Chapter 9

General discussion and future perspectives
INTRODUCTION

Agents targeting neovascularization are well established in the treatment of cancer. In the vast majority these agents are used in the advanced setting in combination with chemotherapy. Although there is much ongoing research exploring new targets that are specific for pathological angiogenesis, currently most approved therapies target the pivotal VEGF-VEGFR pathway. Usually, the benefit of anti-angiogenic therapy in the oncology arena is limited to only a few months addition in progression free survival. In addition, only a small subset of patients respond to therapy and adaptive resistance often develops after a few months of treatment. Monoclonal antibody therapy is given with high frequency, which is burdensome for patients. To circumvent these disadvantages and to maximize benefit of anti-angiogenic therapy alternative strategies are being developed and novel drug combinations are being investigated. This thesis has focused on the development of a therapeutic vaccine targeting VEGF in an attempt to improve currently applied anti-VEGF therapies. The main advantages of this approach include (i) sustained and stable suppression of VEGF, (ii) reduction of hospital visits, (iii) potential of enhanced activity of induced polyclonal antibodies. In addition, we addressed combination strategies targeting angiogenesis with other treatment modalities important in the treatment of cancer.

Part I – Immunotherapy approach to VEGF inhibition

Part I of this thesis is dedicated to combating the process of angiogenesis through immunotherapy, with the focus on a therapeutic vaccine targeting the pro-angiogenic growth factor VEGF. In addition, a biomarker study was performed with the aim to predict efficacy of active- and passive immunotherapy to inhibit VEGF.

The thesis started with an overview of previously described vaccination strategies targeting proteins or cells associated with angiogenesis (Chapter 2). In this review we show that there is a plethora of vascular proteins investigated for immunotherapeutic targeting. In addition, there are studies describing vaccines using and targeting directly the cells important for angiogenesis (i.e. HUVECs). These strategies aim to improve the outcomes of currently clinically applied anti-angiogenic therapies and cancer vaccines targeting tumor cell antigens. Both these treatment modalities have important disadvantages which an active immunotherapeutic approach targeting vascular antigens could circumvent. Such an approach combines the advantages of targeting easily accessible vascular antigens, and active immunotherapy. The vaccine could be administered at much lower frequency and it utilizes the interesting relationship between the systems of angiogenesis and immunity.

We showed that there is an abundance of pre-clinical studies investigating anti-angiogenic vaccination strategies. However, the majority of these studies focused on soluble pro-
angiogenic factors and their respective receptors and in particular the VEGF pathway as target to induce angiostasis through immunotherapy. We discussed the promise of this rather novel treatment strategy and encouraged the clinical development of anti-angiogenic vaccines. Since publication of the review more encouraging clinical data on anti-angiogenic vaccination strategies were reported. Wagner et al showed the results of a pilot clinical study investigating the safety of ValloVax, a vaccine consisting of placenta-derived endothelial cells (1). They reported three patients with cancer whom received ValloVax. Vaccination was well tolerated and serological responses against several angiogenic proteins were observed. In addition, more studies were published on the vaccine CIGB-247, developed by the group of Gavilondo. They showed that off-trial continued vaccination in surviving patients was safe and that the induced immunological responses were long lasting (2,3). A pilot study of peptide vaccines for VEGFR1 and VEGFR2 in patients with high grade glioma showed promising immunogenicity along with good tolerability (4). Finally, a phase I dose-escalation trial studying the safety and immunogenicity of VXM01, an oral T-cell vaccine targeting VEGFR2, reported an encouraging safety profile along with vaccine specific T effector cell responses in patients with advanced pancreatic cancer (5,6). Remaining challenges in the field of anti-angiogenic vaccination strategies are (i) the requirement of a powerful adjuvant to boost the immune response against self-antigens, (ii) the identification of targets that are solely expressed in the proximity of the tumor and (iii) the search for biomarkers that could predict treatment efficacy. In addition, it is to be expected that anti-angiogenic vaccines should be administered as a combined vaccine targeting multiple vascular antigens simultaneously and/or together with other treatment modalities, such as radiotherapy and immune checkpoint inhibition in order to achieve maximum clinical benefit.

We developed a therapeutic vaccine targeting proangiogenic growth factor VEGF, named hVEGF_{26-104}/RFASE. In contrast to other vaccination strategies, this peptide based vaccine stands out in particular because it is designed to perfectly mimic the 3D bevacizumab binding site on VEGF which ensures the induction of neutralizing antibodies. In addition, there is no need for coupling to a carrier protein in order to break immune self-tolerance and induce high titers of neutralizing antibodies. In this thesis the preclinical development of hVEGF_{26-104}/RFASE is described, with in-vitro studies as well as murine and rat studies in chapter 3, and a large non-human primates safety and immunogenicity study in chapter 4.

In chapter 3 we showed that correct peptide design of VEGF derived peptides is vital for the induction of neutralizing cross-reactive antibodies against endogenous VEGF. Several peptides were constructed with large variations in design complexity. Since the monoclonal antibody bevacizumab is able to biologically neutralize VEGF, a peptide was constructed which perfectly mimics the discontinuous binding site of bevacizumab on VEGF (peptide
1; ox-hVEGF

We showed that this particular peptide was able to bind bevacizumab, compete for the binding of bevacizumab to the endogenous protein hVEGF

and inhibit VEGF driven proliferation of Ba/F3-VEGFR2 cells in a bioassay. Other variants of peptide 1, which were either linear in structure, only exhibited the β5-turn-β6 loop of VEGF or lacked the cysteine-knot structure which is crucial for 3D protein conformation of VEGF were able to induce high titers of cross-reactive antibodies in varying degrees, however these antibodies appeared to be non-neutralizing as evidenced by competition ELISA studies and Ba/F3-VEGFR2 bioassays. In vivo studies revealed that only immunization with peptide 1 resulted in the induction of cross-reactive neutralizing antibodies against hVEGF with potent anti-tumor activity. In contrast immunization with peptides derived of the β5-turn-β6 loop or a peptide lacking SS-bonds important for cysteine-knot formation did not result in tumor growth inhibition. These data clearly show that the structure of a peptide for vaccination purposes is essential for success.

The safety and immunogenicity of vaccination with peptide 1 (from here on referred to as hVEGF

), which was administered together with RFASE adjuvant in cynomolgus macaques, is reported in chapter 4. We found that immunizations, which were given in doses that were to be used in the clinical study, were well tolerated and devoid of adverse events that have been reported with VEGF suppression (i.e. proteinuria, hypertension, increased bleeding tendency and venous thromboembolic events). Importantly, cross-reacting antibodies were detected in all but one animals that received hVEGF

 together with RFASE adjuvant. As it was the case with the earlier pre-clinical studies, no decrease in serum VEGF levels were noted after immunization with hVEGF

/RFASE in this study. This could possibly be due to the formation of hVEGF

 – antibody immune complexes, which reduces the ability of the antibodies to compete for the binding of VEGF present in the serum to the capture antibody in the ELISA. However, the antibodies do appear to have proper VEGF neutralizing properties, since they compete well with bevacizumab for binding to VEGF and inhibit the VEGF driven Ba/F3-VEGFR2 cell proliferation. Although some evidence was found for cellular immunity induced by hVEGF

/RFASE, the main mechanism of action of the vaccine is thought to be the induction of humoral immunity.

Treatment efficacy of bevacizumab is extremely difficult to predict and thus far a reliable biomarker is lacking. Although there are some reports, showing a relationship between the blood bevacizumab concentration, treatment efficacy (7) and adverse events (8), pharmacokinetics are currently not being used to guide therapy. In chapter 5 we described a bioassay which could potentially be used as a biomarker for the efficacy of vaccines targeting VEGF as well as for monitoring purposes of the VEGF neutralizing properties of the anti-VEGF monoclonal antibody bevacizumab. In this bioassay we make use of Ba/F3-VEGFR2 cells, which are murine pre-B cells transfected to express VEGFR2, which are therefore
dependent on VEGF for proliferation and survival. In contrast to ELISA, the 3D-structure of proteins is thought to remain intact in the Ba/F3-VEGFR2 bioassay. We hypothesized that this translates to a more suitable biomarker, because it has a closer resemblance to the actual tumor vasculature in patients. We showed that this VEGF dependent cell proliferation can be inhibited by bevacizumab in a dose-dependent manner. In this study we also investigated the properties of serum samples of patients which received bevacizumab as part of their cancer treatment to inhibit VEGF driven Ba/F3-VEGFR2 cell proliferation. To a variable extent this appeared to be the case. Interestingly, the degree of cell proliferation inhibition showed no correlation with the serum bevacizumab concentration, indicating that there might be other factors involved in VEGF neutralization. However, a significant correlation was revealed for platelet corrected serum VEGF levels and Ba/F3-VEGFR2 cell proliferation inhibition. In conclusion, the Ba/F3-VEGFR2 bioassay appears to reproductively monitor the VEGF neutralizing effects of bevacizumab containing serum samples. Whether the degree of cell proliferation inhibition correlates with clinical efficacy, should be assessed in future studies.

A phase I dose-escalation clinical trial was initiated to study the safety and immunogenicity of hVEGF_{26-104}/RFASE in patients with advanced cancer. The study design and preliminary results are presented in chapter 6. When administered in doses up to 250 µg hVEGF_{26-104} and 40 mg RFASE adjuvant, this immunization strategy has an attractive safety profile. The most frequently reported adverse events were local injection site reactions and fatigue, which were both of low grade and easily manageable. Of the patients included in the study so far, only one patient (VEGFVAC08) displayed an evident antibody response against hVEGF_{26-104}, however these antibodies appeared not to be able to cross-react with hVEGF_{165}, since no antibodies against hVEGF_{165} were detected. In the cynomolgus macaques study no differences in antibody titers between hVEGF_{26-104} and hVEGF_{165} were observed. However, a possible explanation for the discrepancy in immunoreactivity in this patient could be that these antibodies are directed towards epitopes on hVEGF_{26-104}, which are cryptic on hVEGF_{165}, which results in the inability of the induced antibodies to cross-react and neutralize hVEGF_{165}. The lack of seroconversion in any of the other patient may be due to (i) insufficient dosing of hVEGF_{26-104}, (ii) insufficient dosing of RFASE adjuvant, (iii) inadequate adjuvant properties of RFASE in man.

In future dose cohorts of the phase I clinical trial, dosing of hVEGF_{26-104} will be further escalated to assess if higher doses are required for breaking immune self-tolerance. In addition, correlative studies will focus on the anti-angiogenic and immunomodulatory effects of hVEGF_{26-104}/RFASE. The potential of other vaccine adjuvants as possible partner of hVEGF_{26-104} will also be explored. Adjuvants of interest that have shown efficacy in clinical vaccine trials in patients with cancer include Monophosphoryl lipid A (MPLA), CpG 7909,
GM-CSF and Montanide ISA51 (9–12). Once the optimal vaccination schedule for maximal VEGF neutralization has been identified, a phase II safety and efficacy study will be initiated in patients with metastatic colorectal cancer (mCRC) receiving standard of care first line (capecitabine and oxaliplatin) or second line (irinotecan) chemotherapy. Another treatment modality of much interest for combination purposes with hVEGF_{26-104}/RFASE is immune checkpoint inhibition. The promise of targeting VEGF and immune checkpoints PD-1, PD-L1 or CTLA-4 simultaneously is shown in several phase I clinical trials (13–15). Especially in tumor types which are known to be susceptible for VEGF inhibition (i.e. renal cell carcinoma and non-small cell lung carcinoma), there is merit for further investigation of this approach.

Part II – Combination treatment strategies

As mentioned before, targeting the system of angiogenesis alone, even though it is being targeted with passive- or active immunotherapy, will probably not be sufficient to induce significant tumor regression in the clinic. In part II of this thesis we described two studies in which angiogenesis was targeted together with other systems that mediate cancer progression, the coagulation system and immune system.

Multiple reports have shown that the dysregulated hemostatic system in patients with cancer contributes to tumor progression. Hemostasis and angiogenesis are processes which are closely connected, especially in the context of malignancies (16). It has been suggested that part of the tumor growth promoting and metastatic potential of coagulation factors is due to activation of pro-angiogenic factors (17) and that inhibition of coagulation could have antiangiogenic effects as well (18). Although there is strong evidence available showing that anticoagulant therapy reduces the incidence of venous and arterial thromboembolic events in patients with cancer, it appears that it does not lead to a reduction in mortality (19,20). However, one could hypothesize that in tumors that are extremely susceptible to angiogenesis inhibition, targeting both systems simultaneously could result in additive anti-tumor effects. On the other hand this could also have implications on the effects of anticoagulation, which could potentially lead to an increase in bleeding events. In chapter 7 we investigated the safety and tolerability of combined therapy with the low molecular weight heparin (LMWH) dalteparin and the tyrosine kinase inhibitor (TKI) sunitinib in patients with advanced renal cell cancer. Dalteparin was given in dose-escalation, with the lowest dose corresponding to prophylactic therapy and the highest corresponding to a therapeutic dose usually given in patients with a venous thromboembolic event (VTE). We show that this combination treatment strategy is safe and devoid of increased incidence in bleeding. However, during exposure to both drugs simultaneously we did observe elevated anti-factor Xa levels, indicative of increased activity of dalteparin. Although this did not translate to an increase in bleeding events, one should remain cautious of this
complication in future studies investigating similar combination treatments. This also holds true for treatment of a VTE in patients with cancer who are being treated with sunitinib. Whether this encouraging safety profile holds true for other angiogenesis inhibitors (i.e. bevacizumab, ramucirumab, caboctinib) is unknown and this should be assessed in future studies. It is to be expected that more patients with cancer and thrombosis will be treated with a direct oral anticoagulant (DOAC), like rivaroxaban and edoxaban. Recent studies comparing LMWH and DOAC in cancer-related VTE indicate that treatment with DOACs might be associated with a slight increase in bleeding events (21–23). The safety profile of these new anticoagulants in patients treated with antiangiogenic agents as well as the potential clinical benefit of this combination therapy warrants further evaluation.

In chapter 8 we investigated the potential of combined treatment of an allogeneic tumor cell vaccine with the immune checkpoint inhibitor ipilimumab to induce immune reactivity against angiogenic factors. The ability of doing so by autologous tumor cell vaccines was revealed in earlier studies by Dranoff et al. in patients with melanoma as well as acute myeloid leukemia (AML)/myeloid dysplastic syndrome (MDS) (24,25). However, this was not shown previously for an allogeneic vaccine nor with combined immune checkpoint inhibition. Inhibition of CTLA-4 will allow for a reduction in immunosuppression and will facilitate seroconversion against self-antigens, among which will be vascular antigens as well. Results from a phase I study in patients with melanoma already suggest that antiangiogenic monoclonal antibody therapy (bevacizumab) might aid in the effect of immunotherapy (13). This could indicate that this is true for antiangiogenic antibodies induced by active immunization as well. Cancer cell vaccines could induce anti-angiogenic antibodies directly by breaking immune tolerance against angiogenic factors produced by the vaccine itself. Alternatively, humoral immunity against vascular targets could be elicited indirectly through cell death of tumor cells as well as endothelial cells, consequently leading to the release of angiogenic factors. In this study, we assessed seroconversion against players involved in angiogenesis in patients with metastatic castration resistant prostate cancer who participated in a phase I clinical trial in which prostate GVAX (an allogeneic cancer cell vaccine consisting of two prostate cancer cell lines transduced to produce GM-CSF) together with the CTLA-4 inhibitor ipilimumab (26). Earlier, we already noticed seroconversion against prostate specific membrane antigen (PSMA), a protein that is expressed on prostate cancer cells as well as the tumor-associated vasculature of several tumor types (26,27). In this study we showed that this combination strategy elicited antibody responses against multiple vascular antigens in several patients. Although the number of patients included in this study is relatively small, the data indicate that the addition of ipilimumab to prostate GVAX is important for the induction of antibodies, since the rate and strength of seroconversion in the dose cohort with the lowest dose of ipilimumab (0.3 mg/kg) was low. Interestingly we found an association of the number of antibody responses
and patients’ survival, indicating that the induction of humoral immunity is important for the efficacy of this treatment strategy and that its effect does not solely rely on Th1 skewed immune activation. In particular, antibody responses against angiopoietin-1 (Ang-1) and basic fibroblast growth factor (bFGF) were associated with improved overall survival in these patients. These interesting results warrant further investigation to assess the value of Ang-1 and bFGF as targets for the treatment of prostate cancer. In conclusion, we show that immunoreactivity against angiogenesis does not only occur with autologous GVAX vaccines, but also with an allogeneic GVAX vaccine, such as prostate GVAX. In addition these results contribute to the large amount of exciting data already published on the interesting relationship of the immune system and angiogenesis and the promise of modulating both systems simultaneously (28).

Concluding remarks
In this dissertation we have studied novel ways of targeting angiogenesis in cancer, by using immunotherapy and combination treatment strategies. We described the pre-clinical development of a therapeutic vaccine targeting the pro-angiogenic factor VEGF. The phase I clinical trial investigating this vaccine is currently ongoing and will provide us insights on its safety and immunogenicity in patients with advanced cancer. Future studies will focus on identifying the group of patients which are likely to benefit most of this treatment and finding an optimal combination treatment partner. The crosstalk between the processes of angiogenesis and other systems vital for tumorigenesis and metastasis formation raises multiple possibilities for combined treatment approaches, for which we provided examples with anticoagulation and immunotherapy. Taken together, the research described in this thesis has provided knowledge on alternative strategies on targeting angiogenesis in cancer and deserves further investigation to help improve the clinical efficacy of angiogenesis inhibitors.
REFERENCES


