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RECOMBINANT ADENOVIRUS VECTORS FOR GENE THERAPY AND CLINICAL TRIALS

(A REVIEW)*

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In the last decade adenovirus (AdV) vectors have emerged as promising technology in gene therapy. They have been used for genetic modification of a variety of somatic cells *in vitro* and *in vivo*. They have been widely used as gene delivery vectors in experiments both with curative and preventive purposes. AdV vectors have been used in the experimental and in some extent in the clinical gene therapy of a variety of cancers. The combination of recombinant AdV technology with chemotherapy (pro drug system) seems to be promising, too. AdV vectors offer several advantages over other vectors. Replication defective vectors can be produced in very high titers (10^{11} pfu/ml) thus allowing a substantially greater efficiency of direct gene transfer; they have the capacity to infect both replicating and nonreplicating (quiescent) cells from a variety of tissues and species. Several important limitations of adenovirus mediated gene transfer are also known, such as the relatively short-term (transient) expression of foreign genes, induction of the host humoral and cellular immune response to viral proteins and viral infected cells, which may substantially inhibit the effect of repeated treatment with AdV vectors, the limited cloning capacity and the lack of target cell specificity. However, the well-understood structure, molecular biology and host cell interactions of AdV-s offer some potential solutions to these limitations.

Keywords: adenovirus, adenovirus genome, recombinant adenovirus vectors, clinical trials

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Characteristics of adenoviruses essential for vector construction

Adenoviruses have a linear double stranded DNA genome of about 35 kbp. The DNA is packaged in an icosahedral protein capsid, which is about 70–90 nm in diameter. The capsid is composed of 252 capsomers, 240 of which are hexons and 12 of which are pentons located at the 12 vertices of the capsid. A rod-like structure called fiber protrudes from each penton base [1]. Each DNA strand has inverted terminal repeats (ITR) of 100–140 bp necessary for viral replication. Their length is dependent on serotype. The genome is divided in early (E) and late (L) regions expressed before or after replication of the viral DNA, respectively (Fig. 1). There are six early regions: E1A, E1B, E2A, E2B, E3 and E4 that are transcribed from individual promoters (except E2). The two transcription units of E1 region (E1A and E1B) are the first part of the genome to be expressed following virus entry into a cell via endocytosis. In the first step of the entry the fiber binds to a specific receptor, then the penton base protein binds to integrins on the cell surface. This latter step promotes the internalization of the virion. Both E1A and E1B proteins are involved in the control of viral gene transcription. E1A proteins are required for virus replication, while the E1B proteins increase and decrease host mRNA transports and prevent apoptosis induced by E1A. It is worth mentioning, however, that the oncogenic AdV type 18 has been isolated from a urological tumor [3]. The presence of antibody to the early non-virion antigens of oncogenic AdV type 12 has been confirmed in more than 50% of the patients with urological tumors, in contrast to the 18% incidence in other urological patients with no tumors, and 4% incidence in patients suffering in some internal disease. Antibodies against the complete virions have been found in 15% and 2%, respectively in the same groups. These data seem to suggest that the genes of the early AdV proteins should be present and operate (expressing the early /onco/ proteins) in fairly high proportion of patients with tumors of urogenital tract, since induction of the non-virion antigens and antibodies against them could not be possible without their effect [4].

The genes of the E2A region encode the DNA binding protein and the E2B encode the DNA polymerase and the terminal protein. All of these proteins are directly implicated in the viral replication. The E3 region encodes proteins that interfere with the host immune response against virus infection. This region is not required for AdV replication [5]. Proteins encoded by the E4 region play a role in viral RNA transport and stability, viral DNA accumulation and transcriptional regulation of E2 and E4 [6].

Fig. 1. Transcription map of the adenovirus genome showing the major transcription units. The thick horizontal line represents the 0–100 map units (mu) of the linear double stranded DNA. The genome is divided in six early (E) and five late (L) regions, respectively, expressed before of after the replication of the viral chromosome

Adenovirus vectorology

Since the early description of the development of AdV vectors for the expression of heterologous genes by Berkner in 1986 [7] several hundreds or even more different adenovirus vectors have been constructed to date and tens of thousands of scientific articles have been published about them. AdVs are now being increasingly investigated as potential mammalian expression vectors for gene therapy and for recombinant vaccines. Modifications of the vector backbone, the introduction of helper-dependent AdV vectors, the generation of hybrid AdV vectors, the targeted gene delivery by tropism-modified adenoviral vectors, the combination of these vectors led to the development of "Adenovirus vectorology" as a distinct research field of gene delivery techniques.

FIRST-GENERATION ADENOVIRUS VECTORS

Foreign genes could be inserted into the AdV genome as replacements for the E1 and E3 regions. These vectors are referred to as *first-generation vectors*. It may be advantageous to retain some E3 sequences to ensure adequate levels of E3 expression [8]. The E1-deleted vectors are replication-defective and can be propagated in 293 cells, which is a human embryonic kidney cell line containing an integrated copy of AdV E1 region and supplying the E1A gene products *in trans* [9]. The doubly deleted E1–E3 AdV vectors have a cloning capacity of 8.3 kbp [2]. Figure 2 shows a typical strategy to construct E1- and E3 AdV type 5 recombinant vector [5] by *in vivo* homologous recombination. Efficient procedures were developed to select recombinant viruses [10].

Fig. 2. Construction of an E1 replication defective adenovirus vector by homologous recombination. The transgene ( ) is inserted in a plasmid (------) between the 0–1.26 mu left end of the AdV containing the LTR and packaging sequences ( ) and an AdV genome fragment from 9.4 to 17 mu ( ) to permit *in vivo* homologous recombination. AdV vectors are generated after cotransfection of the ClaI-linearized chimerical plasmid and the ClaI-restricted AdV genome ( ) into 293 cells. (delE1:E1-deletion, delE3: E3 deletion,: *in vivo* recombination) [5]

First-generation AdV vectors have been used successfully for many short-term applications for genetic modification of a variety of somatic cells (lung, liver, muscle, blood vessels, brain) *in vitro* and *in vivo* [11]. In animal models short-term expression is sufficient for AdV vector based vaccines to protect against numerous pathogens such as Hepatitis B, HSV, HCMV, VSV, HIV, Parainfluenza virus type 3, Rotavirus,

Coronavirus, Malaria, Leishmania [2, 5, 12]. For the construction of recombinant vectors AdV types 2, 4, 5 and 7 have been used in these experiments.

Extensive studies were carried out concerning of cystic fibrosis [15] and of cancer research demonstrating the efficacy of short-term AdV mediated gene therapy. In the field of immunotherapy tumor antigen or interleukin genes were carried by the vectors [13, 14]. The molecular therapy of cancers means the use of tumor suppressor genes in the construction of AdV vector for the restoration of their functions [16, 17, 18]. The "pro drug" system means the combination of AdV vector technology with chemotherapy. In these cases a suicide gene, most often Herpes simplex virus thymidine kinase is induced into the tumor cells by the AdV vector, which makes sensitive the tumor, cells to ganciclovir [19, 20, 21, 22]. The efficacy of short-term AdV-vector expression was demonstrated also by blocking proliferation and by preventing the growth of new vessels to the tumor [23, 24].

Nevertheless there are at least four limitations outlined in the Introduction associated with the use of the first generation AdV-vectors. To overcome all the problems and obstacles it was necessary to construct other different types of AdV vectors and in addition to modulate the host immune response.

Second-generation vectors and the circumvention of the immune response

The most serious obstacles in the use of AdV vectors for long-term gene therapy is the induction of the host immune response and the relatively short transgene expression. The humoral and cellular immune response to viral proteins and viral infected cells substantially limits the level of gene transfer and eliminates AdV-infected cells especially in case of repeated treatment with the same AdV vector. In patients exposed to AdVs the pre-existing immunity to AdV-vectors could block even the effect of the first treatment, too [2, 25, 26]. Further crippling of the vector backbone was thought to be useful in the improvement of first generation vectors to decrease the host immune response and to increase the duration of transgene expression. Another risk of the first generation vectors also became known, namely that replication competent adenoviruses (RCA) may emerge by homologous recombination between identical sequences in the 293 chromosome and the viral genome, that may progressively outgrow the recombinant virus during propagation. To solve this latter problem novel A549-derived and other complementation cell lines was developed as well [34, 35]. For construction new AdV vectors the inactivation/deletion of E2A, E2B and E4 regions have been targeted in addition of deletion of the E1 region [36]. Generally these vectors

are referred too as *second-generation vectors*. Ablation of E2A or E2B in AdV vectors improved transgene persistence and decreased inflammatory response in mouse liver [37]. E4 region is a complex regulatory unit that encodes 7 polypeptides, subvert endogenous gene expression at different levels (transcriptional and post-transcriptional). E4 ORF3 and E4 ORF6 proteins especially show redundant activity. Several groups have studied the approach of crippling viral gene expression by deletion of the E4 region [2, 35, 36, 38]. Different vector systems have been developed to decrease or block the viral gene expression. With the deletion most of the E1 and E4 regions it is possible – and it proved to be advantageous – to retain some part of the E4 for the *in vitro* high level expression of transgene and for reduction of the level of viral proteins. The *in vivo* obtained results, however, are conflicting concerning the effect of E4 deletions on the persistence of transgene expression and on the immune response, therefore further studies are needed [2, 39, 40]. Another way to block the immune response could be the transient immunosuppression of the host with immunosuppressants concurrent with AdV vector injection, which permits vector readministration, and may increase the transgene expression [27, 28]. Agents that target specific cell types or interactions necessary for immune activation have been also studied. Inactivation of CD4 cells was shown to increase the duration of transgene expression. Cellular and humoral anti-AdV immune responses can be blocked by inhibition of the interaction between T cells and antigen-presenting cells. Antibody to CD40 ligands inhibits both types of immune responses and facilitates repeated administration of AdV vectors. Transient immunomodulation with anti-CD40 ligand antibody and CTLA4 Ig with concurrent AdV vector administration enhanced persistence and secondary gene transfer into mouse liver [2]. Circumvention of anti-AdV humoral immune defenses against repeated AdV vector administration by changing the adenovirus serotype ("sero-switch") was also shown to be useful [29, 30]. However, the AdV types used sequentially should be well chosen because of the many intertype specific (IT) epitopes found in different numbers and combinations on the different AdV serotypes. The panels of monoclonal antibodies raised against 3 different AdV types recognized 18 different bi- and multilateral IT specific epitopes in addition to the genus and type specific epitopes on the 21 hexon types examined. Immune response of the host could be induced not only by the genus and the type specific epitopes, but also by the several IT specific ones. For the elimination of harmful immune response 31 AdV type pairs as well as small groups of types were determined showing the loosest antigenic relationship to each other. These pairs suggested to chose for preparation of second or multiple recombinant vectors. For instance, the most frequently and worldwide used vector for the experimental gene therapy is the HAdV type 5. For the second or multiple administration AdV vectors

could be used prepared from (i) bovine AdV type 3 which has no at all identical IT specific epitopes with type 5, although the number of the different epitopes on the two types is 15, (ii) HAdV type 35 which has 5 identical epitopes, but the two types contain 13 different epitopes, (iii) bovine AdV type 2 having 5 identical epitopes, and the two types contain 11 different epitopes, (iiii) HadV type 12 having 6 identical epitopes with type 5 and the two types contain 11 different epitopes on their hexons [31, 32, 33].

Third-generation vectors helper-dependent ("gutless") vectors

Several groups reported the construction of AdV vectors with all or nearly all of the transcription units removed from the viral backbone except the ITRs and the packaging signal. Such vectors could be referred as *third-generation vectors*. In all these cases the viral proteins required for replication should be provided *in trans* by using a replicative transcomplementation approach [2, 36, 41]. For example, the LTU can be provided *in trans* within a helper adenovirus. This strategy extends the cloning capacity of the vector up to 37 kb and decreases the immune response to the minimal level possible. As these vectors are propagated in the presence of a helper virus (*helper-dependent vectors*) extensive purification is required to reduce the amount of contaminating helper virus of the production batch. Because of its smaller density the mini-adenovirus i.e. the helper-dependent adenovirus (HDAdV) vector can be separated from the *wt* or first generation helper AdV by CsCl density gradient centrifugation. Construction of a helper-dependent mini-adenovirus is shown in Fig. 3 [36, 41]. The transcomplementation of the LTU is also possible *in cis* before its removal from the backbone of an AdV vector during viral amplification. Specific excision of large adenoviral helper sequences has been achieved with the loxP specific Cre recombinase from bacteriophage P1 resulting in a minivirus genome of about 9 kb that retained in addition to the transgene, a single loxP site and the E4 region of AdV genome [2, 36, 42]. Construction of a helper-dependent AdV vector directly from a first-generation vector is shown in Fig. 4. An alternative and more effective Cre-mediated system has also been developed in which the packaging element was flanked by loxP sites in a first-generation helper virus. Transfection of 293 Cre cells with HDAdV vector followed by infection with the helper virus resulted in selective excision of the packaging element from the helper virus and preferential packaging of the HDAdV vector [2, 43, 44]. After density gradient centrifugation the helper virus contamination decreased to as low as 0.01 % and *in vivo* administration of the vector

resulted in high expression levels for nearly a year. However, the maintenance of stability and the prolonged expression need appropriate structural design and production methodology to be implemented [2, 45, 46].

Fig. 3. Construction of helper-dependent "mini-adenovirus" with "mini-plasmid" carrying minimal AdV genome including ITRs, packaging signal and E4. The plasmid generates the mini-adenovirus in 293 cells with E4 defective helper AdV. The presence of E4 in the mini AdV and its absence in the helper virus ensure its propagation [36, 41]

Hybrid vectors, vector chimeras

Because of the relatively short-term expression of foreign genes associated with AdV vectors due to their non-integrating DNA, chimerical vector systems have been developed that combines the high efficiency *in vivo* gene delivery characteristics of AdV vectors with integrative capacities derived from retroviruses or from Adeno Associated Viruses (AAV) [47, 48]. In AdV-Retrovirus system this was accomplished by rendering AdV vector-infected target cells into retroviral producer cells by AdV-mediated delivery of retroviral packaging functions with one vector. By this way the locally elaborated retroviral vectors can infect neighboring parenchyma cells via an integrative vector as shown in Fig. 5 [47].

Fig. 4. Construction of helper-dependent AdV vector from a first-generation vector. A first-generation AdV vector was constructed with a transgene expression cassette ( ) upstream of a loxP Cre recombinase site (black arrowhead) in place of E1, and a second loxP site in place of E3. Amplification of this virus in Cre expressing derivate of 293 cells resulted in the excision of all viral sequences between E1 and E3 and generated the mini HDAdV vector shown, which was replicated and packaged by transcomplementation directly from the first-generation vector [2, 36]

In AdV–AAV hybrid – one of the first hybrid vector – a first-generation AdV vector contained *lacZ* flanked by AAV ITRs in the E1 region and when a plasmid carrying AAV *rep* gene was conjugated to the first hybrid in infected cells efficient *lacZ* expression was shown. The AAV *rep* gene is necessary for AAV replication and rescues from the AdV genome [49]. In an other AdV–AAV system, site-specific transgene integration (in human chromosome 19) was observed in cells coinfected with one HDAdV vector encoding AAV *rep* gene, and another HDAdV vector encoding a transgene flanked by AAV ITRs [48, 50].

Fig. 5. Local generation of retroviral vectors at target organ site by AdV-retrovirus hybrid vectors. AdV vectors encoding retroviral vector and packaging functions accomplished *in vivo* gene transfer to target cells, rendering them retroviral producer cells. The locally produced retrovirus virions can thus directly infect neighboring cells [47]

Genetic engineering of the adenovirus capsid targeted vectors

There are two AdV coat proteins, which interact with distinct cellular receptors during the infection process. The fiber protein mediates the viral attachment to a cellular receptor. Following the attachment to cells, the penton base binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors mediating AdV internalization into the host cells by receptor-

mediated endocytosis. The widespread distribution of the cellular receptor for the fiber protein however precludes the targeting of specific cell types, because non-specific transduction of untargeted tissues significantly reduces the efficiency of gene delivery to the target tissue and may allow unwanted side effects [51, 52]. To overcome this limitation alterations in the interaction between AdV and its cellular receptors have been explored. Both AdV proteins involved in receptor binding have been modified either genetically or immunologically to alter target cell specificity [2, 36].

The specific receptor recognition domain of the fiber protein is in the knob portion. Modification of this region may alter the original tropism of the virus. Method has been developed to introduce physiologic ligands with a linker into the structure of AdV fiber protein to redirect the tropism of the AdV virion in targeted manner [51, 53]. The antiknob antibody blocks the normal binding of the knob to its cellular receptor. Furthermore if such an antibody was chemically conjugated to a ligand recognizing a specific cell surface receptor it should also redirect the AdV vector to a particular cell type (Fig. 6). This has been proved by experiments with folate receptors, which are over expressed on the surface of several malignant cell lines (ovarian, lung and breast cancers). Folate conjugated Fab fragment of an antiknob monoclonal antibody complexed with an AdV vector is able to redirect AdV infection to target cells [54, 55]. Coupling of Fab fragment to fibroblast growth factor also successfully redirect AdV vector to cells expressing ligand receptor [56]. The genetically modified fiber at the carboxyl terminus or H1 loop can also alter the tropism of AdV vectors [51, 57].

Cells lacking the α_v integrins on their surface (nasal epithelial cells, muscle cells, monocytes, lymphocytes) are mainly resistant to AdV infections. If the wild type AdV integrin specific RGD sequences are changed to target cell specific sequences the modified proteins of penton base recognize the target cells′ integrins and promote their infectability [58, 59]. In order to achieve AdV infection of cells without the fiber receptor (endothelial and smooth muscle cells) an AdV vector has been constructed (Fig. 7) in which the recognition sequence – the FLAG peptide epitope, DYKDDDDK – for a specific antibody is incorporated into the receptor-binding domain in the penton base. Complexing of this AdV vector with a bispecific antibody, comprising an antibody to the FLAG recognition sequence and an antibody to α_v integrins resulted in the targeted infection of cells lacking the AdV fiber receptor [54, 58, 59, 60, 61].

Fig. 6. Redirection of the tropism of adenovirus from its fiber receptor to the specific receptor of the target cell. This is possible with the introduction of a ligand with a linker into the fiber knob (upper left part), which specifically links to the surface receptor of the target cell. An antiknob antibody which blocks the binding of the virus to the fiber receptor and a ligand conjugated to the antibody recognizing the target cell receptor also redirect the adenovirus vector to a particular cell type (lower right part)

Adenovirus vectors in clinical trials

In the last twenty years genes of many disorders have been identified and diagnostic tests were developed. Hopes for gene transfer led to nearly four hundred gene-therapy clinical trials (for cystic fibrosis, immune deficiencies, cardiovascular diseases, cancer treatment, etc.). Preclinical *in vitro* and *in vivo* studies led to improvement of the AdV vectors used for transfer, but generally the effects obtained did not yet meet expectations. In the majority of the cases, either the gene correction was not enough to

reverse the clinical abnormalities, or it was limited by rapid extinction of transgene expression [62, 63].

Fig. 7. Targeting adenovirus with bispecific antibodies to α_v integrins of host cells. Monoclonal antibody directed against α_v integrins is linked to another monoclonal antibody directed against the FLAG peptide epitope. Bispecific antibody is then added to AdV vector containing the FLAG epitopes incorporated into the penton base [59]

Lately, these trials have been extended to the treatment of AIDS and to cardiovascular diseases. Preclinical studies are in progress to evaluate the possibility of increasing the number of clinical trials for cardiopathies, and of beginning new gene therapy programs for autoimmune diseases, neurological illnesses, allergies, regeneration of tissues, to implement procedures of allogenic tissues or cell transplantation, and for the development of innovative vaccine design known as genetic immunization [63]. In the following part of this paper the use and exploitation of different recombinant AdV vectors in clinical trials conducted worldwide for the treatment of cancer, monogenic disorders and other diseases are shown on the base of several selected papers without any demand of completeness.

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). Mutations in the CFTR gene cause a loss of function of CFTR chloride channel and as a consequence the CF as a disease in electrolyte transport will be developed. There are a variety of clinical manifestations of CF, but the lung disease is the major cause of morbidity and despite of current standard therapy the median survival rate is below 30 years. CF was subsequently considered to be a candidate for gene therapy when the gene responsible for the disease was isolated and the research focused on the *in vivo*

approaches for gene transfer that could be delivered into the airway via aerosols. Soon after one of the first demonstration of complementation of CF cells using recombinant AdV vectors [64] clinical trials have been initiated [15, 65, 66, 67, 68] using a variety of protocols based on nasal and/or lung-directed gene transfer. Experiments on the development of recombinant gene constructions, gene delivery by AdV vectors into epithelial lung cells, gene expression and on the safety of gene therapy procedures were relatively successful. Clinical trials of CF, however, showed that some unaccounted physiological peculiarities of lung tissue of the patients as diminished the effectiveness of the gene transfer, longevity of CFTR gene expression and in some cases unexpected immunological complications arise during clinical trials. For the time being an intensive attempt to overcome these problems in gene therapy of CF should be undertaken [69, 70, 71, 72, 73]. Nevertheless at present it is conceivable to think that gene therapy represents a way to treat or even prevent the respiratory manifestation of CF.

In the recent years the idea of using gene therapy as a modality in treatment of diseases other then genetically inherited monogenic disorders has taken root. This is particularly obvious in the field of *oncology*. The vast majority of current gene therapy trials are for treatment of *cancer patients*, due to the recognition of gene alterations in cancer and the critical need for improvement of cancer therapy. Clinical trials include strategies that involve immunotherapy, induction of drug sensitivity in tumor cells or resistance to chemotherapy of critical host tissues and compensation for tumor suppressor loss or ablation of oncogenes. AdV vectors have been targeted to tumor-specific and tissue-specific antigens, such as epithelial growth factor receptor, c-kit receptor, and folate receptor. Targeted gene expression has been analyzed using tissue-specific promoters such as breast-, prostate- and melanoma-specific promoters, and disease-specific promoters such as carcinoembryonic antigen, HER-2/neu, Myc-Max response elements. Expression could be regulated externally by the use of radiation-induced promoters or tetracycline-responsive elements. Gene expression could be targeted at specific conditions, such as glucose deprivation and hypoxia [74, 75]. Another paradigm involves dendritic cells, potent antigen-presenting cells that play a critical role in the initiation of antitumor immune responses [76]. Using AdV vector transferring a gene encoding a specific tumor antigen this approach could be relevant in treating micrometastases present at the time of primary detection of many malignances.

Brain tumors theoretically could be treated by targeting their fundamental molecular defects. During the malignant progression of astrocytic tumors several tumor suppressor genes are inactivated, and numerous growth factors and oncogenes are over expressed progressively. The p16/Rb/E2F pathway is the frequent target of genetic alterations in gliomas. Herpes simplex virus thymidine kinase (HSV tk) gene therapy

using AdV/tk vector combined with ganciclovir (GCV) medication seems to be a potential method for the treatment of malignant glioma, glioblastoma multiforme, gliosarcoma, anaplastic astrocytoma as it was shown in clinical trials involved 7 and 13 patients, respectively, with advanced recurrent brain tumors. From the results it is clear that gene therapy strategies for brain tumors are promising but more critical research is required, mainly in the field of vectors, to effective amelioration of patients with brain tumors [21, 22, 77].

Head and neck cancer patients are excellent candidates for gene therapy that offers new approach for local control and the possibility to enhance other treatment modalities as well. Tumor suppressor genes, suicide genes and genes whose products enhance immunocompetence can be delivered by AdV vectors. In a clinical trial involving 15 resectable but historically noncurable patients the results support the use of AdV-p53 gene transfer as a surgical adjuvant in patients with squamous cell carcinoma of the head and neck (SCCHN). Although the patient population with advanced or recurrent incurable SCCHN is the standard choice for establishing the safety of novel therapies the greatest chance of eventual success with currently available gene therapy strategies will most likely be in the patients with less advanced stages of the disease. Gene therapy in conjunction with the development of molecular diagnostics may provide the means for preventing the malignant progression of premalignant head and neck lesions upon early diagnosis [78, 79, 80]. A novel intervention strategy for SCCHN (and other tumors lacking p53) could be the use of AdV ONYX-015, which is an adenovirus with E1B 55 kDa gene deleted, engineered to selectively replicate in and to lyse p53-deficient cancer cells while sparing normal cells. In a phase II trial of a combination of intratumoral ONYX-015 injection (Adenovirus therapy) with cisplatin and 5-fluorouracil in patients with SCCHN by six months none of the responding tumors had progressed, whereas all non-injected tumors treated with chemotherapy alone had progressed [81].

Metastatic breast cancer and melanoma patients have been involved in a phase I trial of direct injection of an E1 and E3 deleted AdV vector encoding interleukin-2 into subcutaneous deposits of melanoma or breast cancer. 23 patients were injected at seven dose levels. The trial confirmed the safety of use of AdV vectors and demonstrated successful transgene expression even in the face of pre-existing immunity to AdV [14]. A HSV tk expressing AdV vector in combination with escalating doses of ganciclovir have been also used in a phase I study in patients with cutaneous metastatic malignant melanoma [82].

Non-small-cell lung cancer (NSCLC) is linked with p53 mutations. The p53 protein is a transcription factor that triggers cell cycle arrest and apoptosis in response to certain cellular stress and DNA damage. About 50 per cent of human tumors carry

p53 mutation; therefore attempts at p53 replacement are logical approaches to therapy in these diseases. Clinical trials of 15 and 28 NSCLC patients showed that the use of intratumoral injection of wild-type p53 complementary DNA (AdV–p53) is safe, well tolerated, feasible and biologically effective, results in transgene expression and seems to mediate antitumor activity [83, 84]. Another phase I and phase II trial demonstrated that P21 gene expression appears to be an indicator for the activity of AdV-p53 gene therapy [85]. Complementation of the gene therapy with simultaneous cisplatin/vinorebline treatment was also examined in phase I and II trial studies including 21 patients with advanced NSCLC [86]. Cellular and humoral immune responses induced by AdV vectors were also studied in lung cancer patients [87, 88].

Malignant mesothelioma (MM) is a fatal neoplasm refractory to all forms of standard anticancer therapy. In a phase I dose-escalation clinical trial including 21 MM patients AdV mediated HSV tk/ganciclovir gene therapy was conducted. The study demonstrated that intrapleural administration of the vector was well tolerated and resulted in detectable gene transfer when delivered at high doses [89]. In an other phase I clinical trial including also 21 MM patients it was demonstrated that replication-defective AdV-tk vector administered intraperitoneally induced significant humoral and cellular immune response that induced no obvious adverse clinical squeal [90].

Chronic lymphocytic leukemia (CLL) cells can be made to express CD40-ligand (CD154) by transduction with a replication-defective AdV vector. *In vitro* transduced and bystander leukemia cells become highly effective antigen-presenting cells that can induce CLL specific autologous cytotoxic T lymphocytes. The immunological and clinical responses have been studied to infusion of autologous AdV-CD154-CLL cells in patients with CLL. Based on promising results, this approach may provide a novel gene therapy strategy for patients with CLL [91].

Colorectal cancer (CRC) is about the second commonest cause of cancer death. Death is commonly due to liver metastases and consequent progressive liver dysfunction. About 50% of CRC tumors have p53 alterations. Several gene therapy clinical trials in colon carcinoma are ongoing or in the approval process. Different gene therapy approaches can be employed such as tumor suppressor gene replacement with wild-type p53, enzyme/prodrug system, and immune gene therapy based on cytokine or tumor antigen expression to induce tumor immunity. Replication-deficient recombinant AdV vectors are predominantly used and can be administered by infusion via the hepatic artery for the regional gene therapy of malignant liver tumors. The patients studied generally have incurable metastatic CRC of the liver. Clinical trials provide data for design of future studies with respect to dose, form and frequency of administration, to evaluate the biological efficacy, efficiency and stability of gene transfer. Colon cancer gene therapy is likely to take new directions such as use as

adjuvant to radical surgery, rather than attempts to treat end-stage disease. Another direction might be either prophylactic gene-based immunization against a panel of well-characterized tumor antigens or the combination of gene therapy with conventional anticancer treatment such as radiotherapy and chemotherapy [16, 17].

Primary and secondary liver tumor patients with inresectable tumors were involved in phase I and phase II clinical studies with a recombinant mutant AdV with E1B 55 kDa deletion (d11520). The treatment was well tolerated when administered intratumorally, intraarterially or intravenously. The combination of d11520 and 5-fluorouracil infused into the hepatic artery was also well tolerated. However, in order to achieve better clinical response, further improvement in the vector design will be needed [16, 92].

Ovarian cancer (OC) gene therapy strategies employed various viral and nonviral vectors. Thus far AdV vector has been the most promising vector. Gene therapy approaches include different suicide gene strategies, replacement of wild type p53 and the use of an anti-erbB-2 antibody-encoding AdV vector. Novel advances in gene therapy approaches include refinement of vector targeting, the use of site-specific promoters and conditionally replicative AdV vectors. Phase I study of 14 patients with AdV HSV tk/GCV showed that the treatment was feasible in the context of human OC. In another suicide gene therapy trial including 10 patients acyclovir (ACV) or valacyclovir (VCV) were used as enzymatic substrate. It was concluded that replacing ACV by VCV would offer a cost-effective alternative. Another clinical trail of 10 patients with advanced epithelial ovarian cancer showed that i.p. delivery of AdV HSV tk/ACV and concomitant topotecan chemotherapy was well tolerated without lasting toxicity and side-effects were independent of dose of the AdV vector. In a phase I trial of 15 patients with recurrent ovarian cancer an anti-erbB-2 single chain antibody encoding AdV vector was used to treat erbB-2-overexpressing OC. The results demonstrated the feasibility of this strategy. In general it could be concluded that although several clinical trials have documented the relative safety of the used treatments, few significant clinical responses have been effected. Nevertheless gene therapy strategy does appear promising for the treatment of OC because advances in this field are occurring rapidly [19, 20, 93, 94, 95].

Bladder cancer (BC) frequently shows mutations in the p53 gene. AdV mediated p53 gene transfer is growth-inhibitory to BC cells. The AdV-p53 vector was administered intravesically to patients with BC in phase I trial, and provided basis for phase II and phase III trials [96].

Prostate cancer (PC) represents an accumulation of genetic mutations that causes prostate cells to loose the ability to control their growth, therefore gene therapy approach could be the replacement of tumor suppressor genes p53 and p16 with AdV

vectors containing wild type genes. Alteration in the p16 gene and its protein expression often occur in PC. Prostate tumors injected with AdV-p16 vector expressed p16 for up to 14 days and markedly suppressed tumors *in vivo* [97, 98]. A phase I clinical trial including 18 patients with PC was the first to demonstrate the safety of AdV/HSV-tk plus GCV gene therapy in human PC and the first to demonstrate the anticancer activity of gene therapy. In a further clinical trial 52 patients received intraprostatic injections of AdV/HSV-tk vectors followed by intravenous ganciclovir or oral valacyclovir injections. All toxic events after multiple or repeated injections were mild and resolved completely once the therapy course was terminated. It was concluded that direct injection into the prostate of AdV/HSV-kt gene followed by i.v. GCV is safe even in repeated cycles [99, 100].

Cardiovascular disease is the leading cause of death in the western part of the world and gene therapy approaches to several cardiovascular disorders including restenosis after angioplasty, therapeutic neovascularization, and bypass graft restenosis have been proposed. Phase I clinical trials have been reported, but convincing efficacy data in humans do not yet exist [101]. A six-month assessment of a phase I trial of angiogenic gene therapy for the treatment of *Coronary artery disease* was also reported. In this trial, 21 patients were given direct myocardial injection of AdV vector expressing the human vascular endothelial growth factor (VEGF) 121 cDNA to induce therapeutic angiogenesis. In 15 patients the vector was used as an adjunct to conventional coronary artery bypass grafting and in 6 patients as sole therapy via minithoracotomy. This study suggested that these methods appear to be well tolerated and initiation of phase II evaluation of therapy appears warranted [102]. It is worth mentioning that in a phase I trial including 30 patients with inoperable coronary artery disease direct myocardial transfer of naked DNA-encoding VEGF165 was used with promising success [103].

Wound healing is a particularly attractive approach for gene therapy. AdV-mediated gene transfer in wound healing is a relatively new application of this vector, but it seems to be a useful tool in the study of the role of specific cytokines in normal and impaired wound healing. Phase I trials are in progress with the AdV vector carrying platelet-derived growth factor A or B (PDGF) to study the local and systemic toxicity and the feasibility of using the maximum tolerated dose in chronic venous leg ulcers and chronic nonhealing diabetic foot ulcers [104, 105, 106].

The first gene therapy death

On 17 September 1999 an 18 years old boy with an inherited enzyme deficiency died four days after receiving AdV based gene therapy for treatment of partial ornithine transcarbamylase (OTC) deficiency. He was the first patient in a gene therapy trial to die of the therapy itself. He received a massive dose of vector – 38 trillion virus particles, the highest dose in this 18-patient trial – to try to get enough functioning OTC genes into his liver via hepatic blood vessel. The vector invaded not just the intended target (the liver), but also many other organs. The patient developed acute respiratory distress syndrome and died two days later of multiple organ failure due to anoxia. In spite of severe investigation, it remained a mystery why the patient died in consequence of the therapy. However, a list was presented of problems that need to be addressed to improve AdV safety. Clinicians have been urged to adopt a common index for dosing patients and a standard measure of virus particle concentration, database should be assembled on vectors and their effects, clinicians should be screen patients more carefully and researchers should collect better data on the fate of vectors in the body. The Recombinant DNA Advisory Committee (RAC) called for measuring transgene expression in cells and tissues, better assessment of immune status before and after dosing and studies of vector biodistribution [107, 108].

Conclusion and perspectives

The potential therapeutic application of gene transfer technology seems to be enormous. Many of the studies in inherited diseases and in cancer gene therapy clinical trials have provided information of critical importance for the design of efficient clinical protocols. Clinical trials have been extended to the treatment of many diseases. There are about thirty currently active gene therapy protocols for the treatment only of HIV-1 infection in the USA. These programs aim to confer protective immunity against HIV-1 transmission to individuals who are in risk of infection, to develop preventive or therapeutic vaccines for patients with AIDS and other infectious diseases. There is a proposal to begin in utero gene therapy clinical trials for the treatment of inherited genetic disorders. Gene therapy represents one of the most important developments in oncology, however, before this can be realized as standard treatment the technical problems of gene delivery and safety must be overcome. The early generation vectors are now likely to be phased out for most diseases. Researchers could turn to “gutless” vectors, which cannot reproduce, give much greater gene expression and are far less

inflammatory. Despite the latest significant achievements reported in vector design it is not possible to predict yet to what extent and when gene therapy will be effective.

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