Use of Replicating Oncolytic Adenoviruses in Combination Therapy for Cancer

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ABSTRACT
Oncolytic virotherapy is the use of genetically engineered viruses that specifically target and destroy tumor cells via their cytolytic replication cycle. Viral-mediated tumor destruction is propagated through infection of nearby tumor cells by the newly released progeny. Each cycle should amplify the number of oncolytic viruses available for infection. Our understanding of the life cycles of cytolytic viruses has allowed manipulation of their genome to selectively kill tumor cells over normal tissue. Because the mechanism of tumor destruction is different, oncolytic virotherapy should work synergistically with current modes of treatment such as chemotherapy and radiation therapy. This article focuses on oncolytic adenoviruses that have been created and tested in preclinical and clinical trials in combination with chemotherapy, radiation therapy, and gene therapy.

INTRODUCTION
Cancer remains one of the top causes of death in adults and children. Progress has been made in the overall survival of cancer patients after the emergence of better imaging and diagnostic techniques, improved understanding of the molecular processes that cause cancer, and additional knowledge of treatment using combined chemo- and radiotherapy. However, survival has not improved with current chemotherapy and radiation regimens in patients diagnosed with metastatic disease and certain tumors such as malignant neoplasms of the brain, pancreas, and liver. This is usually due to tumor cells developing genetic mechanisms that override apoptosis caused by chemotherapy and radiation damage to their DNA. This confers resistance to treatment through clonal expansion of genetically resistant tumor cells. Much effort has been directed toward finding alternate pathways that would complement therapeutic induction of apoptosis, overcome multidrug resistance, and ultimately improve overall cure rates. Although the dream of developing a single curative drug per cancer type drives the field, the reality is that combination therapy will be the most effective way of improving survival.

The use of viruses that specifically kill tumor cells while sparing normal cells, known as oncolytic virotherapy, has reemerged over the past 7 years (1, 2). Viruses have evolved to maximize their ability to enter cells, use the machinery of the host cell to replicate and package their own genome and lyse the cells to release their progeny and propagate the viral replicative cycle. Wild-type viruses have been discovered that have tumor-specific cytolysis, such as Newcastle disease virus, vesicular stomatitis virus, vaccinia virus, reovirus, and autonomous parvovirus (3). Furthermore, with the advances of molecular biology and understanding the function of viral genes, it has become possible to genetically re-engineer other viruses to make them selectively target tumor cells. Much of this work has been carried on in adenoviruses and herpesviruses (4, 5), which can also function as therapeutic gene delivery vehicles.

Several biological features have made the adenovirus an attractive virus for oncolytic virotherapy. It is a common pathogen. Viral replication leads to host cell destruction, and a 1000-fold amplification of the viral load is achieved in a single replicative cycle, allowing spread of viral infection. Another biological feature that makes adenoviruses attractive for cancer therapy is that high-titer viral stocks (1010 plaque-forming units/ml) can be produced, which can then be additionally concentrated 100-fold. The viral genome does not integrate into the cell genome, so the risk of recombination and mutation is low. The genome is also large enough to accommodate the incorporation of foreign genes. The technology to manipulate the viral genome is also readily available so that recombinant adenoviruses can be made tumor selective.

This review will describe the mechanisms by which oncolytic adenoviruses that target cancer cells have been designed and their use in combination with chemotherapy, radiation therapy, and gene therapy including the results of clinical trials.

ADENOVIRUS BIOLOGY
Over 40 different serotypes of adenoviruses have been discovered with serotypes 2 and 5 being the most extensively used in developing oncolytic adenoviruses. These viruses primarily infect the epithelial tissue lining the respiratory tract. The most common symptoms are mild upper respiratory symptoms such as nasal congestion and rhinorrhea (runny nose), cough, and conjunctivitis. Adenovirus (type 2 and 5) infection occurs...
through binding of the adenoviral fiber to cellular receptors such as the coxsackie-adenovirus receptor or integrins. Once bound, the virus is internalized via receptor-mediated endocytosis and brought to the nucleus. During transport, disassembly of the viral capsid takes place allowing the viral DNA to enter through the nuclear pores. Once the viral genome enters the nucleus, viral transcription begins.

Adenoviral transcription occurs in three phases: early, intermediate, and late (6). During the early phase, the host cell is transformed into an efficient producer of the viral genome. Because most cells are in a quiescent state, viral proteins must interact with the proteins of the host cell to activate cell replication and division. The first gene that is transcribed in the viral genome is E1A, and its product binds with many cellular proteins such as the pRb, p107, and p130 family of proteins. This interaction prevents these cellular proteins from blocking E2F transcription factors, leading to the initiation of DNA replication and the cell cycle (7). Because the E1A protein will activate the cellular repair or apoptotic pathway mediated by p53-dependent and independent pathways, expression of the adenoviral E1B gene products prevents early death of the host cell thus allowing viral replication to occur unimpeded. The E1B-55kD protein binds to p53 and induces its degradation, whereas the E1B-19kD protein functions similarly to the antiapoptotic factor Bcl2 (8, 9) thereby preventing the death of the infected cell and allowing viral replication to occur. Other proteins in the early phase of the viral replication cycle are encoded by the E2 (proteins needed in viral DNA synthesis), E3 (host immune response modulation and cell lysis), and E4 (regulation of DNA replication, mRNA transport, and apoptosis) gene clusters. The viral structural proteins and the proteins necessary for assembly of the virion are encoded by genes expressed during the intermediate and late phases of viral replication. Once viral progeny assembly is complete, new viral particles (>1000/cell) are released by cytolysis.

METHODS TO RENDER CYTOLYTIC ADENOVIRUSES TUMOR SPECIFIC

Tumor specificity has been achieved in oncolytic adenoviruses in 3 main ways: (a) altering viral genes that attenuate replication in normal tissue but not in tumor cells; (b) by placing viral genes that initiate viral replication under the control of promoter sequences that are active in tumor cells; and (c) by the modification of viral coat proteins that function in host cell infection. Included in this review is a table of current oncolytic adenoviruses and their progress toward clinical trial (Table 1).

Attenuating Viral Replication in Normal Cells but Not Tumor cells via Gene Mutation

Replication of the adenovirus genome after infection relies on the ability of the virus to effectively take over the machinery of the host cell. The elucidation of the molecular mechanisms underlying this phenomenon has led to the creation of modified adenoviruses that contain viral gene mutations, which lead to selective viral replication within tumor cells but not in normal cells (Fig. 1).

The first adenovirus gene used in such a strategy is E1B. Adenoviral replication activates cellular growth arrest and apoptosis through p53-dependent and -independent mechanisms. The E1B-55kD protein plays a critical role in viral replication by binding to and inactivating the p53 tumor suppressor protein. Mutant adenovirus dl1520 (also known as ONYX-015 and CI-1042) contains a deletion in the E1B gene, preventing the formation of a functional E1B-55kD protein. With this mutation, dl1520 was expected to replicate only in p53-deficient cells (2) as the loss of E1B activity was expected to prevent viral replication in normal cells due to growth arrest. Expression of the E1B-19kD protein is not affected by the mutation, thus protecting the cell from apoptosis. This was the first oncolytic virus to take advantage of the high percentage of tumors having some mutation that causes the loss of p53 function (10–12). Initial preclinical studies showed that dl1520 was effective in decreasing tumor size (2, 13, 14).

However, more recent studies show that dl1520 was able to replicate in cells with a wild-type TP53 gene (7, 15–20). There are several explanations for these conflicting results. There are other proteins that affect p53 activity in cells, such as p14ARF and mdm2 (2, 21, 22). p14ARF down-regulates mdm2, which is an ubiquitin ligase that stimulates p53 degradation. Cancer cells with a loss in p14ARF gene expression due to gene deletion or with overexpression of mdm2 after gene amplification will create a p53-null phenotype thereby allowing dl1520 replication in tumors containing a wild-type TP53 gene. Another explanation involves the adenoviral E4orf6 gene product, which also binds to p53 and inhibits p53-mediated apoptosis, possibly allowing E1B-55kD-deficient adenoviruses to replicate in cells with wild-type p53 (23).

Another avenue that has been used to make adenoviruses tumor specific is altering the conserved region-2 in E1A. In wild-type adenoviruses, the E1A viral protein works to overcome the pRb-mediated inhibition of cell cycle progression. E1A binds with pRb, resulting in the release of Rh-bound E2F transcription factors, which then activate the cell to progress from the G1 to the S (DNA synthesis) phase of mitosis. E1A-CR2 mutants such as dl922–947 (24) and Δ24 (25) have small deletions in this conserved region, which prohibit binding to pRb. Many tumors have a loss of regulation of the G1-S checkpoint leading to tumor progression. The preclinical studies showed that dl922–947 preferentially replicated in several different tumor lines and not in normal tissue. An antitumor effect was also demonstrated in xenograft models regardless of the specific defect leading to the dysregulation of the G1 to S-phase transition. Although this specific modification appeared effective, concerns have been raised if this group of replication competent adenovirus mutants would be able to replicate in normally proliferating tissue, which have physiologically down-regulated proteins that normally inhibit progression from G1 to the S phase. This concern could restrict the use of these adenoviruses to intratumoral administration to prevent potential toxicity to other organs in the body making it unsuitable for the treatment of metastatic disease. Clinical trials will be needed to evaluate if this potential toxicity will occur.

More recent generations of adenoviruses have combined E1A and E1B alterations in an effort to additionally improve the selective killing of tumor cells (26). The oncolytic adenovirus AxdAdB-3 carries mutations in both the E1A and E1B-55kD gene regions limiting its replication to p53- and pRb-deficient
### A. Deletion targeting

<table>
<thead>
<tr>
<th>Oncolytic strain</th>
<th>Tumor specific</th>
<th>Modifications</th>
<th>E3 status†</th>
<th>Cells infected</th>
<th>Clinical trial</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>dl1520</td>
<td>Pan</td>
<td>Deleted E1B-55kD</td>
<td>Deletion</td>
<td>p53 null/mutant</td>
<td>Phase I–III (2, 71, 75, 103–109)</td>
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<tr>
<td>Δ24</td>
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<td>Deletion</td>
<td>pRb null/mutant</td>
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<tr>
<td>d932-947</td>
<td></td>
<td>Deleted E1A CR-2</td>
<td>Deletion</td>
<td>pRb null/mutant</td>
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</tr>
<tr>
<td>AxDAdB-3</td>
<td></td>
<td>Deleted E1B-55kD, mutated E1A</td>
<td>Deletion</td>
<td>p53 + pRb null/mutant</td>
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<tr>
<td>dl1331</td>
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<td>Deletion in VAI RNA-coding</td>
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<td>Ras positive</td>
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<td>Adl118</td>
<td>Breast</td>
<td>E1B-55kD, E1B-19kD deletions</td>
<td>Deletion</td>
<td>p53 + pRb null/mutant</td>
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### B. Transcriptional targeting

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<th>Promoter</th>
<th>Gene controlled</th>
<th>E3 status</th>
<th>Cells infected</th>
<th>Clinical trial</th>
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<td>Ad5TRE-E1A</td>
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<td>Tetracycline controlled</td>
<td>E1A, E1B</td>
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<td>p53 null/mutant</td>
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<td>Adv-TERTp-E1A</td>
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<td>Telomerase reverse transcriptase (TERT)</td>
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<td>Deletion</td>
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<td>TEhRT-Ad</td>
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<td>TERT</td>
<td>E1A</td>
<td>Intact</td>
<td>TERT+</td>
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<tr>
<td>HYPR-Ad</td>
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<td>Ar6pAE2E3F</td>
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<td>E2F antagonist</td>
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<td>p53 null/mutant</td>
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<td>1) E1A</td>
<td>Intact</td>
<td>Estrogen receptor and TERT</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2) PSA</td>
<td>2) E4</td>
<td>Intact</td>
<td>Estrogen receptor and replicating</td>
<td>No (30)</td>
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</tr>
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<td>1) Rat prolin</td>
<td>1) E1A</td>
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<tr>
<td></td>
<td></td>
<td>2) E2F-1</td>
<td>2) E4</td>
<td>Intact</td>
<td>Estrogen receptor and replicating</td>
<td>No (30)</td>
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<td>Ad.ERE2</td>
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<td>pS2 promoter (estrogen responsive)</td>
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<td>MUC1+</td>
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<td>Ad.DF3-E1</td>
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<td>DF3/MUC1</td>
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<td>Osteocalcin +</td>
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<td>AdhOC-E1</td>
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<td>Bidirectional osteocalcin promoter</td>
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<td>PSA+</td>
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<td>PSA</td>
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<td>PSA+</td>
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<tr>
<td>CV764</td>
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<td>PSA</td>
<td>2) E1B</td>
<td>Intact</td>
<td>PSA+</td>
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<tr>
<td>CN706</td>
<td>Liver</td>
<td>AFP</td>
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<td>Deletion</td>
<td>PSA+</td>
<td>Phase I (33)</td>
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<tr>
<td>AvE1a04i</td>
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<td>Deletion</td>
<td>PSA+</td>
<td>Phase I (33)</td>
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<tr>
<td>AdTyrwt</td>
<td>Melanoma</td>
<td>Tyrosinase enhancer</td>
<td>E1A</td>
<td>Intact</td>
<td>Tyrosinase+</td>
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<td>AdTyrΔ24</td>
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<td>E1AΔΔ24 CR-2</td>
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<td>Tyrosinase and pRb null/mutant</td>
<td>No (40)</td>
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<td>AdTyrΔ2Δ24</td>
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<td>Tyrosinase enhancer</td>
<td>E1A Δ2(NH₂ terminus) E1A Δ24 CR-2</td>
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<td>Tyrosinase and pRb null/mutant</td>
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<tr>
<td>KD1-SPB</td>
<td>Lung</td>
<td>Surfactant B</td>
<td>1) E1A, 01, 07</td>
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<td>Surfactant B + and pRb null/mutant</td>
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<td>E1A</td>
<td>Intact</td>
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<td>Midkine</td>
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<tr>
<td>AdE3-1AL3B</td>
<td>Ovarian</td>
<td>1AL3B promoter</td>
<td>E1A</td>
<td>Intact</td>
<td>1AL3B+</td>
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<td></td>
</tr>
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</table>

### C. Modified Tropism

<table>
<thead>
<tr>
<th>Oncolytic strain</th>
<th>Tumor specific</th>
<th>Modification</th>
<th>E3 status</th>
<th>Cells infected</th>
<th>Clinical trial</th>
<th>Reference</th>
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<td>AdS3/Δ24</td>
<td>Ovarian</td>
<td>Incorporation of serotype 3 knob and retaining AdS shaft and tail</td>
<td>Intact</td>
<td>pRb null/mutant</td>
<td>No (116)</td>
<td></td>
</tr>
</tbody>
</table>

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* Revised and reprinted with permission from Ref. 1.
† Refer to cited reference for extent of E3 deletions for each virus.

Abbreviations: HIF, hypoxia-inducible factor.
This mutant adenovirus was as effective as an E1B-55kD-deleted adenovirus, AxdAdB (similar to dl1520), in preventing growth of gallbladder cancer cells and xenografts. AxdAdB-3 replicated less effectively in normal tissue compared with AxdAdB, proving that combining E1A and E1B alterations conferred improved selectivity. Although its selectivity and activity were demonstrated, this specificity may limit the use of this virus, because p53 and pRb mutations may not be found in all of the tumor cell types. The potential limitations described for E1A and E1B altered adenoviruses described above would also apply to AxdAdB-3. Because this mutant carries two deletions, its attenuation may affect its ability to efficiently replicate and spread before achieving its desired cytopathic effect in cancer patients (see below).

Another pathway that has been used to confer tumor selectivity is the interferon pathway. One of the proteins in this pathway is protein kinase R. This kinase, when activated by viral double-stranded RNAs, phosphorylates the α subunit of eIF-2α, which inhibits viral replication (27, 28). Many viruses including adenoviruses have evolved mechanisms that inhibit protein kinase R (27). One mechanism is the production of viral-associated RNAs, which bind to protein kinase R and block double-stranded RNA binding. The Ras oncogene also down-regulates protein kinase R. dl1331 is a mutant adenovirus created by deleting a region in the viral-associated RNA-coding region and thereby inactivating the viral-associated RNAs (29). Therefore, dl1331 replication becomes dependent on tumor cells with high expression of Ras to inactivate protein kinase R, thus conferring tumor selectivity. Whereas dl1331 showed antitumor activity, this virus was attenuated compared with wild-type Ad5. Another limitation is that Ras mutations occur in only 30% of tumors, and thereby potentially restricting its use to a subgroup of tumors. One point brought up is whether a Ras mutation is always needed to activate this virus, because tumor-specific alterations to other proteins within the Ras pathway may lead to constitutive activation of the pathway and subsequent protein kinase R inhibition. More preclinical data will be needed to test if this hypothesis is true.

Use of Overactive Cellular Promoters to Direct Lytic Viral Replication in Tumor Cells

Another method to direct viral specificity to tumor cells is the regulation of viral replication via cellular promoters that are overactive or reactivated in certain tumor cells, sometimes referred to as “tumor-specific” promoters (Fig. 2). Certain tumor types, such as germ cell tumors, prostate cancer, and hepatocellular carcinoma, express proteins that are not normally present in the human body after fetal development. If present, these secreted proteins allow the clinician to have a “tumor” marker that can be followed clinically to look at response to therapy. Other tumors, like breast carcinoma, can have mutations that make these tumors highly sensitive to normally secreted hormones (estrogen). Certain oncolytic adenoviruses (Table 1) have been created to take advantage of the expression of these tumor markers or dependence on normally secreted hormones by placing the E1A gene under the control of the promoters derived from the genes of interest (will now be referred as “tumor-activated” promoters). For example, Ad.ERE2 targets estrogen receptor-positive breast cancer by placing the E1A gene under an estrogen responsive element from the pS2 gene promoter (30). Another breast cancer targeted adenovirus, Ad.DF3-E1, places E1A under the control of the DF3/MUC1 gene promoter...
target MUC1+ cancer cells. The oncolytic adenovirus, AvE2a04i (32), was created to target α-fetoprotein (AFP)-expressing hepatocellular carcinoma by placing E1A under the control of the AFP gene promoter. CV706 targets prostate cancer by placing E1A under the control of a promoter derived from the prostate-specific antigen (PSA) gene (33). AdhOC-E1 targets prostate tumor cells by using a promoter derived from the osteocalcin gene (34, 35), the product of which is also secreted by prostate tumor cells.

Newer generations of oncolytic adenoviruses have been developed to additionally restrict viral replication to tumor cells. Ad.ERE2, AdEHT2, ADEHE2F, ONYX-411, and KD1-SPB place the E1A and specific E4 genes under a “tumor-activated” promoter (30, 35–39). Others have combined transcriptional targeting with viral gene mutations to restrict viral replication. One example is the use of a tyrosinase gene promoter to drive expression of an altered E1A gene product deficient in binding to the p300 transcriptional activator and the pRb family for the treatment of melanoma (40). In the treatment of bronchoalveolar lung carcinomas, KD1-SPB places transcriptional control of a specific E4 and an attenuated E1A gene under a surfactant gene promoter (41). Ad5TRE-E1A is an E1B-deleted oncolytic adenovirus, which also has an E1A gene controlled by a tetracycline-responsive promoter that allows exogenous control of oncolytic activity by the concomitant administration of doxycycline (42).

One major limitation with the use of these tumor-activated promoters to regulate adenovirus replication is that they are not always active in all tumor types. As a general feature, tumors usually exhibit some aberrant form of cell growth due to dysregulation of the cell cycle. E2F-1 is an important transcription factor that activates genes that promote the transition from G1 to S phase of the cell cycle. E2F-1 is an important transcription factor that activates genes that promote the transition from G1 to S phase of the cell cycle. Tumor cells appear to frequently have constitutively high levels of E2F-1 expression and activity due to a disruption of pathways that control its expression. The AdE2F-1RC virus was created to take advantage of this by placing the E1A gene under the regulation of the E2F-1 gene promoter. This virus was shown to induce cytolysis selectively in tumor cells but not in nonproliferating normal cells (43). However, side effects could occur with such viruses, because viral replication may happen in proliferating normal tissue such as bone marrow, skin, and gastrointestinal mucosa. An alternative strategy was used in Ad 01/PEME (44). This virus was designed with a two-step mechanism to prevent replication in normal tissue. An E2F protein antagonist, composed of the E2F DNA-binding domain and a pRb transrepressor domain, was placed under the control of a p53-responsive promoter. This strategy confers selectivity to tumor cells, as a cell generating a functional p53 protein (i.e., normal cells) would express the E2F protein antagonist leading to inhibition of viral and cellular E2F-dependent promoters thus preventing the initiation of viral synthesis. The loss of a functional p53 protein (tumor cells) would allow viral replication to proceed. Newer studies have shown that telomerase activity may also serve as a general marker of neoplastic cells. Its activity in normal cells is restricted to fetal tissue, whereas it is elevated in tumors. Adv-TERTp-E1A was developed by placing the E1A gene under the control of a promoter derived from the gene.
encoding telomerase reverse transcriptase (TERT), the rate-limiting catalytic subunit of telomerase (38, 45).

Hypoxia occurs in virtually all solid tumors as they outgrow their blood supply. Hypoxia augments cellular levels of hypoxia-inducible factor, a transcription factor that regulates target genes through the binding of hypoxia-responsive elements. These include the genes that encode for erythropoietin, the proangiogenic factor vascular endothelial growth factor, and the cascade of glycolytic enzymes necessary for cell survival in low oxygen conditions. Oncolytic adenoviruses were created by placing the regulation of the E1A gene under a hypoxia-inducible factor-responsive promoter recently. These viruses showed cytopathic effects only in cells that were hypoxic (46) or that constitutively expressed hypoxia-inducible factor due to a mutation in the von Hippel-Lindau disease (47). These viruses are best used in conjunction with traditional methods such as chemotherapy and radiation, which are more efficient on normoxic tumor cells.

How Attenuated Is too Attenuated?

It is unknown if newer generations of adenoviruses are more effective, because there are few studies comparing different adenoviruses, and these newer viruses must overcome the same obstacles. Some concerns have been raised that as oncolytic viruses become more attenuated, their ability to achieve sufficient cytotoxic effect to reduce tumor growth may not be reached (48, 49). Because many viral proteins (such as E1A, E1B-55kD, and E4) have more than one function, mutations within a viral gene to attenuate the virus may result in needing an increased dose to obtain a measurable antitumor effect in tumor cells.

In addition to its role in the degradation of p53, the E1B-55kD protein has several other important late-phase functions. For example, E1B-55kD is not only involved in blocking p53 apoptosis, but it is also involved in nucleocapsid export of late transcripts that are needed to maximize viral replication. These functional regions of the E1B-55kD protein appear to overlap. By deleting a portion of the gene to create an attenuated virus, the efficiency of viral replication may also be affected. E1B-deleted adenoviruses have also been shown to be cold sensitive. It is interesting to note that E1B-attenuated viruses replicate as well as wild-type adenovirus at higher temperatures (39°C). It has been shown that the expression of an inducible heat shock protein (HSP) or exposing the cells to heat shock improves the oncolytic efficacy of a replication competent adenovirus (50).

The mechanism by which HSP compensates for E1B-55kD loss is unknown. One way to inactivate p53 binding but not the transport of late viral gene transcripts is to cause a point mutation in the E1B gene region that disrupts E1B binding to p53 but does not affect the late-phase function of E1B. R240A (ONYX-051) and H260A (ONYX-053) were created by a single amino acid point mutation (50). Both mutant adenoviruses showed poor activity in binding to p53. Both viruses partially preserved the late functions of wild-type E1B-55kD. Although the antitumor effect was modest compared to dl1520, the ability to disrupt one viral function while retaining other functions in the same location of the gene show promise to creation of the next generation of oncolytic adenoviruses.

Another method that is used to attenuate oncolytic adenoviruses is a deletion in the E3 region. Proteins encoded by this viral genome region are important in protecting viral replication in the infected cell from cytotoxic T cells and death-inducing cytokines (51). Loss of these proteins could affect how quickly the body will respond to a viral infection thereby preventing an oncolytic adenovirus from achieving its maximal effect (52). Not all of the oncolytic adenoviruses have a deletion in the E3 region (Table 1), and additional clinical trials will be needed to compare antitumor efficacy among adenoviruses that have a full E3 region or a partially deleted E3 region, because an immune response may only be demonstrated in humans.

Because many viral gene functions are derived from a small set of genes, attenuation of viruses should be approached cautiously so that maximum viral replication efficiency can be maintained despite limiting its selectivity. As our understanding of the roles of each gene is elucidated, newer generations of oncolytic adenoviruses will hopefully maximize replication efficiency while maintaining their ability to selectively target tumor cells.

Targeting Viral Infection to Tumor Cells through Alterations in Viral–Host Cell Interactions

The above approaches have relied on the ability of the adenovirus to infect a number of different tissue types and then restrict viral replication once it has entered the cell. There are two main drawbacks to such methods. First, not all of the tumor cells will express the particular receptor that will allow oncolytic adenoviruses to infect the cell (53-55). Another problem is that a nontargeted tissue type (typically the liver) may strongly express the particular receptor for viral binding and serve as a physiologic “sink” by binding a majority of the desired dose of the virus. To overcome these limitations, certain laboratories have been able to alter the structural viral envelope proteins to retarget the virus to tumor cell surfaces and direct the virus away from its normal receptor (Fig. 3).

Two different interactions take place that allow infection of a cell by an adenovirus (6). The attachment of adenoviruses type 2 and 5 to a cell is initiated by interaction of the knob domain of the viral fiber protein with cell surface proteins such as the coxsackie-adenovirus receptor (53, 56). After attachment, the Arg-Gly-Asp (RGD) peptide sequence within the viral pentaenon protein interacts with α5β1 and αvβ3 integrins, which then leads to the internalization of the virus by receptor-mediated endocytosis. Not all of the adenovirus serotypes use coxsackie-adenovirus receptor, and the cellular receptors for these viruses are currently unknown. Tumor cells express varying amounts of viral-binding receptors (57–64), and during oncogenesis, expression of these receptors can be lost (65). Furthermore, oncolytic virotherapy will create selection pressure for survival of tumor cells that do not express viral-binding receptors.

Δ to AdΔ24 was created by incorporating an additional RGD motif (RGD-4C) into the fiber knob (66). This allowed the virus to attach directly to the α5β1 and αvβ3 integrins on the cell surface. This modification allowed improved infection of glioma cells, because >50% of glial cells express coxsackie-adenovirus receptor, whereas >50% express integrins. This virus improved the survival of mice-carrying glioma xenografts. Adv-E1Bdb-F/K20 incorporated lysine residues into the fiber protein to allow binding to heparin sulfate receptors, which are
more widely expressed. Adv-E1BdB-F/K20 also carries a defective E1B gene (67, 68).

To improve Ad5 infection of renal carcinoma cells (low levels of Ad5-binding coxsackie-adenovirus receptor expression and high expressions of Ad3-binding receptors), the fiber protein was switched to contain an Ad3 knob instead of an Ad5 knob. Modification of a virus tropism by borrowing cell binding properties of other serotypes or viral species is known as pseudotyping. This fusion fiber protein was able to augment infectivity of the pseudotyped adenovirus in renal cell carcinoma cells in vitro and in vivo (63).

Another modification is the development of a polymer that coats the adenovirus and allows incorporation of desired targeting ligands. This polymer not only broadens the types of tissue the adenovirus can infect but also helps it resist antibody neutralization (69). This system is useful to augment initial infection by replicative adenoviruses, but viral progeny will not have this polymer coat. Further studies are also needed to evaluate whether altering the viral coat will prevent the liver from absorbing most of the injected dose of adenovirus.

These studies provide evidence that the tropism of replication competent adenoviruses can be altered leading to an enhancement of antitumor activity. However, the improved infective capabilities of these adenoviruses may cause infection of non-neoplastic tissue. Unfortunately, to date there have been no clinical trials to show how these variables will affect the efficacy of an oncolytic adenovirus.

Efficacy, Safety, and Routes of Administration

To date, dl1520, CV706, and CV890 have been evaluated in clinical trials. dl1520 has undergone extensive testing and been found to be relatively safe in multiple modes of delivery, including intratumorally, intraperitoneally, intra-arterially, or intravenously (70). Flu-like symptoms were the most common reported toxicity. The incidence of flu-like symptoms appears to be associated with the mode of delivery being highest among patients receiving intra-arterial or intravenous injections. Patients receiving intratumoral injections have also developed flu-like symptoms, most probably due to a partial injection of virus into a blood vessel within the tumor. The flu-like symptoms are thought to be due to an increase in inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukins (IL-1 and IL-6), and interferon γ (71, 72). Other reported side effects of adenovirus administration are transient elevation of liver transaminases and reversible hyperbilirubinemia (71, 72). There has been no clinical evidence of progressive hepatotoxicity from the administration of oncolytic adenoviruses. This was an initial concern, because 1 patient has died after receiving a hepatic intra-arterial infusion of $4 \times 10^{13}$ particles of a replication-deficient adenovirus for the treatment of ornithine transcarbamylase deficiency (73).

Therapeutic response to dl1520 treatment has been variable depending on the mode of delivery with intratumoral injection of virus resulting in the best antitumor response. Administration of dl1520 via intratumoral injections was performed on patients with advanced head and neck squamous cell carcinoma (74, 75). Patients either received single daily dose ($n = 30$) or fractionated twice daily dosing of dl1520 ($n = 10$). Of patients receiving a single daily dose of dl1520, 14% achieved a partial or complete response versus 10% of the patients receiving fractionated daily doses, which was not significant in this small study. This response correlated with TP53 gene status of the tumor with
58% (7 of 12) of p53 mutant tumors showing regression compared with no response among the p53 wild-type tumors. Furthermore, necrosis was confined to treated tumor tissue with no damage to adjacent normal tissue. Viral spread was documented in tumor tissue 5–14 days after treatment, even in the presence of high neutralizing antibody titers.

Other modes of viral delivery have been less effective compared with intratumoral injections. A clinical trial was performed that examined intra-arterial administration of dl1520 (72) in patients with metastatic gastric carcinoma to the liver. The response to viral therapy was modest with no complete responses and 7 of 28 patients having some measurable decrease in size of the tumor. However, 3 patients who had refractory disease to chemotherapy (5-fluorouracil) had a minor response with administration of dl1520. These patients also had delayed viremia. The decrease in tumor size along with delayed viremia occurred despite having high neutralizing antibody titers. Overall, the addition of dl1520 along with 5-fluorouracil proved to be safe, with 2 patients developing reversible direct hyperbilirubinemia (grade 3/4). During this study, 1 patient suffered from a severe inflammatory reaction after hepatic intra-arterial administration of dl1520. Unique to this patient was the absence of IL-10 and a 20-fold increase in IL-6 (72), and this patient recovered from this reaction. The results of intra-arterial administration of oncolytic adenoviruses have also proven safe in the treatment of cancer.

Another study looked at the feasibility of intravenous administration of oncolytic adenoviruses. A Phase I trial was performed in patients with metastatic carcinoma to the lung (71). dl1520 was infused intravenously in escalating doses of $2 \times 10^{10}$ to $2 \times 10^{13}$ particles. Because there were no dose-limiting toxicities, the intravenous administration of adenoviruses proved feasible. Although there were no documented responses with dl1520 alone, tumor stabilization (6.5+ months) was noted in patients who had failed chemotherapy alone supporting that the combination of oncolytic virotherapy and chemotherapy could be synergistic (see below).

Although the routes of administration of oncolytic adenoviruses have had mixed responses, there have been several positive results from these trials. The treatment is tolerated with acceptable toxicities that have been reversible. Whereas intratumoral treatment alone has appeared to only modestly reduce tumor size, the potential synergy with chemotherapy is very promising. Because not all sites are amenable to intratumoral therapy, intravenous administration would be the ideal solution, because it can reach nonoperable sites and multiple metastatic sites. There is evidence to support that the key to efficient tumor control is dependent on the quantity of virus that reaches the tumor (76). Two limiting steps would be neutralizing antibodies and sequestration of viral particles in organs, such as the liver, not affected by tumor. The response of intra-arterial and intravenous injections appears not to be affected by neutralizing antibodies. What may be affected is the number of viral particles reaching a given tumor. Neutralizing antibodies could potentiate increased clearance by the liver. It is unclear from the above studies whether the toxicities experienced by the patients with i.v. administration were noted with each injection of dl1520. Ongoing clinical studies with Newcastle disease virus show that a process called “desensitization” occurs where toxicities become less severe and less frequent with subsequent treatments thereby allowing higher doses of virus to be injected. One possible reason is that desensitization is related to immune tolerance such as giving allergy shots to a patient. Although there may be release of neutralizing antibodies, the inflammatory response may be modified so that patients are less symptomatic with subsequent doses of the virus. Work using 01/PEME adenovirus showed that intravenous injection was 1000-fold less efficient in the prevention of tumor growth in mouse xenograft models compared with intratumoral injections and that this limitation could be overcome by giving a higher initial dose of adenovirus (76). Being able to give higher doses in longer infusions has improved tumor response with Newcastle disease virus (77). Newer clinical studies are needed to determine whether these recent insights are also applicable to adenoviruses. Other potential studies could combine the polymer coating system (above) with oncolytic adenoviruses that would target the desired tissue, possibly bypassing the liver and neutralizing antibodies and thus improve the initial infection rate of tumor cells. Additional investigation is also needed in attenuating the virus for normal tissue but not affecting the efficiency of viral replication.

**THE ROLE OF ONCOLYTIC ADENOVIRUSES IN COMBINATION CANCER THERAPY**

Current limitations of chemotherapy and radiation therapy are their inability to effectively treat metastatic disease. As with other modalities of treatment, oncolytic virotherapy, by itself, has not been effective in complete tumor eradication in both preclinical animal models and clinical studies. It appears that the best chance for complete tumor eradication lies with combining its mechanism of action with current treatment strategies of chemotherapeutics and radiation therapies and the emerging field of clinical gene therapy. The theory, based on combination chemotherapy, is that attacking tumor cells through different mechanisms of action will prevent tumor cells from having time to develop resistance to treatment. There have been several clinical trials looking at the benefit of adding oncolytic adenoviruses to radiation therapy, chemotherapy, and gene therapy.

**Oncolytic Adenoviruses and Chemotherapy**

Despite using multiagent chemotherapy at maximal tolerated doses along with surgical resection, tumor recurrences are still common. Oncolytic virotherapy has shown promising results in combination with chemotherapy in preclinical models (Table 2). The addition of oncolytic adenoviruses appears to augment tumor kill when added to treatment with chemotherapy (12, 34, 75–78). The order in which chemotherapy and oncolytic adenoviruses are administered may also affect the efficacy of tumor cell death (76, 78). One interesting result suggests that chemotherapy could reduce the amount of viral particles needed to help eradicate the tumor (75).

The addition of oncolytic adenoviruses to chemotherapy has already entered Phase II trials with promising results. One such trial evaluated the use of intratumoral dl1520 injection in combination with cisplatin and 5-fluorouracil therapy in patients with recurrent squamous cell carcinoma of the head and neck. Sixty-three percent of patients (19 of 30) had a measurable...
decrease in tumor size (>50%). Eight of 30 (27%) had complete responses (no measurable disease), whereas 11 of 30 (36%) had partial regression [decrease of 50–100% in tumor area; (78, 79)]. This is an improvement when compared with historical data of a 14% measurable response (>50%) in patients treated with dl1520 alone and 30–40% response for chemotherapy alone (80). The combination therapy was well tolerated and did not lead to an apparent increase in toxicity. Most importantly, it was also shown that viral replication was not inhibited by this chemotherapy regimen (78). In addition, the response did not correlate with initial tumor size, presence of pretreatment neutralizing antibodies, TP53 gene status, or prior treatments. Another Phase II trial looked at the combination of dl1520 with leucovorin and 5-fluorouracil in patients with gastrointestinal carcinoma metastatic to the liver. Although results were modest (11% with complete response and 15% with partial responses), overall safety was established. Despite having an initial increase in tumor size on imaging, 43% of the patients had significant tumor shrinkage months after the initiation of viral therapy and a decrease in serum tumor marker levels. This suggests that inflammation associated with viral infection may mask the cytolytic effect of viral replication and tumor shrinkage (68–73).

In summary, these studies on combination oncolytic adenovirotherapy and chemotherapy have shown greater antitumor efficacy without an increase in toxicity. Although the mechanism of the enhanced effects of combining oncolytic virotherapy and chemotherapy is unknown, several hypotheses exist. One is that the virus may increase the cell killing effects of chemotherapy through its ability to induce p53-dependent and -independent apoptotic pathways. In support of this, combination treatment with CV787 and docetaxel shows increased p53

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* Revised and reprinted with permission from Ref. 1.
expression in LNCaP cells (81). Because most chemotherapeutic agents are immuno-suppressive, this may allow a higher dose of viral particles to reach the tumor when injected systemically (intravenously or intra-arterially) and maintain viral spread intratumorally due to a decrease in the production of neutralizing antibodies. Additional clinical trials will be needed to study the timing of when to administer oncolytic adenoviruses within a chemotherapeutic regimen to achieve the best antitumor response.

**Oncolytic Adenoviruses and Radiation Therapy**

Oncolytic virotherapy kills tumor cells by a mechanism different from radiation therapy. Radiation therapy-induced tumor cell death is dependent on directly damaging DNA or indirectly through the formation of oxygen radicals that disrupt cellular pathways. This dual mechanism is different from how oncolytic viruses destroy tumor cells. This has led to several preclinical trials looking at the role of combining these two modalities (Table 2). Intra-arterial injections of dl1520 in conjunction with total body irradiation (5 Gy) were evaluated in subcutaneous human malignant glioma xenograft models that were either p53 mutant or p53 wild-type (82). Significant tumor growth delays were noted in the p53 mutant tumors receiving combination therapy versus monotherapies (30 days versus 10–15 days). There was also an increase in the number of partial and complete responses in the combined treatment group compared with animals treated with radiation or dl1520 alone. Enhanced antitumor action was also shown with combination dl1520 and radiation therapy in xenograft models of human colon cancer in the same study. Both tumor-free survival and average tumor growth delay were higher in the combined treatment group compared with rodents receiving radiation or dl1520 monotherapies (13). Enhanced antitumor efficacy was also shown in combination studies involving Ad5-Δ24RGD, CV706, and AdFGR (83–85). Of note, 10⁶ plaque-forming units of Ad5-Δ24RGD in conjunction with radiation therapy achieved similar antitumor effects as 10⁷ plaque-forming units of Ad5-Δ24RGD alone, suggesting that reduced viral treatment doses can be used in combination therapy and still attain a comparable therapeutic effect than oncolytic adenovirus alone.

In summary, these preclinical studies in combining oncolytic adenoviruses with radiation therapy provided several important findings. Radiation did not appear to impair viral replication (13, 82, 86, 87). However, this will need to be additionally studied when fractionated doses of radiation are given rather than a single dose. There also appears to be no additive toxicities noted in the virus plus radiation-treated groups compared with the monotherapy groups (13, 87). Furthermore, these preclinical studies point to enhanced antitumor effects when compared with monotherapy. Another interesting finding is, like in combination with chemotherapy, the order in which oncolytic adenoviral therapy and radiation therapy are administered may be important. Two of the studies showed (12, 83) that radiation could potentiate the cytolytic effect of the adenoviruses. More studies will be needed to evaluate whether this finding is more universal or is restricted to particular experimental conditions, cell lines, or oncolytic adenoviruses. These preclinical studies support moving forward the incorporation of oncolytic adenoviruses into radiation treatment into clinical trials.

**Oncolytic Adenoviruses and Gene Therapy**

Many oncolytic adenoviruses have the cloning capacity for a small transgene. Addition of an adjuvant therapy transgene could augment killing the infected tumor cells as well as non-infected tumor cells nearby, a property referred to as the bystander effect (Table 2). One group of therapeutic genes that have received much attention is the prodrug-activating genes, also known as suicide genes. In this system, tumor cells are transduced with a gene that codes for an enzyme that converts a prodrug into a toxic agent (83). The most studied prodrug-activating gene system involves the prodrug gancyclovir and the HSV thymidine kinase (HSV-tk) gene. HSV-tk monophosphorylates gancyclovir, which is then converted into gancyclovir triphosphate by other cellular kinases. This toxic metabolite inhibits both viral and cellular DNA synthesis and can spread to nontransduced tumor cells via gap junctions, thereby enhancing the killing effect beyond the infected cell (bystander effect). The antitumor effect of incorporating this system into adenoviruses (IG,AdSE1,E3TK and Ad.TKREC) has been mixed, with some studies showing increased antitumor effect when compared with virus alone (84, 88, 89), whereas others have noted or other side effects (90–92). The mixed findings could be due to gancyclovir inhibiting viral replication. Another reason for the variable effect could be that the number of gap junctions vary among different tumor subtypes (93) leading to a weaker bystander effect. The intracellular accumulation of activated gancyclovir due to lack of spread through the gap junctions would then lead to inhibition of viral replication dominating over viral-mediated oncolysis.

Another replication competent adenovirus, AdFGR (also known as Ad5-CD/TKrep), uses a double-suicide transgene approach. This virus is E1B-55KD null and contains a cytosine deaminase-tk fusion gene (94). Cytosine deaminase converts 5-fluorocytosine into 5-fluorouracil, which has antitumor activity. The antitumor effect of this virus is enhanced when used in combination with gancyclovir (intravenously) and 5-fluorouracil (orally) in vitro and in vivo compared with virus alone or in combination with a single prodrug (94, 95). Furthermore, the addition of radiation therapy to gancyclovir, 5-fluorouracil, and vireotherapy led to greater tumor growth delay and an 80% (4 of 5) complete response that was superior to other treatment strategies (95). The double prodrug system of AdFGR has entered Phase I clinical trials in patients being treated for recurrent prostate cancer. The adenovirus was administered intraprostatically followed by administration of 5-fluorouracil and gancyclovir 2 days later (96). There was no dose-limiting step, because it was found safe at all of the levels, and no maximum tolerated dose was reached. Therapeutic response was documented with a reduction in prostate-specific antigen and histologic evidence. AdFGR has also been studied in a Phase I trial in conjunction with radiation therapy in newly diagnosed intermediate- and high-risk prostate cancer patients (85). Transgene expression was observed 3 weeks after injection. The mean prostate-specific antigen half-life was significantly lower in patients receiving >1 week of prodrug therapy compared with radiation therapy alone (0.6 versus 2.4 months). Although prom-
ising, additional investigation is needed as to how both 5-fluorouracil and activated gancyclovir will affect oncolytic viral replication, because timing and dosing of each agent is still being investigated. Another produg converting enzyme that looks promising for clinical trials is carboxylesterase. This enzyme converts the chemotherapeutic agent irinotecan into a more potent chemotherapeutic agent, SN-38 (97). A recent study looked at dl1520 expressing the transgene for carboxylesterase. This produg converting enzyme strategy has antitumor effects in both in vitro and in vivo studies.

The incorporation of cytokine genes has also been investigated. Ad.DF3E1 is modified to constitutively express TNF-α using the cytomegalovirus promoter (Ad.DF3E1/cytomegalovirus-TNF; Ref. 31). TNF-α is an inflammatory cytokine that has antitumor effects. Viral replication was not affected by expression of TNF-α. Furthermore, treatment with Ad.DF3-E1/ cytomegalovirus-TNF had a prolonged tumor regression, whereas Ad.DF3E1 (lacks TNF-α transgene) or adenovirus cytomegalovirus-TNF (replication deficient) led only to suppression of tumor growth. Besides TNF, the human interferon consensus gene has also been incorporated into an oncolytic adenovirus (Ad5/interferon). This also appears to potentiate the oncolytic effect of the adenovirus (98). However, the antiviral activity of interferon was not examined in this study.

Another area of cancer research is the use of tumor vaccines. Although not capable of stimulating a robust immune response, tumor cells do have antigenic peptides that are immunogenic and could potentially induce tumor-specific immunity. One method to overcome this lack of immunogenicity is the use of HSPs. HSPs have a variety of functions. One main function is to act as chaperone proteins that aid in the folding and translocation of cellular peptides (99). The HSP70 family also helps in the presentation and processing of antigen to CD4+ T cells by antigen-presenting cells. It is thought that because HSPs act as chaperone proteins, overexpressing HSPs within tumor cells may enhance the immunogenicity of normally weak tumor antigens by presenting the HSP-bound tumor peptides to antigen-presenting cells via the major histocompatibility complex class I pathway. Initial vaccines were not practical, because the vaccine needed to be prepared ex vivo with processed tumor cells. As an alternative approach, the gene for HSP70 was incorporated into Ad-E, a E1B-55kD deficient virus, to make Ad-HE (100). This virus effectively impeded the growth of the viral-infected tumor as well as in the contralateral untreated tumor. Ad-HE was also able to prevent tumor growth after rechallenging the rodents with a second injection of tumor cells thereby demonstrating a vaccination effect.

Many malignant neoplasms have lost the function of p53. Although many oncolytic adenoviruses use p53-dependent pathways to cause cell death, several studies have shown that replicating adenoviruses kill cells more rapidly when expressing p53 (19, 101). A conditionally replicative adenovirus, AdΔ24–53, was created to express p53 in p53-deficient tumor cells during viral replication (102). The expression of p53 enhanced the killing potential of these oncolytic adenoviruses. The ability to express p53 in otherwise deficient cells will need to be studied in the broader context of multimodal therapy, because it could also improve the killing efficiency of chemotherapy and radiation therapy.

These studies have demonstrated various ways in augmenting the antitumor effect of oncolytic adenoviruses via the incorporation of transgenes that encode proteins with antitumor activity. Larger studies will be needed to see if these initial results are applicable to a broad range of tumors. The preclinical antitumor activity of cancer therapy Ads carrying immune modulator transgenes are currently performed in immunocompromised rodents using xenografted human tumor cells. Therefore the full potential of these Ads will not be evident without clinical studies.

CONCLUSIONS AND FUTURE DIRECTIONS

With the overall safety oncolytic adenoviruses have shown in preclinical and clinical trials and studies suggesting that combining these viruses with conventional therapies has an enhanced antitumor effect, oncolytic virotherapy has proven to be a viable treatment modality to specifically kill tumor cells. Significant advances have been made in targeting viral infection and replication to tumor cells. These have relied on our improved understanding of the mechanisms of cancer formation and viral replication. Continuous progress in elucidating these mechanisms will be additionally exploited by oncolytic virotherapy. Preclinical trials have highlighted some strengths and limitations of oncolytic adenovirus treatment. More clinical trials are needed to bridge the gap in preclinical testing and antitumor response in humans, because there is no good animal model to simulate the response of a human to an oncolytic adenovirus infection. Newer imaging techniques like positron emission test imaging may help to better understand the dynamics of viral replication in tumors and within the human body without relying on pathological specimens. Finding reporter probes that can be detected by positron emission test scanners will aid in monitoring the spread of oncolytic adenoviruses in vivo. Additional investigation is also needed to establish where in the scheme of treatment oncolytic viruses will have the most benefit. Additional work is needed to improve our ability to deliver adenoviruses intravenously to improve its ability to treat metastatic disease without being sequestered in the liver or subclinical disease that is not detected by current imaging techniques. If clinical studies show significant improvements in survival, the doses of chemotherapy and radiation could be reduced to spare the potential late effects (secondary malignancies, organ toxicities, and neuro-cognitive deficits), which is particularly important in the pediatric oncology setting as more children survive. Other studies are needed to combine different oncolytic viruses to harness the benefits of different antitumor mechanisms and determine what effects they will have on the tumor. The field of oncolytic virotherapy has now matured beyond the initial euphoria. Valuable lessons have been learned that will re-ignite enthusiasm in the further development and clinical testing of second and third generations of these agents. As with other anticancer agents, it is clear that the inclusion of oncolytic adenoviruses into multimodal cancer treatment with chemotherapy, radiation therapy, and gene therapy will be most effective in improving the overall survival of cancer patients.

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