Adenovirus targeting for gene therapy of pancreatic cancer
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GENERAL DISCUSSION

The mortality rate of pancreatic cancer (PC) almost equals its incidence rate. This dismissal prognosis primarily results from its late diagnosis when the tumor has accumulated genetic disturbances that fuel an aggressive phenotype, and has become irresectable. Surgical intervention therefore is only available for a minority of patients. Even after resection the 5-year survival rate is less than 20%. Palliative gemcitabine-based chemotherapy may offer relief of complaints but gain of survival is questionable. The knowledge of the molecular defects underlying PC should allow the development of molecular targeted therapies, or even better; patient tailored intervention. Currently, clinical trials for PC primarily focus on gemcitabine-based therapy, radiotherapy, small molecules that target the EGFR and Raf/Mek/Erk pathway, anti VEGF therapy, and vaccination to boost immune response to mutant proteins expressed in these cancers (www.clinicaltrials.gov). At present only one trial in the US investigates the efficacy of Adenoviral vector mediated gene therapy using Ad5-yCD/mutTKSR39rep-ADP, a suicide gene approach using cytosine deaminase and herpes simplex thymidine kinase. The few trials using the E1B mutant CRAd Onyx-015 failed due to several reasons including poor transduction due to CAR deficiency of PC, poor replication of the virus due to loss of functional E1B and hampered lateral spread throughout the tumor. It is worth mentioning the fact that prior to these trials Onyx-015 was never tested in a proper animal model for PC. Therefore, vectorologists were sent back to the drawing board to develop second generation Adenovirus vectors in order to overcome the poor transduction efficiency. In addition novel pre-clinical models must be developed that better reflect the interaction of adenovirus with a tumor that would more reliably predict the efficacy in patients.

Having access to fresh PC and normal human pancreas specimens we decided to develop a method to culture fresh human pancreatic cancer (and non-malignant) specimens for gene transfer experiments. Using a tissue slicer we were able to process minimal amounts of fresh tissue obtained from resection material. The whole procedure, from operating room to final read out analysis, however, turned out to be extremely difficult. Rapid processing of the material was required to obtain high quality specimens. This was highly dependent on the concerted action of many involved. Although the Academic Medical Center is a primary care referral centre for PC patients, the number of high quality specimens suitable for our studies was low. Still, we for the first time show that pancreatic slices can be kept in culture for at least 3 days while retaining moderate to good viability. This period is sufficiently long for Adenoviral transduction and expression of encoded (reporter) genes to read out transduction efficacy and to perform an immunohistochemical analysis of the quality of the specimens. It became apparent that the viability and size of these slices varied. Therefore we developed a wildtype virus, which can be used as an internal control to correct
for these slice-specific properties. This application is of importance for pancreatic cancer research, since pancreatic tumors are highly heterogeneous with regard to receptor expression (chapter 3). The co-infection with a wildtype virus that has its native tropism could correct, at least in part, for variation between slices with regard to viability, transducibility and receptor expression. The targeting to control ratios now could be used to determine in this ex-vivo setting the effect of targeting strategies applied.

In our experiments we used GFP and dsRED as reporter genes in our vectors. For detection in a fluorometric readout system more sensitive candidates are available. The fluorometric readout system requires a certain minimal amount of fluorescence, which, especially in case of smaller slices, appeared not sufficient after transduction with GFP/dsRED reporter viruses. For future studies it seems therefore better to use vectors encoding luciferase as a reporter gene to increase sensitivity. In this system co-infection with an internal control is possible by using both Renilla and firefly luciferase as reporter genes.

In our study we used this ex vivo technology to determine the targeting efficacy towards several pre defined tumor antigens. From the literature potential targets, receptors that had increased expression on PC compared to normal human pancreas, were selected. We next sought for peptides known to specifically bind these receptors. An important advantage of this strategy is that such receptors are well characterized and have a known tissue distribution pattern which allows selection of tumor specific candidates. One relevant limitation of this strategy is that not all optimal receptor ligands can be incorporated into the HI loop of the viral fiber. Especially the incorporation of complex proteins in the fiber knob interferes with proper trimerization and function of the fiber knob. For this reason short amino acid sequences that bind to a certain receptor are the best suitable candidates for insertion into the HI loop. Identification of all potential candidates is feasible when using phage display techniques using a cell line expressing the selected cellular receptor. Upon identification the potential candidate peptides must be inserted at the correct site of the Adenoviral genome which requires several cloning and virus propagation steps. Since this process is time consuming and in the end may result in an inactive Ad vector due to interference of the peptide with knob function, Miura et al developed a system to produce adenoviral libraries (1). These libraries do display a large variety of peptides on the HI loop and can be used for positive selection of an Adenoviral vector with high affinity for target cells. Using this approach this group selected a CAR-ablated CRAd with an SYE peptide on the human pancreatic cell line AsPC-1 and subsequently tested this CRAd in a mouse model (2). This in vivo study showed a potent anti tumor activity of this vector. However, the authors were not able to identify which receptor was involved in binding of this CRAd. Therefore it remains unclear whether human PC tumors express this unknown cellular receptor and even its expression on the original cell line can not be confirmed. Because of the large
difference between cell lines grown in vitro and a cancer cell present in a tumor in vivo, cell lines do not seem the best option for screening of such Adenoviral libraries. PC tumor explants obtained from resection specimens followed by immediate culturing ex vivo seem a much more attractive option for selection of targeted adenoviral vectors using several rounds of screening. This system can also be used for negative screening by using normal human pancreas and liver. For identification of tumor specific peptides that target PC it would therefore be of interest to screen such an Ad library using this ex vivo culturing system.

Several groups evaluated infectivity-enhanced CRAds by using comparable technology in order to proceed to clinical trials. Infection of primary organotypic glioma spheroids with Ad5-Delta24RGD induced cell kill of small tumor nodules (3), with viral spreading toward the center of the three dimensional model (4). Infection of ovarian cancer tissue slices with Ad5/3cox-2 (5) and Ad5-Delta24RGD (6) revealed a “tumor-on, liver-off” phenotype. Ad5-delta24RGD entered two clinical trials recently for evaluation of safety and/or efficacy in glioma and ovarian cancer patients (Clinicaltrials.gov, identifiers NCT00805376 and NCT00562003, respectively). It would be of interest to see to what extent these improved pre-clinical models did predict the clinical efficacy of these novel infectivity enhanced vectors and if the latter are able to overcome the hurdles that were encountered previously.

In chapter 3 we describe the development and pre-clinical evaluation of a new infectivity enhanced vector that targets the EphA2 receptor highly expressed on PC. We utilized the peptide YSA, that was previously identified as a ligand for this receptor, for expression in the HI loop. The resulting vector Ad-YSA transduced human PC cell lines with a greater efficiency than wildtype virus. This transduction was CAR-independent and fibroblasts not expressing the EphA2 were non-permissive. Cultured endothelial cells, which express high levels of EphA2, were maximally transduced. These data, including those from the YSA peptide inhibition experiments, clearly demonstrate EphA2-mediated gene transfer. Experiments using tissue slices, however, seemed less convincing in terms of transduction efficacies. Whereas we obtained targeting indexes up to 20 fold in cultured cells, these ex vivo studies demonstrated only a maximum of 7.2 fold increase with a median of 4.1-fold. Because this vector also appeared almost twofold more efficient in normal pancreas infection the tumor specific increase of Ad-YSA seems modest (2-fold) although still significant. Therefore these data raise doubts about the efficacy and the tumor specificity of Ad-YSA and its receptor EphA2. Since there are no similar ex vivo studies comparing the increased transduction both in tumor and in adjacent (next to the tumor) normal tissue, we compared our results to the targeting index obtained in spheroids. In a study targeting both the alpha integrins and EGFR on a set of primary glioma spheroids a median increase of 5.9 fold was seen (7) In this study a combination of the RGD peptide and a bispecific antibody against EGFR was used. Hence,
the targeting potency of Ad-YSA found in such an ex-vivo system seems in line with that of Ad-RGD, a frequently used infectivity enhanced vector that targets integrins, which are expressed on normal tissues also (8). As such, Ad-YSA may therefore be a welcome addition and warrants further evaluation in the setting of replication competent Adenovirus and other solid cancers that are known to widely over express EphA2.

The somewhat lower targeting index of ex vivo studies compared with cultured cell experiments seems to reflect the actual in vivo interaction between virus vectors and host determinants. Immunohistochemical localization of reporter protein in transduced tissues revealed that infection and penetration is confined to the superficial layers of cancer specimens. This is in accordance with observations of others (4) and points out to the presence of host anatomical barriers that are present in normal and malignant tissue (9). In tumors these include extra cellular matrix (ECM) components such as matrix proteins and basement membrane. Tumor nodules are encapsulated by an extensive amount of ECM containing collagen and laminin. The presence of blood vessels is scarce. Most probably contact of metastases with blood vessels is an important factor for efficient Ad transduction in vivo. Due to these barriers, for instance, systemic application of a retargeted vector in a mouse breast cancer model resulted in transduction of only 8% of metastases (10). In a colorectal cancer mouse model, systemic injection of adenovirus did not transduce metastases in the liver. Blood vessels were confined to the tumor stroma. Therefore, a virus particle needs to pass several layers of normal cells before encountering malignant cells of a solid tumor (11). For PC this seems very relevant. One of the hallmarks of PC is the strong fibrotic/desmoplastic reaction. This probably results from a (partly defensive) host reaction to the growing tumor. This fibrotic reaction results in deposition of extracellular matrix that primarily contains collagen type I, decorin, lumican and versican (12;13). We and others believe that the presence of these anatomical barriers severely limits replication competent Adenovirus penetration (14). Therefore it seems that resolution of this extracellular matrix seems needed to increase the efficacy of vivo therapy for PC.

Treatment of target tissue with various substances such as ethanol enhances gene transfer in target tissue (15). Also pretreatment with proteases such as trypsin or collagenase/dispase did enhance the effectiveness of Ad-HSV-tk/GCV gene therapy for glioblastoma (16). These data suggest that the use of matrix metallo proteinases (MMPs) seems an option since they specifically degrade extracellular matrix components. MMPs can be organized in four main subgroups: collagenases (MMP 1, 8, 13); gelatinases (MMP 2, 9); stromelysins (MMP 3, 10, 11, 18); membrane-bound MMPs (MMP 14-17). MMP-1 and MMP-8 preferentially cleave collagen type III and I, respectively (17). Especially, MMP-8 seems an attractive candidate for modification of PC fibrosis since it is able to ameliorate experimental rat liver fibrosis (18). Insertion of the MMP-8 gene behind one of
the late promoters can be used to produce this protease in cancers upon viral replication which than will resolve collagens hindering viral spread. A potential risk of this approach seems that a protease may increase cancer metastasis. However, since MMP-8 expression does not seem to correlate with tumor size and differentiation nor with patient survival the risk for this protease seems limited (19-21). Cheng et al constructed an Ad-MMP8 to enhance lateral spread of the virus and performed co-infection with a wildtype, thus replication competent, Adenovirus in a PC mouse model (22). Indeed, intra-tumoral injection of Ad-MMP8 in combination with wildtype virus reduced tumor growth and reduced presence of collagen especially in the necrotic areas where Adenovirus replication localized. Hence, it seems certainly a promising approach to arm a CRAAd with the MMP-8 gene in order to improve viral spreading and oncolytic potency in PC.

In addition to such physical barriers an as yet underestimated characteristic of Adenovirus seems to complicate its efficient spreading capacity. A striking observation during the Ad life cycle is that after completion of production of novel virus particles, cells produce large amounts of fiber molecules for secretion. This has been suggested to be important for proper virus assembly or re-infection. A recent study determined the impact of Ad type 5 fiber protein secretion on virus propagation efficacy. This revealed that these fibers bind and mask CAR on neighboring non-infected bystander cells, thereby preventing local re-uptake and propagation of the virus (23). This mechanism, which also applies to CD46 binding Adenoviruses, would explain why Adenovirus fails to completely eradicate tumors despite ongoing viral replication. From the evolutionary standpoint this feature serves Adenovirus to escape from the initial infection site to other tissues and to allow its shedding into the environment so as to re-infect the next host.

In addition to lateral spreading through the tumor environment, expression of CAR and alternative receptors on target cells are the next major determinants of efficient gene delivery. At the start of this project our group showed that the expression of CAR was low on several human pancreatic cancer cell lines. This led to the conclusion that alternative cellular receptors such as integrins and EGFR should be targeted to obtain efficient transduction of pancreatic cancer (24). With the current knowledge we can now state that this conclusion was drawn too easily. In chapter 3 we show that PC, especially moderately and well differentiated tumors, express high levels of CAR. This is in line with the findings of others who found reduction of CAR expression in poorly differentiated PC only (25). Since poor differentiation relates to poor survival for this patient group advanced therapies such as adenoviral gene therapy using an infectivity enhanced vectors seems the only option. The presence of CAR suggests that for well differentiated tumors an Adenoviral vector with its native tropism may be effective. The majority of our specimens however showed a mixed staining pattern indicating a heterogeneous expression pattern not only of CAR but also of the alternative cellular receptor EphA2. Hence, these observations point out that a vector that can use both
CAR and an alternative receptor for cell entry may be capable to transduce the different cancer cells present in a tumor with sufficient efficiency. This indicates that for intratumoral applications introduction of a small targeting peptide in the HI-loop, that binds to an alternative cellular receptor, but does not interfere with CAR binding seems the better option.

In this thesis we identified the EphA2 receptor as a potential cellular receptor for tumor specific entry of Adeno vectors and subsequently focused on further development of this targeting strategy. When compared to normal pancreas tissue, pancreatic cancer cells and lymph node metastases express high levels of this receptor (26-28). EphA2 is a membranous receptor tyrosine kinase that is involved in regulation of cell growth, survival, angiogenesis, and migration. Various cancers over express EphA2 and higher expression levels associate with aggressive growth characteristics and poor clinical outcome. The role of EphA2 in carcinogenesis and tumor progression however is controversial since it seems to be involved both in tumor progression and in silencing. EphA2 is present in high amounts in tumor cells in a non-tyrosine-phosphorylated state and localizes to the membrane at the leading edge of invasive cancer cells. Interestingly, upon activation by ephrinA1, EphA2 induces signaling events that are more consistent with tumor suppressor functions (29). The ambivalent function of EphA2 was recently elucidated (30). In the absence of its ligand EphA2 is a substrate for the cancer promoting protein Akt, which is an oncogenic signaling molecule. EphA2 (serine) phosphorylation leads to a proliferative phenotype including cell polarization and migration. The ephrinA1 ligand stimulates phosphorylation of EphA2 at the tyrosine residues and dephosphorylation of the serine residue. Upon this change in phosphorylation EphA2 becomes a negative regulator of Akt and thus impairs the invasive phenotype of the tumor. Likewise, activation of EphA2 with a monoclonal antibody also inhibits malignant behavior (31). Initial studies pointed out that EphA2 signaling resulted in a p53-dependent induction of apoptosis but recently it was demonstrated that this receptor is capable of inducing p53-independent apoptosis (32). Binding of the peptide YSA also results in activation of EphA2 and causes tyrosine phosphorylation leading to signaling that impairs aggressive behavior (33). Therefore it is tempting to speculate about the potential of our retargeted Ad-YSA to activate this receptor since this could evoke pro apoptic signals shortly after cell entry of the Adenoviral vector. It would therefore be of interest to examine whether the YSA peptide augments oncolytic potency of a CRAd.

In addition to its role in cancer cells, EphA2 also plays a critical role in the process of tumor angiogenesis. In tumor xenografts EphA2 was present on the endothelium of tumor blood vessels where it seems to function as a pro-angiogenic protein (34). This renders EphA2 an even more attractive target for novel anticancer therapies since targeting it may also have anti-angiogenesis effects. Furthermore, the presence of EphA2 on tumor vasculature may also result
in binding of YSA targeted Ad vector upon systemic administration and in that way targets this vector to the tumor.

We performed some EphA2 stainings as described in chapter 3. In normal pancreatic tissue several cell types expressed EphA2 including islets of Langerhans, exocrine- and ductal cells, fibroblasts, nerve- and muscles cells. The EphA2 expression varied between cancer specimens with a predominant cytoplasmic staining. In some cancers a membranous staining was seen. In our study we did not specifically focus on differences in expression levels between non-malignant en malignant tissue. However, we were not able to find a clear relationship between expression of EphA2 and Ad-YSA targeting efficacy. Most probably this lack of correlation is due to heterogeneity within tumor specimens. The cytoplasmic staining was unexpected since it functions as a receptor. However, a similar expression pattern was reported by others (35). This cytoplasmic expression may also explain the lack of tumor specificity. It may be that the form expressed in these cancer cells indeed remains cytoplasmic and therefore may not be accessible for binding YSA. Since the available immunohistochemical stainings do not discriminate between the two phosphorylation (activation) states, it maybe that one of these localizes to the cytoplasm, explaining the staining in cancer cells. In any case, the outcome of our ex vivo targeting experiments with Ad-YSA does reveal a higher affinity for PC, which does suggests an increased presence of the accessible EphA2 form. Because there is no PC specific tumor marker available for co localization experiments, we were not able to determine exactly which specific cell types were transduced in our ex-vivo studies. Therefore our data do not conclusively answer the question whether EphA2 is a truly tumor specific antigen.

To study the interaction between Ad-YSA and EphA2 and to evaluate if this vector targets PC in vivo we performed a mouse study. To determine to what extent Ad-YSA targets to normal tissue including pancreas and to a human PC cancer we administered Ad-YSA systemically to nu/nu mice with a human PC tumor growing subcutaneously. To reduce liver tropism we chose to use a vector in which the native Ad binding sites were ablated. Chapter 4 describes the development of this doubly-ablated EphA2 retargeted vector (Ad/ΔF(FG)ΔP-YSA). We demonstrated that the YSA peptide was able to redirect Adenovirus from CAR to EphA2 and to transduce PC cells in vitro. Since we have already demonstrated that the cell entry of Ad YSA is mediated via the YSA peptide, it seems that EphA2 mediated the cell entry of Ad/ΔF(FG)ΔP-YSA. Because the YSA peptide is internalized upon binding, it seems possible that Ad-YSA may not be dependent on integrin binding for cell entry as is the case for wt Adenovirus (33). This may especially be relevant for doubly ablated vectors that lack the most prominent native integrin binding site.

One of the hurdles in systemic application of Adenoviral vectors to treat cancer is their liver tropism. Upon systemic administration a large part of injected dose is rapidly cleared by hepatic Kuppfer cells exerting an inflammatory reaction that may
even be fatal. Therefore, detargeting from the liver and making Ad only available for tumor transduction seems very relevant. Several methods have been tried including ablation of the CAR and integrin binding sites. Although this did lower liver transduction to some degree not all deletion mutants were effective and liver detargeting did not occur in all animal models (36). Apparently other binding sites are used upon ablation of the most prominent ones. A recent study indicated that blood factor X binds Adenovirus and allows it to transduce hepatocytes (37;38). Elimination of the Factor IX binding site had a major impact on the tropism of Adeno for hepatocytes and reduced liver uptake (38). At higher vector doses however other native receptors such as heparan sulphate proteoglycans can mediate low affinity uptake by hepatocytes (39). This underscores the need to ablate several native Adeno binding regions for effective liver detargeting.

Our results using a double ablated retargeted vector, injected intravenously or injected in the peritoneum, did not result in significant liver detargeting. This was not entirely unexpected since our doubly ablated vector originates from the first generation ablated viruses that only display a small reduction in their affinity for the liver. The Ad-/-ΔF(FG)ΔP-YSA displayed a lower transduction of the liver while transduction of subcutaneous PC xenografts was comparable to that of the doubly ablated control virus. Due to the large spread in transduction levels between individual animals, however, no significant increase in tumor/liver ration was seen for this retargeted vector.

To our surprise the doubly ablated vector was still capable of transducing the CAR negative Capan cell xenografts in this model. This suggests that other receptors, such as proteoglycans that are known to be present in PC, seem able to mediate vector uptake (40). Recent in vivo studies revealed that tumor transduction by Adenovirus also depends on vitamin K dependent coagulation factors (41). Treatment with warfarin to block these factors completely inhibited tumor transduction. In vivo, warfarin treatment also reduced transduction of liver and spleen (42). This suggests that these factors are major determinants of adenoviral entry in normal and in tumor cells in vivo. Incorporation of the RGD peptide into the HI loop was not able to rescue tumor transduction via integrins. This again suggests that blood factors primarily dictate liver and tumor transduction, which renders the role of the fiber knob less important. In the near future vectors will be generated with mutations in the hexon region that do abolish binding to blood factors. Although this will prevent liver transduction it will also affect tumor cell transduction, necessitating the presence of a high affinity peptide ligand or single chain antibody to compensate for cell entry mediated by binding to blood factors.

Our high affinity peptide ligand YSA however did not result in increased targeting to PC in vivo when compared to the doubly ablated virus. As seen with the RGD virus, this may be due to the binding of blood factors that dictate biodistribution and tumor cell entry. Alternatively, Ad-/-ΔF(FG)ΔP-YSA may have
been trapped in the tumor microenvironment or in the liver sinusoids and was subject to degradation. Also, EphA2 receptors may not be accessible in vivo. Our data therefore do not provide an answer to the questions raised about tumor specificity of the EphA2 receptor. The transduction data of the organs suggests that the YSA peptide does not have a major impact on adenovirus biodistribution. The total amount of transduction however does seem reduced when compared to the doubly ablated vector. This is rather unexpected and suggests that insertion of this peptide results in increased uptake and degradation of the vector by macrophages, or in increased clearance of particles by Kuppfer cells in the liver or, alternatively, YSA was not able to target any tissue at all. In conclusion, chapter 4 leaves several questions unanswered and but does show that PC tumors are certainly not refractory to adenovirus infection, not even to doubly ablated vector, upon intravenous administration. A human study, in which intravenous administration of Ad-p53 (Advexin) in patients with advanced cancer led to transduction of tumor lesions in 86% of patients, seems to support our findings (43). Most likely virus propagation rather than initial tumor cell transduction is the rate limiting step in CRAd cancer gene therapy.

The aforementioned chapters have described several limitations of Adenovirus vectors for efficient propagation in tumor environment. To overcome these hurdles several steps of genetic engineering need to be performed including ablation of several binding sites and expanding tropism, which may eventually result in a highly truncated virus that has disturbed oncolytic potency. Therefore Adenovirus subtypes with natural tropism for tumor cells may be much more useful for PC gene therapy. In chapter 5 we describe the development of vectors with fiber chimera carrying fiber knobs from subgroup B. We show that Ad5.fib16 and Ad5.fib50 have excellent tropism for PC compared to normal human pancreas and hepatocytes, suggesting a favourable “tumor on/liver off” phenotype in vitro and ex vivo. In order to evaluate the potency of these fiber chimera, in vivo studies need to be initiated using replication competent Ad5.fib16 to infect PC xenografts. Ad16 almost exclusively utilizes CD46 as a cellular receptor (44). Since this receptor is upregulated on various tumors including PC it seems an attractive target. We did however not examine whether every single tumor cell expresses CD46. If non-permissive CD46 negative cells prevail, it may be an option to alter tropism of a subgroup B virus. Matsui et al (45) showed that the fiber knob of Ad35 subgroup B virus is permissive for incorporation of a foreign peptide into the HI loop. This approach has been used in vivo as well (46). Subgroup B viruses display features, such as lower seroprevalence and liver tropism, that make them outperform their subgroup C (Ad5) family members. In addition, the utilization of serotypes with natural tropism for human cancers would safe elaborate genetic engineering to alter tropism of other serotypes. In case of uniform CD46 expression in and among PC and proven infectivity enhancement in vivo, Ad5.fib16 would be the vector of choice compared to Ad-YSA.
Pancreatic cancer continues to have a bad prognosis requiring novel interventions. It is ironic to note that during the preparation of this thesis, instead of a novel virus, a 13 year old virus inhibitor nelfinavir (in combination with chemoradiotherapy) surprisingly induced several partial responses, which made the patients eligible for surgical resection (47).

The concept of infectivity enhancement of Adenoviral vectors will need to prove itself in upcoming clinical trials utilizing intratumoral administered RGDCOX2CRAdF and 5/3COX2CRAdF for PC. In case of proven safety, one or both agents will likely be combined with surgical resection, followed by perioperative virus administration in the tumor region, and concurrent chemoradiotherapy given its reported synergistic anti tumor effects (48;49). Metastases are likely to be approached by intratumoral injection and if not feasible, via intra-arterial administration (50). Eventually the best intervention for PC will consist of multimodality treatment.
REFERENCE LIST


