Chapter 8

Summary, discussion and future perspectives
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Summary

Increasing evidence suggests that therapy-resistance in solid tumors is partly due to the protective effect of the microenvironment on neoplastic cells. Cross-talk between tumor cells and their microenvironment is partly coordinated by G-protein coupled receptors (GPCRs) which are implicated in many facets of solid tumors, including cancer cell proliferation, production of angiogenic factors and metastasis [1-3]. Therefore, in this thesis we aimed to study two membrane bound receptors, namely formyl-peptide receptor-1 (FPR1) and chemokine receptor-4 (CXCR4). Both receptors are highly involved in tumor cell migration, triggered by cognate ligands present in the tumor microenvironment.

Chapter 1 provides a general introduction to glioblastoma (GBM) and GPCRs including the aim of the thesis and the chapter outline. Despite the available treatment options of surgical resection and radio-chemotherapy, GBMs invariably show recurrence. A number of treatment options are available after recurrence, amongst which the anti-angiogenic treatment with bevacizumab has been extensively explored. However therapy resistance develops soon, which is partly due to neovascularization. To better understand the course of neovascularization following bevacizumab therapy, we performed in Chapter 2 a literature review about the recruitment of bone marrow derived cells (BMDC) involved in the process of therapeutic resistance. GBMs are highly vascularized tumors characterized by rapid and invasive tumor growth, typically followed by oxygen depletion and neovascularization, all resulting in a network of disorganized, tortuous and permeable vessels. Acute hypoxia following prolonged vascular endothelial growth factor (VEGF-A) depletion induces the recruitment of BMDC, which may contribute to treatment refractoriness. These cells may act as vascular progenitors by integrating into the newly formed vessels or as vascular modulators by releasing pro-angiogenic factors. BMDC recruitment plays a prominent role in the
refractoriness to anti-VEGF treatment. Therefore bevacizumab therapy might benefit when combined with other treatment modalities targeting the BMDC recruitment as compared to single agent use. As such, this chapter also elaborates on possible anti-VEGF combination strategies, including targeted therapy against Tie-2 and/or its two ligands angiopoietin 1 and 2 (Ang1/2), CXCR4, placental derived growth factor (PIGF) and platelet derived growth factor-β (PDGF-β), to improve GBM treatment outcome.

Tie-2 has been originally described together with Ang1 and Ang2 for its involvement in vessel stabilization and destabilization. However recent studies reported certain types of monocytes marked by the expression of Tie-2 and therefore dubbed Tie-2 expressing monocytes (TEM). In Chapter 3 we semi-quantitatively evaluated the expression of Ang1, Ang2 and Tie-2 in a series of 50 immunostained GBM patient samples. Tie-2 expression was correlated with patient survival and angiogenic data (consisting of tumor microvessel density, tumor cell proliferation and tumor cell apoptosis data). By using immunofluorescent double staining of Tie-2 with glial acidic fibrillary protein (GFAP) or with CD163, we determined whether Tie-2 was expressed by glial tumor cells or microglia/tumor associated macrophages. Hundred percent of GBMs were positive for Ang1 and 98% were positive for Ang2. Semi-quantitative Ang1 and 2 scores did not correlate with patient survival or angiogenic data. We observed a high Tie-2 expression on TEMs and to a lesser extent on endothelial cells (vascular compartment) and tumor cells. In addition Tie-2 expression negatively correlated with overall patient survival (P = 0.05) but not with angiogenic data. Overall this chapter describes the presence of TEMs in a larger series of GBM and the negative correlation of Tie-2 expression with patient survival, suggesting the importance of the angiopoietin/Tie-2 system in GBM.

Conspicuous evidence indicates that in tumors, CXCR4 highly contributes to the microenvironment mediated cancer therapy resistance [4]. Therefore the inhibition of CXCR4 with AMD3100 is deemed an interesting approach
for cancer therapy. However results from several different (pre-)clinical studies on AMD3100 treatment in different contexts show a dichotomous function of this inhibitor. On the one hand it was previously reported for its anti-metastatic effects in ovarian [5] and breast cancer [6], oral squamous carcinoma [7] and for sensitizing cells to treatment when combined with chemotherapy in gliomas and prostate cancer [8,9]. On the other hand it is used to stimulate hematopoietic CXCR4-expressing stem cell mobilization for autologous stem cell transplantation in non-Hodgkin’s lymphoma and multiple myeloma patients [10,11]. In Chapter 4 we studied the effects of CXCR4 inhibition combined with irradiation in human prostate cancer cells and investigated whether AMD3100 treatment also affects mobilization of tumor cells. In an in vitro co-culture with stromal cells we observed that the CXCR4 inhibitor AMD3100 sensitized prostate cancer cell lines PC3-Luc and LNCaP to irradiation. Combined treatment of mice xenografted with luciferase-expressing PC3-luc cells with radiotherapy and AMD3100, resulted in reduced tumor volumes at the fourth week of treatment as compared to either treatment alone. Immunohistochemically stained prostate cancer xenografts from irradiated mice showed higher CXCR4 and CXCL12 expression as compared to controls. However, bioluminescent imaging of blood samples from these animals revealed that AMD3100 also mobilized tumor cells as measured at days 14 (83-fold increase, P < 0001) and 21 (33-fold increase, P < 0001) in comparison with baseline measurements. Although not significantly, AMD3100 also increased the number of metastases as assessed at termination. In conclusion this chapter shows that AMD3100 transiently enhances prostate cancer radiosensitivity, but induces cancer cell mobilization and slightly increases metastasis.

FPR1 is member of the GPCR network involved in cancer biology which is highly present in grade IV astrocytoma (GBM). It is expressed by the human GBM cell line U87 in which ligand induced activation of FPR1 promotes tumor cell motility, growth and angiogenesis [12,13]. In Chapter 5, we aimed to
further define the role of FPR1 in GBM. We performed an immunohistochemical evaluation of FPR1 expression in tumor samples of 32 patients diagnosed with astrocytomas grades I-IV (8 patient samples for each grade). FPR1 was detected in 29 out of 32 (90%) tumors. In addition we performed in vitro experiments with U87 GBM cell line and a FPR1 transfected human promonocytic cell line U937-FPR. In calcium mobilization assays, activation of U87 and U937-FPR cells with a bacterial derived agonist, fMLF, could be inhibited with Chemotaxis Inhibitory Protein of S. aureus (CHIPS) up to 80% (U87) and 10-fold (U937-FPR). Activation of U937-FPR cells exhibited upregulation of calcium mobilization when stimulated with mitochondrial derived agonists fMMYALF (3-fold) and fMLKLIV (4-fold) which could be completely inhibited with 1 µg/mL CHIPS. Migration induced by fMLF could be inhibited with CHIPS up to 100% (U87) and 86% (U937-FPR). Migration induced by fMMYALF and fMLFKLIV on U937-FPR reached up to 75% and 77% inhibition respectively by CHIPS. In addition U87 stimulated with fMLF induced phosphorylation of AKT and ERK1/2 as measured with Western blot and the production of VEGF as measured by ELISA, could be inhibited with CHIPS. Finally in vivo CHIPS treatment versus vehicle treatment improved survival of mice bearing U87 subcutaneous xenografts (P = 0.0019). The results of this study indicate that formylated peptides, including those of mitochondrial origin, present in the microenvironment of necrotic cells, activate FPR1 and that all responses could be inhibited with CHIPS. Therefore FPR1 might be a target of interest for the development of novel therapies to improve treatment results for GBM patients. The overall high expression pattern of FPR1 in GBM samples prompted us to further investigate the expression profile in a large panel of GBM patient specimens. Therefore in Chapter 6 we evaluated the FPR1 expression in an extended panel of human GBMs. We investigated the possibility to elicit agonist induced FPR1 activation in GBM cell lines and to inhibit these responses with CHIPS. All 178 GBM patient specimens expressed FPR1.
Activation of FPR1 in U87 cells by fMLKLIV and fMMYALF was measured by increased calcium mobilization, AKT and ERK1/2 phosphorylation, and ligand-directed migration. All responses could be inhibited by CHIPS. FPR1 mRNA and functional activity could not be detected in any of the 8 primary human GBM cell lines (dubbed GG 1/6/7/9/12/13/14/16) tested. However FPR1 was expressed in all tumor samples from which the GG cell lines were originally isolated. In addition immunofluorescent staining of GBM slides revealed FPR1 expression on microglia/tumor associated macrophages (CD68+/CD163+ cells) and glial tumor cells (GFAP+ cells). Finally brain sections of orthotopic xenografts of GG cell lines revealed specific FPR1 staining. In conclusion FPR1 is widely expressed in GBM and can be activated by human mitochondrial-derived agonists in U87 cells. Although FPR1 expression could not be detected in GG cell lines in vitro, when engrafted in mouse brains these cells show FPR1 expression. This implicates that the microenvironment potentially plays a role in modulating FPR1 expression.

FPR1 was highly expressed in GBM samples and CHIPS was a potent inhibitor of formylated peptide induced responses, therefore CHIPS remains a potential interesting drug for the treatment of GBM patients. CHIPS has been previously tested in a small phase I clinical trial as an anti-inflammatory drug. However due to pre-existing circulating anti-CHIPS antibodies, side effects were observed upon intravenous administration. Given the immunogenicity effects of CHIPS, the quest for less immunogenic CHIPS variants containing the same FPR1 binding affinity was investigated in Chapter 7. We used an Escherichia coli expression system to develop a number of CHIPS-mutants. Calcium mobilization assays with neutrophils revealed that the substitution of 3 amino acids (aa) at position 69-71 and deletion of aa 7-56 (α-helix) or aa 7-30 (first spacer), affected the binding capacity of CHIPS to FPR1. Pre-treatment of neutrophils with CHIPS-based and FLIPr-based N-terminal peptide fragments containing the first 6 to 20 aa inhibited calcium mobilization. However peptide fragments were on average 10,000 times less
potent than the CHIPS protein. Finally with a less immunogenic CHIPS variant called CHIPS-JC, FPR1 inhibition could be achieved with calcium mobilization assays but not migration assays.

**Discussion and Future Perspectives**

The exploration of new therapeutic options, which aimed at overcoming treatment resistance of solid tumors, has primarily focused on targeting tumor cells and proven very difficult to achieve. The notion that therapy resistance is largely conferred by the microenvironment in solid tumors has turned it into an important potential target for new anti-cancer treatment approaches.

**Cut the cross-talk: isolating tumor cells from their microenvironment**

Tumor cells communicate with their environment by modulating the surrounding cells to induce enhanced growth and survival. As presented in Chapter 2, many different factors produced by cancer cells induce the recruitment of bone marrow cells thereby contributing to therapy resistance. As presented in Chapter 3, the extra-endothelial expression of Tie-2 in GBM and its negative correlation with patient survival led us to the conclusion that Tie-2 expression is a negative prognostic factor in these tumors. Tie-2 in GBM has been previously reported to mediate therapy resistance. Tumor stem cells isolated from surgical samples of human glioblastoma and treated with a panel of cytotoxic and chemotherapeutic drugs were highly resistant when they expressed Tie-2 [14]. Furthermore in an intracranial glioma mouse model in which Tie-2 positive glioma cells, when co-transplanted with endothelial cells, regulated tumor cell invasiveness [15]. In a mouse mammary tumor virus (MMTV) pyMT mammary carcinoma mouse model a conditional Tie-2 knock down in TEMs was investigated. The knock down of Tie-2 prevented the interaction of TEMs with blood vessels even inducing regression of the vasculature [16]. All together these findings strongly
indicate that Tie-2 is involved in the cross-talk between neoplastic cells and the TEMs surrounding their environment. Future research should be directed towards targeting these monocytes. Specifically Tie-2 could be a valid target to test, by performing intravital imaging of mice expressing Tie-2-GFP under the control of a lentiviral vector. This way it might be possible to directly observe the effects of counteracting the recruitment of these bone marrow cells. More importantly a recent study showed that targeting tumor associated macrophages in a PDGF-β-driven glioma mouse model can be re-educated and used to oppose tumor growth [17]. Therefore by combining the inhibition of Tie-2 with the re-education of TAMs we could use the microenvironment to isolate the communication of tumor cells with their surrounding and neutralize them at the same time. Important readouts in this context would be the effects on vasculogenesis, on the balance of Ang1/2 expression and tumor growth. Overall resetting the micro-environment and defeating the cross-talk between tumor cells and their surrounding might possibly counteract tumor evasion to therapy.

**Evaluating FPR1 as a drug target in GBM**

FPR1 is highly upregulated in GBM and involved in many aspects of malignant cell activity in vitro and in vivo [12,18]. In Chapter 6 the observation that FPR1 expression was absent in all primary GBM cell lines led to the conclusion that FPR1 expression might be dependent on microenvironmental factors. Such a factor could be interleukin-8 (IL-8), as several studies reported that IL-8 might influence the expression of FPR1 by neutrophils [19,20]. In cancer, IL-8 plays an important role as a regulatory factor in the microenvironment and is involved in resistance to chemotherapeutic drugs and angiogenesis [21]. IL-8 receptor and FPR1 activation induce the nuclear translocation of STAT3 [12,21] while HIF1α is upregulated by FPR1 activation [12]. Furthermore both STAT3 and HIF1α are transcription factors of genes encoding for VEGF-A. Given that IL-8 has also been implicated in the upregulation of FPR1, the
The possibility of an existing feedback loop arises. To date the only existing FPR1 studies with intracranial models are performed with U87 and compared to U87 containing siRNAs against FPR1. In none of the existing preclinical mouse models have intracranial tumors been treated with drugs targeting FPR1. Therefore a next step would be the treatment of orthotopic U87 glioma mouse models with CHIPS. In addition to gain a larger repertoire of in vitro cell models expressing this receptor it will be necessary to investigate how the microenvironment affects the expression of FPR1. Overall more preclinical insights into the commitment and function of FPR1 in GBM are necessary in order to further define its role as a target for anti-cancer treatment.

The hurdles of AMD3100 and CHIPS

An important hallmark of solid tumors resides in their heterogeneous nature characterized by the presence of various elements including tumor (stem) cells, endothelial cells, pericytes and tumor associated immune cells. Furthermore, multiple zones can be identified in GBM including a necrotic core, surrounded by a contrast enhancing bulk of tumor tissue, which in turn is surrounded by an infiltration area of individual tumor cells alternated by normal brain cells. Hypoxic as well as normoxic zones are present in the bulk of tumor. Although the blood brain barrier (BBB) is disrupted in the tumor bulk, the function of the BBB at the infiltration zone may vary. All these aspects will influence the microenvironment and the cellular make-up of the tumor at different zones. Targeting the microenvironment may thus affect the various regions of the tumor differently.

CXCR4 is present on tumor cells and several types of tumor associated immune cells (Chapter 2)[22,23] including TEMs and TAMs [4], while FPR1 is highly expressed by tumor cells and TAMs (Chapter 6) [24-26]. Therefore, targeting tumor cells and their microenvironment at both levels can be achieved by the CXCR4 inhibitor AMD3100 or the FPR1 inhibitor CHIPS.
CXCR4 is highly present in various types of solid tumors and has been extensively described as a target for therapy. However in Chapter 4 by detecting increasing numbers of circulating tumor cells (CTCs) in a prostate cancer model after AMD3100 treatment we showed for the first time that CXCR4 inhibition in solid tumors causes unwanted effects. In addition previous findings showed that the administration of AMD3100 induces hematopoietic CXCR4-expressing stem cell mobilization and is useful for autologous transplantation in non-H Hodgkin’s lymphoma and multiple myeloma patients [10,11]. Given that AMD3100 could cause the mobilization of tumor cells, potentially resulting in enhanced metastasis formation, the presence of CTCs should be closely monitored in future clinical studies with AMD3100. This was recently subject of investigation in a phase I clinical trial on a new CXCR4 inhibitor (LY2510924). The authors reported an increase in CTCs in 6 out of 42 treated patients with various types of solid tumors. Interestingly in two out of the three enrolled prostate cancer patients, the CTC count after treatment was increased [27]. In this clinical trial no GBM patients were enrolled and to date it remains unknown whether AMD3100 treatment could induce CTCs in GBM.

FPR1 is another interesting target for therapy as its blockade can affect a great number of FPR1 downstream pathways related to the activation of this receptor [28]. FPR1 has a very potent and specific inhibitor called CHIPS, but interaction between FPR1 and CHIPS in the context of cancer has not yet been extensively investigated. In Chapter 5, our in vivo study showed slightly improved survival of animals with subcutaneously implanted human tumors following treatment with CHIPS. CHIPS’ serum half-life is short and therefore the use of osmotic pumps for drug administration instead of intraperitoneal administration might guarantee a more stable drug level for treatment of mice. However CHIPS has side effects as observed in a phase I study in human subjects. Therefore finding the proper window for treatment, in which CHIPS variants have sufficiently reduced immunogenicity, is essential
for future use of CHIPS in the context of cancer therapy. Specifically reduced immunogenicity would enable the use of higher concentrations of CHIPS in order to reach similar FPR1 activity as CHIPS wild type, but with less side effects.

Generally a potential hurdle for GBM treatment is achieving the delivery of sufficient tumor drug levels across the BBB. Although the BBB is disrupted where the bulk of tumor cells reside, it is mostly intact at the site where infiltrated tumor cells are located [29-31]. In Chapter 2 we discussed the possibility to combine bevacizumab with AMD3100 to obtain better treatment outcome in GBM patients. AMD3100 is a ~500Da bicyclam of which the ability to cross the BBB is not yet known. Currently in an ongoing phase I trial in recurrent-high grade glioma patients, the ability of AMD3100 to cross the BBB (NCT01339039, ClinicalTrials.gov) is addressed as a subquestion, although further specifications on the exact methods are not provided.

In chapters 5, 6 and 7 we investigated the possibility to apply CHIPS as an anti-GBM drug. However the ability of CHIPS to freely cross the BBB has not yet been investigated but given the size of the protein (14.1 kDa) this may be a problem [32]. Various techniques exploiting delivery vehicles to carry therapeutic agents across biological barriers are emerging as a novel therapeutic strategy [33,34]. For instance one of these consists of attaching the treatment compound to other structures. This creates a pro-drug with improved lipophilic characteristics, which facilitates the diffusion through the BBB [35]. Also, microbubble focus ultrasound is an emerging technique currently under investigation and extensively described [36,37]. This system causes the temporary disruption of the BBB allowing the conveyance of large molecules across the barrier. Therefore it might be a suitable method for CHIPS and AMD3100 to achieve sufficient drug delivery at sites where the BBB could still be intact, thus changing the microenvironment at the infiltration zone of GBM.
Conclusions

This thesis partly elucidates the role of FPR1 and CXCR4 in interacting with elements lodging in the tumor microenvironment. In resistance to cancer treatment, a bypass system originating from BMDC recruitment can play a role. To circumvent resistance this aspect could potentially be exploited in combination therapies in GBM. Specifically the upregulation of CXCR4 and its presence on Tie-2 expressing monocytes suggest an important role of these two receptors in tumor resistance to therapy. However targeting CXCR4 as anticancer treatment with its inhibitor AMD3100 still requires careful investigation as it induced CTCs in the animal model.

FPR1 is highly expressed in GBM and directly interacts with mitochondrial peptides possibly originating from necrotic tumor microenvironment. Antagonizing FPR1 in a preclinical setting by using CHIPS, even suggested a survival benefit. However the use of CHIPS as a therapeutic drug necessitates the construction of a less immunogenic variant, which warrants further research. Overall understanding the regulatory mechanisms of FPR1 and CXCR4, operative within the tumor microenvironment might contribute to the development of better strategies for a more successful cancer treatment. Furthermore their inhibitors CHIPS and AMD3100 require additional insight into the proper application of these drugs as therapeutic agents.
Chapter 8 | Summary, discussion and future perspectives

References


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