Experimental treatment modalities for osteosarcoma
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Summary and Discussion
9.1 Summary

The goal of this thesis was to explore novel treatment modalities for high-grade osteosarcoma. As described in chapter 1, osteosarcoma patients have an overall 5-year survival rate of 50-70% when treated with a combination of surgery and multi-agent chemotherapy. Osteosarcoma patients presenting with metastases have long-term survival rates of 10-30%. Complete surgical removal of metastases is essential, because hardly any patient survives without complete surgical removal of their metastases. In spite of several clinical trials combining different chemotherapeutics and some new treatment modalities the 5-year survival rate has not increased over the last two decades. Clearly, new treatment modalities for osteosarcoma are needed. That virotherapy could be of use for the treatment of osteosarcoma was supported by the finding that the intratumorally injected Ad5-Δ24RGD, designed to selectively replicate in tumor cells with disrupted pRb-pathway, caused significant osteosarcoma tumor growth regression in mice (1). The work presented in this thesis evaluated experimental treatment modalities like virotherapy and the small-molecule Nutlin for the treatment of osteosarcoma and its lung metastases.

In chapter 2 we discuss a hurdle for effective virotherapy with conditionally replicative adenoviruses (CRAds), i.e., adenovirus binding to osteosarcoma cells. This is hampered by low to absent levels of the native adenovirus receptor (CAR) on osteosarcoma cells. Although others have suggested the use of adenovirus with native binding capacity for osteosarcoma treatment, we advocate the use of retargeted adenoviruses to guarantee efficient tumor cell infection. In this chapter we show that a CRAd with expanded tropism towards integrins, broadly expressed on primary osteosarcoma tumor cells is more effective than virus with native tropism.

It is to be expected that new treatment modalities such as adenoviral therapy will be tested in the clinic in combination with conventional therapies. For osteosarcoma, doxorubicin and cisplatin are widely used chemotherapeutic agents. Promising results have been obtained in preclinical experiments that demonstrated synergy between adenoviral therapy and chemotherapeutic agents like doxorubicin and cisplatin. In chapter 3 we show that combination of the Ad5-Δ24RGD with doxorubicin or cisplatin indeed resulted in synergistic cell kill on osteosarcoma cell lines. Strikingly, on osteosarcoma primary cells such a synergistic effect was not observed. In contrast, chemotherapeutic agents reduced CRAd
mediated cell killing. Importantly, the virus did not reduce the effect of chemotherapy. The adverse combination effect on primary osteosarcoma cells was linked to slow population-doubling time, reduced viral replication and absence of chemotherapy induced G2 phase cell-cycle arrest. Based on these results we propose to use chemotherapeutic agents and CRAd therapy in intermittent administration schemes to obtain maximal anti-tumor effect.

Next to adenoviral cell entry, cell killing potency of the CRAd is of importance. Clinical trials with adenoviral vectors for the treatment of different cancer types and its metastases have indicated relative safety but modest clinical effect, urging the development of more potent adenoviral therapy approaches (2-5). In chapter 4 we evaluated the effect of combining a small-molecule antagonist of MDM2 (Nutlin) with the p53-expressing adenoviral vector Adp53, with the CRAd AdΔ24, or its p53-expressing derivative AdΔ24-p53 on cell killing properties and adenoviral replication. Combination of Adp53 and Nutlin increased cell kill on p53-wild type and p53-deficient tumor cells. Moreover, cell-killing properties of CRAds increased up to 1,000-fold when combined with Nutlin. Enhanced cell kill correlated with accelerated viral progeny burst. These findings suggest that combination of oncolytic adenoviruses and Nutlin is promising to treat osteosarcoma.

As mentioned before, osteosarcoma metastases (usually confined to the lungs) predict a poor prognosis. This warrants a systemic approach. Therefore, in the last 4 chapters we focus on systemic delivery of adenoviral vectors and the limitations encountered. As a proof of principle we injected, in, Ad5-Δ24RGD intravenously into mice bearing human osteosarcoma lung metastases (chapter 5). It appeared that repeated intravenous administration of this CRAd resulted in a significant decrease in tumor mass and a reduced number of osteosarcoma lung metastases. However, lung metastases were not completely eradicated.

An approach to develop a more potent CRAd is via insertion of toxic transgenes in the adenoviral genome. A key feature herein is that toxic transgene expression should be confined to cancer cells, especially relevant for systemic administration where adenovirus could also infect normal cells. Genes under control of the major late promoter would theoretically be expressed only during viral replication. Since CRAds exhibit cancer cell-specific replication properties, replication dependent expression should be cancer cell-specific. To test this, we constructed the CRAd AdΔ24.SA-Luc as shown in chapter 6. This CRAd carries a luciferase gene regulated by the major late promoter through alternative splicing via an
inserted splice-acceptor (SA) site. We observed high level and replication-dependent transgene expression in cancer cells. However, transgene expression was also present, albeit at a much lower level, early in the adenoviral replication cycle, suggesting that this approach would be more suited for transgenes requiring high expression levels than for highly toxic transgenes.

Another way to protect normal tissues from systemic CRAd delivery is detargeting, i.e., deleting native binding sites from the viral capsid. Ablation of CAR and integrin binding sites reduces infection of non-targeted cells, thereby possibly reducing toxicity and increasing the viral dose available for tumor cell infection. Therefore, we constructed a CAR- and integrin-binding deficient CRAd targeted towards the epidermal growth factor receptor (EGFR), which is abundantly expressed on primary osteosarcoma cells (chapter 7). This newly developed CRAd showed a highly selective targeting profile for EGFR-positive tumor cells. Ablation of CAR and integrin binding-sites resulted in decreased infection of human liver as assessed on ex-vivo human liver slices. However, human liver cells were found to express EGFR. Therefore, EGFR-targeted CRAds appear not more suitable for systemic administration than a native CRAd. Importantly, our CRAd design allows relatively simple exchange of binding specificity. Furthermore, development awaits identification of more stringent cancer-specific targeting molecules.

The adenovirus type 5 capsid further carries a binding site for heparin sulphate glycosaminoglycans (HSG). As reported in chapter 8 we constructed a new virus with ablated tropism for CAR, integrins and HSG. To this end, the adenovirus fiber was replaced by a chimeric molecule comprising the fiber tail domain and the reovirus σ1 oligomerization domain. The integrin-binding motif in the penton base was mutated and a polyhistidine tag was added as a model targeting moiety. In comparison to the control virus, the new virus showed a 10,000-fold reduced transduction of mouse liver cells in freshly isolated liver slices. Intravenous administration of this virus resulted in a significant reduction in liver transduction and increased blood persistence. This virus thus represents a prototype of a useful adenovirus design for systemic delivery applications.
9.2 Discussion

The observation that naturally occurring viruses caused tumor regression prompted research on virotherapy for cancer. Safety was a major issue and till the discovery of recombinant DNA technology that allowed genetically engineering of viruses, little progress was made. Clinical trials for oncolytic viral therapies are predominantly performed with the DNA viruses: adenovirus, herpes simplex virus type one and vaccinia virus; and RNA viruses: reovirus and Newcastle disease virus (6). Advantages of adenovirus are: high achievable virus titers, relatively easy genetic manipulation and a mild virulence pattern of wild-type adenovirus. That safety features are a major concern for clinical applicability of adenoviral therapy is illustrated by the death of a patient treated for ornithine transcarbamylase deficiency with replication-defective adenovirus. Most likely his death resulted from adenovirus induced massive cytokine release inducing disseminated intravascular coagulation and multiorgan failure (7). The immunologic response to adenoviruses might pose problems, but considerable safety data has accumulated from several clinical trials.

The great challenge for treating osteosarcoma patients is to cure them from metastases. Especially for patients with non-operable osteosarcoma recurrence (5-year survival: 0%) new treatment options are needed. To be successful, the anticancer agent should reach and kill all metastases and is therefore likely to be administered systemically. Relatively few adenoviral particles administered this way will reach the cells of interest because of physical barriers in the body and nonspecific uptake by other cells. Conditionally replicative adenoviruses, in contrast to non-replicating adenoviruses, are capable of amplifying the viral input dose and are therefore promising for systemic treatment. The vast majority of clinical trials for oncolytic adenoviral therapy tested the utility of ONYX-015 (8). Although these trials showed relative safety of the adenoviral vector, minimal anticancer response was observed. Efficacy was increased by combination with chemotherapeutic agents that resulted in objective tumor response in some cases (9, 10). Failure to prove clinical effect challenged development of more potent replicating adenoviral vectors. Recent data suggests that the AdΔ24 CRAd is more potent than ONYX-015 making it appealing to test this CRAd in future clinical trials (11, 12). It is likely that gene therapy will not be applied as a single treatment, but will be combined with established therapies. Strikingly, our in vitro experiments suggested that combination with cisplatin and doxorubicin is best
not administered simultaneously. This in contrast to combination with Nutlin that showed increased cell kill when combined. These approaches, sequential administration of chemotherapy and virus or a combination of Nutlin and virus should be explored in an in vivo setting to assess effectiveness. Combination experiments might reveal if adenoviral tumor penetration and subsequent infectivity is increased and the role of tumor matrix as a possible infection barrier is decreased.

A concern for adenoviral osteosarcoma treatment is the low to absent CAR expression on osteosarcoma cells making these cancer cells refractory to adenovirus infection (13, 14). To infect osteosarcoma cells and minimize infection of normal cells, CRAds with altered tropism should be engineered. Several strategies to retarget adenoviral vectors have been employed. Two different approaches were used in this thesis. One has an integrated RGD-motif in the adenoviral fiber knob and the other approach uses a bi-specific antibody produced by the adenovirus itself to achieve tumor cell targeting. The bi-specific antibody approach has the theoretical advantage of the versatility of this technique to switch antibodies for different tumor types. However, this two-step targeting procedure might be less effective in systemic administration applications. Following injection into the blood stream relatively few viral particles will infect tumor cells with subsequent low levels of antibody production. It remains to be investigated if this will be significant to support intratumoral virus spread. In this regard, adenoviruses with genetically modified capsid proteins might prove to be more efficient.

Besides targeting viral particles to specific tumor receptors, ablation of native receptor binding sites seems essential, because of the adenovirus promiscuous tropism. More than 90% of systemically administered adenovirus transduces the liver (15-18). Even following local delivery of adenoviral particles to solid tumors a significant amount of virus is detected in the liver (19). CAR binding site ablation did not substantially change biodistribution or hepatotoxicity of systemically delivered adenovirus (18). Ablation of CAR as well as αv-integrin binding sites was found necessary to reduce liver transduction (more than 700-fold) (15). Based on these observations we constructed a CAR and integrin binding ablated CRAd with tropism for the epidermal growth factor receptor (AdΔ24P*F*-425S11). In a pilot experiment, we injected athymic nude mice intravenously with this CRAd with or without precomplexation with the bispecific EGFR-specific antibody 425-S11 and control mice with the parental CRAd AdΔ24-425S11 (that has native plus EGFR-
Specific tropism) with or without 425-S11 precomplexation. Unfortunately, viral genome copy numbers as analyzed by quantitative PCR analysis in mouse liver did not differ between the four treatment groups (data not shown). Furthermore, another study showed that CAR and αvβ3 and αvβ5 integrin expression does not correlate with vector distribution after systemic adenovirus delivery (20). These findings put the discussion of the pivotal role of receptor specific uptake via CAR and integrins and the need for binding site ablation in a different perspective. However, vectors with abolished tropism for CAR, integrins and heparin sulphate glycosaminoglycans (the latter by replacing the fiber shaft domain with that derived of Ad serotype 35) resulted in a spectacular 15,000-30,000-fold decrease of liver transduction. Moreover, coagulation factors (factor VII, IX and X), protein C and complement component C4BP have been shown to play an important role for hepatocyte transduction with adenovirus serotype 5 (21, 22). These binding affinities are likely to be located in the fiber knob domain as demonstrated for coagulation factor IX. Pretreatment with the anticoagulant warfarin resulted in diminished liver transduction rescued by adding the coagulation factor X (22). In chapter 8 we report the construction of an adenoviral vector that lacks all known binding sites. This vector was made by replacing the adenovirus fiber with a chimeric molecule consisting of the fiber tail domain and the reovirus spike trimerization domain and by mutating the penton base RGD motif. Removal of the fiber knob eliminated putative binding sites for blood factors (VII, IX and X), protein C and complement component C4BP. The new virus indeed exhibited strongly reduced binding to liver cells and human erythrocytes. In addition, in vivo biodistribution was improved although liver detargeting was only 6-fold. Interestingly, transduction of the lungs by the new vector was undetectable. Effective lung detargeting is an attractive feature for the treatment of osteosarcoma lung metastases. Future experiments should focus on retargeting the new adenovirus to specific receptors on osteosarcoma cells or tumor vasculature. In general, the effect of abolishing native tropism of the adenovirus seems to hold great promise but the question if this detargeting effect is maintained when combined with adequate tumor retargeting is so far unanswered.

A different and complementing approach to improve adenovirus biodistribution and reduce toxic side effects is based on diminishing non-specific (non-receptor mediated) adenoviral uptake by liver and other organs. This will extend the half-life of the adenovirus in the bloodstream, resulting in more adenovirus available for infecting target cells. Several approaches to overcome non-specific uptake of
the adenoviruses have been tested, including pre-treatment with 6% hestastarch and GdCl₃ to deplete Kupffer cells. This resulted in prolonged bloodstream persistence and (less than 10-fold) reduced uptake by liver and other organs (23, 24). Even without the above-described improvements, CRAds have achieved systemic anticancer effects in animal models. For example, the CRAd Ad-OC-E1a, carrying the essential adenoviral gene E1a under the osteocalcin promoter has shown encouraging results for the treatment of osteosaroma lung metastases in vivo (25). We found that infectivity-enhanced, but not native binding site ablated, Ad5-Δ24RGD was capable of inducing regression of osteosarcoma primary subcutaneous tumors and osteosarcoma lung metastases in vivo (1) (osteosarcoma lung metastases; chapter 5 of this thesis). This CRAd is designed to selectively replicate in cancer cells with dysfunctional pRb control. It has been reported that this mutation still allows some replication in normal cells and tissues like keratinocytes, normal brain explants and hepatocytes tested in vitro (26-28). A recent report stated that intravenously delivered Ad5-Δ24RGD in cotton rats caused substantial hepatic toxicity at a dose of 1 x 10¹¹ viral particles but not at 1 x 10⁸ viral particles (29). Safety data in humans are not yet available. Recently, a clinical trial treating ovarian cancer with this virus was started and a clinical trial for the treatment of glioblastoma is planned for the near future (30). Safety data on loco-regional administration from these studies will be important to better predict feasibility of systemic or loco-regional delivery of Ad5-Δ24RGD to treat metastatic osteosarcoma.

Systemic administration will be the most suited for diffuse metastasized disease. However, the majority of osteosarcoma metastases are located in the lungs. This allows loco-regional application methods such as isolated lung perfusion and aerosol inhalation. These routes of administration could allow high doses of adenoviral vectors with presumed fewer side effects, because liver and other organs are bypassed. If these administration routes are safe for lung and upper airway tissues, in case of inhalation therapy, and show significant anti-tumor effects awaits experimental verification. A potential disadvantage of inhalation therapy is spread of the adenoviral vector to the environment via exhalation. Furthermore it is speculated that via this inhalation technique genetically modified adenoviral vectors might encounter wild-type adenoviruses. It is hypothesized that this might lead to recombination of these modified adenoviruses with wild-type adenoviruses.
All adjustments to the procedures and modifications of the virus to increase safety should outweigh the potentially associated reduction in treatment efficacy. Careful experimental evaluation is needed. However, these efforts should not slow down the process of developing the current generation of promising CRAds like Ad5-Δ24RGD for clinical applicability.

**Perspectives**

We have shown that Ad5-Δ24RGD is capable of inducing tumor regression of primary subcutaneous OS tumors and pulmonary osteosarcoma metastases in preclinical models. The next step is to optimize the treatment of lung metastases bearing mice with Ad5-Δ24RGD with or without chemotherapy (in intermittent administration schemes). Prolonged administration of intravenous Ad5-Δ24RGD with or without chemotherapy in a survival experiment will elucidate if this treatment results in prolonged survival. If prolonged survival is observed one might proceed by evaluating the treatment in a clinical trial. An advantage is that by that time safety data of this particular virus will have been collected by two clinical trials planned for the near future (30). One of these trials in planned in the Netherlands. Legislation demands strict application rules to prevent genetically modified agents to be released into the environment. When this hurdle has been taken and application is regarded safe, other trials with replicating agents are more likely to follow. A major requirement to successfully initiate a clinical trial with Ad5-Δ24RGD for osteosarcoma is collaboration with other institutes to reach adequate numbers of osteosarcoma patients. Most likely patients with (non-operable) recurrent osteosarcoma, given their poor prognosis, are included first. Besides trying to reach the clinic with Ad5-Δ24RGD for osteosarcoma metastases preclinical testing should continue in order to develop more potent adenoviral therapeutic regimens. So far, systemically administered CRAds to combat metastatic cancer have shown modest local efficacy in recent clinical trials (3, 5, 10).

Enhanced delivery to tumor cells, in our case predominantly lung metastases, can be achieved via detargeting strategies or different administration routes by simply bypassing scavenger sites. Therefore isolated lung perfusion to increase therapeutic efficacy is attractive and certainly to be tested in the context of oncolytic viral therapy. Preclinical studies should reveal if lung tumor nodules are efficiently killed alongside with limited toxicity to normal lung tissue. Ad5-Δ24RGD is the vector of choice because native ablated adenoviral vectors as
shown in chapter 7 exhibited reduced cell killing properties without guaranteed reduced toxicity. In case of diffuse metastatic disease where isolated lung perfusion is not feasible, detargeting strategies could reveal their potential.

An exciting avenue that is worth exploring is to use certain cells types with intrinsic tumor targeting properties as carriers for the virus. In elegant experiments, antigen-specific T-cells have been used to deliver viruses encoding cytokines or suicide genes to sites of metastasis (31). Recently, naïve T-cells have been successfully employed to target oncolytic vesicular stomatitis virus to lymph nodes harboring metastatic cells (32). These methods are well suited for metastatic disease and obviate the need for high titer injection of the virus in the bloodstream. This warrants exploring whether T-cells can be used as “homing devices” in the context of the treatment of osteosarcoma metastatic disease using Ad5-∆24RGD.

References


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