Adenovirus targeting for gene therapy of pancreatic cancer
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SUMMARY AND CONCLUSIONS

Patients suffering from pancreatic cancer have a poor prognosis. Particularly due to late discovery of disease, 5 years after diagnosis only 5% of the patients is still alive. Surgical resection of the tumor is the only chance of survival but is only available for 20% of pancreatic cancer patients. Resection of the tumor including duodenum and part of the biliary system (pancreaticoduodenectomy, Whipple’s operation) is not a guarantee for survival. The 5 year survival after this operation is 15 to 20%. For patients that are not eligible for surgical resection palliative chemotherapy is available, but gemcitabine only offers relieve of complaints. The effect on survival is subject to discussion but probably is minimal. In order to treat the majority of pancreatic cancer patients more effectively new therapies such as gene therapy are being developed.

Aggressive forms of gene therapy such as virotherapy are promising. Virotherapy makes use of the lytic life cycle of virus. Following infection virus particles use the host cell machinery to amplify. After replication the cell lyses thereby releasing an amplified amount of virus particles. These are able to infect neighboring cells and start a new lytic cycle. Oncolytic viruses replicate efficiently in tumor cells while their replication is blocked or at least strongly impaired in normal cells. Theoretically the replication of an oncolytic virus in a tumor will continue until total eradication of all malignant cells.

Human Adenovirus (Ad) is a frequently used virus for virotherapy. Initially wt virus, that is able to replicate in normal cells, was used. To increase safety and specificity genetic engineering of the genome was applied to generate virus with tumor specific replication, called conditionally replicating Adenoviral vectors (CRAds). Onyx-015 and delta24 are the most prominent CRAds generated. These were designed to replicate specifically in tumors harboring mutations in the p53 and retinoblastoma tumor-suppressor-proteins, respectively. Defects in these genes are present in the majority in human cancers including pancreatic cancer. Both Onyx-015 and delta24 proved to be effective in killing pancreatic cancer cells in pre-clinical in vitro and in vivo models. However the results of two clinical trials in which pancreatic cancer patients were treated with Onyx-015 were disappointing. No virus replication or cell death was observed following intratumoral injection. Additional studies proposed this resulted from low expression of the primary cellular receptor for Adenovirus, the Coxsackie- and Adenovirus receptor. Low expression would complicate transduction and thus hamper the replication and spread in the tumor rendering it refractory to Ad. One possibility to circumvent this problem is the usage of alternative cellular receptors on tumor cells. Retargeting CRAd to receptors such as integrins, which are expressed at high levels on cancer cells, enhances tumor cell transduction.

Integrins are internalizing membrane proteins that, after Ad binds to CAR, mediate virus uptake into the cell. The RGD motif that localizes to the hexon
protein of Ad is crucial for its binding to integrins. By incorporating the RGD peptide in the protruding fiber knob of Ad, Ad-RGD binds directly to integrins resulting in CAR-independent infection of tumor cells. Since these proteins are also present on non-malignant cells, targeting them will not be tumor specific enough. For this reason several research groups endeavor targeting tumor specific antigens to enhance transduction specificity.

In this study we aimed to develop an Adenoviral vector that targets pancreatic cancer-specific antigens. In addition we aimed to develop a new model that better predicts the clinical activity of the novel recombinant Ad than current pre-clinical models.

Chapter 2 describes the development of an ex vivo system to evaluate the efficacy of recombinant Ad in primary human pancreatic tumors. A tissue slicer was used to process freshly isolated pancreatic cancer specimens to 'tissue slices'. In this model tumor tissue architecture is preserved in its native state, including vasculature and extracellular matrix components. This type of culture has obvious benefits because these features can not be easily modeled. Therefore it is expected to more reliably predict clinical efficacy of Ad vectors compared to existing preclinical models. Using this slicer also allowed culturing of freshly obtained normal human pancreas tissue slices that served for control experiments. Pancreatic tissue slices could be cultured for up to 3 days with preservation of viability and morphology. This period is sufficiently long for performing transduction experiments with several viral vectors. To correct for slice viability and size, co-infections with a second reporter virus were performed. Immunohistochemical analysis following transduction of tissue slices revealed that only the peripheral cell layers were transduced by Ad. This finding clearly points out to the presence of physical barriers in tissues that will limit virus penetration in tumors, such as extracellular matrix components. The tissue slice system proved to be a good method to process minimal amounts of fresh pancreatic tissue to perform ex vivo transduction experiments.

Chapter 3 describes the selection of several pancreatic cancer specific antigens (EphA2, Thomsen Friedenreich antigen, neurotensin receptor, VEGFR-II) as potential alternative cellular receptors for targeting with recombinant Ad vectors. Genetic engineering was applied to incorporate peptide ligands for each of these receptors into the fiber knob of Ad. The capability to transduce pancreatic cancer cells via the aforementioned antigens was determined for all targeted vectors, Ad-YSA/SWL, Ad-p30, Ad-NT, Ad-K237 respectively. Of these candidates Ad-p30 could not be produced most likely because of interference of the p-30 peptide with fiber knob trimerization. Ad-NT could be grown to high titers but did not target the neurotensin receptor. Ad-K237, targeted to the VEGFR-II, infects pancreatic cancer cell lines 4 to 5-fold more efficient than wildtype virus. Two different ligands, YSA and SWL, were used to target the EphA2 receptor with high affinity. Ad-YSA enhanced pancreatic cancer cell transduction 7 to 20 fold
while EhpA2 negative fibroblasts were not infected. This points out the tumor specific transduction by Ad-YSA. The transduction of Ad-SWL appeared less specific. Based on these experiments we selected Ad-YSA for targeting pancreatic cancer slices in our ex vivo system. Transduction studies in slices obtained from 12 patients revealed that compared to wt virus, Ad-YSA transduces pancreatic cancer 4.1 fold more efficient. The targeting efficacy however varied extensively between specimens. Most likely this is due to heterogeneous expression of CAR and EphA2 in tumor tissues. This research revealed that pancreatic cancers do express CAR and therefore suggests that alternative factors such as extracellular matrix components are critical determinants of effective tumor transduction. We detected the EphA2 receptor both on cancer cells and non-malignant cells. However, these immunohistochemical studies did not discriminate between the activated, present in cancer, and the non-activated form of EphA2. From the effects seen on transduction we could conclude that the novel recombinant Ad-YSA does target pancreatic cancer via the EphA2 receptor. In addition to decreased CAR expression more factors influence the efficacy of adenoviral gene therapy for pancreatic cancer. The incorporation of a receptor-binding peptide into the fiber knob therefore is only one of the solutions for more efficacious tumor transduction and will have to be accompanied with additional virus modifications. Because of the presence of EphA2 receptor on various malignant tumors and tumor endothelium, the Ad-YSA vector seems to be a good candidate to target pancreatic cancer and other malignancies.

In chapter 4 we describe the in vivo distribution profile of Ad-YSA and its potency to target pancreatic cancer in vivo. In addition to incorporating YSA into the fiber knob both native receptor binding domains of our vector were ablated. Presence of these in Ad-YSA allowed entry of this vector via CAR and integrins. In vitro transduction of pancreatic cancer cells by the resulting doubly ablated and retargeted virus Ad-/ΔF(FG)ΔP-YSA was 4.5 to 7.6 fold increased compared to the non targeted vector. Synthetic YSA peptide inhibited this increased transduction pointing out to EphA2-mediated cell entry of this vector. Subsequently, we administered Ad-/ΔF(FG)ΔP-YSA intravenously and intraperitoneally to immunodeficient mice on which subcutaneous human pancreatic cancers were grown. Incorporation of the YSA peptide did not target healthy tissues. Compared to the control virus (Ad-/ΔF(FG)ΔP) only 15% of reporter protein production in the liver was found upon Ad-/ΔF(FG)ΔP-YSA infection. This suggests increased degradation of Ad-/ΔF(FG)ΔP-YSA. Possibly, ablation of native binding sites in combination with the incorporation of the YSA peptide resulted in higher uptake and degradation by macrophages. This may have occurred via a specific uptake of virus particles. Anyhow, Ad-/ΔF(FG)ΔP-YSA did not target pancreatic cancer in vivo. The ablated Ad-/ΔF(FG)ΔP, however, was able to transduce pancreatic cancer in vivo, which suggests CAR and/or integrin-independent uptake. Possibly, heparan sulphate proteoglycans or binding to blood factors mediate its tumor
transduction. From this study we conclude that Ad-YSA does not target healthy tissues but that for better tumor transduction additional capsid modifications need to be implemented.

Chapter 5 describes an alternative method to target pancreatic cancer. We developed fiber chimeras by replacing the fibers of Ad type 5 for those of other Ad serotypes. A library screen using Ad vectors based on Ad5 and carrying fiber molecules from 16 other serotypes revealed that Adenoviruses containing fibers from serotype 16 and 50 showed increased gene transfer in pancreatic cancer cells. We proceeded with these two chimeras and implemented them in a gene-directed prodrug activation system using cytosine deaminase. This showed both were effective in eradicating primary pancreatic tumor cells. Subsequently transduction efficacy of primary pancreatic cancer by Ad-5/16 was evaluated in our novel ex-vivo model system. Analogous to the in vitro experiments, the studies with tissue slices revealed enhanced transduction of pancreatic cancer, while infection of normal pancreatic tissue and human hepatocytes was diminished. As seen with Ad-YSA previously, the transduction efficacies in tissue slices were lower compared to those in cultured cells in vitro. In conclusion, we advocate that Ad-5/16 is a promising vector to target pancreatic cancer specifically while leaving normal tissue unharmed.