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Preclinical targeting of the tumor microenvironment

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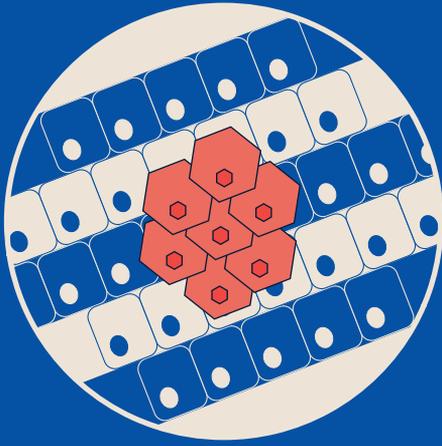
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Chapter 7

Summary, discussion and future perspectives

Summary

Currently, the treatment of cancer patients is mainly based on systemic therapy, next to local surgery and radiotherapy. Current systemic treatment options for cancer patients consist of systemic therapy with chemotherapy, hormonal treatment, targeted agents or immunotherapy. Despite our continuously increasing knowledge of driving oncogenes and growing insight in crucial oncogenic signaling pathways, metastatic disease remains mostly incurable and the main cause of all cancer induced deaths. Therefore, there is a continuous ongoing search for new treatment strategies that may increase the effect of systemic treatment.

Over the past few decades, the development for new treatment options in cancer research was mainly focused on the cancer cells. However, the importance of the tumor microenvironment as a contributor to tumor growth and metastatic disease has become more evident. Furthermore, the tumor microenvironment is an important regulator of cancer cell related drug sensitivity. This provides the rationale for targeting not only the cancer cells, but also the tumor microenvironment, in order to aim to improve cancer treatment. To enable accurate targeting, it is essential to identify key targets present in the tumor microenvironment. Two potential targets present in the tumor microenvironment are transforming growth factor (TGF)- β and vascular endothelial growth factor (VEGF). In addition, early evaluation of drug effects is of major interest to support drug development. ImmunoPET, molecular imaging of radiolabeled antibodies, can non-invasively provide information about the presence of specific targets in the tumor and microenvironment of a patient. This information can potentially be used for patient selection and as a biomarker for evaluation of treatment response.

This thesis aims to describe the development of microenvironmental factors as potential markers of tumor response and their use as markers for molecular imaging. In preclinical co-culture models, drug effects on tumor cell - microenvironment interactions are studied for TGF- β . In addition, the development of a new tracer based on an existing antibody is described for TGF- β . For VEGF, the effects of a VEGF targeted therapy on the tumor microenvironment and tumor drug uptake are studied in preclinical animal imaging models with already more established tracers.

Chapter 1 provides a general introduction and outline of this thesis. In **chapter 2** contains a literature review about TGF- β expression and activation mechanisms as potential targets for anti-tumor therapy and tumor imaging. Sources used to identify data for this review were PubMed and ClinicalTrials.gov. Articles published in English between 1985 and 2011 were included. One of the most interesting targets in the metastatic setting

is TGF- β . TGF- β can promote tumor growth, invasion and metastasis. However, TGF- β also has a physiological, opposing role: maintaining tissue homeostasis and suppression of tumor progression. The window of effective TGF- β targeting is therefore evidently small, which poses a clear challenge in selecting patients at the right time. Despite this complexity, several TGF- β inhibitors are currently in clinical development, modulating TGF- β production, activation or signaling. Still, specificity and long term toxicity remain unclear, emphasizing the importance of careful monitoring of clinical trials. Development and application of these drugs in the clinic, requires adequate insight in, and evaluation methods for the role of TGF- β during tumor invasion and metastasis. In this review, presently available methods for clinical evaluation will be discussed, such as an *ex vivo* stimulation assay, TGF- β response signature and molecular imaging techniques. Future clinical trials incorporating the validation of these evaluation methods will show which method will be most predictive and suitable for clinical application.

Given the importance of the tumor microenvironment as a contributor to tumor growth, metastatic disease and cancer cell drug sensitivity, finding targetable microenvironmental factors, such as stromal derived soluble factors, is of interest. Assessing treatment efficacy of this approach requires new tools, which can evaluate direct effects on the microenvironment and indirect effects on the cancer cells. Such a model should mimic the human situation, by consisting of both human cancer and human stromal components. The use of traditional mouse models would fall short, since host stromal infiltration into the human tumors occurs to a high extent in xenografts from cell lines as well as patient material. The chorioallantoic membrane (CAM) model potentially offers a solution because it allows evaluation of the direct interactions between human tumor cells and human stromal cells in an immune deprived *in vivo* setting. In **chapter 3** we investigated the mechanism behind the anti-cancer effect of modulating the microenvironment with zoledronic acid. In 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) survival assays, it is shown that the breast cancer cell lines MCF-7, SUM-149, MDA-MB231, SCP2 and H2N are insensitive to zoledronic acid (half maximal inhibitory concentration (IC_{50}) > 85 μ M), whereas the human stromal cell line Hs27a was very sensitive (IC_{50} = 8 μ M) to zoledronic acid. In an *in vitro* co-culture model, SCP2 breast cancer cells were cultured alone or co-cultured in the presence of human Hs27a stromal cells and treated with increasing doses of zoledronic acid. In cultures with cancer cells only, cell death remained stably low throughout the increasing concentrations used (~ 10%). However, when SCP2 breast cancer cells were co-cultured with Hs27a stromal cells, zoledronic acid did induce cancer cell death up to 44% for the highest concentration of zoledronic acid (500 μ M, P <0.01). In an *in vivo* co-culture CAM model, zoledronic acid did not reduce size and weight of tumors of SCP2 or MCF-7 tumors compared to placebo.

However, co-culture tumors of SCP2 or MCF-7 mixed with Hs27a stromal cells made these tumors sensitive to zoledronic acid as shown by a decrease in size and weight compared to placebo treated co-culture tumors (Size: 32 vs 56 mm³ $P < 0.05$, weight: 23 vs 43 mg, $P < 0.01$ for SCP2 and size: 28 vs 54 mm³ $P < 0.01$, weight: 37 vs 24 mg $P < 0.01$ for MCF-7). Furthermore, zoledronic acid decreased TGF- β 1 excretion levels when breast cancer cells were co-cultured with Hs27a stromal cells in a dose dependent matter. The addition of TGF- β in the *in vitro* co-culture model, counteracted the induced cell death by zoledronic acid in the presence of stromal cells ($P < 0.001$). In addition, supernatant of Hs27a stromal cells treated for 48 hours with zoledronic acid reduced active TGF- β signaling in SCP2 cells. These data show that the presence of stromal cells is required to exert an anti-breast cancer effect and that this is mediated via TGF- β .

Chapter 4 describes the preclinical development of ⁸⁹Zr-labeled TGF- β antibody fresolimumab as a PET tracer. Fresolimumab, which neutralizes all mammalian active isoforms of TGF- β , was radiolabeled with ⁸⁹Zr for PET to analyze TGF- β expression, antibody tumor uptake, and organ distribution. Tumor uptake and organ distribution of ⁸⁹Zr-fresolimumab was assessed in a human TGF- β transfected Chinese hamster ovary xenograft model, as well as in a subcutaneous and a metastatic human triple negative breast cancer model (MDA-MB231). In all tumor models, tumor uptake of ⁸⁹Zr-fresolimumab was similar to the non-specific control antibody ⁸⁹Zr-IgG for both the *in vivo* PET as the *ex vivo* biodistribution data. In addition, distribution of ⁸⁹Zr-fresolimumab in most organs was similar to ⁸⁹Zr-IgG, except for liver and kidney where ⁸⁹Zr-fresolimumab uptake was higher. Active TGF- β is known to be rapidly cleared by the liver, with a half-life of 2-3 min. Enzyme-linked immunosorbent assay (ELISA) of liver samples could only detect latent TGF- β , while tumor samples showed both active and latent TGF- β . However, immunohistochemistry for pSMAD2 on liver tissue confirmed presence of active TGF- β . Thus, the high liver uptake of ⁸⁹Zr-fresolimumab was likely caused by specific binding to active TGF- β and rapid hepatic clearance of active TGF- β bound to ⁸⁹Zr-fresolimumab. Furthermore, high uptake of ⁸⁹Zr-fresolimumab was seen at sites of tumor ulceration and in scar tissue, which resemble physiological processes in which TGF- β is highly involved. This study shows that ⁸⁹Zr-fresolimumab is feasible for preclinical imaging and quantification of fresolimumab tumor uptake and organ distribution.

Several preclinical and clinical studies indicate that antiangiogenic drugs, including the VEGF-A antibody bevacizumab, lead to tumor vessel normalization in addition to their antivascular effect. In the process of tumor vessel normalization, the architecture of the remaining vasculature is largely restored. This can encompass a marked influence on intratumoral delivery of monoclonal antibodies and might have implications

when bevacizumab is combined with other monoclonal antibodies. In **chapter 5A** we investigated the effect of tumor blood vessel normalization induced by bevacizumab on antibody tumor uptake. In mouse xenograft models of human ovarian and esophageal cancer (SKOV-3 and OE19), we evaluated antibody uptake in tumors by PET imaging 24 and 144 hours after injection of ^{89}Zr -trastuzumab, ^{89}Zr -bevacizumab or ^{89}Zr -IgG before or after treatment with bevacizumab. PET images obtained at 24 and 144 hours after tracer injection visibly showed a lower tumor uptake of ^{89}Zr -trastuzumab, ^{89}Zr -bevacizumab and ^{89}Zr -IgG during bevacizumab treatment in the SKOV-3 model. Already on the 24 hours PET images, standardized mean tumor uptake values (SUV_{mean}) decreased with 38%, 16% and 27% for respectively ^{89}Zr -trastuzumab, ^{89}Zr -bevacizumab and ^{89}Zr -IgG. This effect was even more pronounced at 144 hours, with a reduction in ^{89}Zr -trastuzumab tumor uptake of 41% and a similar reduction in ^{89}Zr -bevacizumab tumor uptake of 43%. Tumor uptake of ^{89}Zr -IgG was also lowered, although to a lesser extent namely 28%. Placebo treatment did not affect ^{89}Zr -IgG uptake in the tumor. In the OE19 model, bevacizumab treatment also decreased the tumor uptake of ^{89}Zr -trastuzumab with 34% at 24 hours and with 39% at 144 hours. In addition, *ex vivo* biodistribution data of both ^{89}Zr -IgG groups showed a lower tumor uptake when animals were treated with bevacizumab compared to placebo. Bevacizumab treatment reduced tumor vessel density in all bevacizumab treated groups, compared to placebo. Moreover, bevacizumab treatment induced vessel normalization of the remaining vessels. In placebo treated tumors 68% of the tumor vessels had no pericyte coverage and only 7% of the vessels were fully covered with pericytes. After bevacizumab treatment pericyte coverage was absent in only 10% of the tumor vessels, whereas 75% of the vessels were fully covered. *Ex vivo*, IgG-800CW was mainly present in the extracellular matrix and bevacizumab treatment relatively reduced the accumulation of IgG-800CW compared to placebo, matching the PET results. **Chapter 5B** further discusses the dose dependency of vessel normalization in response to comments received regarding our work in chapter 5A.

Registered drugs targeting the VEGF pathway are small molecule tyrosine kinase inhibitors (TKI) targeting the VEGF receptors (VEGFR), an antibody directly targeting VEGF and an antibody targeting VEGFR2. In **Chapter 6** a review is given on preclinical and clinical results of registered VEGF pathway targeting agents. In particular, the interplay between these VEGF pathway targeting agents, vessel normalization and tumor drug delivery is discussed. VEGF pathway targeting agents have been combined with other anticancer drugs, improving efficacy in a few cancer types. Vessel normalization induced by VEGF pathway targeting agents influences tumor drug uptake. Preclinical and clinical studies have shown a decrease in tumor delivery of radiolabeled antibodies and two chemotherapeutic drugs, following bevacizumab treatment. The decrease in vessel

pore size during vessel normalization might explain the decrease in tumor drug uptake. Moreover the addition of bevacizumab to cetuximab or panitumumab in colorectal cancer patients or to trastuzumab in breast cancer patients, did not improve efficacy. However, combining bevacizumab with chemotherapy did increase efficacy in a few solid tumor types. Novel biomarkers to select patients who may benefit from combination therapies, such as the effect of an angiogenesis inhibitor on tumor perfusion, requires innovative trial designs and large clinical trials. Small imaging studies with radiolabeled drugs could be used in the interphase to gain further insight in the interplay between VEGF targeted therapy, vessel normalization and tumor drug delivery.

In conclusion, this thesis describes the possibilities and consequences of targeting factors present in the tumor microenvironment. For TGF- β , the development of a new tracer based on an existing antibody is described. Furthermore, the mediating role of TGF- β excreted by stromal cells to exert an anti-breast cancer effect has been identified. On the other hand, the detrimental effects on tumor drug uptake following VEGF targeted therapy illustrate consequences of targeting the tumor microenvironment.

Discussion and future perspectives

Apart from surgery, radiotherapy and systemic treatment with chemotherapy and hormonal treatment, over the last years targeted therapies and immunotherapies have become available. Despite the development of these new treatment options, metastatic disease remains the major cause of cancer related deaths (1).

Tumor heterogeneity and the tumor microenvironment

A major hurdle in the treatment of metastatic disease is the heterogeneity in tumor characteristics between patients. Moreover, with respect to a single patient and even single lesions, it is increasingly clear that there is often also major heterogeneity of characteristics across and within tumor lesions. In Chapter 4 and 5 we visualized TGF- β , VEGF and HER2 with ^{89}Zr -fresolimumab, ^{89}Zr -bevacizumab and ^{89}Zr -trastuzumab respectively. Insight in heterogeneity within one patient would require biopsies of all identified lesions. ImmunPET can non-invasively provide information about the presence of a specific target in the primary tumor, metastases and tumor microenvironment of a patient. With ^{89}Zr -bevacizumab primary breast tumors, neuroendocrine tumor lesions and renal cell cancer lesions were identified (2, 3). Furthermore, this technique can be used as read out of drug effects. We demonstrated with ^{89}Zr -trastuzumab that it is possible to show reduction in HER2 by HSP90 inhibition and visualize heterogeneity in response within one tumor lesion in breast cancer patients (4). The visualization

of TGF- β , VEGF and HER2 illustrates that it is possible with the help of immunoPET to depict and analyze the expression of specific factors present in the tumor or tumor microenvironment. These techniques can potentially support future studies to provide insight in tumor characteristics within a patient.

Possibilities of targeting the tumor microenvironment

Targeted drugs mainly focus on one target or pathway in the tumor, whereas tumors rarely depend on a single pathway. It has become clear that a tumor does not solely exist of a cluster of epithelial cells, but has its own heterogeneous microenvironment. The tumor microenvironment is increasingly acknowledged as an important contributor to tumor growth and metastatic spread (5). By additional targeting of a patient's tumor microenvironment, hurdles of heterogeneity and drug resistance might be addressed. In chapter 3 we showed that the anti-breast cancer effect of the bisphosphonate zoledronic acid on breast cancer tumors grown in the CAM model is controlled via TGF- β excretion by stromal cells. This study supports the possibility to target the tumor via the tumor microenvironment. Future preclinical studies should further explore possibilities of targeting the tumor microenvironment with other agents. In chapter 4 we described preclinical imaging of TGF- β with ^{89}Zr -fresolimumab. It might be of interest to study TGF- β expression in the tumor microenvironment with ^{89}Zr -fresolimumab to select breast cancer patients in the metastatic setting to adjust intensity of zoledronic acid therapy.

Consequences of targeting the tumor microenvironment

The cells of the tumor microenvironment can be divided in three classes: angiogenic vascular cells, immune cells and mesenchymal cells. Up to now, angiogenesis is one of the most studied components of the tumor microenvironment. As described in chapters 5 and 6, antiangiogenic drugs can initiate vessel normalization in the tumor vasculature. In chapter 5A we showed that bevacizumab induced vessel normalization led to a decrease in tumor delivery of other monoclonal antibodies. These results illustrate the possibility to use immunoPET for the evaluation of interactive effects of new combination therapies. Early implementation of small preclinical and clinical imaging studies during drug development, would allow visualization of the distribution of the labeled drug, provide serial information on whole body drug distribution, and could guide rational trial design for combinatorial studies.

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