impede the biodistribution of viruses inside tumors, as well as the potency and kinetics of the oncolytic effect. Incorporating therapeutic genes with immunostimulatory, pro-apoptotic or anti-angiogenic functions is one option to increase the efficacy of OV (Table 1). Cancer-initiating (stem) cells and components of the tumor stroma are being investigated as potential targets for OV. Moreover, combining OV with standard chemotherapy, radiotherapy or biological therapies is being investigated intensively following promising results that have been obtained in patients. This review summarizes the most relevant advances described in the field of virotherapy in recent years.

Table 1. Selected oncolytic viruses adapted as vectors for the expression of therapeutic genes.

[insert table 1 here]

[legend] **Ad** adenovirus, **ECM** extracellular matrix, **MV** measles virus, **Pox** poxvirus, **VSV** vesicular stomatitis virus

The regulation of viral replication

Incorporating target sequences for endogenous micro (mi)RNAs into the 3'UTRs of essential viral genes reduces the production of the corresponding viral protein and, consequently, reduces viral replication in cells expressing the specific miRNA. This approach has been used to inhibit the replication of wild-type Ad in healthy livers [2, 3]. Binding sites for the hepatocyte-specific miRNA-122 were introduced into the 3'UTR of the early viral gene *E1A* in mice, resulting in the strong inhibition of viral replication in hepatocytes and a concomitant reduction in liver damage, without decreasing the oncolytic potency of the virus in cancer cells that did not express miRNA-122 [2, 3]. A parallel strategy was used to avoid the replication of HSV-1 in normal cells. Multiple copies of complementary target sequences for miR-143 or miR-145 (which are expressed in normal cells, but are downregulated in prostate cancer cells) were inserted into the 3'UTR of the *ICP4* viral gene [4]. Selective viral replication was observed in prostate cancer cells, with a > 80% reduction in tumor volume in mice bearing LNCaP human prostate tumors. Although miRNA-mediated inhibition appears to be a general method that is useful for the selective reduction of virus replication, some viruses, such as VSV, are relatively resistant to this approach. Nevertheless, following the introduction of four tandem copies of the neuronal miR-125 target sequence into the 3'UTR of the VSV polymerase (*L*) gene, the neurotoxicity of the virus was reduced in mice [5]. Interestingly, the post-transcriptional regulation of viral genes by miRNAs can be used in combination with classical promoter replacement strategies to achieve an optimal control of viral replication. This concept was demonstrated by the controlled production of Ad E1A using the chromogranin-A promoter and miR-122 target sequences [6]. A double layer of genetic regulation was also obtained for the essential HSV-1 gene *ICP27*, with the androgen-responsive *ARR(2)PB* promoter and the 5'UTR of *rFGF-2* controlling transcriptional and translational expression of the gene, respectively [7]. Furthermore, RNAi technology has been used to obtain an apparent p53-restricted oncolytic Ad (OAV). In this elegant approach, an miRNA network directed against essential Ad genes was expressed under the transcriptional control of p53 [8]. Non-cancerous cells with an intact p53 activate the antiviral miRNA response and block the progression of infection.

Various tumor-specific promoters have been used for the construction of OV. Several of these promoters, such as the *SPARC* promoter, is active not only in cancer cells, but also in tumor-associated stromal cells [9]. This property is important to prevent tumor-associated stromal cells from acting as a barrier and preventing virus spread inside the

tumors. Transcriptional control of replication is also a versatile method to target cancer-initiating cells, and can be used in cases in which detailed information regarding gene expression profiles is available. Using this approach, an oncolytic HSV-1 that had replication regulated by the nestin promoter demonstrated the ability to destroy neuroblastoma-initiating cells [10]. An alternative strategy, based on specific viral gene deletions, led to the identification of a multimutated HSV-1 virus (G47 δ) that was active against glioblastoma stem cells [11]. Interestingly, deletion of the *ICP6* gene was neutral with respect to replication in these cells, but elimination of the neurovirulence factor γ 34.5 had a negative effect, which could be reversed by the simultaneous deletion of the α 47 gene.

The deletion of specific genes combined with alterations in the regulation of viral gene expression is usually more effective in the control of OV replication than through the use of either strategy alone. For example, deletions in the pRB-binding CR2 domain of the Ad *E1A* gene, eliminate the ability of E1A to interference with the pRB pathway but do not affect its function as a pan-activator of virus transcription [12, 13]. However, the deletions prevent the sequestration of pRB by E1A, thereby precluding the elevation of free E2F in quiescent cells infected by the virus. Therefore, placing this *E1A* mutant under the transcriptional control of optimized *E2F1* or hypoxia-responsive promoters can produce highly tumor-specific OAVs.

Strategies to increase the oncolytic effect of oncolytic viruses

The good safety record of OVs that has been established in clinical trials suggests that there is the possibility to develop less attenuated, more potent agents with an increased likelihood of eliciting a clinically relevant antitumor effect. This possibility has prompted researchers to investigate new approaches to increase the oncolytic effect of viruses and to re-evaluate the methods used to obtain tumor specificity. For example, IFN type I (IFN-I)-sensitive viruses, such as VSV and NDV, replicate preferentially in cancer cells, at least in part, because the cells have altered IFN pathways. In fact, the incorporation of IFN β as a transgene [14] or mutations in the VSV matrix (*M*) gene that induce increased the production of IFN in infected cells [15] were reported to increase the safety of the virus. However, attenuation also affects the ability of viruses to replicate in some cancer cells, and methods to counteract this effect are being investigated. For example, treatment with the VSV-M δ 51 virus in combination with the mTOR inhibitor rapamycin reduced the induction of IFN-I and increased the antitumor effect of this virus, without any evident toxicity [16]. In addition, the inhibition of NK and NK T-cell (NKT) function by virus-mediated expression of the *UL141* gene from human cytomegalovirus enhanced the replication of VSV in the tumors of immunocompetent rats following intra-arterial administration of the vector, resulting in prolonged survival [17]. Furthermore, expression of the IFN antagonist gene *NS1* from influenza virus in NDV favored the formation of syncytia, the lytic activity of the virus and the overall efficacy of the treatment in a syngeneic murine melanoma model, similarly without signs of toxicity [18].

The oncolytic effect of viruses can be enhanced by potentiating specific viral functions, while leaving the overall biology of the virus relatively unaffected. For example, a point mutation in the *F* gene of NDV was demonstrated to improve the fusogenic activity of the virus and to increase antitumor effects in a multifocal liver cancer model in Buffalo rats, without damaging the surrounding hepatic parenchyma [19]. In addition, incorporating the MV wild-type *N*, *P* and *L*

genes into the oncolytic Edmonston strain resulted in a virus with increased rates of replication and transcription, both of which were relatively insensitive to the inhibitory effect of IFN α [20]. Another approach that can be used to increase the potency of the oncolytic effect involves the use of gain-of-function mutants, which can be discovered by the *in vivo* bioselection of randomly mutagenized viruses. This method has recently been applied to Ad; one of the mutants identified exhibited a truncation in the E3/19K protein that changed its cellular localization from the endoplasmic reticulum to the plasma membrane, where the protein acted as a viroporin and induced membrane permeabilization [21]. The incorporation of this mutation into Ad increased the release of progeny and potentiated the antitumor effect.

Methods to improve biodistribution of oncolytic viruses

Infecting an adequate proportion of cancer cells in a solid tumor is challenging. In addition to the immune system, OVs encounter other important obstacles to infection, including the low expression of viral receptors in target cells, physical barriers, binding to blood components and sequestration by the liver. Several methods are being evaluated to improve the biodistribution of oncolytic viruses.

[sub] Virus retargeting

The discovery that hepatocyte infection by Ad type 5 (Ad5) requires the binding of coagulation Factor X to the major component of the viral capsid (hexon protein) led to the construction of a virus mutant in the hypervariable loop 5 of the hexon protein that avoids this interaction [22]. Using luciferase as a reporter gene, the mutant was demonstrated to exhibit a significant reduction in liver transduction in immunocompetent and immunodeficient mice. Reducing sequestration of the virus by the liver should increase the number of viral particles available for infecting other tissues, including tumors. However, the bioluminescence levels of human tumor xenografts in mice infected with the mutated virus were not affected [22]. These results indicate that liver detargeting should be combined with efficient strategies to increase the affinity of Ad for tumor cells. One such approach involves the incorporation of peptides into the HI loop of the fiber knob; this modification is compatible with capsid integrity, and expands the tropism of the virus. For example, an Ad incorporating a peptide derived from the VP1 capsid protein of the foot-and-mouth disease virus was efficiently retargeted to infect carcinoma cells expressing the $\alpha_v\beta_6$ integrin [23]. Compared with wild-type Ad, the retargeted Ad exhibited up to 50-fold increases in coxsackievirus- and Ad-receptor-independent transduction, and up to 480-fold increases in cytotoxicity against a panel of $\alpha_v\beta_6$ -positive human carcinoma lines. An alternative strategy that allows Ad particles to avoid clearance by the liver, protects the particles from neutralizing antibodies and permits tumor retargeting of the virus is the coating of the virus with hydrophilic polymers, such as poly(hydroxypropylmethacrylamide). Moreover, the addition of an anti-EGFR antibody (cetuximab) to the coated virus achieved preferential infection of human ovarian cancer cells in a xenograft model [24].

Tumor retargeting of an oncolytic HSV-1 virus was recently demonstrated by the genetic modification of glycoprotein D (gD), which is an important mediator of virus entry into cells [25]. The chimeric gD protein displaying a single-chain antibody against the HER2 receptor reduced the toxicity of HIV-1 and resulted in the preferential infection of HER2-overexpressing human tumor xenografts in mice. A parallel strategy was used to direct MV to infect cells expressing

the urokinase-type plasminogen activator receptor (uPAR), which is overexpressed in various malignancies [26]. The domains of uPA that are implicated in binding to the uPAR were incorporated into the MV-H protein. Importantly, the retargeted MV efficiently infected not only breast cancer cells, but also the tumor vasculature in a breast cancer xenograft model in mice. Thus, this tropism may enhance the biodistribution and antitumor effect of the virus.

[sub] Carrier cells

A variety of cells have been proposed as carriers for OV, based on their ability to penetrate solid tumors and areas of cancer dissemination, such as lymph nodes. Carrier cells can simply transport the virus over their surface or inside cellular compartments, or can be permissive for OV replication, which provides the advantageous possibility of delivering more particles than the quantity originally loaded. In all cases, a desirable property of carrier cells is to protect the virus from neutralizing antibodies and other antiviral elements present in the circulation.

The combination of the OV and carrier cell used depends on each particular application. In general terms, cells from solid tumors will support high levels of virus amplification, but are trapped in the small capillary beds of the lung and other organs. Cells of hematopoietic origin exhibit wider biodistribution and may exert additional immunostimulatory effects against tumors. Moreover, infection with an OV may enhance the functions of the immune cells and, in some cases, the additional immunostimulatory effects are entirely responsible for the antitumor effect, as described recently for T-cells loaded with reovirus in a murine model of melanoma [27]. A cooperative effect between viral oncolysis and immunotherapy is also possible, and the balance between antiviral and antitumor immune responses determines the outcome of these immunovirotherapy strategies. For example, in C57Bl/6 mice bearing lymph node B16tk melanoma metastases, mature dendritic cells (DCs) loaded with reovirus were more effective antitumor agents than T-cells if mice were pre-immunized against the virus [28]. In another study, adoptive cell therapy in immunocompetent mice using melanoma-specific T-cells loaded with VSV resulted in improved antitumor responses compared with virotherapy alone, and the approach could be optimized without increasing toxicity by the addition of biological therapy (eg, IL-2 conditioning and regulatory T-cell [Treg] depletion) [29].

Stem cells from various origins have recently been considered as popular carriers for OV because of their natural tropism and ability to infiltrate solid tumors, including large lesions [30]. The capacity of mesenchymal stem cells (MSCs) to support the amplification of several OV, including Ad and MV, makes the use of this cell type an appealing option. Although the detailed biodistribution of viruses following systemic administration has not been fully characterized, intra-arterial administration of MSCs loaded with an OAV has recently been described as leading to the infection of orthotopic glioma xenografts and increased survival in mice [31]. In another study conducted in passively immunized mice bearing ovarian cancer xenografts in the peritoneum, MSCs were demonstrated to protect OV from neutralizing antibodies and to allow the infection of cancer cells [32]. MSCs were loaded with MV and, in this case, cell-to-cell heterofusion was responsible for the transfer of viruses. Similar results were obtained using irradiated myeloma cells as carriers for MV in a disseminated myeloma xenograft model in mice [33]. However, these studies were conducted in immunodeficient mice, and it is unclear if these, or other carrier cells, are similarly efficient in the

transfer of non-fusogenic viruses in immunocompetent, pre-immunized animals. Other stem cells, such as neural and adipose-derived stem cells, have recently been used as carriers for OAV [34] and myxoma virus [35], respectively. The cells were permissive for virus replication, and demonstrated the ability to migrate toward orthotopic glioma xenografts in mice following intracranial injection. However, although the use of carrier cells is a promising and conceptually appealing approach, the reality is that only a small fraction of systemically administered carrier cells in tumor-bearing animals reach their targets [36]. Nevertheless, in a recent exploratory clinical trial, the infusion of MSCs loaded with the ICOVIR-5 OAV obtained a strong partial remission in one out of four children with refractory stage IV neuroblastoma, and subsequent standard chemotherapy achieved a durable complete response in this individual [37]. In addition to systemic administration, local or regional delivery of OV-loaded cells may be advantageous [32]. In the setting of local or regional delivery, the main objectives are protecting the virus from neutralizing antibodies to enable the efficient re-administration of OV, and eliciting potential vaccination effects if tumor cells are used as carriers.

Incorporating therapeutic genes into oncolytic virus vectors for cancer gene therapy

An approach to increase the efficacy of OVs involves incorporating therapeutic genes into the vectors. Genes with pro-apoptotic, cytotoxic, anti-angiogenic or immunostimulatory functions may have potential in cancer gene therapy.

[sub]Pro-apoptotic and cytotoxic genes

Various genes that promote pro-apoptosis and cytotoxicity have been incorporated into OVs to increase the oncolytic effect. For example, in one approach, the pro-apoptotic genes *TRAIL* and melanoma differentiation-associated gene 7 (*MDA-7/IL-24*) were incorporated into an OV, which subsequently replicated in, and was released from, transduced cells, causing considerable amplification of the oncolytic effect of the virus [38-40]. In another approach, the sodium/iodide symporter (*NIS*) gene was introduced into an OAV with replication restricted to cells with an active Wnt signaling pathway [41]. Although restricting viral replication to cells with such an active pathway reduced the intrinsic oncolytic effect of the OAV, expression of the *NIS* transgene resulted in the accumulation of the systemically administered radioisotope ^{131}I in transduced cells. Moreover, a clear improvement in the antitumor effect of the *NIS*-expressing OAV was observed compared with an equivalent virus that did not express *NIS*. In addition, *in vivo* monitoring of transgene expression and spread of the virus was possible by PET/CT imaging following administration of the $^{99\text{m}}\text{TcO}_4^-$ radiotracer [41]. The antitumor effect was also enhanced when *NIS* was expressed in an oncolytic MV [42].

The gene-directed enzyme/prodrug therapy (GDEPT) has been hampered by the inefficient tumor transduction of replication-defective vectors. Therefore, the adaptation of OVs as vectors for these genes has increased research interest in this approach. In addition, the development of improved versions of prodrug-converting enzymes, such as a fusion between the yeast cytosine deaminase and uracyl-phosphoribosyltransferase, ensures the efficient elimination of infected and neighboring cells upon administration of the prodrugs [43]. The benefit of combining a replication-competent vector with the expression of the enzyme is demonstrated by several lines of evidence. First, the addition of the prodrug increases the antitumor effect of these vectors, indicating that both mechanisms of action (direct

oncolysis and drug cytotoxicity) are at least additive [44]. Second, OV carrying these genes have shown enhanced antitumor effect versus replication-deficient vectors in xenograft models of cancer [45].

[sub] Anti-angiogenic genes and extracellular matrix proteases

In principle, the kinetics of transgene expression by OVs do not favor their use as vectors to express anti-angiogenic genes, because duration is too short, especially in immunocompetent hosts [13]. However, recent preclinical data have contradicted this prediction. For example, an oncolytic Pox expressing the single-chain antibody GLAF-1 against the pro-angiogenic factor VEGF demonstrated an improved therapeutic effect compared with the parental virus in human xenograft tumors in mice [46]. Furthermore, the production of GLAF-1 could be detected in the serum of animals for at least 1 month following infection; however, no information was available regarding the immunocompetence of the mice. Recently, another study used Pox to deliver a soluble form of VEGFR-1 fused to the Fc tail of human IgG (VEGFR-1-Ig) [47]. The fusion protein was detected for at least 2 weeks in athymic mice compared with 3 days in immunocompetent mice, suggesting VEGFR-1-Ig is expressed for a significantly shorter time period in immunocompetent animals. In both cases, the virus armed with VEGFR-1-Ig exhibited a superior antitumor effect compared with the unarmed control virus expressing only a luciferase reporter gene. However, the difference in antitumor effects between the armed and control viruses was only apparent at moderate to low doses of vectors, including systemically administered doses. [47]. This confers high relevance to this results, because gene therapy approaches that show efficacy upon systemic administration have more chances to be effective against advanced cancer in patients.

The expression of proteases that degrade the extracellular matrix (ECM) of tumors can enhance the biodistribution of OVs. This approach is gaining popularity, as an increase in the antitumoral activity of OVs has been observed in xenograft models treated with OAV expressing enzymes like hyaluronidase. This increase correlated with a wider distribution of the virus in tumors, from 6 to 13% of the total viable tissue [48]. In iaddition, initial concerns regarding the possibility of stimulating the formation of distant metastases have not been demonstrated [48]. Hyaluronidase acts on glucosaminoglycans causing a transient increase in the penetration of molecules up to 200 nm in diameter, without affecting vascular permeability [49].This means that most viruses, but not tumor cells, can increase their dispersion. An alternative to enzymes is decorin, a proteoglycan that decreases the diameter of collagen fibrils and enhances the remodeling of the ECM. Treatment of melanoma xenografts with an OAV expressing decorin caused a drastic inhibition of primary tumor growth and reduced the appearance of metastasis, with a significant prolongation of the survival of animals [50].

[sub] Immunostimulatory genes

The importance of the immune system in the response to various treatment modalities is being investigated intensively. Virotherapy is probably the most extreme example of a treatment approach in which the interaction of the therapeutic agent with the immune system is unavoidable. Strategies can be designed either to minimize the immune response in favor of viral oncolysis, or to use the immune system to aid the anticancer effects of OVs.

In one approach, addition of the immunosuppressive agent cyclophosphamide enhanced replication of VV and prolonged survival in a model of intracranial glioma in immunocompetent rats [51]. Moreover, even OV_s that are thought to rely on the IFN system for their tumor specificity, such as NDV, are now being produced with genes that block the IFN response [18], or are being combined with the immunosuppressive agent rapamycin to increase their efficacy, as described for VSV [16]. However, if the manipulations increase the biodistribution of the virus, then viral replication must be tightly controlled in order to avoid side effects (see *The regulation of viral replication* section).

An alternative approach uses OV_s to stimulate an efficient immune response against cancer cells. This strategy could overcome important limitations shared by both virotherapy and cancer gene therapy, such as biodistribution, efficacy of transduction and persistence of vectors in immunocompetent hosts. OV_s may contribute to the reversion of the immunosuppressive environment of tumors, as these agents are recognized as danger signals by the immune system and strongly activate innate and adaptive responses. However, OV_s may also polarize the response against strong viral antigens to the detriment of tumor antigens [52] [53]. It is clear from clinical experience that the most frequent outcome of natural viral infections or the administration of OV_s to patients with cancer is the stimulation of antiviral immune responses that result in efficient elimination of the virus, but not regression of tumors [54]. In reports of tumor responses to natural infections or vaccination with attenuated viruses (reviewed in reference [1]), the relevance of the immune system is unclear. However, some preclinical studies indicate a prominent role of the immune system in the antitumor effect of OV_s [55-57]. Therefore, virus-mediated stimulation of anticancer immune responses is possible, but unraveling this potential may require specific modifications to the viruses. OV_s may function simultaneously as adjuvants and vectors for the expression of genes that stimulate virus-mediated anticancer immune responses. An example of such an approach is OncoVEX GM-CSF (BioVex Inc), an HSV-1 virus that has been attenuated by deletion of the *ICP34.5* and *α 47* genes and has been adapted as a vector for expressing GM-CSF. This OV replicates selectively in and destroys tumors, and also induces an immune response to kill cancer cells throughout the body. The efficacy of OncoVEX GM-CSF was evaluated in a phase II clinical trial in patients (n = 50) with metastatic melanoma. The agent was injected intratumorally, with repeated administrations at 2-week intervals for up to 24 treatments [58]. The treatment was well tolerated and achieved a 26% response rate, which included eight complete responses of non-injected lesions [58]. An analysis of tumors undergoing regression demonstrated reversion of the immunosuppressive microenvironment of tumors, with increases in melanoma-specific T-cells and reductions in Tregs, suppressor T-cells and myeloid-derived suppressor cells [59]. Trials of a Pox expressing GM-CSF (JX-594; Green Cross Corp/Jennerex Inc) revealed objective responses in patients with melanoma and liver cancer, some of whom exhibited objective responses in non-injected lesions and in the presence of neutralizing antibodies (reviewed in reference [60]). JX-963 (Jennerex) is an evolution of JX-594, with an additional deletion of the vaccinia growth factor (*VGF*) gene to improve tumor selectivity. The virus demonstrated antitumor efficacy against hepatic tumors in a rabbit tumor model following systemic administration, without evidence of organ toxicity [61].

Other immunostimulatory approaches that have demonstrated promising results in preclinical studies include the expression by OAV_s of the cytokines IL-12 [13] or GM-CSF [62], the costimulatory ligand CD40 [63], the chemokine

RANTES [64] and the antimicrobial peptide β -defensin 2 [65]. In addition, the expression of IFN β using a replication-competent VSV increased the safety and efficacy of the virus in a mesothelioma model in mice [14]. These types of OV s can be used in combination with biological therapies to induce robust antitumor responses. For example, administering dendritic cells increased the efficacy of an OAV expressing *IL-12* and the costimulatory molecule *41BBL* in a murine model that was non-permissive for viral replication [66]. However, responses to 'immunovirotherapy' are difficult to predict, and require extensive testing in relevant models. For example, the use of highly immunogenic, replication-competent vectors is not always the best choice for the expression of immunostimulatory molecules, as described for *CD40L* in a syngeneic melanoma model in mice [53]. In this study, the authors found that the immune system reacted preferentially against VSV antigens instead of tumor antigens, despite the potent costimulatory signal provided by CD40L.

Combining oncolytic viruses and standard therapies

Multiple preclinical studies have demonstrated the benefit of various combinations of OV plus standard therapies for the potential treatment of cancer, suggesting that combined treatment strategies will increase the feasibility of the clinical use of OV s in the near future. The most studied regimens consist of OV s in combination with chemotherapeutic agents, including 5-fluorouracil [67], cisplatin [68], oxaliplatin [39], gemcitabine [69] temozolomide [70] and taxanes (eg, docetaxel and paclitaxel) [71]. Evidence also suggests that OV s can cooperate with radiotherapy [68]. In combined treatment regimens, the sensitization of cancer cells to apoptosis, together with the stimulation of immunological responses, are considered responsible for the increased antitumor effect. These mechanisms of action should be considered in order to establish the most favorable combination regimen. For example, OAV s designed to express transgenes that stimulate apoptosis [39] or with selective deletions of viral genes [69] are particularly suited for this purpose. In addition, OV s can be designed specifically to increase their activity in response to radiotherapy by using radio-inducible promoters for the control of viral functions [72].

Conclusion

Different aspects of virotherapy have undergone progress in recent years. For example, methods to regulate gene expression, such as RNAi technology, have been applied to improve the control of viral replication. However, perhaps more importantly, advances have been made regarding issues that limited the efficacy of OV s in early clinical trials. Attempts to improve virus biodistribution and tumor penetration, and to protect these agents from neutralizing antibodies, have used carrier cells, some of which demonstrate intrinsic antitumor activity. The optimal combination of carrier cell and OV remains to be defined, but recent clinical observations are encouraging. There is also a trend in research efforts within the field to increase the potency of OV s, even if this potency would result in a moderate reduction in tumor specificity. Poxs, particularly vaccinia-based OV s, which naturally possess favorable biodistributions and potent oncolytic effects, have attracted substantial interest in the past 2 years. Progress has continued on the incorporation of therapeutic genes into OV s and, despite all of the limitations of OAV s, this group of viruses continues to be the most frequently used platform. A greater understanding of the immunological aspects of virotherapy, particularly with respect to reovirus and VSV, is preparing the field for further developments that will attempt to avoid

immune reactions against OV_s or will use this response for tumor destruction. Currently, 'virocentric' and 'immunocentric' approaches are divergent, but the development of methods to inhibit antiviral responses selectively may reconcile them. A rational optimization of virotherapy will be aided by the development of mathematical models. At present, these models cannot integrate all of the variables encountered *in vivo*, but are able to produce reasonable predictions regarding the influence of individual parameters, such as the immune system, the duration of the viral life-cycle, the spread of the virus and tumor architecture [73-75]. Thus, recent advances in virotherapy are providing not only improved OV_s, but also better strategies to integrate these agents into multimodal treatment strategies against cancer.

Table 1.

Function of transgene	Name of transgene	Type of virus	Name of oncolytic virus	Reference
Pro-apoptotic and cytotoxic	<i>TRAIL</i>	Ad	SG235-TRAIL	[38]
	<i>IL-24</i>	Ad	Ad.sp-E1A(δ 24)	[40]
	<i>NIS</i>	Ad	AdIP2	[41]
	<i>FCU1</i>	Ad	Ad5/3-D24FCU1	[43]
	<i>TRAIL</i>	Pox	VVTRAIL	[39]
Anti-angiogenic and ECM degradation	<i>NIS</i>	MV	MV-NIS	[42]
	<i>sPH20</i>	Ad	ICOVIR17	[48]
	<i>VEGFscAb</i>	Pox	GLV-1h68 derivatives	[46]
Stimulation of immune system	<i>sVEGFR-1</i>	Pox	vvdd-VEGFR-1-Ig	[47]
	<i>IL-12</i>	Ad	Ad-DH-scIL12	[13]
	<i>GM-CSF</i>	Ad	TOAO2	[62]
	<i>CD40L</i>	Ad	AdEHCD40L	[63]
	<i>RANTES</i>	Ad	Ad-RANTES-E1A	[64]
	<i>BD2</i>	Ad	Ad-BD2-E1A	[65]
	<i>IL-12 and 4-1BBL</i>	Ad	Ad- Δ B7/IL12/4-1BBL	[66]
	<i>GM-CSF</i>	HSV	JS1/34,5-/47-/GM-CSF	[58]
	<i>EGFP</i>	Pox	vvDD-EGFP	[51]
	<i>GM-CSF</i>	Pox	JX-963 (Jennerex Inc)	[61]
	<i>δM51</i>	VSV	VSV(δ M51)	[16]
	<i>INFβ</i>	VSV	VSV-IFN β	[14]
	<i>CD40L</i>	VSV	VSV-CD40L	[53]

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Reference annotations

- of outstanding interest
- of special interest

Reference [7] Lee *et al*

- Demonstrated that expression of the essential gene ICP27 could be controlled using an androgen-responsive promoter in conjunction with the 5'UTR from the rFGF-2 gene. Therefore, the strict regulation of viral gene expression, rather than the deletion of neurovirulence genes, may result in an HSV-1 with stronger oncolytic effects.

Reference [11] Wakimoto *et al*

- Demonstrated that gene deletions in HSV-1 correlated with the ability of the attenuated virus to kill glioblastoma stem cells.

Reference [13] Bortolanza *et al*

- Describes an OAV in which the CR2 domain of the E1A gene was partially deleted, and in which the E1A and E4 genes were regulated simultaneously at the transcriptional level. Viral-mediated expression of the cytokine IL-12 achieved antitumor effects in an immunocompetent tumor model in Syrian hamsters that supports Ad replication

Reference [21] Gros *et al*

- *Identified a mutation in the E3/19K gene of Ad that changed the cellular localization of the protein from the endoplasmic reticulum to the plasma membrane; the associated acquisition of viroporin-like functions accelerated the release of viral progeny.*

Reference [22] Shashkova *et al*

- *Demonstrated that a mutation in hypervariable loop 5 of the Ad hexon protein inhibited the binding of the capsid to blood factors, and reduced liver transduction and toxicity in both immunocompetent and immunodeficient mice.*

Reference [25] Menotti *et al*

- *Describes the genetic modification of the receptor-binding protein gD from HSV-1 with an anti-HER2 single-chain antibody for targeting the virus to carcinoma cells. This approach achieved the preferential infection of cells expressing HER2.*

Reference [26] Jing *et al*

- *Demonstrated that incorporating the receptor-binding domains of uPA into MV-H achieved the preferential infection of uPAR-overexpressing carcinoma cells and tumor vasculature.*

Reference [28] Ilett *et al*

- *Investigated the use of different carrier cells as vehicles for reovirus in naïve or pre-immunized mice harboring lymphatic melanoma metastases. Mature dendritic cells were more efficient antitumor agents than immature dendritic cells or T-cells in reovirus-immunized mice.*

Reference [31] Yong *et al*

- *Demonstrated that intra-arterially administered MSCs loaded with an OAV were able to migrate and to transfer the virus to intracranial glioma xenografts in mice.*

Reference [58] Senzer *et al*

- *Demonstrated the antitumor efficacy of an HSV-1 OV encoding GM-CSF in patients with advanced melanoma. Intralesional administration of the virus led to the regression of some uninjected tumors.*

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