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## Review

# Toward a New Generation of Conditionally Replicating Adenoviruses: Pairing Tumor Selectivity with Maximal Oncolysis

FRANK A.E. KRUYT<sup>1</sup> and DAVID T. CURIEL<sup>2</sup>

### ABSTRACT

Conditionally replicating adenoviruses (CRADs) represent a promising new platform for the treatment of cancer. CRADs have been demonstrated to kill tumor cells when other therapies fail, indicating that their anti-tumor properties are complementary to, and distinct from, those of standard treatments such as chemotherapy and radiation. In clinic trials CRADs have shown encouraging results, demonstrating mild side effects when administered at high doses and via different routes, including intratumorally, intraperitoneally, and intravenously. Tumor-selective replication has been detected, although as a single agent the efficacy appears to be limited. Interestingly, combined treatment with radiation or chemotherapy has been found to enhance CRAD efficacy considerably. To date, the molecular mechanisms underlying adenovirus-mediated oncolysis, and the way in which chemotherapy enhances oncolysis, are not well understood. A fuller knowledge of these processes will open up new strategies to improve the cell-killing potential of CRADs. Here, we discuss several possibilities that may lead to CRADs with enhanced oncolytic activity. These approaches include strategies to functionally couple tumor targeting and optimal oncolytic activity, and ways to further increase tumor cell disruption at later stages of infection to facilitate the spreading of virus throughout the tumor mass. In addition, improved methods to evaluate the efficacy of these agents in animal models, and in the clinic, will be required to systematically test and optimize CRAD efficacy, also taking into account the influence of tumor characteristics and the administration route.

### OVERVIEW SUMMARY

The recognition that genetic alterations are at the basis of the development of cancer has initiated the design of therapeutic approaches, known as cancer gene therapy, aimed at correcting the primary genetic defect in tumor cells. Both nonviral and viral strategies have been used for gene correction or the introduction of therapeutic genes into tumor cells. Viruses have been particularly popular because of their natural ability to infect cells. Initially, nonreplicating viral vectors were used; however, limitations related to their inability to infect all cancer cells, and to the relatively low levels of therapeutic gene expression obtained, have shifted

attention to the use of replicating viruses as the treatment platform. In this respect, genetically modified adenoviruses that selectively replicate in cancer cells have been generated and tested in clinical trials. Although promising, several hurdles have been encountered that need to be addressed to expand their therapeutic potential.

### CONDITIONALLY REPLICATING ADENOVIRUSES

**T**HE CONCEPT OF USING replication-competent adenoviruses for the treatment of cancer, also known as adenoviral ther-

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apy, originated in the 1950s. The knowledge that adenoviruses could eliminate cancer cells *in vitro*, as a consequence of their reproductive cycle leading to cell lysis ("oncolysis"), resulted in clinical studies in which various wild-type adenoviral serotypes were examined for their effect on cervical cancer patients (Smith *et al.*, 1956). In the studies, no significant toxicity was reported after intratumoral injection or intravenous administration and a moderate tumor response was observed. It was not until 1996 that this concept regained attention via the use of a genetically engineered adenovirus with tumor-selective replication characteristics, leading to the first conditionally replicating adenovirus (CRAD) that was developed for cancer therapy (Bischoff *et al.*, 1996). It is now broadly recognized that these agents have beneficial properties when compared with their nonreplicating counterparts that were initially used for cancer gene therapy.

In this regard, cancer gene therapy with nonreplicating adenoviruses, although promising in preclinical models, has not resulted in successful treatments in the clinic. Armed with various therapeutic genes, including prodrug-converting enzymes and tumor suppressor genes, these agents have not demonstrated the expected beneficial effects *vis à vis* the eradication of cancer cells in the human clinical context. The main reason for the poor clinical therapeutic effect of these agents is related to the relatively small number of cancer cells in the tumor mass that are transduced by these vectors *in vivo*. As a consequence, the levels of expression and the dispersion of the therapeutic genes limit the clinical effect.

The new therapeutic platforms provided by CRADs are expected to overcome these limitations by their ability to increase the input dose of the therapeutic gene and, moreover, by the oncolysis and eradication of cancer cells during replication. In clinical trials, CRADs have been shown to be promising and safe agents. On the other hand, in clinical trials as single agents their antitumor effects have been somewhat disappointing. Thus, these clinical studies have been of great importance for defining the current limitations of the system. In this review, we address these limitations and focus on advancing several strategies that may improve the tumor-killing properties of CRADs, resulting in the development of agents with enhanced therapeutic potential.

### CRADs IN HUMAN CLINICAL TRIALS

The most studied CRAD so far is the one originally generated in the laboratory of Arnold Berk (Barker and Berk, 1987), designated *dl1520*, and for the first time used by Frank McCormick as a selective vector (Bischoff *et al.*, 1996). In this CRAD, also known as ONYX-015 and more recently renamed CI-1042 (Pfizer, Groton, CT), the viral gene encoding E1B-55kD, which is required for binding and subsequent inactivation of the p53 protein, has been deleted. Being part of a cellular antiviral mechanism, the p53 protein would normally trigger a cellular response leading to cell cycle arrest and early death of the host cell, thereby preventing replication and spreading of the virus. In cancer cells that lack functional p53, E1B-55kD would be dispensable, thus resulting in selectivity, a concept that has been confirmed in preclinical studies (Heise *et al.*, 1997). However, on this basis reduced replication and cy-

topathogenicity have been reported for this CRAD when compared with wild-type virus (Bischoff *et al.*, 1996; Harada and Berk, 1999). The clinical tests of this oncolytic agent in various cancer types, including head and neck and pancreatic cancer, have been evaluated extensively in reviews in terms of safety and efficacy (Alemany *et al.*, 2000b; Kim, 2001). The overall conclusion is that adenoviral therapy is a safe method when applied via various routes. Further, tumor-selective replication has been documented, thus validating the concept *in vivo*. Of note, no evidence was obtained for the expected reduced efficacy in patients with pre-existing antibodies against adenoviruses (Ganly *et al.*, 2000; Nemunaitis *et al.*, 2000). In fact the immune response is generally considered to be a factor that can increase the antitumor effect of the therapy. However, CRADs as a single agent have demonstrated limited efficacy, with an overall response rate of approximately 15 % in patients receiving the agent. Interestingly, the efficacy could be significantly enhanced by combined treatment with chemotherapy.

The results obtained in these studies have been helpful in determining the limitations of the current generation of CRADs, and in determining which aspects need to be addressed in order to develop a new generation of improved agents. In this respect, critical problems that have been encountered involve the following: (1) infectivity of cancer cells by adenovirus, (2) tumor selectivity of CRADs in relation to efficacy, (3) oncolytic activity or cell death-inducing ability of CRADs, (4) accessibility of tumor for virus internalization and spreading, and (5) methods to evaluate CRAD efficacy in animal models and in the clinic. In the following sections we elaborate in more detail on these aspects of CRAD efficacy.

### ADENOVIRAL INFECTION OF CANCER CELLS

Apart from the favorable characteristics of CRADs compared with nonreplicating adenovirus vectors, some problems are common to both approaches. A major issue is the fact that cells can be resistant to adenoviral infection because of the lack of the primary receptor for viral entry, the coxsackievirus-adenovirus receptor (CAR) (Douglas *et al.*, 1996; Wickham *et al.*, 1996). It has been noted that primary tumor cells often express relatively low levels of the CAR, resulting in poor infectivity, which in the case of CRADs will also affect the lateral dispersion of the virus in tumor tissue. This has been demonstrated by analyses of the oncolytic activity of wild-type adenovirus in a pair of tumor cell lines that differed only in CAR expression levels, demonstrating that low CAR levels strongly reduced viral replication and oncolysis in monolayer cultures and murine tumor models (Douglas *et al.*, 2001). To circumvent this, CAR-independent entry pathways have been identified that can bypass this deficiency, such as the use of the RGD motif in the fiber knob of the virus, which facilitates binding and entry via integrin receptors that are abundantly expressed on tumor cells (Dmitriev *et al.*, 1998; Krasnykh *et al.*, 2000). CRADs have been generated to contain fiber knobs with intact CAR-entry capability, and an additional integrin-entry capability resulting in more effective antitumor characteristics by enhanced infectivity (Suzuki *et al.*, 2001). Additional strategies have been explored to obtain tumor-specific entry of adenoviruses, involv-

ing the modification of the viral coat or the use of secondary targeting moieties, approaches that have been reviewed in more detail elsewhere (Curiel, 1999; Wickham, 2000).

### SELECTIVITY OF CRADs

Exploitation of replicating adenoviruses as a new modality for cancer gene therapy has led to the use of novel ways to obtain tumor selectivity. The so-called type 1 CRADs that are now available make use of the frequent inactivation of tumor suppressor genes in cancer that occurs as part of the process leading to malignant transformation (Curiel, 2000). Examples are mutations or deletions in the *p53* and retinoblastoma (*Rb*) genes; their protein products are known to interact with, and to be modulated by, adenoviral gene products as an essential step in virus propagation. However, these strategies to obtain selectivity often occur at the expense of efficacy, as the adenoviral reproductive cycle is a highly orchestrated process. At the molecular level, completion of the infectious cycle relies on the timely expression of a set of regulatory proteins that interact with essential endogenous cellular pathways that determine cell viability in order to facilitate viral DNA replication, expression of adenoviral genes, and, finally, disruption of the cell and the release of new viral particles (Yeh and Perricaudet, 1997). Specifically, the viral genome encodes eight transcriptional units that are activated in a timely way at different phases of infection, referred to as immediate-early (E1A), early (E1B, E2, E3, and E4), intermediate (IX and IVa2), and late genes encoding structural proteins for the capsid and the internal core. The early genes are mainly regulatory proteins that set the stage for viral DNA replication, thereby blocking cellular antiviral strategies such as the activation of cell death programs and the downregulation of immune response stimulatory proteins, strategies that are shared by other mammalian DNA viruses (for review see Wold *et al.*, 1999; Mahr and Gooding, 1999). At all stages of infection adenovirus proteins control various cellular processes by interacting with multiple host cellular proteins; many of these interactions are not yet completely understood or remain to be identified.

On infection, the immediate expression of E1A, and its binding to Rb, leads to the release of the transcription factor E2F, which forces the host cell to enter the S phase of the cell cycle in order to facilitate the coreplication of the viral genome (Flint and Shenk, 1997). Infection, E1A expression, and the unscheduled entry of the cell into the S phase inflict cellular stress signals leading to the activation of cell cycle checkpoints and the onset of suicide pathways or programmed cell death (PCD), including apoptotic cell death. Of note, the p53 protein plays an important role in the activation of apoptosis in the infected cell. In opposition to these processes, the adenoviral proteins E1B-55kD and E4orf6 work in concert to bind to p53, causing its degradation and thereby facilitating host cell survival (Steeenga *et al.*, 1998). The exploitation of the interaction between E1B-55kD and cellular p53 to obtain tumor selectivity with *dl1520* has caused considerable controversy. It has been reported that the wild-type virus grows more efficiently in cells expressing wild-type p53, compared with *dl1520* in p53-mutated cells, suggesting that either functional p53 is required for effective replication and/or that interaction between E1B-55kD and p53 has a favorable effect (Ridgway *et al.*, 1997; Hall *et*

*al.*, 1998; Dix *et al.*, 2000). In addition, several groups have demonstrated that the host range specificity of *dl1520* in *in vitro* models is independent of p53 status (Goodrum and Ornelles, 1998; Rothmann *et al.*, 1998; Harada and Berk, 1999; Turnell *et al.*, 1999). One reason for these discrepancies may be that in most studies cells derived from various cancer types were compared in examining the relationship between p53 status and *dl1520* replication, rather than isogenic cell systems to exclude the involvement of other genetic factors. In this regard, Rogulski and co-workers (2000) showed that in an isogenic colorectal cancer cell model *in vivo* *dl1520* replication occurred in both p53 wild-type and mutant cells, although with significantly higher antitumor activity in p53-deficient tumors. Another cause for these differences involves the multifunctional properties of p53 and the particular function being inactivated in the cancer cell studied. In a study of hepatocellular carcinoma cell lines, it was found that an intact transcription activator function of p53 in mutants leads to increased susceptibility for *dl1520* when compared with p53 mutants in which this function was disrupted (Zhao *et al.*, 2001). In addition, other factors in the p53 pathway have been identified that affect the oncolytic properties of *dl1520*, such as deletions in the *INK4a/ARF* locus, which occur at high frequency in cancer cells (McCormick, 2000). This locus encodes two proteins, p14<sup>ARF</sup> and p16<sup>INK4a</sup>, that are part of the pRb and p53 pathways, respectively. On transcriptional activation by E2F, p14<sup>ARF</sup> can promote the degradation of MDM-2 in a manner similar to E1B-55kD, leading to stabilization and activation of p53. In this way, p14<sup>ARF</sup> links the pRb with the p53 pathway and thus connects adenovirus-dependent activation of the pRb route with the p53 response. It has been shown that a functional p14<sup>ARF</sup>-p53 pathway is required to inhibit ONYX-015 replication and that disrupted p14<sup>ARF</sup> function facilitates replication (Ries *et al.*, 2000). Also, in mesothelioma cells with wild-type p53, mutated p14<sup>ARF</sup> enabled ONYX-015 replication whereas restored expression of functional p14<sup>ARF</sup> significantly increased resistance to oncolysis (Yang *et al.*, 2001). These studies illustrate the relative lack of knowledge about the cellular mechanisms underlying the tumor selectivity of type 1 CRADs, such as ONYX-015, and the ongoing identification of relevant cellular factors.

Another promising CRAD, not yet tested in the clinic, is Ad-Δ24, which makes use of the function of the E1A protein to bind to Rb in order to trigger cell cycle progression into the S phase (Fueyo *et al.*, 2000). Independently, a similar CRAD has been developed, designated *dl922-947* (Heise *et al.*, 2000a). These CRADs are designed for selective replication in tumor cells that have a deficiency in the Rb pathway, which is the case in the majority of cancers, by deleting from conserved region 2 (CR2) of E1A the sequence encoding eight amino acids that are required for binding to pRb and related pocket proteins (Dyson *et al.*, 1992). In addition, an infectivity-enhanced variant containing the RGD targeting motif in the fiber knob of the virus has been generated with even more favorable antitumor characteristics in lung and prostate cancer cells (Suzuki *et al.*, 2001). Both CRADs are effective in eradicating various types of cancer cells in preclinical studies, in most cases more effectively than *dl1520* or wild-type virus (Heise *et al.*, 2000a). However, a more recent study employing an organotypic model, derived from human primary keratinocytes to examine the effect of various E1A mutants on replication and their potential to kill

cells, indicated that Ad- $\Delta$ 24 is not as selective as anticipated (Balague *et al.*, 2001). While studying the complementary activity of human papillomavirus (HPV) E6 and E7 proteins toward the E1A deletion mutants, normal and E6,E7-expressing keratinocytes facilitated the replication of Ad- $\Delta$ 24 as efficiently as wild-type adenovirus. In addition, a CRAD carrying an additional deletion in CR1, a region also known to be involved in binding to members of the Rb pocket protein family, demonstrated substantial selectivity for HPV protein-expressing cells with strongly reduced but not completely abrogated replication in normal cells.

The superior antitumor activity of Ad- $\Delta$ 24 is likely to be due to the fact that only a small, but specific, mutation in E1A was introduced without altering other functions of E1A. In a similar way, an improved version of *d11520* may be generated by making more precise mutations in the E1B gene. Apart from binding to p53 and E4orf6, E1B-55kD is known to facilitate the transport of late viral mRNAs from the nucleus to the ribosomes and a nuclear export signal has been identified in this protein controlling nuclear/cytoplasmic export (Kratzer *et al.*, 2000). Impaired mRNA transport in the E1B-55kD-deleted CRAD *d11520* is probably the cause of its reduced potency when compared with wild-type adenovirus or Ad- $\Delta$ 24 (Harada and Berk, 1999). A report describes the identification of an E1B-55kD mutant, R240A, that fails to degrade p53 but has retained its E4orf6-binding and mRNA-transporting potential (Shen *et al.*, 2001). According to expectation, this mutant in the context of a replicating adenovirus was demonstrated to enhance replication and to be effective in a broader range of cell types when compared with *d11520*.

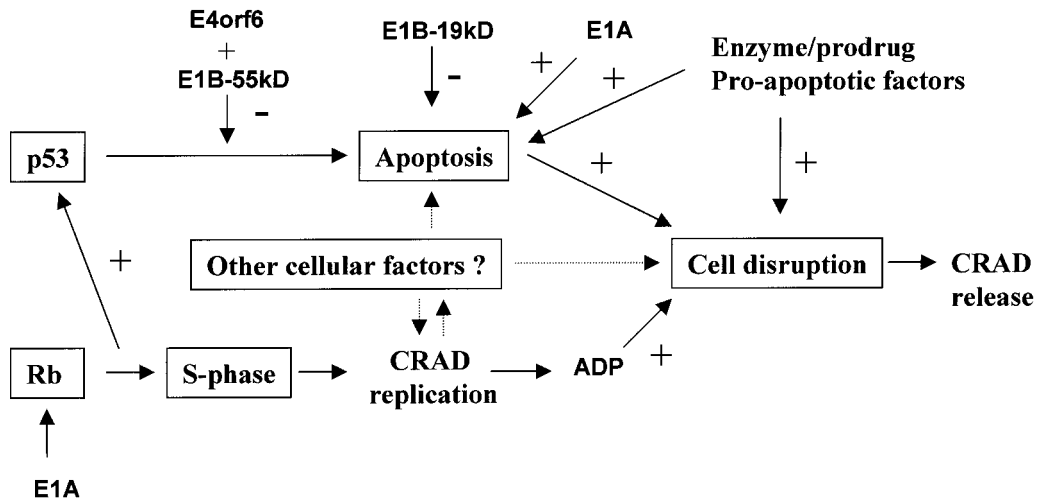
The above-described studies of the selectivity and efficacy of CRADs indicate that it is possible to generate CRADs with improved specificity and activity by introducing more precise mutations in adenoviral genes that affect only the critical and desired functions facilitating tumor preferentiality. To derive such CRADs, a better knowledge of the function of the viral genes will be required. On the other hand, it is also evident that the development of such CRADs may actually be at the expense of selectivity of these agents, thereby compromising safety issues for applications in the clinic. Additional safety measures will need to be incorporated if true selectivity cannot be obtained in this way, for example, by making use of tumor/tissue-specific promoters (TSPs). Indeed, apart from CRADs that make use of mutations in adenoviral genes that can be rescued in cancer cells, CRADs have been generated in which essential adenoviral genes are driven by TSPs, also known as type 2 CRADs (Curiel, 2000). The use of TSPs that direct the expression of genes essential for viral replication, leading to the transcriptional targeting of viruses to cancer cells, has been widely exploited (for review see Gomez-Navarro and Curiel, 2000; Kim *et al.*, 2001). To drive E1A expression, multiple promoters have been used for specific cancers, such as the prostate-specific antigen (PSA) promoter for prostate cancer and the  $\alpha$ -fetoprotein (AFP) promoter for hepatocarcinomas (Rodriguez *et al.*, 1997; Alemany *et al.*, 1999). An additional favorable feature of the use of TSPs is the prevention of potential replication at unwanted sites in the body, such as in the liver, where adenoviruses accumulate via specific and nonspecific interactions. There is an ongoing quest for promoters that display "tumor-on" and "liver-off" features. Several have been

employed to mediate tumor-specific expression of suicide genes, including the cyclooxygenase 2 (Cox-2), midkine (Mk), and telomerase reverse transcriptase (hTert) promoters, which may also be suitable for use in the context of a CRAD (Adachi *et al.*, 2000; Majumdar *et al.*, 2001; Yamamoto *et al.*, 2001).

## CRAD-INDUCED CELL DEATH

Intensive research has revealed that PCD is a genetically controlled process, with many cellular factors involved in sensing and balancing survival and death-inducing stimuli. This balance can be disturbed in various ways, including by cytotoxic agents, radiation, growth factor withdrawal, and virus infection. The molecular events triggering PCD, as well as the accompanying cellular characteristics, can vary greatly between different cell systems and in relation to the type of stimulus, classic apoptosis being one of them.

Several adenovirus-encoded gene products have been found either to block or activate cell death, and the coordinated and timely expression of these factors leads to optimal conditions for generating progeny viruses. Among the inducers of cell death are the E1A proteins, the E4 region-encoded proteins orf4 and orf6/7, and the E3-11.6kD protein, also known as the adenovirus death protein (ADP), whereas E1B-19kD, E1B-55kD, and E4orf6 suppress cell death (for review see Braithwaite and Russel, 2001). Except for ADP, the gene products encoded by the E3 region act to prevent cell death induced by external stimuli such as factors from the immune system (for review see Wold *et al.*, 1999). ADP appears to be the only adenovirus-encoded protein that directly affects cell lysis, whereas the other gene products modulate cell survival through existing pathways present in the host cell. The latter include the p53 pathway and the Bcl-2 family of pro- and antiapoptotic proteins that control mitochondria integrity, a crucial factor in the regulation of PCD (Kroemer, 1997; Green and Reed, 1998). In this regard, a window of opportunity for enhancing the antitumor potential of CRADs is at the final stage of the reproductive cycle, which involves the lysis of the host cell and spreading of viral progeny (see also Fig. 1). Oncolysis of cancer cells, when compared with lysis of the natural host cells of adenoviruses, that is, cells of the upper and lower respiratory tract, may be suboptimal because of cancer cell-specific genetic alterations. Cancer cells are often resistant to therapeutic treatment, either at the start or during the course of treatment, and one may argue that the molecular alterations conferring resistance will also lead to cancer cells being refractory to adenovirus-induced cell death. Thus far, only the enhancing effect of ADP on adenovirus-induced oncolysis has been established in this regard; ADP is transcribed at low levels at early stages from the E3 promoter, whereas at later stages the major late promoter facilitates high expression levels of ADP (Tollefson *et al.*, 1992). The mode of action of ADP, an integral membrane glycoprotein, is not well understood (Scaria *et al.*, 1992); however, adenoviruses lacking functional ADP have been shown to kill cells and release progeny virus more slowly than wild-type adenovirus (Tollefson *et al.*, 1996). Doronin and co-workers (2000) have shown that overexpression of ADP, in the context of a CRAD with small deletions in the E1A region, is more potent in eradicating tumor cells than versions lacking ADP.



**FIG. 1.** Interactions between adenovirus-encoded proteins and cellular factors that facilitate CRAD replication and host cell disruption; possible enhancement of CRAD efficacy by stimulating cell disruption at late stages of infection. The currently identified major players are indicated, with cellular factors boxed. E1A expression induces forced entry into the S phase, resulting in activation of the p53 pathway leading to cell death. This is counteracted by E1B-55kD and E4orf6 that bind to, and inactivate, p53, whereas E1B-19kD provides an additional block to apoptosis by maintaining mitochondrial integrity through inhibition of proapoptotic Bcl-2 family members. Balancing of the cell death status of the host cell by the virus may involve other, yet unidentified factors, in the mean time allowing the virus to replicate. At later stages of replication the balance shifts toward an excess of cell death-inducing stimuli, either directly mediated by viral proteins (e.g., ADP) or through modulation of the cellular apoptotic machinery. In general, cancer cells have acquired genetic rearrangements or mutations during malignant transformation that make them cell death resistant. The inclusion of cell death-inducing factors in CRADs, such as genes encoding prodrug-converting enzymes or pro apoptotic factors, and on expression at later stages of infection may facilitate the disruption of cancer cells, thus enhancing viral release and dispersion. See text for additional details.

The notion that altering the cell death-inducing properties of adenoviruses may lead to improved oncolytic potential has also been tested by deleting adenoviral genes that have an anti-apoptotic function, such as that encoding the Bcl-2 homolog E1B-19kD. Realizing that cancer cells often have developed cell death-inhibitory mechanisms during malignant transformation, Sauthoff and co-workers (2000) have used a viral mutant in which E1B-19kD has been deleted; this mutant was shown to be more effective at tumor killing than wild-type virus. This variant displayed a more rapid release of viral particles from infected tumor cells in monolayer compared with the wild-type virus, likely due to enhanced PCD, and was more potent in a lung cancer xenograft model in mice (Harrison *et al.*, 2001).

Another strategy involves the use of cytotoxic genes or proapoptotic genes in the context of a CRAD. Combining the prodrug-enzyme suicide gene strategy, ganciclovir (GCV)-thymidine kinase (TK), with an E1B-55kD-deleted virus was found to be more effective in killing tumor cells than control virus without TK (Wildner *et al.*, 1999). Interestingly, GCV treatment failed to enhance the efficacy of a replicating adenovirus expressing functional E1B-55kD in combination with TK, whereas E1B-55kD-deleted viruses showed an increased cytopathic effect after GCV treatment, perhaps because of the already optimal conditions and enhanced baseline oncolytic activity of the previous agent (Wildner and Morris, 2000). In addition, the incorporation of the proapoptotic tumor necrosis factor (TNF) gene driven by the cytomegalovirus (CMV) promoter in a replicating adenovirus, rendered breast cancer specific by employing the *MUC1* promoter, showed enhanced on-

colytic activity compared with the TNF-deleted version (Kurihara *et al.*, 2000).

Although these approaches in the models described above appeared to be of utility, it is realized that the optimal effect conferred by an incorporated cell death-inducing gene may depend on the timing of its onset of expression. Effective replication of the adenovirus is dependent on the coordinated and timely expression of adenoviral genes; inappropriate timing of expression of a death-inducing gene may be counterproductive to the cycles of infection. This is illustrated by the complex interactions observed between the oncolytic effect of a replicating vaccinia virus and the cytosine deaminase (CD)-5-fluorocytosine (5-FC) enzyme-prodrug system (McCart *et al.*, 2000). The investigators found that the tumor response-enhancing effect of 5-FC was virus dose dependent; at low multiplicities of infection (MOIs) the prodrug enhanced the response whereas at higher MOIs (>0.1) a decrease in efficacy was evident.

A likely hypothesis is that the induction of expression of cytotoxic genes in a replicating virus would be most effective at later stages of infection in order to enhance the outburst of viruses in cell death-resistant cancer cells. Papers from Hermiton and colleagues have dealt with this issue by inserting toxin genes in the E3 region in place of the native adenoviral genes, in consideration of the fact that the majority of genes in this region are not essential for viral replication *in vitro*. They found that the replacement of E3-6.7/gp19K-, ADP-, and E3B-encoding regions with toxin genes resulted in a timing of expression similar to that of the replaced viral genes, thereby maintaining normal expression of the resident adenoviral genes

(Hawkins and Hermiston, 2001; Hawkins *et al.*, 2001). Although the various viruses containing as replacements a cDNA encoding CD or TNF- $\alpha$  were not evaluated in detail for their oncolytic activity, the expression of these genes was stronger than that obtained with the CMV promoter, probably because of the high copy numbers of viral DNA at later stages. Moreover, deletion of ADP resulted in a longer survival of the infected cells accompanied by attenuated protein synthesis leading to increased production from the inserted gene (Hawkins and Hermiston, 2001). These studies appear to indicate that the E3 region is an attractive locus for inserting potent death-inducing genes in the CRAD genome, thereby not interfering with viral replication but facilitating the disruption of possibly death-resistant cancer cells to obtain optimal dispersion.

### CRADs AND CHEMOTHERAPY

Several investigators of various *in vitro* and *in vivo* model systems, as well as in the clinic, have reported additive or synergistic effects between chemotherapy and CRADs. In a phase II trial for patients with recurrent head and neck cancer, dl1520 (ONYX-015), in combination with cisplatin and 5-fluorouracil, had the strongest antitumor effect when compared with the separate treatments (Khuri *et al.*, 2000). Studies to characterize this interaction in nude mouse-human tumor xenograft models indicated that this synergism was independent of the route of administration and p53 status. However, the order of administration of the agents appeared to be crucial; treatment with ONYX-015 first, or simultaneous exposure to cisplatin and the virus, is superior to cisplatin followed by ONYX-015 (Heise *et al.*, 2000b). Contrary to the lack of involvement of p53, You *et al.* (2000) found in monolayer lung cancer cultures that cells with nonfunctional p53 were at least 10 times more sensitive to ONYX-015 cytolysis than cells with wild-type p53, and chemotherapy with taxol and cisplatin was able to enhance oncolysis only in p53 mutant lung cancer cells. Synergistic effects have also been observed in prostate cancer cells, both *in vitro* and *in vivo*, on treatment with CV787, a prostate cancer-specific replicating adenovirus and the taxanes paclitaxel and docetaxel (Yu *et al.*, 1999). In addition, radiotherapy has also been found to enhance the antitumor activity of ONYX-015 in xenograft mouse models (Rogulski *et al.*, 2000). Using isogenic cell lines with wild-type or mutant p53, the authors observed no effect of irradiation on viral DNA replication in monolayer cultures. However, *in vivo* little oncolytic activity was found in p53 wild-type tumors, with radiation having no enhancing effect, whereas in mutant p53 tumors the already higher antitumor basal level was further increased after irradiation.

Classically, synergy is defined as greater than additive therapeutic effects when compared with the therapeutic efficacy of each drug alone. The molecular mechanisms underlying the synergy between replicating adenoviruses and chemotherapy are currently unknown. For the *in vivo* tumor context, this interaction may involve the enhanced activity of the immune system due to chemotherapy-dependent tumor cell damage or may perhaps be due to antiangiogenic effects elicited by these cytotoxic agents. In addition, it could be envisioned that chemotherapy may alter the structure of the tumor mass, thereby facilitating the penetration and spreading of virus throughout the

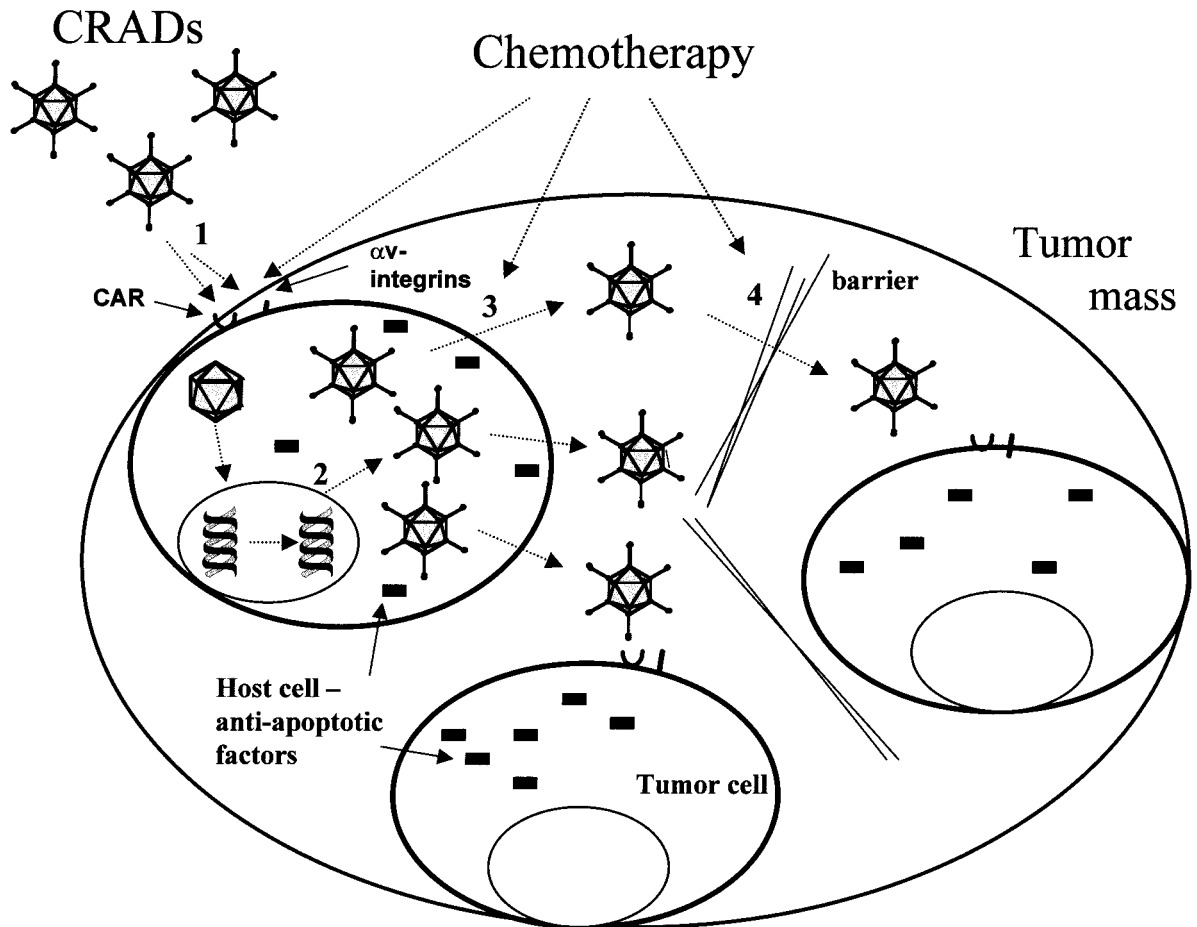
tumor (see also Fig. 2). However, interaction between the two types of anticancer agents can also occur at the cellular level in the absence of an immune response and a structural context as, for example, illustrated by the synergy found between ONYX-015 and subtoxic concentrations of paclitaxel or cisplatin in lung cancer cell lines and primary lung cancer cells (You *et al.*, 2000) and CV787 in prostate cancer cells (Yu *et al.*, 1999).

Additive or synergistic interactions between drugs may depend on dissimilar mechanisms of action of the drugs, thereby targeting two independent pathways. In this respect, the molecular mechanism(s) underlying the enhancing effects of chemotherapy and irradiation on CRAD efficacy may be due to interactions between viral genes and stress-activated host cellular factors leading to enhancement of the tumor cell-killing effect of CRADs. For example, the two major mRNAs transcribed from the E1A region, E1A 12S and E1A 13S, encode proteins of 243 and 289 residues (243R and 289R) that can induce apoptosis via both p53-dependent and -independent mechanisms (White, 1998). Apoptosis induced by 243R was found to require the presence of functional p53 that correlated with deregulation of Bcl-2 and Bax, whereas the 289R variant could trigger apoptosis independent of p53. However, more recently, 243R has been reported to induce apoptosis also independent of p53 (Putzer *et al.*, 2000). E1A expression has also been found to enhance the sensitivity to apoptosis induced by ionizing radiation and various cytotoxic agents in murine embryonic fibroblasts, keratinocytes, and human ovarian cancer and leukemia cells (Stiewe *et al.*, 2000 and references therein). CRAD-mediated E1A expression may thus contribute to synergistic effects with chemotherapy or radiation, although also other adenovirus genes may be involved, which currently remains to be investigated. Results from such studies may be translated into the generation of CRADs with enhanced activity.

On the other hand, although much is known on the molecular basis of cancer cell resistance to chemotherapy-induced cell death, the potential effect on the oncolytic activity of CRADs has currently not been addressed. Interestingly, in a study with cisplatin-sensitive and -resistant p53 mutant-expressing ovarian cancer cell lines, ONYX-015 displayed preferential replication in cisplatin-resistant cells in *in vitro* and *in vivo* models (Ganly *et al.*, 2001). The restored expression of wild-type p53 in the sensitive cell line resulted in early onset of apoptosis that probably formed the basis for the observed reduction of viral production. These findings provide more evidence for the concept that early onset of cell death is detrimental for virus production whereas cell death resistance may delay or block viral release.

### TUMOR CHARACTERISTICS AND OTHER BARRIERS TO CRAD EFFICACY

In the ideal situation, a CRAD should be applied intravenously and, on reaching the tumor site(s), infect tumor cells and spread throughout the tumor to eradicate all cancer cells, even when dealing with advanced metastatic disease. Although these features have been challenging to meet for all anticancer therapies, CRADs encounter several barriers that are particular to this class of agents. In this respect, apart from difficulties encountered by viral particles entering a tumor via the blood



**FIG. 2.** Schematic representation of several obstacles for CRAD efficacy and the possible stages at which combined treatment with chemotherapy may sort its beneficial effect. On administration of CRADs, a first hurdle may be difficulties to pass the extracellular matrix of the tumor or the lack of adenoviral receptors on tumor cells (1). Combined treatment with chemotherapy may loosen the tumor structure, thus facilitating CRAD penetration. The potential absence of the CAR may be circumvented by exploiting other virus entry routes, including allowing primary entry via the  $\alpha_v$ -integrin pathway by incorporation of the RGD motif in the fiber knob of the virus. After infection, the replication of CRADs may be influenced by both viral and cellular factors, such as gene deletions in the CRAD genome, in order to obtain tumor selectivity and/or host cell factors such as p53 and additional, yet to be identified factors (2). After viral assembly, cellular mechanisms conferring cell death and/or chemotherapy resistance that are common to cancer cells may counteract cell death induced by CRADs and hamper the release of virus (3). At this stage chemotherapy may work in concert with CRADs to enhance tumor cell killing, resulting in improved dispersion of the virus; however, it may also act to disrupt possible existing intratumoral barriers (4). On the other hand, chemotherapy may increase the antitumor potential of CRADs via indirect phenomena such as a possible costimulatory effect on the immune response induced by dying tumor cells or potential antiangiogenic effects of the drugs (see text for more details).

stream, a process about which little is known, adenoviruses are known to be cleared rapidly by the liver on systemic administration, as illustrated by the 90% clearance rate detected within 24 hr in both immune-competent and -deficient mice (Worgall *et al.*, 1997). In addition, mainly because of clearance by Kupffer cells in the liver, the adenovirus half-life is approximately 2 min (Worgall *et al.*, 1997; Alemany *et al.*, 2000a).

The role of the immune system in adenovirus vector efficacy is not clear. There may be dual effects of the immune system. On the one hand, interference with viral function may occur via neutralizing antibodies or macrophage-mediated phagocytosis; on the other hand, activation of a virus-induced tumor-specific cytotoxic T lymphocyte (CTL) response may help to eradicate

tumor cells. In addition, interactions between the growing tumor, the amount of replicating viruses, and antiviral immune responses are highly complex and nonlinear. An attempt has been made to describe these interactions in a mathematical model (Wodarz, 2001). In this model, the outcome of viral therapy is dependent on the balance between several host and viral parameters, including the growth and death rates of infected and noninfected tumor cells and the speed of viral replication. Such a model predicts optimal antitumor activity at the highest possible level of oncolytic activity and in the absence of an immune response. Also, a high growth rate of the tumor is expected to reduce efficacy and conventional treatments such as chemotherapy may be combined to decrease tumor growth, al-



lowing the virus to eliminate as many tumor cells possible (Wodarz, 2001).

Other characteristics of the tumor mass may also obstruct spreading of the CRAD, such as the architecture of the tumor. In general, it has been noted that established tumors appear to be more difficult to eliminate by CRADs than when tumor cells are premixed with virus before injection in mice for xenograft models or when treatment is started early after tumor growth is detected. "Older" tumors are more difficult to eradicate because of infiltrating macrophages and fibroblasts resulting in connective tissue formation, the ingrowth of blood capillaries, the tumor matrix, and the presence of necrotic areas, all of which impose blocks to the spreading of CRADs. It is currently unknown to what extent these obstacles interfere with CRAD efficacy but they will need to be addressed.

### VALIDATION OF CRADs IN ANIMAL MODELS AND IN THE CLINIC

It is important to measure CRAD infection, replication, and selectivity to evaluate the efficacy of the agent. A major limitation in assessing the efficacy of CRADs in animal models is the inability of CRADs derived from human serotypes to replicate in nonhuman tissue. CRAD activity can thus be determined only in human xenografts models, predominantly in mice, in terms of antitumor effect and general toxicity of the virus. On this basis, determination of CRAD selectivity remains unaddressed. The molecular basis of the inability of human CRADs to replicate in nonhuman host cells is currently unknown. Until this is resolved and animal cells can be modified to become replication permissive, an alternative may be the generation and use of species-specific CRADs, such as a murine adenovirus in mice. In the clinic, viral infection has been determined in tumor biopsy samples by *in situ* hybridization with adenoviral DNA. Replication was demonstrated by testing blood from patients, using a quantitative polymerase chain reaction (PCR), for the presence of adenovirus sequences on day 3 after treatment, on the assumption that this reflects replicating virus because the initial inoculate at this time will be cleared from the blood. Determining infection and replication of adenovirus in tumor biopsies by histologic analysis has its drawbacks. Biopsies provide only a small amount of tissue, thus increasing the chance for false negatives, not to mention the ethical and practical matters associated with acquiring samples. For these reasons PCR is the method of choice, although both methods can be complementary.

The use of noninvasive methods to determine infection and replication is an important area of research that may help to evaluate CRAD efficacy. Methods that are currently being developed are adenovirus imaging systems for *in vivo* detection of infected cells, such as by incorporation of a transgene expressing the receptor for somatostatin subtype 2 (SSTR2) into the virus, allowing detection with intravenously administered radiolabeled tracer (Rogers *et al.*, 1999). The HSV-TK cassette has been used in combination with this approach, allowing the detection of TK on exposure of cells to <sup>131</sup>I-labeled 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracil (<sup>131</sup>I-labeled FIAU) with γ-camera imaging (Zinn *et al.*, 2001). Other strategies employ photon-emitting reporter genes, including luciferase and green fluorescent protein in conjunction with a charged-

coupled device (CCD) camera for *in vivo* imaging (Honigman *et al.*, 2001 and references therein).

### FUTURE DIRECTIONS FOR IMPROVEMENTS

The current limitations to CRADs being effective single anticancer agents occur at multiple levels, including macrocellular, cellular, and molecular. Effective systemic delivery of these agents, being preferable in more advanced metastatic cancer, is hampered by clearance of the virus and binding/uptake by non-tumor cells. The development of strategies to detarget the liver and other organs, in order to obtain the best possible ratios of tumor to nontumor targeting, is crucial for this application. This will require ongoing efforts to modify the viral coat to detarget the liver while at the same time increasing tumor specificity via tumor-targeting approaches such as the incorporation of targeting domains in the fiber knob or the use of targeting moieties, including bispecific single-chain antibodies. At the same time these methods will bypass the often-occurring CAR deficiency of cancer cells. These approaches may be used in conjunction with TSPs, which can drive both essential adenoviral genes and/or therapeutic genes to obtain liver-off/tumor-on characteristics. Enhancing replication and oncolytic properties of CRADs to overcome possible structural barriers in the tumor will be necessary to improve tumor-eradicating activity. The optimization between selectivity and oncolytic activity by means of small and subtle gene deletions and/or modifications, coupled with the use of TSPs, will probably provide the best and safest adenoviral platform to build in additional improvements. The combined use of enzyme-prodrug strategies or proapoptotic genes with such CRADs, thereby ensuring onset of expression at later stages of replication, may improve oncolysis and spreading of the virus in the tumor mass and may help to overcome structural barriers. Moreover, for additional rationalized approaches to improve CRADs more basic research on the factors involved in adenoviral oncolysis will be required, including the unraveling of the mechanism(s) responsible for synergy between chemotherapy and CRADs that may be instrumental for designing better agents. Studies to understand the way in which rodent cells block adenovirus replication may lead to the development of transgenic mouse models that are permissive for CRAD replication, thus providing better model systems. Alternatively, other species-specific adenoviruses may be used to examine CRAD characteristics. Finally, the incorporation of reporter genes in CRADs to allow noninvasive imaging techniques will be helpful in determining CRAD efficacy.

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