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ONCOLYTIC VIRUSES FOR THE THERAPY OF BRAIN TUMORS AND OTHER SOLID MALIGNANCIES: A REVIEW

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1. ABSTRACT

In spite of significant advances in the understanding of molecular processes in tumor biology that have led to the development of oncologic therapeutic strategies, the prognosis for several types of tumors (such as brain, pancreas, or hepatic malignancies) remains dismal. Without question, a strong need exists for continued investigations in new agents and new therapeutic regimens.

The realization that several genes used by viruses in their lytic life cycle interact and/or complement the function of genes employed by cells in cellular events linked to cell cycle progression, apoptosis, and/or metabolism immediately suggests the development of treatment strategies wherein viral mutants could be employed as selective anticancer agents. Such viruses (designated as oncolytic viruses) can selectively grow in tumor cells, produce viral progeny in those cells, lyse them and release this progeny that can then infect additional cells in the tumor mass. A theoretical advantage of oncolytic viruses (OV) is that their numbers should augment within the tumor mass, a property that is lacking with drugs or radiation treatments. Additionally, Ovs' mode of tumor killing differs from standard anticancer agents, providing the possibility for synergistic interactions in multimodal tumor therapies.

In this review, we will describe the development of OVs and briefly review the life cycle of their wild-type (wt) counterparts. We will also summarize published results from OV clinical trials and attempt to provide a perspective on research in this area.

2. INTRODUCTION

In spite of significant advances in the understanding of molecular processes in tumor biology that have led to the development of oncologic therapeutic strategies, the prognosis for several types of tumors (such as primary brain, pancreas, or hepatic malignancies) remains dismal. Without question, a strong need exists for continued investigations in new agents and new therapeutic regimens. It is likely that no single treatment, but rather a combination of different treatments with different anticancer modalities can achieve this goal. New anticancer agents could be used in tumor therapy without inducing cross-resistance with current standard drug-based and radiation-based approaches.

Historically, several strains of viruses were used in an attempt to treat tumors. For instance, an Italian physician published in 1911 a report demonstrating spontaneous regression of cervical carcinoma after injection

with a live rabies vaccine (1). After this report, several types of wt viruses continued then to be anecdotally used in tumor therapeutics: the strategy of employing an agent that could itself grow within a tumor and destroy it seemed propitious for therapeutic exploitation (2). However, the advent of modern chemotherapeutics and radiotherapeutics in the 1950s provided defined dosing and known chemical structures to the treatment of patients, an alternative that was more appealing than the use of undefined cellular lysates containing potentially pathogenic organisms, thus leading to a general absence of interest in the field of viral-based therapeutics. Interestingly, as gene therapy based on the use of viral vectors to deliver cDNAs into cells, became an active area of research in the late 1980s, exploitation of viruses as cancer-killing agents started to make a comeback in terms of research efforts. In fact, in 1991 Martuza et al. described the use of a herpes simplex virus (HSV) mutant (dlsptk) that appeared to destroy tumor cells in a relatively selective fashion (Table 1) (3). Since that report, numerous other types of genetically engineered viral mutants have been described and the field has advanced to the point of human clinical trials.

Additional advancements in this field have arisen from knowledge acquired in molecular virology. It is now evident that the life cycle of a virus involves multiple interactions between viral and cellular proteins/genes (for example the binding of the adenoviral E1B55Kd factor to p53 tumor suppressor protein), which maximize the ability of the virus to proliferate within the infected cell. Exploitation of such interactions has led to the design of interesting and sometimes complex strategies to selectively kill tumor and spare normal cells. The "ideal" OV should include all the following characteristics: a) specificity for tumor cells, b) rapid and effective lysis and propagation within a tumor, c) low or bearable toxicity, d) facility in engineering, e) facility in large scale production, f) genomic stability, g) possession of "fail-safe" mechanisms for inactivation, and h) absence of potential spread to the general population and/or unwanted generation of toxic, undesirable mutants that could pose danger to the general population. The potential of current OVs for fulfilling all these criteria exists, but further manipulation of viral genomes to find better OVs will remain an active area of investigative development. Like the pharmaceutical quest for better anticancer drugs that target novel cancer processes, a virologic quest for improved OV that target such oncogenic events appears poised for interesting discoveries.

In this review, we plan to provide an overview of the different types of OVs, tested in preclinical and clinical models. We first describe the biologic features of wt strains of viruses, investigated for oncolytic tumor therapy. We then review the different modifications, made in these viruses, in order to improve their specificity for tumor cells and therapeutic efficiency. Finally, we will describe the current state of clinical trials in oncolytic viral therapy and provide a perspective in areas of future research.

3. VIRUSES EMPLOYED FOR THERAPY

A large variety of both RNA and DNA viruses have been tested as potential anticancer agents. In general, most of the tested viral species are relatively innocuous and endemically present in the general population, although it is evident that in particular cases (such as immunosuppression) most viral species can provoke illnesses of various severities. The modern history of oncolytic viral therapy shows that investigators have worked with either attenuated, yet wt, viral strains whose tumor specificity was a priori unknown or genetically engineered mutants whose tumor specificity was thus endowed by the engendered mutation(s). Examples of the first type include reovirus, Newcastle disease virus (NDV), vesicular stomatisis virus (VSV), vaccinia virus and autonomous parvovirus (APV), while adenovirus, herpes simplex virus (HSV) and poliovirus represent example of the second type.

3.1. Wild-type replication competent viruses that display tumor specificity

3.1.1. Reovirus

WT reovirus is mildly pathogenic and appears to widely spread in the human population, (approximately 70% of the human population is serologically positive). It displays tropism for epithelial cells of the respiratory and intestinal tracts and in neonates/children it is associated with mild respiratory and enteritic illnesses. Structurally, it is composed of an icosahedral capsid, which surrounds a core containing the double stranded RNA genome. Its genome encodes for a total of eleven proteins (4). Because of its double stranded RNA structure and the lack of dispensable genes, routine genetic manipulations of the virus remain difficult and have not been achieved so far. In fact, virological studies have employed the isolation of naturally occurring mutant strains for functional genetic analyses.

The oncolytic properties of reovirus were first observed by Klein et al. (Table 1), who noticed that mice infected with this virus developed humoral immunity to transplanted tumors (5). Even more interestingly, it was shown that mouse NIH 3T3 cells, which generally are impervious to reovirus replication, lost this resistance upon transfection with activated Ras. This suggested that reovirus could selectively replicate in cells with an activated Ras pathway (6). These data were confirmed by experiments performed in SCID mice showing that a single injection of reovirus was sufficient to induce a 65-80% tumor regression of transplanted U87 human glioblastoma cells, which exhibit an activated Ras pathway (6, 7). Indeed, the presence of reovirus transcripts induces activation of the double stranded activated protein kinase (PKR), which in turn inhibits RNA translation (8). Since other groups have shown a relation between the Ras pathway and PKR, it is postulated that activated Ras inhibits PKR activity and allows translation of reovirus transcripts (9). Clearly the identity of the factors and molecular events that link the Ras pathway to PKR remain to be elucidated. Given the high frequency of activated Ras pathway in human tumors, reovirus represents an attractive agent for oncolytic viral therapy and phase I/II clinical trials in patients with advanced lung, breast and colon cancer are in preparation (Table 1) (10).

3.1.2. Newcastle Disease Virus

NDV is a negatively stranded RNA virus responsible for Newcastle disease, a respiratory infection in avians. Although the molecular basis for the preferential

Table 1. Oncolytic viruses in pre-clinical and clinical models.

Viruses	Oncolytic strains	Tumor targeting mutations	Cythopatic mutations	Permissive cells	Tumors[1]	Clinical trials	Injection methods**	References
Viruses with innate tumor specificity								
reovirus	wt	none	none	ras + EGFR +	lung breast colon glioma	phase I	intra-tumoral	5-7,10
NDV	wt	none	none		bladder SCCHN ovarian	phase III	ex vivo	11-16
NDV	PV701	attenuated strain	none			phase I		138
VSV	wt	none	none	IFN-gamma -	melanoma	no	no	17-19
vaccinia	wt	none	none	broad	melanoma	phase I	intra-tumoral	22
H-1 APV	wt	none	none	p53 -		phase I		28-30
AAV	wt	none	none	p53 -	colon	no	no	137
Deletion retargeting								
HSV-1	dlsptk	deleted HSV-TK	none	replicating	glioma	no	no	3
HSV-1	hrR3	deleted *UL39* (ICP6)	none	replicating	glioma liver	no	no	66-69
HSV-1	R3616	deleted *gammma-34.5* (ICP34.5)	none	ras +	glioma	no	no	70
HSV-1	G207	deleted *UL39* and *gamma-34.5*	none	ras +/replicating	glioma	phase I	intra-tumoral	76-83
HSV-1	NV1020	deleted single copy of *gamma-34.5*	none	replicating	colorectal	phase I	intratumoral	127
adenovirus	dl1520[2]	deleted *E1B-55kD*	none	p53 - controversial	SCCHN[3] colorectal ovarian pancreatic	phase I-III	intra-tumoral intra-peritoneal intra-arterial intra-venous	53,57,58
adenovirus	dl922-947	deleted E1A-CR2	none	pRb -	carcinomas	no	no	54-56
polyovirus	PV1 (RIPO)	IRES substitution	none	CD155 +	glioma	no	no	59
Transcriptional retargeting: gene-promoter								
HSV-1	d120	*ICP4-albumin* enhancer	none	albumin +	HCC[4]	no	no	98
HSV-1	Myb34.5	*gamma-34.5-Bmyb* promoter	none	replicating	liver	no	no	102,103
adenovirus	AvE1a04i	*E1A-AFP* promoter	none	AFP +	HCC	no	no	104
adenovirus	CN706	*E1A-PSA* promoter	none	PSA +	prostate	phase I	intratumoral	106
adenovirus	Ad.DF3-E1	*E1A-DF3/MUC1* promoter	none	MUC1 +	breast	no	no	107
adenovirus	CV764	*E1A-PSA* promoter *E1B-kallikrein* promoter	none	PSA + kallikrein +	prostate	no	no	110
adenovirus	CN787	*E1A-probasin* promoter	none	PSA +	prostate	phase I	intravenous	111
adenovirus	HYPR-Ad	*E1-HIF* responsive elements	none	hypoxic	glioma	no	no	abstract
Modifications enhancing therapeutic efficiency								
HSV-1	rRp450	deleted *UL39*	insertion *CYPB21*	replicating	glioma	no	no	118
HSV-1	NV1023	deleted single copy of?*gamma-34.5*	insertion *IL12*	replicating	colorectal	no	no	127
HSV-1	R8305	deleted *gamma-34.5*	insertion *IL4*	replicating	glioma	no	no	128
HSV-1	R8307	deleted *gamma-34.5*	insertion *IL10*	replicating	glioma	no	no	128
HSV-1	R47-delta	deleted *gamma-34.5*	deleted *alpha-47*	replicating	glioma	no	no	129
HSV-1	G47-delta	deleted UL39 and *gamma-34.5*	deleted alpha-47	replicating	glioma	no	no	129
adenovirus	Ad.TKRC	deleted *E1B-55kD*	insertion *HSV-TK*	controversial	lung	no	no	113,114
adenovirus	FGR	deleted *E1B-55kD*	insertion *CD/HSV-TK*	controversial	prostate	phase I	intratumoral	120
adenovirus	01/PEME	insertion antagonist E2F under p53 control	*E3-11.6kD* under control of MLP	p53 -	PC3	no	no	96
adenovirus	adl118	deleted *E1B-55kD*	deleted *E1B-19kD*	controversial	breast	no	no	122,123
adenovirus	ONYX 300	deleted *E1B-55kD*	deleted *ADP*	controversial		no	no	124-126
adenovirus	ONYX 305	deleted *E1B-55kD*	*CD* in *ADP* site	controversial		no	no	124-126
adenovirus	ONYX 320	deleted *E1B-55kD*	*TNF* in *ADP* site	controversial		no	no	124-126
adenovirus	Ad5/IFN	deleted *E3*	*INF*-con1in *E3*	p53 -	breast	no	no	135
VSV			insertion *HSV-TK*			no	no	20
VSV			insertion *IL-4*			no	no	20
vaccinia	v-GM-CSF	none	*GM-CSF* insertion	broad	melanoma	phase I	intra-lesional	22

[1]The tumors indicated are the models used in pre-clinical and clinical studies, whereas the injection method includes only clinical trials, [2]dl1520 is also called ONYX15, [3]SCCHN= head and neck squamopus cell carcinoma, [4]HCC= hepatocellular carcinoma

replication of NDV in tumor cells is not known, studies appear to show preference for cells with activated Ras (Table 1) (11, 12). NDV has been used as a direct oncolytic agent that is injected into tumor masses in animal models (11, 12). One characteristic of viral infection consists of the induction of innate and acquired humoral and cellular responses, leading to production of cytokines known to have anticancer effects, such as interferons and TNF-alpha (13). Therefore, NDV has also been used to infect tumor cells *ex vivo*, and the subsequent infected cell lysate is used as an autologous vaccine (14-16). This vaccination stratagem is now being tested in a phase III clinical trial (Table 1) (14-16).

3.1.3. Vesicular Stomatitis Virus

VSV is an enveloped single-stranded RNA virus that is not endemic to the human population. It naturally infects cattle, horses and swine causing vesiculation and ulceration of the tongue and oral epithelia. The recent discovery that in mice it replicates in carcinoma and melanoma cells but not in primary epithelial cells (Table 1) evidenced its potential use as an oncolytic virus in tumor therapy (17). The molecular basis of its apparent tumor specificity has been recently linked to the IFN-gamma pathway, which is often defective in tumor cells (17). Murine experimental tumor models provide evidence for inhibition of VSV's anti-cancer potential when animals are treated with IFN-gamma (17). The connection between IFN-gamma and VSV is most probably mediated by PKR (18). Indeed, treatment of normal cells with IFN-gamma activates PKR and prevents viral replication, whereas tumor cells defective in IFN-gamma pathway components become permissive to viral replication and retain their permissiveness even in the presence of IFN-gamma (19).

Thus, IFN-gamma represents a potential mean to pharmaceutically inhibit further replication by this oncolytic virus, after completion of tumor therapy. Even though it has a small genome coding for only 5 genes, new recombinant strains of VSV carrying the *thymidine kinase* (*HSV-TK*) gene or the *interleukin-4* (*IL-4*) gene have been recently engineered (Table 1) (20). Unlike other RNA viruses, VSV has not reached the stage of testing its safety/effectiveness in clinical trials because of the toxicity induced in mice models. Natural attenuated strains of this virus have not been isolated and new recombinant strains carrying the five VSV genes in different positions are being studied as a mean to decrease the toxicity of this virus (21).

3.1.4. Vaccinia Virus

Vaccinia virus is the attenuated strain of smallpox virus and its historical role linked to Jenner's smallpox vaccine has rendered it one of the most widely known and used agents in human illness. In terms of oncolysis, both wt as well as a vaccinia virus mutant have been tested in phase I clinical trials (Table 1) (22). It is a relatively safe virus, with generally tolerable side effects. The most serious adverse events linked to use of this virus (in its vaccine setting) have been associated with immunocompromised patients (23). Vaccinia is a cytoplasmic virus that uses its own polymerases to replicate and transcribe viral DNA, which thus exists in an extrachromosomal state. Therefore, the chance of recombination with the host genome and generation of viral mutants is decreased. Moreover, distribution of this virus in the human population, because of use of similar mutant strains as vaccines in the past, should minimize the risk of dangerous epidemics. Vaccinia virus infects most mammalian cells and appears to induce tumor regression, not only through direct oncolysis but also through its elicitation of strong immune responses. The molecular explanation of the tumor selectivity of this virus has yet to be elucidated, but seems to be correlated to the increased permeability of tumor vessels (24). It has been estimated that the vaccinia genome could carry up to 25 kb of exogenous DNA (25). Therefore, engineering additional anticancer functions to the viral genome has been attempted, thus providing enhanced anticancer action (24, 26, 27).

3.1.5. H-1 Parvovirus

Parvoviruses are nonenveloped icosahedral single stranded DNA viruses. Their genome averages 5 kb, but packaging of an additional 15% of genetic material to its DNA content is possible. Three different strains of parvoviruses have been described in nature, but the most promising in tumor therapy is the autonomous rodent parvovirus H1 strain (Table 1) (28-30). It replicates in a broad range of human cells that enter S phase following virus entry. This strain does not enter latency, does not integrate, and exhibits minimal, if any, pathogenicity (28). Thus, these characteristics seem propitious for the use of H1 autonomous parvovirus as a vector for oncolytic therapy. Little is known about the molecular pathways that allow selective killing of tumor cells by this virus, but several studies have shown that mutation of the p53 tumor suppressor is required for the viral replication (29, 30). Currently a phase 1 clinical trial using wt H-1 APV is underway (Table 1).

3.2. Genetically engineered oncolytic viruses

3.2.1 Herpes Simplex Virus

The HSV virion consists of a relatively complex structure that includes an envelope, a tegument and a capsid. The latter surrounds a core where the double stranded DNA is located in concentric layers (31). The binding of the virus to the cell membrane is mediated by attachment of the viral glycoproteins C and B to the cellular heparan sulfate proteoglycan, whereas entrance is mediated by the cell surface receptors HveA and HveC that bind to the viral glycoprotein D (32). HveA belongs to the tumor necrosis factor superfamily and its normal cellular function mediates transcription of several genes involved in eliciting the immune response, whereas HveC is the poliovirus receptor-related protein1, nectin 1 (32, 33). The viral capsid is further transported by the cytoskeleton to nuclear pores and the genome is released into the nucleus. Following circularization of the viral DNA in the nucleus, a tightly regulated temporal program of transcription of HSV genes begins. Based on this temporal program, HSV genes have been divided into three groups: a) immediate early genes encoding factors responsible for the transcription of the remaining viral genes as well for evasion from the immune system, b) early genes responsible for DNA metabolism, and c) late genes encoding for structural proteins of the virion (34).

OVs to date derive from HSV-1 that, in its wt state, is responsible for mucocutaneous lesions of the mouth, face and eyes (35). This virus enters the terminal dendrites of trigeminal nerve branches innervating the face and is transported to the neuronal cell body by retrograde axonal flow. Once in the trigeminal ganglion sensory neuron, HSV-1 can remain in a latent phase, in which the virus genome is transcriptionally silent, except for one coding area (the *LAT* related transcripts), whose function remains unknown. Occasionally, environmental stress factors will induce re-activation of HSV-1 in these neurons, induction of viral gene transcription, followed by entry into the lytic cycle and recurrence of the oral mucocutaneous lesions. Rarely, HSV-1 is responsible for a hemorrhagic encephalitis and meningitis in the brain (36). HSV-1 is endemic in humans and serologic studies reveal that 50 to 80% of the population possesses neutralizing immunity to the virus. The viral DNA remains in an extrachromosomal state and does not integrate in the host genome, thus limiting the possibility of recombination with the host genome and the occurrence of undesired viral mutants.

The major advantage of this virus is its large genome: 152 kB. Indeed, this virus offers an extremely vast possibility of genetic engineering for therapeutic purposes and new very efficient methodologies for this purpose have been recently developed (37). There are also very effective and widely available anti HSV-1 drugs (acyclovir, ganciclovir, valacyclovir) that can be used to curtail viral replication, a property that is not available with other OVs. HSV-1 appears to have generated the largest number of genetically engineered mutants for oncolytic

therapy to date (Table 1) and clinical trials in gliomas and hepatic colorectal metastases have been and are being carried out (38, 39).

3.2.2. Adenovirus

More than 40 different adenovirus serotypes have been described, but the most widely investigated for gene and oncolytic viral therapy belong to serotypes 2 and 5. Several features render adenovirus an attractive candidate for oncolysis. It is endemic in the human population and its pathogenicity is associated only with mild respiratory infections (10). The major advantage of this virus is that it can be produced to relatively high titers (10^{10} pfu/ml), and further concentrated to 10^{12} pfu/ml. Since it does not integrate into the cell genome, but remains in an extrachromosomal state, risks for recombination and disruption of critical endogenous genes or formation of undesired viral mutants are minimized. Moreover, it has a large genome and recombinant technologies to remodel it are relatively easy and widely available. One of the major disadvantages of this virus is represented by its relatively high immunogenicity as well as associated concerns in its use due to toxicity and deaths in patients that were treated at very high doses in gene therapy trials. It is also not clear if the presence of adenovirus neutralizing antibodies and adenoviral immunity in the human population provides a major limitation to its effective and safe use.

Structurally, adenovirus consists of a non-enveloped 65-80 nm icosahedral capsid that encloses a linear double stranded DNA of approximately 38 kD. Viral attachment to the cell surface is mediated by two basic elements of the capsid: the fiber, that extends from each of the twelve vertices of the icosahedral capsid and, through its knob domain, binds the cosxackie-adenovirus receptor (CAR), and the penton, that recognizes the cellular integrins and together with the receptor CAR mediates the internalization of the virus through endocytosis (40-43). Upon internalization, the virus is transferred through the cellular endosomal system to the cytosol, from where it reaches the nuclear pores and its genome is translocated to the nucleus (42, 43). Transcription of the adenovirus genome commences at the *E1* locus, where gene products function by preventing the premature death of the cell during viral replicative cycle and by inducing the transcription of host genes that mediate cellular entry into S phase (44). These gene products include E1A that binds and inhibits cellular factors involved in blocking cell cycle progression, such as the retinoblastoma protein (pRb) and p300/CBP, the E1B-55 kD protein that binds and inhibits p53 thus inhibiting an apoptotic cellular antiviral response, and the anti-apoptotic factor E1B-19 kD, that is a viral homologue of the cellular anti-apoptotic factor bcl2 (45-48). The temporal progression of viral gene expression includes transcription of the *E2* region, followed by transcription of the *E3* region. The latter includes the synthesis of proteins important for cell lysis, such as E3-11.6 kD and for evasion by the virus of immune system factors, such as E3-19 kD, E3 10.4/14.5 kD and 14.7 kD (49-51).

Although one of the largest clinical trials of viral therapy employed a wt adenovirus in the 1950s, the report by Bischoff et al. (1996) in which an E1B-55kD deleted mutant adenovirus was shown to display selective replication in p53-deficient tumor cells resurrected the use of this virus as a form of cancer therapy (52, 53). Since this report, adenoviral mutants in E1A have been shown to target tumor cells with defects in the pRb tumor suppressor pathway and another adenoviral mutant has been recently engineered in which robust viral replication becomes associated with p53 cellular defects (54-56). The E1B-55kD mutant has been tested in clinical trials for a multitude of cancers and recently it has shown encouraging results in a phase II trial in head and neck patients when combined with chemotherapy (Table 1) (57, 58).

3.2.3. Poliovirus

Poliovirus is a non-enveloped DNA virus and it is the causative agent of paralytic poliomelytis. The wt poliovirus selectively targets motor neurons through the interaction with the Ig superfamily receptor CD155 that is present also in glioma cells and its lytic cycle is restricted to neuronal cells through the internal ribosome site entry (IRES) element (59). A mutant form of this virus has been engineered to selectively target glioma cells (see below), but has not been used in clinical trials yet.

3.3. Limits and disadvantages of oncolytic viruses

Even though these novel agents for tumor therapy seem very promising, evaluation of their real effectiveness awaits results from clinical trials. It should be noted that each virus might exhibit or possess limitations that could hamper its use and effectiveness.

OVs such as VSV, reovirus, H1 APV and NDV are difficult to work with genetically and, thus, further genetic refinements to augment oncolytic efficacy and minimize toxicity may be arduous. On the contrary, genetic manipulations of HSV-1 or adenoviral genomes have become relatively standard for a number of laboratories. Nevertheless, safety concerns will always exist with these viruses and their mutant OVs. Furthermore, in many patients, wt adenovirus and HSV-1 may already be present and it is not known if this will pose a risk during tumor treatment with their recombinant OV derivatives. However, it appears that in initial clinical trials evidence for such recombinatorial events has not been observed (see below and Table 1).

Finally, the effects of the immune system on viral infection and propagation may cause deleterious effects to the patient. Such effects have not been fully studied, yet would seem to be an important and fertile area of investigative research.

4. GENETIC MODIFICATIONS FOR ENGINEERING OF ONCOLYSIS

4.1. Mutations to induce viral tumor specificity

There have been three primary types of modifications in the genome of viruses with the objective of increasing their tumor specificity. First of all, retargeting of viral attachment and entry into cells by genetic modifications of viral surface polypeptides responsible for viral entry has been primarily carried out

with adenovirus. Second, appropriate genetic alterations can be carried out to viral genes to attempt to target viral replication to tumor cells. Finally, tumor-specific promoters can be employed to drive expression of viral genes. Each of these genetic alterations has been shown to provide increased specificity for tumor cell versus normal cell lysis.

4.1.1. Retargeting of viral entry

Knowledge about the mechanism of initial viral entry into cells provides several strategies aimed at increasing viral tumor selectivity. For instance, bifunctional antibodies have been used to link viral surface molecules to a desired surface peptide on target cells. This stratagem has been employed with anti-adenovirus knob antibodies conjugated with folate, fibroblast growth factor, or anti-EGFR antibody (60, 61). One problem with this technology is that antibody-ligand binding is potentially unstable. Thus, several genetically engineered viral strains carrying modifications on the structural envelope proteins responsible for virus-cell interactions have been developed (62, 63). It was hoped that such redirected viral mutants would display retargeted characteristics toward tumor cells. However, alterations in viral entry molecules can end up limiting effective infection and entry (64). This has proven to be particularly true with HSV-1 where 11 viral glycoproteins are involved in virus-cell entry, attachment and exit. Because adenoviral entry involves primarily knob interactions with CAR, retargeting of this virus may be more easily accomplished. Even though these strategies of virus retargeting seem appealing, they have not yet been tested in clinical trials. Further research aimed at identifying small tumor-specific peptides may also provide additional tools for more effective retargeting of viral cell entry (65).

4.1.2. Retargeting of viral replication by complementation with cellular factors

Several viral genes are essential for replication in normal cells, but can become dispensable in replicating cells. Thus, a variety of OVs with total or partial deletion(s) of such genes have been engineered. The first deletion mutant was originated from HSV-1 by deleting the *HSV-TK* gene (dlsptk, Table 1) (3). The oncolytic dlsptk exhibited attenuated neurovirulence and an efficient therapeutic profile in glioma models (3). Further, the hrR3 HSV-1 strain containing an *Escherichia coli LacZ* gene instead of the early gene *UL39* was engineered (Table 1) (66, 67). *UL39* encodes for the ribonucleotide reductase ICP6, essential for HSV-1 replication in normal cells, but dispensable in actively replicating host cells (67). The therapeutic potential of this OV was shown in immunocompetent mice with liver metastasis in colon, where a single intravascular injection of hrR3 was able to reduce the tumor and in rats with brain neoplasia (68, 69). Another HSV-1 derived oncolytic strain (R3616) was obtained through deletion of the neurovirulence gene *gamma-34.5* coding for the ICP34.5 factor (Table 1) (70). The latter enzyme is responsible for disrupting the host cells defense mechanism by antagonizing PKR activity (71, 72). Indeed, PKR inhibits, through phosphorylation, the HSV initiation translation factor eIF-2-alpha, essential for the translation of HSV mRNAs, whereas ICP34.5 binds the protein phosphatase 1-alpha and redirects it to dephosphorylate eIF-2-alpha, thus enabling the synthesis of viral proteins (73). Activated Ras pathway downregulates PKR and allows HSV replication also in the absence of ICP34.5 (74, 75). In fact, the R3616 mutant was shown to maintain the capacity to replicate in glioma cells with activated Ras (70). For safety issues further research in HSV oncolytic strains led to the construction of double HSV deletion mutant G207 (Table 1), with disrupted both the *gamma-34.5* and the *UL39* genes (76). The oncolytic potential of this virus was tested in animal models for several tumor types such as colon, ovarian, breast, prostate, head and neck squamous cell cancers, melanoma and bladder (77-83). The results obtained from these pre-clinical studies allowed the use of G207 OV in a phase 1 clinical trial.

The adenovirus mutant dl1520 (also called ONYX-015 and CI-1042) is deleted for the *E1B–55kD* gene, responsible of inhibiting p53-dependent apoptosis, and can replicate only in cells with a disrupted p53 tumor suppressor pathway (Table 1) (53, 84). Replication of this mutant OV was the first to be associated with a defect in a tumor suppressor pathway. This has generated considerable enthusiasm for its preclinical and clinical use, particularly since more than 50% of tumors have a mutant *TP53* gene and even more possess defects in one of the genes that modulate p53 expression (85, 86). In spite of this early enthusiasm, there have been reports that have contradicted the mechanism of dl1520 selectivity, based on finding tumor cells with a normal *TP53* gene that still supported replication of the mutant virus (53, 87-92). Three hypotheses may explain the contradictory results. First, adenovirus E4orf6 that also interacts with p53 may affect replication of dl1520 in wt p53 cells (93). Second, it is hypothesized that E1B-55 kD may have other functions than binding to wt p53, that could allow its replication in wt p53 cells. Finally (and probably the most likely explanation), deletion of *p14ARF* or amplification of *MDM2* may again result in permissive dl1520 replication in wt p53 cells (94). Indeed, p14ARF and mdm2 regulate cellular levels and function of p53 and their expression is frequently altered in human tumors with wt p53, providing an alternative mechanism to p53 inhibition (95). Clinical trials with dl1520 have revealed that it is relatively safe in humans. Ultimately, this may be due to its relatively poor burst size/replication even in permissive mutant p53 cells (58).

Adenovirus dl922-947 (Table 1) has small deletions in the region coding for E1A-CR2 and grows selectively in cells that have disrupted pRb (54). Indeed, E1A-CR2 codes for a protein able to bind and inactivate pRb, thus promoting entry into the cell cycle. Interestingly, this virus strain has a much higher therapeutic efficiency than dl1520. This might be explained by the fact that the E1B-55 kD is completely disrupted in dl1520 therefore, other functions of this protein are lost, whereas only small regions of E1A-CR2 are deleted in dl922-947. Given the multifunctional characteristic of many viral proteins, targeted deletions might be more efficient than whole gene

deletions. In order to overcome the problems caused by gene deletions, Ramachandra et al. have constructed a replication selective adenovirus (01/PEME) expressing an antagonist of the E2F protein under the control of a *TP53* promoter (Table 1) (96). E2F is a transcription factor essential for the activation of the cell cycle and for the transcription of viral replication proteins. E2F activity is inhibited by pRb, but in tumor cells the pRb/E2F complex is frequently deregulated (97). Thus, insertion of an E2F antagonist containing the E2F DNA binding domain and a pRb transrepression domain under the control of a p53 promoter, will inhibit viral replication in wt p53 cells, but not in those disrupted for p53.

Finally, a novel OV was obtained from poliovirus (PV1 (RIPO)). In this case, the deletion consisted of an IRES element, which is essential for virulence in cells of neuronal origin. Gromeier et al. have demonstrated that substitution of the poliovirus IRES element with the IRES element of a rhinovirus type 2, inhibited neuronal infection of this poliovirus mutant, but still allowed infection of glioma cells (Table 1) (59).

4.1.3. Transcriptional retargeting

Another approach to target replicative viruses to tumor cells consists in exploiting tumor-specific promoters to induce the viral lytic cycle. Miyatake et al. have used an HSV-1 mutant deleted for both *ICP4* genes to build a new virus strain (d120) harboring an albumin enhancer/promoter–*ICP4* transgene in the *HSV-TK* locus (Table 1) (98). ICP4 is an essential immediate early gene product for HSV-1 transcription, thus, viruses deleted for this gene can not undergo the lytic cycle (99, 100). Moreover, viruses deleted for *HSV-TK* gene possess poor replicative capacity in non-dividing cells. Hence, this construct guarantees replication of the virus only in replicating cells expressing albumin. In human adults only hepatocytes and hepatocellular carcinomas express albumin and this viral construct was shown to selectively replicate in hepatocellular carcinomas (98, 101). Nevertheless, the presence of albumin expression in normal hepatocytes, poses safety concerns for the clinical use of this OV.

Similarly, the HSV-1 derived Myb34.5 OV has the *gamma-34.5* gene under the control of the E2F responsive cellular *B-myb* promoter (Table 1) and was shown to induce cell lysis only in actively dividing cells (102). The same virus was successfully used in preclinical studies on mice with diffuse liver metastases through intravascular injection (103).

The oncolytic adenovirus AvE1a04i (Table 1) was constructed by engineering the E1A protein (essential for adenovirus replication) under the control of the alpha-fetoprotein (AFP) promoter and can replicate only in hepatocellular carcinomas (HCC) that express AFP (Table 1) (104). AFP is present in about 70-80% HCC and absent in normal cells (105). A similar strategy was adopted to target prostate tumors using the minimal enhancer/promoter of the human prostate specific antigen (PSA) (CN706 virus, currently in phase 1 clinical trials) and to target breast carcinomas using the promoter of the *DF3/MUC1* gene over-expressed in breast and other carcinomas (Table 1) (106-109). CN706 has been further modified (CV764) by adding the human kallikrein promoter to control *E1B* expression, (Table 1) (110). A second generation virus, CN787, derived from the oncolytic mutant CN706 and harboring the *E1A* gene under the rat probasin promoter and *E1B* under the *PSA* promoter is currently in trials using an intravenous mode of administration (Table 1) (111).

Finally, an oncolytic adenovirus containing the *E1* region under the control of a hypoxia inducible factor (HIF) responsive element (HYPR-Ad) and targeting only hypoxic cells was recently constructed (Table 1). This strategy is of relevance considering that all solid tumors have large hypoxic areas. The tumor selectivity of this virus was demonstrated for subcutaneus tumors of human glioblastoma cells in immunocompromised mice (abstract in Neuro-Oncology 3, 2001).

4.2. Further enhancements of the therapeutic potential of oncolytic viruses

Several strategies based on viral genetic engineering have been adopted to enhance the therapeutic efficiency of OVs. The resulting virus strains are listed (Table 1).

First of all, OVs have been engineered to carry cDNAs able to activate pro-drugs (112). The first example of combining oncolytic viral therapy with antitumor pro-drugs was illustrated by treating syngeneic 9L rat gliomas with ganciclovir and an oncolytic HSV expressing the *HSV-TK* gene. These experiments show that combination of OVs with ganciclovir/HSV-TK treatment was much more efficient than either strategy alone (69). Thereafter, Wildner et al. engineered the oncolytic adenovirus dl1520 with *HSV-TK* (Ad.TKRC) and showed again an increased therapeutic efficiency (113, 114). Nevertheless, the combination of oncolytic viral therapy with ganciclovir treatment was not always successful. Indeed, other groups have shown that addition of ganciclovir did not increase the anti-tumor efficiency of an HSV-TK expressing oncolytic adenovirus (115, 116). This might be explained by the fact that ganciclovir kills the virus, thus impeding the viral intratumoral spread. As discussed in Aghi et al., the addition of prodrug-activating gene therapies, which possess both antitumor and antiviral action, may lead to different effects (117). In cells in which the virus replicates well and in which gap junctional communication is relatively poor (i.e. possess a weak bystander effect), the antiviral action of activated ganciclovir predominates over its anticancer action. Therefore, activated GCV would impede the viral oncolytic effect, leading to an antagonistic action. However, if viral replication is relatively poor and gap junctional communication is strong, ganciclovir activation would augment the viral oncolytic effect.

Seeking prodrug-activating genes that convert drugs into metabolites that possess primarily anticancer and not antiviral action, an HSV-based OV was engineered with the *CYP2B1* transgene in the ICP6 region (rRp450) (118). The *CYP2B1* cDNA is able to activate the cyclophosphamide (CPA) and ifosfamide prodrugs into

anti-cancer agents. Combined tumor treatment with rRp450 and one of the prodrugs mentioned above gave strong enhanced antitumor efficiency. This might also be explained by recent discoveries showing that CPA plays an important role in facilitating the intratumoral spread of the virus (119). The best therapeutic effects were obtained by using a combination of multiple pro-drug activating gene therapies with OVs as delivery systems. Examples are given by CPA and ganciclovir treatment in combination with either the rRp450 HSV virus, carrying both *HSV-TK* and *CYP2B1*, or the adenovirus variant FGR, carrying a *cytosine deaminase* gene (*CD*) fused to *HSV-TK* (117, 120). In this regard it is interesting to mention the recent generation of a bidirectional HIF responsive expression vectors that can be used to target hypoxic cells with multiple cytopathic cDNAs (121). To increase the cytotoxicity of the adenovirus derived 01/PEME oncolytic strain a copy of the viral major late promoter (*MLP*) was introduced in the *E3* region to overexpress the viral lytic protein E3-11.6K (96). Similarly, deletion of the anti-apoptotic adenovirus gene *E3-19kD* (adl118) potentates adenovirus intratumoral spread (122, 123).

An opposite strategy of oncolytic therapy was recently tested. This strategy is based in delaying the viral cytotoxicity (instead of enhancing it) in order to increase the time of viral protein synthesis and consequently the expression of the transgene. Thus, two different adenovirus-derived oncolytic mutants were constructed by substituting the ADP gene, involved in inducing cell apoptosis, with either the bacterial CD gene (ONYX 305), or the human tumor necrosis factor (TNF-alpha) (ONYX 320) (124-126).

Additional modifications in viral genomes have been carried out with the intent of activating antitumoral immune responses. For example, an HSV-derived mutant (NV1023) was engineered to express interleukine 12 (IL12) (127). In this example, the presence of IL12 allowed for the same therapeutic effects on colon cancer with a reduced dose of virus (127). The HSV derived R3616 oncolytic mutant was engineered with either IL4 or IL10 gene (R8305 and R8307, respectively) (128). The first one showed a significant increase in anti tumor responses, whereas the second was not as effective (128). Two HSV derived OVs, G47delta and R47delta were obtained by deleting the alpha-47 gene from the G207 and R3616 strains, respectively (129). This deletion was performed with the goal of disrupting viral ability to evade MHC-1 activation (130, 131). Indeed, alpha-47 gene codes for the ICP47 factor which functions as an inhibitor of the transporter associated with antigen presentation (TAP), which in turn is responsible for MHC class I molecules loading (132-134). These viruses were shown to induce CTL responses that led to more efficient tumor regression compared to the parental strains G207 and R3616 (129). Finally, the adenovirus derived OV strain Ad5/IFN was originated by inserting the human consensus IFN gene (IFN-con1) in the E3 region (135), and vaccinia virus mutants have been engineered by insertion of the cytokine GM-CSF (136).

The recent discovery that, the single stranded hairpin DNA adeno-associated virus (AAV) triggers death of cells with disrupted p53, through a DNA damage response opens new horizons for the production of OVs (137). Indeed, the data reported by Raj et al. clearly indicate that viruses can be used to deliver DNA of unusual structure to induce a DNA damage response that kills selectively cells with disrupted p53 (137).

5. CLINICAL TRIALS

Several clinical trials have been performed or are underway using some of the aforementioned OVs (Table 1). The results of these clinical trials are widely described by Kirn et al. and Norman et al. (44, 138). The important questions addressed by these trials are: -1) how safe is the therapeutic strategy in use?- 2) would it be safe to use systemic delivery strategies of OVs to treat patients with metastases, infiltrating malignancies and multiple tumors?- 3) could the combination of OVs with other therapeutic strategies increase anti-tumor efficiency? In order to answer all these questions, the general strategy in use is to sequentially dose-escalate the viruses and, if safety is demonstrated, combination of oncolytic viral therapy with other therapeutic strategies and systemic virus delivery modalities are tested.

Adenovirus derived dl1520 strain was tested in phase 1 and 2 trials with several tumors and four different routes of administration: intratumoral, intraperitoneal, intraarterial, and intravenous (139). In all cases safety was demonstrated, but with little evidence of tumor regression. However, combination of viral delivery and chemotherapy showed a synergistic antitumoral action in head and neck cancers and liver metastasis (57). It is important to notice that, following intra-arterial and intra-venous administration of dl1520, a strong increase in acute inflammatory cytokines, such as IL-1, IL-6, TNF-alpha and IFN-gamma, was observed (140). These may have been responsible for transient febrile events experienced by some patients.

The prostate specific oncolytic adenoviruses CN706 and CN787 were tested through intratumoral and intravenous delivery, respectively, in phase 1 trials. The results with CN787 are not yet published, whereas CN706 was demonstrated to be safe (138).

Treatments of brain tumors present additional difficulties that are not found in other organs. The presence of the blood brain barrier (BBB) and/or blood tumor barrier (BTB) limits the penetration of OVs into the brain tumor. Indeed, treatment of brain tumors with OVs in combination with drugs that increase the permeability of the BBB and BTB has shown to increase significantly the therapeutic efficiency, specially with intravenous and/or intrathecal injections (141, 142). Moreover, recent results indicate that also the pericapillary basement membrane can trap and inhibit the brain entry of several therapeutic agents (142). In pre-clinical models several HSV-1 delivery methods have been analyzed for the treatment of brain tumors, including intratumoral, intravascular, intraartherial and intrathecal, but only the intratumoral delivery has been tested in human patients. In particular, the intrathecal injection of the HSV derived oncolytic strain hrR3 for the

treatment of pediatric disseminating tumors in a rat model has shown an increased anti-tumor effect, yet some toxicity was observed, possibly because of ependymal and subependymal cell infections (143, 144). HSV-derived OVs G207 and HSV 1716 were demonstrated to be safe in phase 1 clinical trials using intratumoral injections (38, 39).

Trials with vaccinia viruses expressing tumor antigen or proinflammatory cytokines have tried to elicit immunostimulatory effects and, in general, have shown evidence of relative safety (136, 145-147). A phase I clinical trial with an attenuated form of NDV (PV701), injected intravenously, has recently been published and has also shown the relative safety of this OV up to a certain dose (139).

An important physiological element that could be responsible for the poor therapeutic efficiency obtained in clinical trials is the tumor interstitial pressure. This is an important factor in determining the fluid movement across the vessel walls and thus the connective delivery of large therapeutic molecules to tumor cells. Stohrer et al. have recently measured the oncotic interstitial pressure of several human carcinomas implanted in nude mice, and have shown that it is significantly greater than the one in normal tissues (148). The increased pressure in these malignancies can be explained with the leaky aspect of tumor vessels (148). Therefore, the delivery of anti-angiogenic agents in combination with oncolytic viral therapy should be considered (149).

6. EFFECT OF THE IMMUNE RESPONSE ON TUMOR ONCOLYSIS AND FUTURE PERSPECTIVES

Several OVs have been constructed and tested in preclinical and clinical models. The results obtained until now are encouraging but clearly additional work needs to be done. The safety of these viruses was demonstrated but their antitumoral efficiency was weak and several questions have risen and are still unanswered. The role of the immune system in oncolytic viral therapy is still almost unexplored. It is well known that humans have developed sophisticated immune strategies to defend themselves from viruses and, on the other hand, viruses have developed strategies to evade the host immune responses (150, 151). Nevertheless, very little is known about the immune molecular pathways elicited by OVs. This subject is particularly important considering that most humans express neutralizing antibodies specific for the OVs currently in use. Indeed, the presence of these antibodies may limit the efficiency of the oncolytic viral therapy. Results obtained from clinical studies have shown a strong induction of cytokines followed by systemic delivery of OVs and experiments performed on rats show that intravascular delivery of the HSV derived hrR3 OV is inhibited by the classical pathway of the complement and anticipate the existence of a natural antibody recognizing hrR3 (119, 139, 152). In the same study, the authors show that inhibition of B cell maturation by CPA treatment significantly increased the therapeutic efficiency of hrR3. Even though CPA is currently used in tumor therapy and seems to be efficient, general inhibition of the immune system can have a negative therapeutic drawback. Indeed, the immune system plays also an important antitumoral role and several OVs have been developed with the goal to induce immune responses. Thus, a very interesting point to examine would be to what extent repressing or, on the other hand, eliciting the immune system can potentate oncolytic viral therapy. Moreover, it would be important to distinguish the molecular pathways that induce viral killing from those that induce tumor cells killing.

These types of studies are limited by the lack of an immunocompetent model for the therapy of human tumors with OVs. Moreover, immune responses induced by OVs are species specific and experiments performed on mice can give only quantitative but not qualitative indications of the immunologic pathways that would be elicited in humans (153). Until now the construction of OVs to be used in tumor therapy was based on the assumption that the therapy will be more efficient if the virus is more cytopathic, if the antiviral immune response is inhibited and if a tumor-specific immune response is elicited. Nevertheless, the data we have today indicate that the relation between viral cytopathic effect or viral induced immunity and a successful tumor therapy is more complex and the above assumptions have been revised with a mathematical model (154). This model anticipates the consequences that could derive following specific viral engineering and enlightens the future perspectives of this antitumoral strategy. Three different scenarios are taken in consideration in this model: the virus induced lysis of tumor cells, a virus specific CTL response, a tumor specific immune response that contributes with the virus to tumor eradication.

The results obtained through the theoretical analysis of these scenarios indicate that, to obtain an optimal therapeutic effect with OVs the growth rate of infected and uninfected cells must be taken in consideration. If the uninfected cells grow quicker than the infected cells, it is better to use a virus with low cytopathic effect, otherwise viral production will be too low compared to the number of cells within a growing tumor. On the other hand, if the infected cells grow faster it is worth to engineer the virus in a way to increase its cytopathic effect.

Similarly, the virus specific immune response can also have positive consequences. If the virus is not very cytopathic and the infected cells grow faster than the non-infected ones, activation of CTL could increase the lysis of infected cells and the release of the virus. Finally, this model strongly encourages the construction of viruses inducing tumor specific immune responses.

In conclusion, the field of OVs appears ready to fulfill its potential: generation of more effective OVs coupled with knowledge of their interaction with cellular factors and host immune responses could provide a series of agents that will enter the oncologic armamentarium.

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