Adenoviral Gene Therapy for Pancreatic Cancer: Where Do We Stand?

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Key Words

Pancreatic cancer · Gene therapy · Adenovirus

Abstract

Background: The prognosis of patients with pancreatic cancer is poor. This is mainly caused by the late diagnosis, the aggressive biology and the lack of effective treatment modalities. Adenoviral gene therapy has the potential to selectively treat both primary tumor and (micro)metastatic tissue.

Methods: This review provides an overview of what has been achieved so far in the field of adenoviral gene therapy for pancreatic cancer. **Results:** Transductional targeting allows decreased toxicity due to vector dissemination to non-target cells and permits delivery with a lower viral dose. It can evade or diminish the immune response, which remains a major problem. Transcriptional targeting evolves quickly but essential drawbacks such as the lack of an efficient animal model delay clinical application. Few clinical trials utilizing adenoviruses have been performed in patients with pancreatic cancer today. Worldwide, only seven phase III trials are being performed investigating adenoviral vectors in cancer patients. **Conclusion:** A clear therapeutic effect of adenoviral gene therapy in pancreatic cancer has not yet been achieved, because the step from bench to bedside has encountered drawbacks. Combinations of the different targeting strategies and techniques to evade the immune system harbor the future for adenoviral gene therapy in patients with pancreatic cancer.

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Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death with approximately 38,000 newly diagnosed patients in the USA each year [1]. The prognosis of patients with pancreatic cancer is poor, indicated by the fact that the incidence reaches mortality and that 5-year survival is low [2]. Several factors cause this poor prognosis. Tumor biology of pancreatic cancer presumably plays the most important role. Pancreatic adenocarcinoma is highly malignant and has the capacity to infiltrate and metastasize early. Furthermore, the late onset of symptoms leads to diagnosis at an advanced stage of the disease in most patients. Anatomy also plays a role because the pancreas is closely surrounded by vital structures such as the superior mesenteric vein and the portal vein. Tumor ingrowth in these structures takes away the chance of a complete resection of the pancreatic tumor. The advanced stages of pancreatic cancer are associated with substantial morbidity such as cachexia and pain resulting in rapid deterioration. Finally, a major determinant in the poor prognosis is the fact that pancreatic cancer is fairly resistant to most treatment regimes tested to date, such as chemo-, radio- or immunotherapy [3–6]. Gemcitabine was tested in a randomized trial and resulted in a significant improved 5-year survival rate of 21% compared to 8% in pancreatic cancer patients who did not receive gemcitabine ($p = 0.009$) [3].

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Fig. 1. Schematic structure of the human adenovirus.

The only treatment option with a curative intention is surgical intervention by means of a pancreatic resection (pancreaticoduodenectomy or Whipple's resection). Due to the high percentage of patients with locally advanced metastatic disease at diagnosis, only 10–20% of patients are eligible for curative surgery [7]. The median survival of these most optimally treated patients is only 17 months [2]. Thus, it is clear that novel treatment modalities that do have an effect on survival of patients with pancreatic cancer are urgently needed. Adenoviral gene therapy has for many years been regarded as a potential new treatment modality. The aim of this review is to provide an update of what has been accomplished in the field of adenoviral gene therapy today and is focused on the application of gene therapy for pancreatic cancer.

Adenoviruses: Structure and Biology

Adenoviruses were first isolated from adenoid tissue in 1953. They can cause a wide range of mainly mild symptoms of the respiratory, gastrointestinal and urinary tract and the eye. Adenoviruses are non-enveloped and have a capsid of 20 triangular surfaces that form the icosahedral structure with a diameter of 80–110 nm. They contain a linear, double-stranded, DNA genome of approximately 36 kb. The most important proteins of the capsid of the adenovirus are the hexon, penton base, protein IX and knobbed fiber (fig. 1). At present, 51 distinct serotypes have been recognized which are divided in six subgenera. Serotype 5 (Ad5, subgroup C) has been studied most extensively and is most frequently used in human clinical gene therapy trials [8]. These serotypes have different tissue tropism, which is determined by capsid proteins and most importantly the fiber knob [9]. In the clinical setting, liver tropism of Ad5 is most relevant due to the substantial side effects that can be caused by liver

damage. Although the mechanisms of this tropism to the liver are not fully known, it is partially caused by the abundant expression of the coxsackie-adenovirus receptor (CAR) on hepatocytes, the fenestration of adenovirus in the liver sinusoids and their clearance by Kupffer cells [10–12].

The process of target cell entry of Ad5 starts with the binding of the fiber knob to the CAR, a cell-cell adhesion protein involved in tight junction formation (fig. 2a) [13]. Thereafter, internalization through endocytosis is mediated by the interaction of cellular $\alpha_v\beta$ integrins and the adenoviral Arg-Gly-Asp (RGD) motif in the penton base [14]. In addition to CAR and integrins, recent studies suggest that other receptors, including heparan sulfate proteoglycans and major histocompatibility complex I, may play a role in Ad5 entry into cells [15, 16]. Once inside the cell, the virus escapes the endosome and translocates to the nuclear pore complex, where the viral DNA is released into the nucleus. Adenovirus transcription occurs in an early and late phase. The early transcriptional genes are E1A, E1B, E2, E3 and E4. E1A leads to activation of the cell cycle and initiation of DNA replication. E1B blocks the apoptotic pathway, induced by E1A activity. E2 is needed for viral DNA replication, E3 modulates the host immune response and E4 regulates DNA replication, mRNA transport and apoptosis. The structural viral proteins are encoded by genes in the late phase of viral replication.

Adenoviral Vectors and Human Gene Therapy

Currently, human adenoviral vectors (mainly based on Ad5) are being used in approximately one quarter of all gene therapy trials, predominantly for the treatment of cancer [17] (<http://www.wiley.co.uk/genmed/clinical/>). They are one of the most attractive therapeutic vec-

Fig. 2. Schematic representation of different strategies of transductional targeting: **a** natural target cell attachment, **b** knob modification, **c** penton modification, **d** fiber-chimeric adenovirus, **e** serotype switch, **f** fiber replacement, **g** application of an adaptor molecule, and **h** chemical modification.

tors for several reasons. They have the ability to infect both dividing as well as non-dividing tumor cells. They are stable, double-stranded DNA viruses that can be manipulated relatively easily. Furthermore, the techniques to produce high-titered preparations of adenoviral vectors are straightforward.

There are three potential hurdles in the application of adenoviral vectors for the treatment of pancreatic cancer. First, pancreatic adenocarcinoma, as well as many other malignancies, does not express adequate levels of CAR, the receptor to which the natural tropism of Ad5 is directed, to permit efficient cell entry [18–20]. Second, expression of CAR on healthy tissue may lead to vector- or transgene-related toxicity through the entry of adenoviral vectors into non-target cells. Third, the innate immune response towards the vector is dose-dependent and

can lead to neutralization of the adenoviral vectors and to a deadly systemic inflammatory response [21]. These potential disadvantages highlight the importance of understanding the role of native adenoviral receptors in gene transfer in vivo and of developing targeted adenovirus vectors with improved efficiency and specificity of gene transfer to pancreatic tumor cells.

Generally, modification of the adenoviral vectors can result in transductional or translational targeting. Transductional targeting implies adenoviral modifications that redirect the adenoviral vector to a ligand specifically expressed on the target cell. Translational targeting implies modifications that restrict gene expression to the target cell. The gene-directed prodrug activation therapy system can be used to achieve local high concentrations of toxic drugs into tumor tissue.

Modification of the Adenoviral Tropism: Transductional Targeting

Because of the described limitations of Ad5 as a gene therapy vector, a lot of effort has been put into the modification of the tropism of the virus. It allows transductional targeting to tumor cells and decreases toxicity due to vector dissemination to non-target cells and permits delivery with a lower viral dose. Several approaches have been developed, such as modifications of Ad capsid proteins, mainly the fiber knob, conjugation-based modifications using an antibody or bispecific fusion protein, and chemical modifications of the adenoviral vector using polymers containing targeting ligands. This strategy can be complex because genetic modifications can lead to destabilization of the fiber, causing loss of trimerization resulting in a non-functional virus.

Genetic Modification of the Fiber Knob

Krasnykh et al. [22] identified the HI loop in the Ad knob monomer as a potential site for genetic modification of the Ad fiber. The HI loop is exposed on the knob of the fiber and has a certain degree of flexibility. It can tolerate peptide insertions of up to 100 amino acids, with minimal negative effects on virion integrity. Incorporation of foreign peptides into the HI loop, the C-terminus of the fiber knob or a combination of both have been investigated extensively [23, 24]. The cloning of the Arg-Gly-Asp (RGD) peptide, originally located at the penton base, into the HI loop of the fiber knob results in vitro and in vivo an improved targeting to $\alpha_v\beta$ integrins of pancreatic carcinoma and also of other human tumors (fig. 2b) [14, 18, 22, 25, 26]. There are also multiple studies that report the insertion of other peptides into the HI loop of the fiber knob, for example those discovered by phage display [27–29]. Although the target of these peptides is often unknown, the viruses show enhanced transduction to multiple cell types including cancer cells. Double modifications of the fiber knob were performed by insertion of a stretch of seven lysine residues (K₇ peptide) into the C-terminus of the fiber knob which target to the heparan sulfates of the cellular surface, while RGD was inserted into the HI loop [24]. These double modified viruses were more efficient than both single modified viruses concerning gene transfer to multiple cancer cells.

Other Capsid Modifications

It is also possible to retarget adenoviral vectors by inserting peptides into capsid protein pIX, penton and hexon, although these are not directly involved in target cell

attachment (fig. 2c). Based on the proposed structure of pIX, a heparan sulfate-binding motif (polylysine) was inserted into the C-terminus which resulted in fiber knob-independent transduction of CAR-deficient cell types [30]. It was also shown that the C-terminus of pIX tolerates the insertion of large peptides, which is important for cell type-specific targeting [31]. Accessibility of the incorporated peptide at the capsid surface was improved by incorporation of a spacer [32]. The RGD motif in the penton base can be replaced by a peptide that can recognize tissue-specific integrins [33]. Vigne et al. [34] showed that the RGD peptide can be incorporated into the hexon leading to targeting of the modified adenoviral vector to vascular smooth muscle cells.

Fiber-Chimeric Adenoviral Vectors

Another approach to modify Ad5 is the substitution of the complete Ad5 fiber knob by fibers derived from other Ad serotypes (fig. 2d). These fibers are mainly from subgroup B because, in contrast to most serotypes from the other subgroups, they use a receptor for initial cell attachment other than CAR [35–38]. Subgroup B adenoviruses, including Ad3, Ad11, Ad14, Ad16, Ad21, Ad35 and Ad50, use other molecules for infection, such as CD46, CD80 and CD86 [39–41]. Several adenoviruses belonging to serotype D (Ad8, Ad19 and Ad37) use sialic acids as the primary receptor [42, 43]. Primary endothelial cells, smooth muscle cells, synoviocytes, hematopoietic stem cells are poorly transfectable by conventional Ad vectors but were efficiently transfected by fiber-chimeric adenoviral vectors [44–46]. For example, the transduction efficiency of intestinal mucosa can be enhanced using an Ad5 virus bearing subgroup B fibers [47]. Recently, improved gene transfer to primary human pancreatic carcinoma cells was observed using Ad5 containing fibers from serotype 16 and type 50 [Kuhlmann et al., submitted]. Thus, both approaches allow Ad tropism to be expanded (or changed) via binding of the modified fiber of serotype 5 or via a fiber from another serotype.

Another approach to enhance infectivity of Ad vectors is called ‘complex mosaicism’. This is a combination of different serotype chimerism and targeting ligand(s) incorporation in the fiber molecule. For example, Borovjagin et al. [48] constructed an Ad containing the RGD-4C peptide into the HI loop, at the carboxy-terminus, or both locales of the Ad3 knob in the context of the Ad5/3 vector. This vector was simultaneously retargeted to integrins and Ad3 receptors, and showed up to 55-fold increase of gene transfer to bladder cancer cell lines. In this

case, most retargeting was due primarily to Ad3 retargeting, but also some integrin-mediated targeting was observed.

Serotype Switch

Using another serotype instead of a fiber-chimeric adenoviral vector could provide additional advantages since most host-neutralizing antibodies are directed towards the penton and hexon capsid proteins (fig. 2e) [49, 50]. In chimeric adenoviral vectors these neutralizing antibodies will still bind to the Ad5 backbone. In the USA, Europe and Asia, preexisting immunity against adenoviral serotype 5 exist in approximately 80% of the population, compared to 1–15% for subgroup B adenoviruses indicating that switching to another serotype may solve the problem of readministration of Ad to humans [51, 52]. Recently, serotypes 11 and 35 have been shown to be possible candidates for pancreatic cancer, because both display enhanced infectivity in pancreatic cancer cells [51, 53, 54].

Fiber Replacement

Switching of the fiber or serotype only results in the redirection of the adenoviral vector to its natural ligand and often to widespread distribution of the virus to the target tissue but also to normal tissue. Replacing the native Ad fiber with a non-adenoviral chimeric protein allows redirecting the virus to specific surface receptor cells that for example cannot be infected by any Ad serotype (fig. 2f). There are several proof-of-principle studies describing the radical replacement of the adenoviral fiber with a non-adenoviral chimeric protein containing a heterologous trimerization motif and a receptor-binding ligand. Krasnykh et al. [55] replaced the fiber with two amino-terminal repeats of the shaft domain of the Ad5 fiber protein genetically fused with a truncated form of the bacteriophage T4 fibrin protein. Van Beusechem et al. [56] constructed knobless adenoviral vectors with a Myc epitope and a 6His tag to mimic the targeting ligands which lead to mature but unstable virions. As an example of successful targeting via fiber replacement, Belousova et al. [57] functionally displayed the human CD40 ligand in the chimeric fiber of the Ad vector, which infected CD40-positive dendritic cells with high efficiency.

Ablation of Natural Tropism

So far, all described modifications do enhance adenoviral transduction of (pancreatic) tumor cells by using additional entry pathways to compensate for low CAR expression. Although modified, some of the viruses de-

scribed above can still bind to their natural receptor. Therefore, the natural adenoviral tropism should be abolished to achieve selective targeting. There are at least three regions that determine this tropism of Ad5: the fiber knob, the fiber shaft and the RGD motif at the penton base. Mutations in multiple loops of Ad5 fiber knob reduce transduction efficiency by ablating the fiber-CAR interaction [58, 59]. In addition, interaction of the penton base with integrins was abolished by deletion of the RGD motif [60]. A single mutation of either fiber knob or penton base does not change the biodistribution of Ad vectors in mice upon tail vein injection [60, 61]. In vivo studies with viruses containing double mutations give conflicting results; some studies report significant reduced liver transduction, while others report no reduction [62–64]. An important cause for this observation is the fiber shaft and in particular its length [65–67]. Shortening of the shaft results in lower liver transduction, possibly via elimination of the motif that binds to the heparan sulfate. A triple mutant containing a CAR-binding ablated mutant fiber knob derived from Ad5, a fiber shaft derived from Ad35 (the fiber shaft of Ad35 does not possess the KKTK motif and is shorter than that of Ad5), a fiber tail and a mutant penton base without RGD motif, both derived from Ad5, mediated a 30,000-fold lower level of liver transduction in mice compared to the conventional Ad5 vector [63].

Application of Adaptor Molecules

Another way to retarget adenoviral transduction can be achieved by complexing bispecific conjugates with the adenoviral vector (fig. 2g). These conjugates have one component directed to a region of the adenoviral vector, often an antibody that binds the fiber knob, and one component directed at a target cell receptor. These conjugates not only change the tropism but also ablate the natural tropism by binding to ligand-binding sites. Many different conjugate types have been generated with affinity to many different cell types. Anti-knob monoclonal antibodies (mAb) have been fused to proteins such as the fibroblast growth factor which enhanced adenoviral transduction of Kaposi's sarcoma [68]. Furthermore, they have been fused with antibody fragments. Fusion with a mAb against epidermal growth factor receptor (EGFR) for example resulted in EGFR-specific transduction of squamous cell carcinoma of the head and neck [69]. Bispecific conjugates directed to other capsid proteins were used by Wickham et al. [70], who targeted an adenoviral vector with a FLAG peptide incorporated into the penton base to integrins using an anti-FLAG/anti-integrins bi-

specific antibody. Bispecific fusion proteins consisting of two single-chain Fv domains were developed to direct the adenoviral knob to EGFR [71]. Other bispecific fusion proteins use the extracellular domain of CAR to bind the knob and a conjugate of truncated CAR and EGF has for instance been used to retarget Ad to pancreatic cancer [18].

Chemical Modification

Human serum is a very hostile environment to most viruses including adenoviruses. This is not only caused by the neutralization of the virus by antibodies, but it is also due to binding to blood cells. Over 90% of a typical virus dose binds to human (but not murine) erythrocytes *ex vivo* [72]. Coating of adenoviral vectors in this respect is promising because it could shield the virus from blood cells. Another advantage of this coating is that one could retarget the adenoviral vector using a ligand-specific peptide coating. The polymer polyethylene glycol (PEG) can be covalently attached to free lysine groups on the surface of the adenoviral vector to shield it from its environment [73]. Thus, covalent attachment of PEG to Ad exhibit antibody evasion activity and long plasma half-life. However, these vectors are less effective in cell entry because of the steric hindrance caused by PEG. Eto et al. [74] modified the PEGylated Ad vector by introduction of the RGD motif into the fiber protein. This RGD-PEG-Ad not only showed up to 200-fold higher gene expression compared to PEG-Ad, but it also retained high activity in the presence of anti-Ad antiserum. Furthermore, RGD-PEG-Ad revealed efficient gene expression in both CAR-positive and CAR-negative cells, but also in *in vivo* experiments. Lanciotti et al. [75] coated the adenoviral vector with a conjugate of fibroblast growth factor-2 and PEG, showing that the transduction of ovarian cancer cells became dependent on the FGF-2 receptor and independent of CAR. In an intraperitoneal model of ovarian cancer, this conjugate mediated increased transgene expression in tumor tissue and at the same time reduced localization of the vectors to non-target tissues compared with unmodified Ad vectors [75].

In conclusion, transductional targeting is a promising strategy to target pancreatic cancer cells. Using different targeting techniques it can enhance cell specificity. Furthermore, it can evade or diminish the immune response, which remains one of the major problems in adenoviral gene therapy. A limitation of this strategy is the fact that it is never 100% specific and that normal cells can also be infected, possibly leading to safety problems when tested in human gene therapy trials.

Gene Expression Modifications: Transcriptional Targeting

Transcriptional targeting can be performed in different ways. An obvious strategy would be the restoration or silencing of a gene specific for pancreatic cancer. However, all types of cancer, including pancreatic cancer, contain mutations in several genes. Furthermore, to effectively treat cancer, every single cell should be hit by an adenovirus which is virtually impossible. Other strategies include conditionally replicating adenoviral vectors (CRAds) which should selectively replicate in tumor cells, leading to oncolysis. They have the theoretical advantage of sparing healthy tissue. Furthermore, the released virus can infect neighboring tumor cells leading to a cascade of tumor destruction and viral replication (bystander effect).

Restoration or Silencing of Pancreatic Cancer-Specific Genes

Many mutations in oncogenes and tumor-suppressor genes are associated with pancreatic cancer. The most commonly mutated oncogene is *K-ras* (in 95% of patients), and the most commonly mutated tumor-suppressor genes are *P16/RB1* (>90%) and *P53* (50–75%) [76]. *In vivo* experiments revealed that intraperitoneal injection of adenoviral vectors expressing antisense *K-ras* RNA suppresses peritoneal dissemination of hamster pancreatic cancer without systemic toxicity [77]. Adenovirus-mediated coexpression of antisense *K-ras* RNA and interferon- α (a cytokine with an anti-growth effect on many malignancies, including pancreatic cancer) has been shown to enhance interferon- α -induced cell death and suppress the subcutaneous growth of pancreatic cancer cells in mice [78]. Using the same strategy, the cancer-associated Sm-like (CaSm) oncogene was silenced, resulting in a significantly decreased proliferation and growth of pancreatic tumor cells *in vitro* [79]. In two studies the wild-type *P53* tumor-suppressor gene was reintroduced into pancreatic cancer cell lines using an adenoviral vector, which resulted in induction of apoptosis and inhibition of cell growth [80, 81]. Another study used adenoviral vectors to reintroduce wild-type *P16* and *P53* into pancreatic cancer cells *in vitro* and into subcutaneous pancreatic tumors in an animal model to determine the effect on tumor growth, resulting in significant growth reduction [82]. Rigg et al. [83] observed tumor invasion after adenoviral introduction of tissue inhibitors of metalloproteinase 1 and 2 (TIMP1 and TIMP2). They demonstrated *in vitro* that the TIMP-expressing pancreatic

tumor cells were significantly less invasive than those cells transfected with a control vector. In vivo, adenoviral delivery of TIMP1 or TIMP2 to nude mice harboring intraperitoneal human pancreatic cancer resulted in prolonged survival compared with control mice. To reach curative treatment, this strategy of restoration and silencing needs a transfection efficiency of 100%, which seems impossible to achieve. Because of the multifactorial genetic nature of the disease, it cannot be expected that restoration or silencing of a single gene can cure a pancreatic cancer cell.

Gene-Directed Prodrug Activation Therapy

This strategy results in the adenoviral delivery of a prodrug activator to the target cell. When the non-toxic prodrug is systemically administered, only cells containing the prodrug activator are able to convert the non-toxic prodrug into a highly toxic drug. This system provides low systemic but high local intratumoral toxicity. Diffusion of the toxic drug to neighboring cells results in killing of cells that were not transduced. This bystander effect makes it possible that transduction of a relatively low percentage of cells will be sufficient to kill virtually all tumor cells. Cytosine deaminase can convert non-toxic 5-FC into toxic 5-FU and has been shown to be effective in a murine pancreatic cancer model [84]. Herpes simplex virus thymidine kinase (HSV-Tk) can phosphorylate systemically administered gancyclovir which eventually leads to cell death. This system has also shown to result in tumor regression in a pancreatic cancer mouse model [85].

Conditionally Replicating Adenoviral Vectors

The first described CRA is based on the fact that the function of tumor-suppressor gene *P53* is inactivated in many malignancies, including pancreatic cancer. The human adenoviral E1B gene encodes a protein that inactivates the cellular tumor-suppressor protein *P53*. Activity of this protein must be blocked in order to allow efficient virus replication. ONYX-015 is an Ad2/5 fiber-chimeric adenoviral vector in which the E1B gene is mutated and therefore absent. This virus is unable to replicate in normal cells but is able to replicate in cells lacking functional *P53*, such as pancreatic cancer cells [86]. In a phase I/II clinical trial with ONYX-015, endoscopic intratumoral injection was assessed in combination with intravenous gemcitabine in 21 patients with advanced pancreatic cancer [87]. Endoscopic injection was feasible and led to few procedure-related complications. Although it was not the aim of the study, it resulted in a partial response

in 2 patients, stable disease in 10 patients and progressive disease in 11 patients. Recent studies have shown that replication of ONYX-015 can be independent of the *p53* status, possibly due to inactivation of *P53* by other viral genes [88]. ONYX-015 replication depends on an unidentified mRNA transport mechanism that is active in many cancer cells but not in normal cells [89]. Because the clinical outcome of patients undergoing monotherapy with ONYX-015 has been disappointing thus far, E1B-mutated vectors have been further developed. This has led to vectors that can be used to carry genes encoding anti-tumor factors. For example, ONYX-015-expressing endostatin, a strong inhibitor of angiogenesis, has been developed [90]. This virus is capable of replicating in and lysing hepatocellular carcinoma (HCC) cells selectively with effective tumor growth suppression and antiangiogenic activity on HCC xenografts in nude mice.

Other oncolytic adenoviral vectors of importance are those with a mutation in the E1A gene. The E1A protein overcomes the inhibition of cell cycle progression mediated by the retinoblastoma (Rb) pathway. E1A binds with the retinoblastoma protein (pRb), resulting in the release of cell cycle activators resulting in the progression from G1 to S phase of mitosis. A 24-bp deletion in the pRb-binding region of the E1A region eliminates binding to pRb and thus results in a virus that cannot overcome the inhibition of the cell cycle by pRb [91]. Since many tumors have defects in the pRb pathway (e.g. Rb mutation, p16 loss, cyclin D amplification), these E1A mutant adenoviral vectors should selectively replicate in Rb-defective cells. Although mutations of the Rb gene are rarely detected in pancreatic cancer, p16 abnormality is present in 90% of patients [92]. An E1A-mutated adenoviral vector containing a small deletion in the E1A region efficiently destroyed pancreatic cancer cell lines, while leaving resting, normal cells unharmed [93]. In order to improve the selectivity, an adenoviral vector has been developed with alterations in both E1A and E1B [94]. This virus effectively and selectively replicates in and causes oncolysis of gallbladder carcinoma in vitro and in vivo with reduced negative effects on normal cells. However, because this mutant Ad carries two deletions, its attenuation affects its ability to efficiently replicate and spread to achieve its desired cytopathic effect.

In addition to CRAs made tumor specific by deletion of the E1 region, another option is to generate a vector in which the genes essential for Ad replication are under control of a tumor-specific promoter (TSP) (fig. 3). In recent years, DNA array studies have discovered many potential pancreatic cancer marker genes which can be used

Fig. 3. Conditionally replicating adenovirus.

for this purpose [95]. Midkine (MK), a heparin-binding growth factor and cyclooxygenase-2 (COX-2), a key enzyme in the conversion of arachidonic acid, are both up-regulated in pancreatic cancer. Wesseling et al. [96] constructed adenoviral vectors with the luciferase reporter gene under control of MK and COX-2 and compared it to the cytomegalovirus promoter. Both genes display a so-called 'liver-off, pancreatic cancer-on' expression and did not show significant liver toxicity. Both vectors achieved moderate to high activity upon transduction of primary pancreatic cancer cell lines. Yamamoto et al. [97] constructed a CRAAd with a cyclooxygenase (COX-2)-driven E1A gene. Infectivity was enhanced by incorporation of the RGD peptide into the HI loop. The CRAAd showed selective cytotoxicity in vitro in COX-2-positive pancreatic cancer cell lines. In a pancreatic cancer xenograft model in mice, intratumoral injection of the retargeted CRAAd showed a strong antitumor effect comparable to that of wild-type adenovirus, while the non-targeted CRAAd

showed limited effect. Other TSPs evaluated for usage in an adenoviral system to treat pancreatic cancer are mucin 1 (MUC1), early growth response gene 1 (Egr-1), and telomerase [98, 99]. Telomerase is a protein of interest because the vast majority of human tumors have telomerase activity, whereas most benign diseases and normal tissues do not express this enzyme. It is upregulated in approximately 95% of human pancreatic carcinoma cells, while it is absent in benign pancreatic tissue [100]. It is a ribonucleoprotein enzyme that maintains telomere length through the addition of TTAGGG repeats [101]. Adenoviral vectors with therapeutic or suicide genes driven by the telomerase promoter (mainly hTERT; human telomerase reverse transcriptase) have shown to efficiently kill different cancer cells in vitro and in vivo, in a telomerase-dependent manner [102, 103]. Recently, a hTERT promoter fragment was used to drive the expression of the E1A gene in oncolytic adenoviral vectors, which revealed tumor-selective oncolysis of cancer cells

in vitro [104, 105]. Although transductional targeting of Ad proved to be promising, Ad vectors with RGD modification still revealed limited expression in normal tissue. To overcome this obstacle in treatment of melanoma, Okada et al. [106] used an AdRGD-mediated HSV-Tk/ganciclovir system where the HSV-Tk gene is under the control of the melanoma-specific tyrosinase (Tyr) promoter or tumor-specific telomerase reverse transcriptase (TERT) promoter. Thanks to transcriptional targeting, AdRGD-Tyr/HSV-Tk or AdRGD-TERT/HSV-Tk has the same effect in gene expression compared to the AdRGD-CMV/HSV-Tk, but with 10 times less vector dosage of the TSP vectors, indicating that these vectors are effective and safe for melanoma suicide gene therapy.

In the last meeting of the American Society of Gene Therapy in Seattle in May 2007, many interesting abstracts were presented concerning replicative adenoviral vectors. Initial experience in a phase I study with a telomerase-specific CRAd suggests that the virus is safe and well tolerated. Pathological examination showed activity at the tumor site in patients with solid tumors [107]. Terao et al. [108] presented an in vitro study using a MK-promotor based CRAd in a bladder cancer model. A significant antitumor effect was shown in cell lines overexpressing MK mRNA. This is interesting because earlier studies showed overexpression of the MK promoter in pancreatic cancer [96]. Because most animal models do not support replication of human adenovirus, Smith et al. [109] developed a canine adenovirus which was studied in a fully immunocompetent dog model with osteosarcoma. The model showed to be safe. Experiments with models like this are essential for the further application of CRAds in humans.

In conclusion, transcriptional targeting is evolving quickly but essential drawbacks delay the clinical application of the developed viruses. For example, the replicating viruses should first be tested in animal models, but the most commonly used mice model cannot be used because adenoviruses do not replicate in the mice. Questions concerning the activity of TSPs in the targeted tissue should be addressed. Furthermore, the efficacy of replicating adenoviral vectors is questioned in a hypoxic environment. Because hypoxia is a pathophysiological condition in cancer, Shen et al. [110] investigated the lytic potential of the replication-competent adenoviral vector. They demonstrate that hypoxia does not alter receptor-mediated viral uptake, but alters the viral replication and cytolysis in a viral- and cell-specific manner. Studies with replication vectors in a hypoxic environment should further address this problem.

A final problem is the biosafety issue when replicating viruses are brought from bench to bedside. What would be the consequences of a recombination of the engineered virus with a related wild-type virus in the population? Unfortunately, most phase I clinical trials do not address the topic of viral shedding and recombination with wild-type virus. Until these issues are investigated sufficiently, maximum safety precautions should be undertaken [111].

Adenoviral Vectors and Chemoradiation

Multiple preclinical trials have investigated the effect of combining adenoviral vectors with chemo- or radiotherapy. Gemcitabine has become widely used in the treatment of advanced pancreatic cancer.

Halloran et al. [112] performed combining experiments with adenovirus-mediated delivery of *p53* or *p16* and 5-fluorouracil (5-FU) or gemcitabine in pancreatic cancer cell lines. They showed that the combination of use of 5-FU or gemcitabine with restoration of *p16* expression resulted in a significant increase in apoptotic cells compared with controls. Furthermore, the combination facilitated the use of chemotherapeutic drugs at lower concentrations than would otherwise be effective. Wack et al. [113] investigated whether pancreatic cancer cells could be sensitized to gemcitabine chemotherapy by cotreatment with an adenoviral hTERT-targeted proapoptotic gene expression vector using the proapoptotic genes *Bax* and TNF-related apoptosis-inducing ligand (*TRAIL*). In cell lines and an orthotopic tumor model, this combined treatment resulted in a significant tumor regression and prolongation of the experimental animal's life compared to gemcitabine alone. Adenoviral vectors can also enhance the efficacy of radiotherapy. Radiotherapy is hampered by tumor hypoxia. Liu et al. [114] used a bacterial cytosine deaminase (bcd) adenoviral gene therapy system which employed the hypoxia-response element promoter to regulate bcd transcription. They also show significantly improved tumor response in cell lines as well as tumor xenografts. These studies show the importance of adenoviral vectors in the improvement of established treatment strategies.

Clinical Trials

Although a lot of experimental work has been performed, only few clinical trials utilizing adenoviral gene therapy have been performed in patients with pancreatic cancer today (table 1a, b). Mulvihill et al. [115] performed

Table 1a. Overview of experimental studies concerning adenoviral gene therapy in pancreatic cancer

Type of Ad vector	Serotype	Type of modification	Gene to be transduced	In vitro/ in vivo	Ref.
Fiber knob modification	Ad5	RGD in HI loop	LUC	In vivo	18
	Ad5	TRAIL gene driven by the hTERT promoter; RGD in HI loop	TRAIL gene	In vivo, in vitro	25
Fiber-chimeric adenoviruses	Ad5/16	Fiber chimera	GFP	In vitro	Sub- mitted
Serotype switch	Ad35	Unmodified	None	In vitro	51, 54
	Ad11	Unmodified	GFP	In vitro	53
Bispecific adaptor molecules complexed with Ad5	Ad5	Epidermal growth factor receptor targeting using bispecific antibody recognizing knob of Ad5 and EGFR receptor	LUC	In vitro	18
Restoration or silencing of pancreatic cancer-specific genes	Ad5	Vector containing antisense human K-ras RNA	Antisense human K-ras RNA	In vivo	77
	Ad5	Vector containing antisense human K-ras RNA and interferon- α	Antisense human K-ras RNA and interferon- α	In vitro	78
	Ad5	Vector containing antisense RNA to cancer-associated Sm-like oncogene (CaSm)	Antisense RNA to CaSm	<i>p53</i> and <i>p16</i>	79
	Ad5	Vector containing the wild-type <i>p53</i> tumor-suppressor gene	Wild-type <i>P53</i>	In vivo	80
	Ad5	Vector containing the wild-type retinoblastoma tumor-suppressor gene	Wild-type <i>Rb</i>	In vivo	81
		Vector containing the wild-type <i>p53</i> and <i>p16</i> tumor-suppressor gene	Wild-type <i>p53</i> and <i>p16</i>	<i>p53</i> and <i>p16</i>	82
Transcriptional targeting	Ad5	Vector containing tissue inhibitors of metalloproteinase 1 and 2 (TIMP1 and TIMP2)	TIMP1; TIMP2	In vitro, in vivo	83
Gene-directed prodrug activation therapy	Ad5	Vector containing cytosine deaminase (CD)	CD	In vitro, in vivo	84
	Ad5	Vector containing thymidine kinase (TK)	TK	In vitro, in vivo	85
Conditionally replicating adenoviruses (CRAd)	Ad5	Midkine and COX2 promotor	Tumor-specific expression of LUC and thymidine kinase	In vitro	96
	Ad5	COX-2 promotor CRAd and RGD-COX-2 CRAd	oncolytic: mutant E1B	In vitro, in vivo	97
	Ad5	Mucin promotor	Tumor-specific expression of somatostatin	In vitro	99

LUC = Luciferase; GFP = green fluorescent protein.

Table 1b. Overview of clinical studies concerning adenoviral gene therapy in pancreatic cancer

Type of Ad vector	Serotype	Type of modification	Gene to be transduced	Trial phase	Ref.
Conditionally replicating adenoviruses (CRAd)	Ad5/2	ONYX-015 (E1B gene absent)	Oncolytic: only replicates in p53 deficient cells, intratumoral injection	I	115
	Ad5/2	ONYX-015 (E1B gene absent)	Oncolytic: only replicates in p53 deficient cells, intratumoral injection	I/II	87
Adenovirus carrying gene with antitumor effect	Ad5	Vector containing human tumor necrosis factor- α under control of the radiation-inducible Egr-1 promoter	TNF- α ; intratumoral injection	I	117
	Ad5	Vector containing interleukin-12 gene	Interleukin-12; intratumoral injection	I	116

a phase I study with CT-guided or preoperative intratumoral injection of ONYX-015 in 23 patients, which was published in 2001. They showed that this is a feasible and well-tolerated therapy, although viral replication was not detectable. Hecht et al. [87] performed a phase I/II trial in 2003 of endoscopic intratumoral injection of ONYX-015 with intravenous gemcitabine in patients with unresectable pancreatic carcinoma. This study also showed that the treatment strategy was feasible and was generally well tolerated. Again, no replication was detectable. In 2004, Sangro et al. [116] performed a phase I trial to evaluate intratumoral injection of an adenoviral vector carrying the interleukin-12 gene in patients with advanced digestive tumors. Seven of 21 included patients were bearing an advanced pancreatic tumor. This study concluded that the treatment was feasible and well tolerated; however, only mild antitumor effects were observed. TNFerade™, a replication-deficient adenoviral vector that expresses human tumor necrosis factor- α under control of the radiation-inducible Egr-1 promoter, was tested in a phase I study and was shown to be feasible and well tolerated in patients with different solid tumors including pancreatic cancer [117]. The therapeutic role of this vector is being investigated in patients with pancreatic, esophageal and rectal cancer. No phase III studies for pancreatic cancer are being performed at this moment.

When results of phase III adenoviral gene therapy trials for other malignancies are summarized, conclusions are disappointing. Worldwide, only 9 phase III trials using adenoviral vectors are being performed at this moment, 7 of them for cancer diseases (<http://www.wiley.co.uk/genmed/clinical/>). Six trials are investigating the therapeutic effect of adenoviral restoration of the mutated *P53* tumor-suppressor gene in carcinoma of the head and neck. One study is investigating replication-competent adenovirus-mediated suicide gene therapy in prostate cancer. Up to now, no phase III study has proved to be more effective than existing treatments for tumors.

Perspectives

In this review, we have focused on the ongoing development of adenoviral vectors for the treatment of pancreatic cancer. We summarized the different strategies that have been explored to improve adenoviral vectors. During the past decade, adenoviral vectors have been modified to target cells of interest, and furthermore they have been modified to restore tumor-suppressor genes or si-

lence gene defects or to specifically replicate in a target cell.

Unfortunately, a clear therapeutic effect has not yet been achieved. Several reasons could explain these poor results. The neutralization of the virus by the immune system and binding to blood cells play an important role, particularly after systemic administration. This could be encountered by intratumoral injection, although this will decrease the systemic effect on possible metastatic tissue. Other reasons are the anatomical barriers in a solid tumor. Viral spread inside the tumor could be hampered by the tightness of tumor cells and the surrounding fibrotic tissue.

To achieve a therapeutic effect in the future, the different strategies of adenoviral improvement should be combined. Ideally, a targeted vector containing a modified capsid should be combined with an oncolytic adenoviral vector. This vector should be altered or packaged to bypass innate and acquired immunogenicity and neutralization by blood cells. Furthermore, it should be administered systemically to reach distant metastatic tissue as well micrometastases. Although preclinical trials have highlighted some benefits and limitations of oncolytic adenoviral treatment, clinical trials are needed to understand the optimal way of delivery and antitumor response in humans, because animal models are often not comparable to human physiology.

Although considerable research and studies are still needed to optimize the transduction efficiency and oncolytic effect of targeted adenoviral vectors, steady improvements have been achieved during the last decade. The development of new generations of oncolytic adenoviral vectors, including various ways of targeting pancreatic cancer, will hopefully provide a new anticancer agent for multimodal pancreatic cancer treatment. Most likely, the best optimal effect in these severely ill patients will be achieved when the virus is administered in combination with existing chemotherapy and radiation therapy.

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