Vascularization in an orthotopic Pleural Mesothelioma model – Characteristics and targeted treatment

PhD thesis

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1. Introduction

Mesothelioma is a relatively rare but aggressive malignancy, with dismal prognosis. Pleural mesothelioma (PM) is the most common form of mesothelioma, affecting 80-85% of all patients. The primary cause of PM is occupational or environmental exposure to asbestos. There is a long latency in the development of PM; 20-40 years can pass from asbestos exposure until the first symptoms appear. Although many countries have issued bans on asbestos usage, approximately 80% of the world's population still lives in countries where asbestos usage is not restricted. Due to the continued mining and usage complicated by the long latency period between asbestos exposure and the onset of the disease, global PM incidence is still predicted to rise in the upcoming decades. PM can originate from either layer of the serosal membrane lining of the thoracic cavity, although it more frequently arises from the parietal layer and then spreads to the visceral pleura. Three main histological subtypes were defined: epithelioid (50-70%), sarcomatoid (10-20%), and a transitional category, biphasic (10-20%). Survival time is greatly affected by tumor histology. Epithelioid tumors have the best prognosis with a median overall survival (mOS) of 14.4 months. Non-epithelioid tumors are considered more aggressive and also chemoresistant. Unfortunately, the currently available treatment options are ineffective, and the mOS of unresectable patients remains between 8-14 months. The therapeutic options for patients diagnosed at an advanced stage are limited; the backbone of the treatment is platinum-pemetrexed chemotherapy (ChT).

In order to improve clinical outcomes, first-line ChT is often complemented with the anti-angiogenic agent bevacizumab. Moreover, the role of immunotherapy in the treatment of PM is also increasing since several clinical trials yielded promising results.

Evidence suggests that angiogenesis is important in PM development, certain pro-angiogenic cytokines are overexpressed both in the tissue and serum samples of PM patients. The vasculature plays an essential role in the advancement of solid tumors and in hypoxia-mediated chemo- and radiotherapy resistance. Tumor vasculature can be very diverse in appearance and in formation. Besides sprouting angiogenesis, the most well-known way of tumor vascularization, several alternative vascularization methods are known today. One of these mechanisms is called vascular co-option. In this process, the tumors gain vasculature without neo-angiogenesis by incorporating the already existing vascular network of the host tissues. Despite the rationale, to this day, a truly effective anti-angiogenic strategy has not yet been developed for PM therapy. The microanatomical steps of PM vascularization and, moreover, how PM evades the effects of anti-angiogenic drugs are largely unknown.

2. Objectives

PM is a rare but fast-growing thoracic malignancy characterized by rapid progression and poor prognosis. Combining antivascular agents with ChT can improve cancer treatment effectiveness. Based on previous research, it has been concluded that angiogenesis is a crucial factor in the biology of PM. So far, bevacizumab (anti-VEGF antibody) is the only molecular-targeted therapy available for the treatment of PM. However, bevacizumab is anticipated to be effective only in tumors expressing high levels of VEGF. Effective personalized treatment methods for PM require further exploration of key vascularization mechanisms and the development of novel anti-angiogenic approaches.

Our research aimed to assess the microanatomical steps of PM vascularization by investigating the vascularization processes of orthotopically implanted human PM nodules and their protein- and gene-expression backgrounds. We also assessed the *in vitro* characteristics of the PM cells according to their motility, invasion potential, and interaction with endothelial cells in spheroid co-cultures. Moreover, we analyzed the role of secreted VEGF-A in tumor-induced vascular plexus formation. Finally, we studied the antitumor and antivascular effects of two anti-angiogenic drugs (bevacizumab and nintedanib) *in vitro* and in orthotopic human PM xenograft models. We compared the effectiveness of these agents in monotherapies and in combinations with conventional ChT regimes.

3. Methods

We used a combination of *in vivo*, *in vitro,* and molecular analytical methods to investigate the process of tumor vascularization during the progression of PM and the effects of anti-angiogenic treatments against PM.

In vivo

In order to be able to study the microanatomical processes of PM vascularization we used an orthotopic cell line-derived xenograft (CDX) model. Under anesthesia, human PM cells were injected into the thoracic cavity of immunodeficient SCID mice. Then, intrathoracic tumors were allowed to grow until the animals became moribund, after euthanasia the diaphragms were excised along with the tumor nodules growing on their surfaces and analyzed by confocal (whole-mount preparation and frozen sections) and electron microscopy.

We also used transgenic cell lines, VEGF-A overexpressing SPC111 (SPC111-RFP-VEGF-A), and SPC111-RFP, SPC111-mCherry and P31-mCherry cells to study the vascularization mechanisms. These cell lines were generated by infection with replication incompetent pseudotyped retroviruses and subsequent antibiotic selection.

In order to study the *in vivo* effects of nintedanib treatment, tumor-bearing mice were randomized into treatment and control groups (n=9/group) when PM nodules reached a macroscopically visible size (28 days after tumor implantation). For the survival experiments, animals with orthotopically growing P31 tumors received 1. nintedanib *per os*

(PO, 50 mg/kg), 2. nintedanib intraperitoneally (IP, 50 mg/kg), 3. solvent PO or 4. solvent IP. Animals were weighed three times a week and euthanized when they showed significant morbidity. Nintedanib was dissolved either in methylcellulose (PO treatment) or in DMSO (IP treatment).

To investigate the effects of treatment on tumor growth, P31 or SPC111 tumor-bearing animals were randomized into the following groups (n=9/group): 1. solvent IP, 2. cisplatin (3 mg/kg, dissolved in 0.9% NaCl IP) and pemetrexed IP (30 mg/kg, dissolved in 0.9% NaCl), 3. nintedanib IP (50 mg/kg, dissolved in DMSO), 4. bevacizumab IP (10 mg/kg), 5. cisplatin and pemetrexed in combination with nintedanib IP, 6. cisplatin and pemetrexed in combination with bevacizumab IP. The treatments started 21 and 12 days after P31 and SPC111 tumor cell inoculation. In both sets of experiments, cisplatin was applied once weekly, pemetrexed and nintedanib were administered five times a week on consecutive days, while bevacizumab was injected twice weekly. Body weight was checked thrice a week. To assess cell proliferation two hours before the mice were sacrificed, 200 mg/kg 5-bromo-2'-deoxyuridine (BrdU) in 0.9% NaCl was injected IP. Tumor nodules were harvested, weighed, and frozen in liquid nitrogen. For comparing two groups, Mann-Whitney U tests were applied. One-way ANOVA with Tukey's multiple comparison test was used for the comparison of more than two groups. The correlations between different parameters were calculated by Spearman correlation test. Kaplan-Meier curves for animals' survival were evaluated and the

log-rank test was used to establish the significance of the difference. All statistical analysis was performed by using GraphPad Prism 5.0 software. P values are given as two-sided and were considered statistically significant below 0.05.

All animal experiments were carried out in accordance with the ARRIVE guidelines and with the animal welfare regulations of the host institutes.

In vitro

For endothelial sprout growth assay, HUVEC, P31, and SPC111 spheroid aggregates were created by seeding cells in aggregation chambers that do not support cell adherence. The chambers were then incubated in EGM-2 medium for 1 day allowing cells to self-organize into spheroid aggregates. These aggregates were collected and embedded in 3 mg/ml fibrin gel. Anisotropy of the sprout arbor growing from endothelial (HUVEC) aggregates in contact with PM spheroids in fibrin gel co-cultures was measured on the basis of sprout morphology and by using a modified Sholl analysis. At least 7 aggregates were measured and averaged for each substrate and each cell line. Statistical analyses were performed using Student's unpaired t-test. To determine 2D and 3D motility, spheroids of SPC111 or P31 cells were transferred onto TC plastic or fibronectin-coated (5 µg/ml) surfaces. Other spheroids were embedded in collagen type I gels (1.7 mg/ml) or in combined collagen/fibronectin gels, which was produced by mixing fibronectin (10 µg/ml final concentration), human factor FXIII (2 U/ml) and thrombin (0.2 U/ml) to 1.7 mg/ml collagen type I gel.

In our *in vitro* studies and molecular analysis, we used 20 PM cell lines, 3 mesothelial cell cultures, and 1 immortalized mesothelial cell line of human origin to test the effects of nintedanib treatment.

Chemosensitivity for nintedanib and cisplatin measured by sulforhodamine B (SRB) assay determining cell viability. PM cells were seeded in 96-well plates 24 hours prior to drug exposure and then treated with different cisplatin and nintedanib concentrations for 72 hours. Clonogenicity was measured by the crystal violet method.

The effects of nintedanib treatment on cell proliferation (BrdU assay), migration (2D videomicroscopy), and apoptosis (TUNEL) were investigated in 5 cell lines (SPC212, P31, M38K, VMC40, SPC111).

Molecular analysis

Isolation of total RNA from cell lines was performed using TRIzol Reagent. Real-time qPCR was performed by the ABI 7500 Fast Real-time PCR system, and the following ABI TaqMan assays were used according to the manufacturer's instructions. COL1A1 (Hs00164004_ m1), expression was measured in P31 and SPC111 cells, PDGFRA (Hs00183486_m1), PDGFRB (Hs00387364_m1), FGFR1 (Hs009 15134_g1), FGFR2 (Hs00256527_m1), FGFR3 (Hs00179829_ m1), VEGFR1 (Hs01052961), VEGFR2 (Hs00911700_m1) and VEGFR3 (Hs01047677_m1) expression profiles were determined in 20 PM cell lines, and in control mesothelial cell cultures. GAPDH (Assay ID: Hs02786624_g1) was used as endogenous control. The relative gene expression was calculated using the ΔΔC(T) method.

Genomic DNA and array CGH analysis were isolated using 4x44K human whole genome oligonucleotide-based arrays (Agilent). Gene dose was categorized into normal, gain, amplification (ampl), loss, and deletion (del). This was done by calculating the mean signal (log2 ratio) of the respective number of oligonucleotides present on the microarray for each gene (2 oligonucleotides for VEGFR2, 3 for PDGFRA, 5 for PDGFRB, 5 for FGFR1, 1 for BAP1, 3 for CDKN2A, 4 for NF2). Borders for gain or loss were set to +0.2 or -0.2 and for amplification or deletion to +1 or -1. In case of partial gene loss or deletion, mean signals for each part of the gene were calculated.

In order to determine VEGF-A protein levels, PM cells were lysed in RIPA buffer, and protein concentration was determined using the Pierce BCA kit. VEGF-A was also measured in the supernatant by ELISA (DVE00) and secretion was calculated as pg VEGF-A/ml.

4. Results

Tumor vascularization in PM

To better understand the mechanisms of tumor vascularization in PM, cells of two human PM cell lines (P31 and SPC111) were injected orthotopically into immunodeficient mice. We found that the early process of vascularization did not differ in the two examined cell lines. Both PM lines induced the early formation of submesothelial microvascular plexuses covering large pleural areas including regions distant from tumor colonies. These microvascular networks developed due to intussusceptive angiogenesis and endothelial sprouting.

During later stages of tumor vascularization, remarkable differences were seen. While P31 nodules incorporated the aforementioned tumor-induced capillary plexuses from the earliest stages of tumor formation SPC111 colonies pushed the capillary plexuses away and thus remained avascular for weeks. In P31 tumor colonies, the tumor cells deposited a high amount of collagenous matrix of human origin, which provided “space” for further intratumoral angiogenesis. On the other hand, the key event in SPC111 vascularization was the deposition of a desmoplastic matrix of mouse origin underneath the tumor colonies. This matrix was continuously invaded by SPC111 cells, and transformed into intratumoral connective tissue trunks, providing a route for endothelial sprouting from the diaphragm. Accordingly, the relative expression levels of COL1A1 mRNA were significantly higher in P31 cells compared to SPC111 (2dCT: $1.25x10^{-1}$ vs. $2.35x10^{-6}$). Based on the

proliferation indices learned from BrdU incorporation assays we concluded that the vasculature of the diaphragm provides the highest level of nutrition for the avascular SPC111 tumors.

We also studied the role of VEGF-A in the vascular plexus formation of PM tumor nodules. The baseline VEGF-A expression profiles of the two examined cell lines differed greatly, P31 cells express a much higher level of VEGF-A (0.791 pg/ml vs. 0.03681 pg/ml). With the help of VEGF-A overexpressing SPC111 cells, we established the importance of secreted VEGF-A in tumor-induced capillary proliferation. Increased VEGF-A production accelerated the vascular plexus formation, leading to the coverage of the entire diaphragmatic surface by capillary proliferation, resulting in the death of the animals after a week of tumor cell inoculation.

We also tested PM cells' *in vitro* 2D and 3D motility and their interaction with endothelial sprouting. P31 tumor cell spheroids permitted endothelial sprouting with minimal spatial distortion in co-cultures of PM and endothelial cells, whereas SPC111 spheroids repealed endothelial sprouts. P31 cells also exhibited significantly higher 2D motility and 3D invasiveness than SPC111 cells *in vitro.*

Effects of anti-angiogenic treatment in PM

To more accurately predict the effectiveness of the anti-angiogenic, receptor tyrosine kinase (RTK) inhibitor, nintedanib, we determined the RTK expression profiles of PM cell lines. Nintedanib's target receptors were (co)expressed in all the 20 investigated human PM cell lines, and

all of the cell lines were double positive for PDGFRB and FGFR1. Also, the FGFR1 expressions were elevated compared to control cells. By genomic profiling of our PM cell lines, we identified the most common mutations in the BAP1, NF2, and CDKN2A tumor suppressor genes.

In our *in vitro* short- and long-term viability assays nintedanib effectively inhibited PM cell growth. PM cells showed a wide range of sensitivity to nintedanib (IC50: 1.6 μM – 5.9 μM), but no association was found between nintedanib sensitivity and histological subtypes or mRNA expression of target RTKs. Upon *in vitro* treatment, nintedanib reduced PM cell proliferation (in a dose-dependent manner) and migration in all of the tested cell lines. Apoptosis induction was observed only in two of the tested cell lines. An additive effect on cell viability was detected when nintedanib was combined with cisplatin at certain concentrations, however, no synergism was evident in either cell line.

We also tested the *in vivo* effects of nintedanib treatment against PM compared to the effects of bevacizumab and standard ChT, in mono- and combinational therapies. In the previously described orthotopic CDX mouse model of human PM, the survival of animals receiving nintedanib PO showed a favorable trend, but no significant benefit. However, nintedanib significantly reduced tumor burden and vascularization and prolonged the survival of mice when it was administered IP. Combined chemo- and anti-angiogenic regimens demonstrated significant *in vivo* tumor growth-inhibitory potential in both cell models when compared to untreated control tumors. However, bevacizumab was effective only

against P31 tumors with high VEGF-A levels, while nintedanib demonstrated significant *in vivo* antivascular and antitumor potential in SPC111 tumors with low VEGF-A levels.

Another key observation in this set of experiments is, that combining nintedanib with standard ChT produces significantly higher responses than ChT alone. These responses were comparable (P31) or superior (SPC111) to those achievable by the combination of bevacizumab and ChT. Moreover, in contrast to bevacizumab, nintedanib monotherapy proved to be a more effective inhibitor of *in vivo* tumor growth than standard ChT in P31 tumors. We also examined the antivascular and antitumoral effects of nintedanib treatment. Morphometric analysis using the endothelial marker CD31 revealed a strong tendency for increased microvessel areas (MVAs) in control P31 tumors with high baseline VEGF-A expression as compared with untreated SPC111 tumors (P=0.067). In accordance with the potent *in vivo* PM growth inhibitory effect of nintedanib, significantly lower MVAs were present in tumors treated with nintedanib (alone or in combination) in both models compared to controls. The robust antivascular effects of nintedanib were accompanied by increased intratumoral necrosis in both models and it was most prominent in the combined nintedanib-ChT groups. Nintedanib monotherapy significantly increased PM cell apoptosis compared with controls (P=0.0317) and decreased proliferation in P31 tumors (P=0.0341). However, no other treatment caused significant changes in tumor cell apoptosis and proliferation rates.

Conclusions

To the best of our knowledge, our study was the first to report two distinct vascularization patterns in orthotopic xenografts of human PM. Moreover, we were the first to test the *in vivo* efficacy of the triple angiokinase inhibitor nintedanib in these orthotopic PM xenograft models. Our findings most likely bear translational relevance and can assist in developing new therapeutic approaches for this deadly malignancy.

We showed that the two tumor models we investigated use significantly different mechanisms to develop intratumoral vasculature. In the invasive growth pattern (P31), tumor nodules vascularize from an early stage by the invasion and co-option of the peritumoral capillary plexuses. On the contrary, in the pushing/desmoplastic growth pattern (SPC111), tumor nodules remain avascular for an extended period of time. Then, the vascularization process starts with the deposition of a desmoplastic matrix underneath the tumor nodules. This matrix enables the endothelial ingrowth from the diaphragmatic vessels to develop a nutritive vasculature.

Significant differences were also seen in the *in vitro* characteristics of the two cell lines. The epithelioid P31 cell line was significantly more invasive, motile, and less repellent to HUVEC sprouts in co-cultures than the biphasic SPC111 cell line.

Secreted VEGF-A has an important role in the regulation of capillary plexus formation on the diaphragmatic surface, as proved by the results obtained from the VEGF-A overexpressing SPC111 transfected model.

Our preclinical testing on the potential of nintedanib as a treatment for PM yielded promising results. We determined that the target receptors of nintedanib are co-expressed on human PM cells. Furthermore, nintedanib inhibits PM cell growth, proliferation, and migration *in vitro*. Moreover, we showed that nintedanib potently reduces tumor growth and vascularization in mice with orthotopically growing human PM xenografts. Interestingly, these *in vivo* antivascular and antineoplastic effects of nintedanib are more robust in tumors with low baseline VEGF-A expression than the effects of bevacizumab.

Our findings offer valuable insights into the formation and vascularization of PM nodules and provide evidence that nintedanib has the potential to inhibit angiogenesis and tumor growth in PM, both *in vitro* and *in vivo*. These results can potentially form the foundation for personalized therapeutic approaches and future biomarker studies in PM patients.

5. Bibliography of the candidate's publications (∑ IF: 70.906)

List of publications that served as a basis for the current thesis

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