Virotherapeutics: conditionally replicative adenoviruses for viral oncolysis

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Viral oncolysis, or virotherapy, is an endeavor to use viruses as therapeutic agents in an effort to exploit their highly evolved qualities of host cell killing and simultaneous multiplication and spread. This review describes the concept of oncolytic adenoviruses, also called conditionally replicative adenoviruses (CRAds), and recent developments—inspired by early clinical results—that aim at the optimization of CRAd efficacy. Molecular strategies applied for the development of oncolytic adenoviruses include (i) the genetic manipulation of the expression and/or function of key regulatory viral proteins in order to restrict viral replication and spread to tumor cells, (ii) the engineering of the adenoviral capsid for efficient and tumor-targeted infection, and (iii) the incorporation of heterologous genes to facilitate combination therapies or tracking of the virus. Initial clinical trials have provided proof-of-concept for adenoviral oncolysis in patients and a favorable safety profile for oncolytic adenoviruses has been demonstrated. In conclusion, adenoviral oncolysis, with its distinct therapeutic mechanism, shows remarkable therapeutic potential. Advanced generations of virotherapeutics are currently in development. *Anti-Cancer Drugs* **14:577–584**

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Introduction

Virotherapy, or viral oncolysis, represents a novel and promising biological strategy for treatment of cancer in a quest to redefine an old enemy as an ally in the war against cancer [1]. Virotherapy research capitalizes on the highly evolved qualities of various viruses to efficiently infect human cells, replicate, kill the host cell to release the progeny virions and to spread. Accordingly, viruses are being engineered for applications in cancer therapy to derive therapeutic agents that specifically kill tumor cells and simultaneously multiply to derive a new generation of anti-cancer 'drugs' (Figure 1). By this means, the patient's body, i.e. the tumor, is transformed into a drug factory. Ideally, therapeutic viruses would spread through the tumor and the patient's body until tumor cells are completely eradicated.

Early clinical virotherapy efforts were inspired by occasional tumor regressions observed in vaccinated or virus-infected patients [2]. These studies were restricted to the application of wild-type viruses, and were abandoned because of unfavorable efficiency and the advent of chemotherapy. Recently, virotherapy has entered a new era in which recombinant viruses are being engineered for anti-cancer therapy [1,3,4]. The powerful tool of recombinant DNA technology, substantial progress in molecular virology and a vast experience regarding virus modification from recent decades of gene therapy research have been the critical impetus for this endeavor. Indeed, novel oncolytic viruses have been rapidly evaluated in clinical trials.

Because of their advantageous properties, adenoviruses have been extensively analyzed and engineered as gene transfer vectors, and represent promising candidates for the development of oncolytic viruses [5,6]. In the following, strategies for the development of therapeutic adenoviral agents with tumor-restricted replication capacity and recent developments to further optimize these agents for anti-cancer applications are reviewed.

The agent: adenoviruses for virotherapy of cancer

Adenoviruses possess several attributes that are favorable for their development as anti-cancer virotherapeutics [7,8]. As required for viral oncolysis, the adenoviral replication cycle is lytic and results in killing of the infected cell. Adenoviruses have a favorable safety profile because of their low pathogenicity, their non-integrating genome and their genetic stability. Also, manufacturing and handling of adenoviruses as drugs benefits from the ease of virus production at high titers and the high stability of adenovirus particles.

A key requirement of any anti-cancer drug is tumor specificity. In contrast to chemicals, viruses as biological

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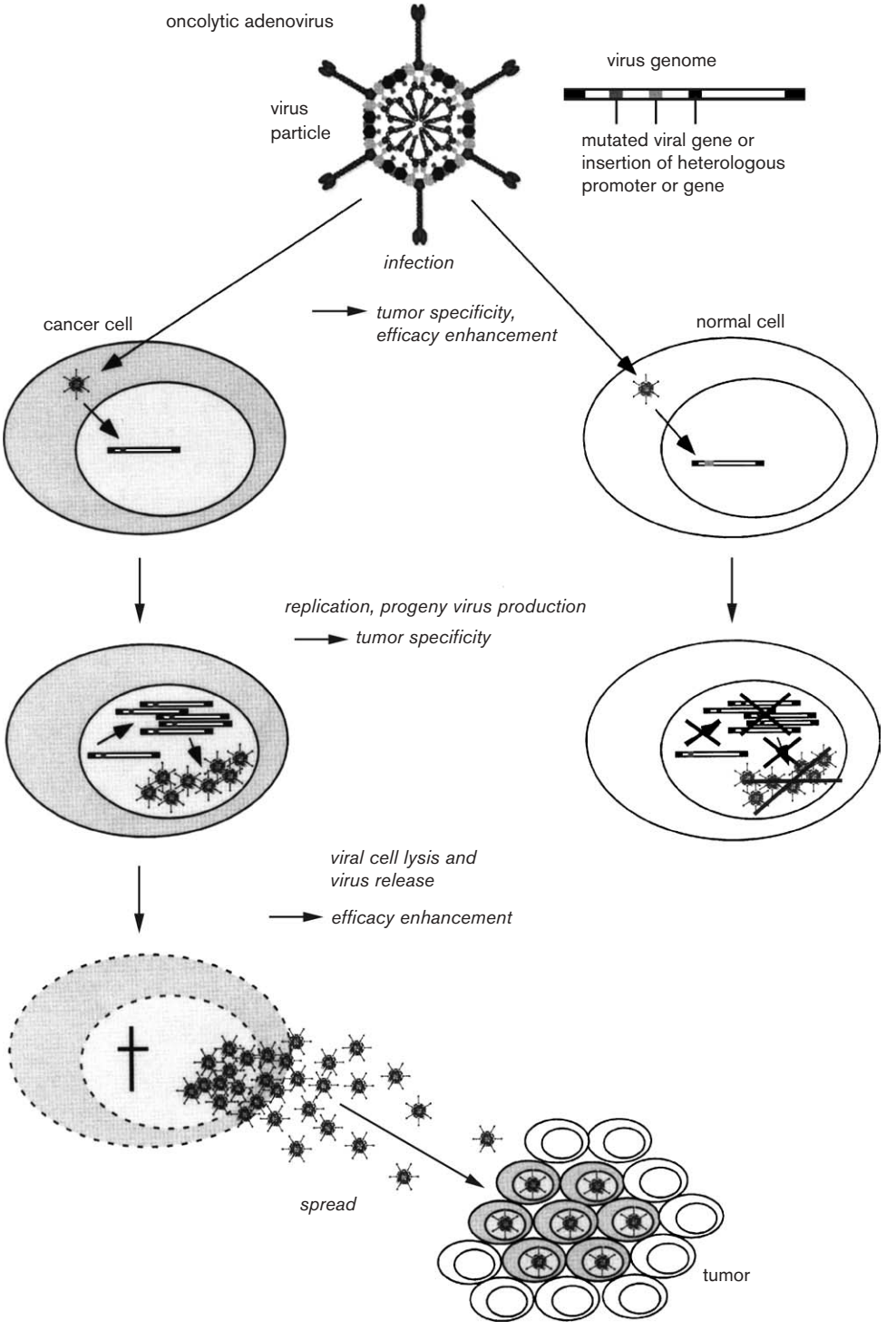
Fig. 1



Adenoviral oncolysis: the concept. Oncolytic adenoviruses are derived from human adenoviruses via genetic modifications. Such modifications include the mutation or deletion of viral genes, or the insertion of heterologous genes or promoters (see Fig. 2). Oncolytic adenoviruses infect tumor cells, replicate their genome, assemble new viral particles and kill the host tumor cells by lysis, resulting in the release of the progeny viruses. Thereby, approximately 10 000 viruses are released from one infected cell. This new virus generation spreads, and starts a new cycle of virus replication and tumor cell killing. Genetic modifications of adenoviruses allow for efficacy enhancement and/or tumor cell restriction at various steps of the viral life cycle, such as viral infection, viral replication and/or virus release/tumor cell lysis. As a consequence, infection of normal cells by oncolytic adenoviruses and/or their replication (as shown here) within these cells is attenuated. Thus, the ideal oncolytic adenovirus represents an efficient and specific anti-cancer agent.

agents allow for the incorporation of targeting 'devices' by molecular modeling at several levels (Fig. 1). For example, 'smart drugs' could be derived from adenoviruses by incorporation of specific cell binding or specific gene expression features which can be achieved by various strategies. In this regard, adenoviruses are suitable candidate viruses, because their genome structure, capsid composition and infection pathway are known in detail [7–9]. This is because adenoviruses have long served as model organisms in molecular biology research. Moreover, a great deal of knowledge concerning adenoviral biology and molecular modeling of adenoviruses has been accumulated in recent decades of gene therapy research, where replication-deficient adenovirus mutants have been exploited as gene transfer vectors [10]. Indeed, 'vectorology', the optimization of vectors with the goal of efficient and targeted gene transfer, has been a major avenue of this field [9]. Thus, molecular modifications that are required for the development of sophisticated viral therapeutics are feasible for adenoviruses and will be described in the following (see also Fig. 2). Importantly, several clinical trials with replication-deficient and replication-competent adenoviruses have already demonstrated a favorable safety profile for these agents [10].

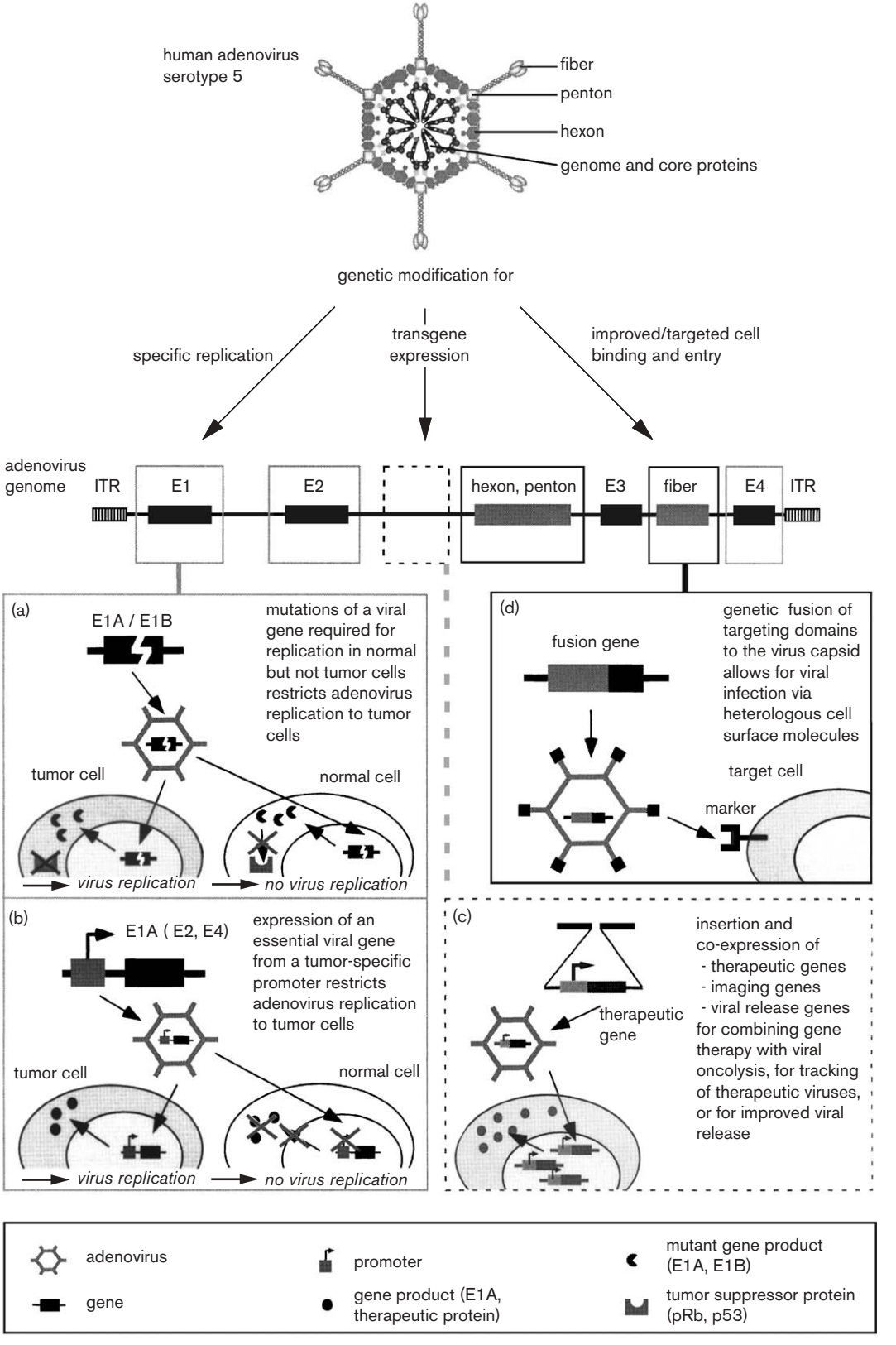
Introduction of tumor specificity: conditionally replicative adenoviruses (CRAds)

Key to the development of 'smart' adenoviral therapeutics has been the implementation of strategies to restrict viral replication to cancer cells. Such adenoviruses with tumor-cell restricted replication capacity were termed CRAds [6]. Most approaches for the development of CRAds have focused on the genetic engineering of E1 (for early 1) genes (Fig. 2A and B). This seems logical since these are the first viral genes expressed after infection of the host cell and encode the key regulators of adenoviral replication. The major tasks of E1 proteins are to induce expression of further viral genes, and to orchestrate modifications of cellular gene expression and protein activity that favor viral replication. With the goal of tumor-restricted replication, adenoviral E1 genes have been genetically engineered by two means: partial or complete deletions (type 1 CRAds, Fig. 2A) or promoter replacement with tumor-specific promoters (type 2 CRAds, Fig. 2B).

The idea of developing type 1 CRAds stems from the observation that cellular processes induced by adenovirus infection often strikingly resemble those acquired during cancerogenesis. Thus the deletion of corresponding viral functions should not influence viral replication in cancer cells, but block viral replication in healthy cells. The first described CRAd published by Bischoff *et al.* in 1996, dl1520 or ONYX-015, does not express E1B55K [11]. One function of E1B55K is to bind and inactivate the tumor-suppressor protein p53. Thus, the rationale for this strategy is the inactivation of p53 in most cancer cells, but not in normal cells, even without adenovirus infection. However, it turned out that this CRAd is strongly attenuated even in tumor cells. This is probably because of the lack of E1B55K functions different from p53 inactivation. A different type 1 CRAd was derived by mutation of E1A. E1A is required to induce viral gene expression and to propel the host cell into S phase which is necessary for productive viral replication. E1A mutant CRAds, such as AdΔ24 (dl922-947), KD1 or CB016 contain specific mutations in individual domains of the protein [12–15]. This strategy aims to ablate S phase induction by the CRAd, which is not required in proliferating (tumor) cells or in cells transformed by tumor viruses such as HPV, but retain transactivation functions that are mandatory for viral gene expression. Future development of type I CRAds will have to consider such multiplicity of functions of manipulated viral proteins and also functional redundancies between different viral proteins in an effort to increase specificity and retain efficiency. Also, differences in cell physiology between distinct tumor types and even between individual tumors of one type must be addressed.

The first type 2 CRAd, CV706, was described by Rodriguez *et al.* in 1997 [16]. This CRAd was engineered to contain an insertion of the prostate-specific antigen (PSA) promoter upstream of the E1A gene for specific expression in prostate cancer cells resulting in a selective cytopathic effect. Critical for the development of CRAds by transcriptional targeting is the availability of promoter fragments with specific activity and the retention of specific promoter activity in the context of the adenoviral genome and during adenoviral replication. To date, type 2 CRAds have been developed for various tumors by exploiting promoters of the genes encoding AFP (hepatocellular carcinoma) [17–19], kallikrein 2 (prostate cancer) [20], probasin (virus CV787; prostate cancer) [21], MUC1 (breast cancer) [22], osteocalcin (prostate cancer) [23], midkine (pediatric solid tumors and other tumors) [24], E2F-1 (pan-cancer) [25,26], tyrosinase (melanoma) [27,28], uroplakin II (bladder cancer) [29], flk-1 or endoglin (tumor endothelium) [30]. In addition, CRAds based on the insertion of hypoxia- and estrogen-responsive promoters (breast cancer) [31,32] and of binding sites for Tcf (colon cancer) [33,34], or for externally inducible transcription factors [35] have been engineered. Moreover, selective cell killing has been achieved or enhanced by targeted expression of viral genes other than E1A, such as E1B [18,20,21,29,30,33,34], E2 [33,34] or E4 [26,31,32,34]. Finally, oncolytic adenoviruses that combine E1A mutants with transcriptional targeting have been reported (type 1/2 CRAds) [27,36].

Fig. 2



Both strategies for genetic engineering of CRAds have clearly shown that tumor-specific viral replication and adenoviral oncolysis are feasible. In cell cultures, various CRAds showed a remarkable specificity profile with several orders of magnitude attenuation in non-target or normal cells versus tumor cells. These results indicate a high therapeutic window for the application of CRAds. The evaluation of CRAd specificity in animal models, however, is hampered by the fact that adenoviral replication is species specific. Nevertheless, efficient anti-tumor activity of various CRAds has been demonstrated in mice bearing human tumor xenografts.

Trials: first clinical data for CRAds

Adenoviral oncolysis proceeded to clinical studies remarkably fast. Clinical trials for both type 1 and 2 CRAds started only a few years after initial description of these agents. To date, results of a significant number of trials have been reported [37]—a field landmark for adenoviral oncolysis. In initial trials, oncolytic adenoviruses were applied i.t. (ONYX-015 to head and neck, and lung cancer patients; CV706 to prostate cancer patients). Subsequently, viruses were applied i.p. to ovarian cancer patients (ONYX-015), into the hepatic artery of patients with liver metastases of colorectal cancer (ONYX-015), and i.v. to prostate (CV787) and other cancer patients. These studies demonstrated that adenovirus injections were well tolerated, even at high titers and after repeated dosing. Virus delivery to tumors after systemic application was achieved. Also, antibody responses did not appear to block biological efficacy after i.t. and i.v. virus injections. Importantly, clinical studies have validated the concept of adenoviral oncolysis *in vivo* by demonstrating tumor-specific viral replication and tumor cell killing. However, durable responses have been rare for oncolytic adenoviruses as single agent. Nevertheless, these clinical observations with advanced tumor patients have been extremely valuable in addressing severe concerns regarding toxicity. Importantly, by revealing the limitations of current oncolytic adenoviral agents these early clinical trials triggered further research efforts aimed at the generation of advanced oncolytic adenoviruses. On-going research aims to overcome major road-blocks of adenoviral oncolysis, with the main focus being on improving the efficacy of viral replication, cell killing, and spread. In addition, new avenues of research on CRAds, i.e. combination therapies, were initiated after the clinical observation of synergistic effects of adenoviral oncolysis and chemotherapy or radiotherapy resulting in tumor reductions in individual patients.

Improving therapeutic efficacy of CRAds: tropism modification by engineering of the viral capsid

The strategy of adenoviral oncolysis takes advantage of the highly evolved replication cycle of adenoviruses. Nevertheless, adenoviruses need to be tailored by recombinant DNA technology for the purpose of their therapeutic exploitation. Key to the realization of adenoviral oncolysis has been the development of CRAds by incorporating features into the adenoviral genome that result in the restriction of viral replication and cell killing to tumor cells. However, the native tropism of adenoviruses has turned out to be a major limitation in their exploitation as anti-cancer agents. In this regard it is of interest to note that the primary cellular receptor for the most commonly applied adenovirus serotype 5, CAR (for coxsackie and adenovirus receptor), is a transmembrane protein involved in cell–cell contacts [38–43]. It has recently been shown that CAR expression is down-regulated in tumors. Indeed, tumors *in situ* and freshly purified tumor cells, in contrast to established tumor cell lines, were shown to be resistant to adenovirus infection due to a paucity of CAR [44–46]. This observation might explain why early clinical gene therapy and virotherapy trials that applied adenoviral vectors have fallen short of their theoretical promising potential which was based on pre-clinical studies in cell lines. For viral oncolysis, a paucity of CAR expression in tumor cells limits both infection and spread of therapeutic adenoviruses with serotype 5 fiber (Fig. 2). Indeed, some freshly purified tumor cells were resistant to killing by wild-type

Genetic engineering of adenoviruses for therapeutic applications in viral oncolysis. Oncolytic adenoviruses are usually derived from human adenovirus serotype 5, a common cold virus. The detailed characterization of the structure, genome and life cycle of this virus allows for genetic engineering as required to generate recombinant viruses optimized for applications in cancer therapy. Such viruses might implement specific replication, transgene expression or improved/targeted cell binding and entry. The adenovirus genome is a linear DNA double strand with inverted terminal repeats (ITRs), sequences required for DNA replication, at its left and right terminus. Adenoviral early genes E1 to E4 are expressed prior to viral DNA replication. Late genes are expressed after DNA replication, and include genes encoding structural proteins of the virus capsid such as hexon, penton and fiber. Tumor-specific adenoviral replication has been achieved by mutations of E1 genes, the key regulators of adenoviral gene expression and replication (A). For example, tumor suppressor proteins (such as p53 or pRb) need to be inactivated by E1 proteins for functional adenoviral replication to occur in normal cells. However, these tumor suppressor proteins might not be expressed or functional in tumor cells *per se*. Hence, corresponding E1 mutants implement a block of virus replication in normal, but not in tumor cells. An alternative strategy is the expression of these genes from tumor-specific promoters (B). Because these genes are essential for adenovirus replication, replication of the resulting viruses is restricted to cancer cells, in which the promoter is 'on' and E1 genes are expressed. Anti-cancer efficacy of oncolytic adenoviruses can be increased by incorporating therapeutic genes or viral release genes into the viral genome (C). Similarly, monitoring of virotherapy is facilitated by the incorporation of imaging genes. Finally, incorporation of cell-binding domains into the adenoviral capsid (e.g. by genetic fusion to fiber, hexon or penton proteins) can result in enhanced or tumor cell-specific viral infection and spread (D).

adenovirus. Bearing these limitations in mind, many gene therapy researchers have endeavored tropism modification of adenoviruses for several years now. For this purpose, bispecific adapter molecules have been developed that bind simultaneously to the virus capsid and to the target [47]. Re-targeted adenovirus infection has been achieved with such adapters *in vitro* and *in vivo*. However, a second strategy for tropism modification of adenoviruses, i.e. genetic capsid modification [48], seems more promising for applications in viral oncolysis. This is because progeny viruses produced in the patient will be tropism modified as well and thus might mediate enhanced virus spread and therapeutic efficiency. Genetic capsid modification faces structural constraints that result from the multimerization of the capsid components that form an intrigued structure through various protein–protein interactions. Nevertheless, tropism modification via genetic capsid modification has been demonstrated by various strategies such as the incorporation of targeting domains into the adenoviral capsid, the swapping of capsid proteins or domains with those of other adenovirus serotypes (that bind to different receptors), or the replacement of capsid proteins with targeting proteins. Recent reports have clearly demonstrated that efficient adenoviral oncolysis is critically dependent on expression of a receptor for adenovirus on tumor cells and that genetic tropism modification results in strongly enhanced adenoviral replication, spread and cell killing in CAR-negative cells [49–53]. Tropism-modified CRAds with augmented infectivity are currently entering clinical trials. On-going research efforts in this field are endeavoring tropism modification with the goal of tumor-specific viral infection. This is not only to secure efficient infection of tumor cells, but in addition to reduce side-effects and virus sequestration resulting from virus-binding to normal cells.

Improving therapeutic efficacy: adenoviral oncolysis in combination therapy and 'armed' CRAds

Pre-clinical and clinical studies have clearly shown that adenoviral oncolysis is feasible, but requires further optimization of therapeutic efficiency of CRAds. However, these studies also revealed that virotherapy lacks cross-resistance with various conventional therapies, i.e. radiation therapy and chemotherapy [37]. Current research in this area is focused on revealing and further optimizing synergistic effects of such combination therapies that result from sensitization of cancer cells for drug- or irradiation-induced apoptosis by virus infection or *vice versa* from enhanced viral release by drug- or irradiation-induced processes. In particular, the specification of drugs that show best compatibility with the molecular mechanism of adenoviral replication and determining at what stage of the adenoviral life cycle the best synergism occurs needs to be further evaluated.

An even more fascinating perspective is the combination of chemotherapy and viral oncolysis into one therapeutic agent: an 'armed' CRAd [54]. Such agents are CRAds that have therapeutic genes incorporated into their genome and have the potential to target different aspects of tumor biology by this means. In order to combine viral oncolysis with molecular chemotherapy [55], transgenes that encode enzymes which activate systemically applied prodrugs have been exploited [56–58]. Different therapeutic genes may be harnessed to incorporate therapeutic strategies other than molecular chemotherapy, such as genetic immunopotentiation, apoptosis induction or anti-angiogenesis [55].

The incorporation of reporter or imaging transgenes into CRAds is an exciting strategy to facilitate non-invasive monitoring of oncolytic adenoviruses in patients. For example, transgene-encoded fluorescent proteins or proteins that trap radioactively labeled molecules [59,60] might allow for the detection of virus-infected cells and thus of virus spread by optical or radiodiagnostic imaging. Alternatively, transgene-encoded marker peptides secreted into the bloodstream by infected cells [61] could facilitate the quantification of virus replication via a blood test. Clearly, tracking and quantification of therapeutic viruses would be extremely advantageous for the field, and significantly improve both the evaluation of clinical studies and future routine clinical applications of oncolytic adenoviruses.

The development of armed CRAds, however, faces several challenges that are currently being addressed [62]. These include the identification of candidate therapeutic genes, the fine-tuning of strength and timing of transgene expression for individual therapeutic or diagnostic applications and tumor types, and the orchestration of the distinct and complex molecular processes of therapeutic or diagnostic gene transfer and adenoviral replication.

Conclusions

Viral oncolysis using genetically engineered adenoviruses represents a novel cancer treatment modality that implements a therapeutic mechanism distinct from conventional therapies for which cancers are often resistant. Moreover, the inherent quality of therapeutic viruses to amplify and spread is advantageous for anti-cancer efficacy. Several conditionally replicative adenoviruses have been designed on the basis of advances of basic research. Their pre-clinical evaluation suggests considerable therapeutic potential for these agents. Oncolytic adenoviruses have been rapidly examined in clinical studies, which confirmed the principle of tumor-specific replication and did not reveal any significant safety problems. However, they also indicated that the first generation of oncolytic adenoviruses lack the efficacy

required to mediate measurable therapeutic effects as single agents. Consequently, in a 'bedside to bench' effort, substantially improved CRAds have been engineered by means of various molecular modifications. Future endeavors need to address the clinical potency of these second-generation virotherapeutics. In this respect, the outcome of studies with tropism-modified CRAds in patients will be of special interest. Moreover, in light of the enormous pace of progress in the molecular characterization of tumor development, the design of more advanced CRAd therapeutics, e.g. tailor-made oncolytic viruses shaped to the genetic characteristics of individual cancers, can be envisioned. Notably, synergistic effects of viral oncolysis combined with conventional anti-cancer therapies have been observed in recent preclinical and clinical studies. These observations indicate that oncolytic adenoviruses, in the context of combination therapies, might enter routine applications sooner than expected. Indeed, the ability of oncolytic adenoviruses to express therapeutic proteins might facilitate the combination of different therapeutic strategies within one viral agent which attacks tumors at multiple levels.

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