Radiotherapy and angiogenesis inhibition: From bench to bedside

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Radiation therapy (RTx) has been used for cancer treatment for over 100 years. Nowadays, more than half of all cancer patients will receive RTx, making it one of the most frequently used treatment modalities in oncology. RTx is based on ionizing radiation (IR) beams that induce DNA damage in cells, resulting in cell death. RTx can be administered in a single dose, usually applied in the palliative setting, or in multiple low-dose fractions, so called fractionated RTx. The latter is mainly used with curative intent. Scientific research and technical developments have greatly contributed to more accurate dose delivery of IR to the tumor tissue while sparing normal tissue. In addition, drugs have been developed that enhance the sensitivity of cells to RTx, so-called radiosensitizers. All this has resulted in increased efficacy of RTx while reducing toxicity.

While DNA damage is considered as the main actor of cancer cell death in response to RTx, there is an increasing interest in the effects of IR on the tumor micro-environment (TME). The TME is affected by IR, and the TME also affects the sensitivity of cancer cells to IR. It consists of different cell types and structures, including, immune cells, supporting tissues and blood vessels. Tumors are known to stimulate the formation of new blood vessels out of pre-existing capillaries. This growth of blood vessels, so-called angiogenesis, is essential for sufficient delivery of nutrients to the growing tumor, and it has been identified as one of the hallmarks of cancer. There are several targeted drugs available for anti-cancer treatments that inhibit angiogenesis. While the clinical benefit of monotherapy with these drugs appears limited, pre-clinical data demonstrate that inhibition of angiogenesis could increase the efficacy of RTx. However, the mechanisms underlying this effect and the effect of RTx on angiogenesis are poorly understood. The aim of the research described in this thesis was twofold; 1) To study the effect of RTx on tumor angiogenesis and 2) To investigate the efficacy of different treatment schedules of the combination treatment of angiogenesis inhibition and RTx.

Chapter 1 provides a review of the literature describing the knowledge regarding the combination of angiogenesis inhibiting drugs and RTx. Pre-clinical studies show encouraging effects on tumor growth inhibition with the combination of both treatment strategies, but the translation to clinical trials is still challenging. While numerous phase I/II trials report encouraging response rates, others fail to show benefit of the combination treatment. The discrepancy between preclinical and clinical responses appears to be due to differences in treatment schedules that are used. Indeed, little is known about dose-scheduling RTx and angiogenesis inhibitors in patients. In addition, the combination therapy has also been shown to cause rare but severe side effects. Based on these observations we recommend that pre-clinical research should focus on dose-scheduling of angiogenesis inhibiting drugs with RTx, to improve efficacy and reduce toxicity. It is also essential that non-invasive methods to monitor oxygenation and pro-angiogenesis signalling in tumors are validated for both prior to therapy as well as during therapy. This will give better insight in how and when to combine RTx with angiostatic drugs.

In order to study the effects of RTx and anti-angiogenic drugs on angiogenesis and

tumor progression, we used several *in vitro* and *in vivo* assays, described in **chapter 3**. For *in vivo* studies, we adapted the chorioallantoic membrane (CAM), which is the highly vascularized embryonic sack in a fertilized chicken egg. The physiologic angiogenesis during embryogenesis allows easy monitoring of the effect of different treatments. In addition, because human cancer cells are easily grafted on the CAM, it represents an affordable and reproducible in vivo tumor model. More importantly, the effect of combining RTx with drugs that influence angiogenesis can be readily monitored with these assays.

To study the interaction between RTx and angiogenesis inhibition the anti-angiogenic drug sunitinib was used. Sunitinib is a tyrosine kinase inhibitor (TKI) known to target several angiogenesis related receptors. Sunitinib is currently FDA-approved for the treatment of advanced renal cell carcinoma, gastrointestinal stromal tumors and pancreatic neuroendocrine tumors. First, we evaluated the current knowledge regarding the combination of RTx with sunitinib. As described in **chapter 4**, this combination treatment shows encouraging response rates in clinical trials, but it remains challenging to apply both treatment modalities with the right schedule and dose. Several (pre)clinical studies suggest that it is mainly maintenance therapy of sunitinib after RTx that enhances the tumor responses. The importance of dose-scheduling is further illustrated in **chapter 5** in which we describe the effects of different schedules of the combination of sunitinib and RTx on angiogenesis and tumor growth. Both single dose RTx (4 Gy) and sunitinib affected angiogenesis in the CAM, which was further reduced when the treatment modalities were combined. Applying different schedules of the combination treatment on HT29 (human colorectal adenocarcinoma cell line) xenografts on the CAM demonstrated that scheduling was important for obtaining the maximal anti-tumor effect. While RTx after 4 days of sunitinib had no additional effect compared to sunitinib alone, RTx given before sunitinib hampered tumor growth. The latter treatment schedule also allowed a dose reduction of sunitinib with 50%. This finding is especially important for the clinical setting, since reducing the dose of sunitinib could reduce side effect.

To further substantiate these findings with more clinically relevant RTx schedules, we continued to study the combination of angiogenesis inhibition with fractionated RTx, as described in **chapter 6**. Using human HT29 xenografts in nude mice, we studied the combination of low dose sunitinib and single dose or fractionated RTx. While monotherapy with low dose sunitinib did not affect tumor growth, the combination with both single dose and fractionated RTx significantly improved tumor growth reduction, as compared to RTx alone. This suggests that RTx made the tumor cells sensitive to sunitinib therapy. In addition, we found that RTx enhances tumor perfusion, reduces hypoxia, and causes repopulation of cancer cells in the central hypoxic area of the tumor. Low dose sunitinib treatment after RTx counteracted these effects.

In order to translate these preclinical findings to the clinical setting, we next aimed to study the effect of fractionated RTx on tumor angiogenesis in patients. To that end, a clinical pilot study is ongoing, of which the preliminary results are described in **chapter 7**. Esophageal cancer patients receiving neo-adjuvant chemoradiation were selected to undergo an extra tumor biopsy during chemoradiation. The first aim is to determine a time-point during

chemoradiation with enhanced expression of pro-angiogenic factors in the tumor. The second aim is to administer low dose of the angiostatic drug bevacizumab at this time point, in order to study its effect on alteration of the tumor vasculature and activation of the VEGF receptor.

To get more insight in the cellular mechanisms involved in the response to RTx, we next conducted a comprehensive gene expression analysis **(chapter 8)**. mRNA deep sequencing and micro-array analyses were performed on irradiated HT29 cells, *in vitro* and *in vivo*. This analysis identified the type I interferon (IFN) pathway as the most significantly enriched pathway after IR. We identified the STING pathway as the activator of IFNβ and IFNλ1 and subsequently the type I IFN response after RTx in cancer cells.

Finally, the main findings of this thesis and the perspectives for future research are discussed in **chapter 9**. Overall, research described in this thesis provides insight in the effects of RTx on tumor angiogenesis and the combination of RTx and angiostatic therapy. We found that RTx elicits a pro-angiogenic response and can enhance the tumor perfusion. In addition, low dose angiostatic drug after RTx can reverse this process and thereby enhance the anti-tumor effects. To further explore whether the findings translate to the patients is the aim of the clinical pilot study and is subject to future research.