Project closing report - ANN128666

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Background Malignant pleural mesothelioma (MPM) is a chest tumor with high mortality. Angiogenesis (the formation of new blood vessels) has long been regarded as critical for malignant tumor growth. Drugs targeting the tumor blood vasculature (i.e. antiangiogenic therapies) are today well established in oncology; however, despite early optimism, the clinical results are both encouraging and disappointing. Resistance to antiangiogenic drugs arises through a range of mechanisms, but one of the clinically most relevant is being non-angiogenic tumor growth (i.e. vessel cooption or incorporation of the host organ capillaries by cancer cells). Another emerging resistance mechanism is related to the connective tissue structure of malignant tumors: Non-functional blood capillaries and irregular blood perfusion result in inadequate antiangiogenic and chemotherapeutic tumor tissue drug distribution. Thus, endothelial and cancer cells distant from functional capillaries are exposed to suboptimal drug concentrations, resulting in therapeutic resistance. The current proposal aimed to better understand these mechanisms.

Results

MPM nodules stimulate pleural angiogenesis both peritumorally and distant from the tumor site. To study the mechanisms of tumor vascularization in MPM under experimental conditions *in vivo*, human MPM cell lines were injected orthotopically into the pleural cavity of SCID mice. Colonies formed by both cell lines were allowed to grow until the animals became moribund. Notably, the number of days elapsed until the mice became moribund was lower in case of the SPC111 cell line (*vs.* P31 cells, 28–35 *vs.* 42–52 days, respectively). MPM nodules at these time points were observable all over the chest cavity including the diaphragmatic (*Figure 1A*) and costal (*Figure 1B*) surfaces of the parietal pleura. In case of both MPM cell lines, the size of the nodules ranged between 1 and 3 mm in diameter. Of note, however, SPC111 cells reached this nodule size slightly faster, thus explaining why the mice inoculated with this cell line became moribund earlier than the ones inoculated with P31. Next, the diaphragm was removed and analyzed by confocal (whole mount preparation and frozen sections) and electron microscopy. Importantly, neither the pattern of mesothelial implantation nor the morphological aspects of proliferation differed between the two cells. Specifically, as shown in *Figure 1C,1D*, both MPM cell lines induced dense, tortuous vascular proliferations bulging into the pleural space and covering large areas of the diaphragm. Regardless of the duration of the experiment, these vascular plexuses were not necessarily confined to regions covered by MPM nodules, but were found throughout the surface of the diaphragm including areas free of tumor colonies (*Figure 1C,1D*). Tumor-independent vascular plexus growth along the pleural surface was observed in both groups and these deposits were homogenous between cell lines.

MPM-induced vascular plexus formation utilizes both endothelial sprouting and intussusceptive angiogenesis (IA). In order to gain a deeper understanding of MPM-induced angiogenesis, we thoroughly examined the development of the aforementioned vascular plexuses. Notably, the growing capillary plexuses elevated above the original diaphragmatic surface but remained covered by the mesothelium (Figure S1A,S1B). 3D reconstruction of CD31 stained samples revealed numerous blind endothelial sprouts and pillars of different sizes within these capillary plexuses (*Figure 2A,2B*). Transluminal pillar formation within vessel lumens is the first step and a characteristic feature of IA. The structure of these pillars was analyzed by electron microscopy. Close to the level of the original surface of the diaphragm the pillars contained collagen bundles, which are basal components of these structures (*Figure 2C*). Farther above the original surface of the diaphragm the pillars contained amorphous ECM, rich in fibronectin, instead of the collagen bundles (*Figure 2D*). Accordingly, at this level, capillaries were embedded in a loose fibronectin-containing matrix, concentrating around the microvessels (*Figure 2E*). This provisional matrix showed signs of maturation, characterized by the appearance of collagen fibers around the vessels (Figure S1C).

The endothelium of the proliferating capillaries contained fenestrations (*Figure 2C* right lower inset) and the capillaries of the vascular plexuses were surrounded by alpha-smooth muscle actin (SMA)-positive pericytes. In contrast, pericytes of the capillaries situated deeper in the muscular layer of the diaphragm were negative for SMA (Figure S1D). In late-stage tumor nodules, SMA positive myofibroblasts were embedded in fibronectin and collagen I matrix (Figure S1E-S1G).

VEGF-A is an important promoter of vascular plexus formation. We also aimed to investigate how VEGF-A over-expression in MPM cells influences vascularization. Our studies to address this aim involved experiments with SPC111 cells stably transduced with human VEGF-A using retroviral constructs. Compared with the control SPC111-RFP cells, SPC111-RFP-VEGF-A cells secreted a much higher amount (1.97 *vs.* 10,750.47 pg/mL, respectively) of VEGF-A (Figure S2). Increased VEGF-A production by MPM cells resulted in accelerated capillary plexus formation. The development of vascular plexuses was observable from day 4 after tumor cell injection and increased dramatically by day 7 (*Figure 2F,2G*). The process culminated in the coverage of the entire surface of the diaphragm by the vascular proliferations leading to the death of the animals (day 7) even without the appearance of macroscopic tumor colonies.

Vascularization of orthotopically growing human MPM xenografts occurs through two distinct mechanisms. We analyzed the development and role of the aforementioned vascular structures in the growth of SPC111 and P31 MPM cell lines in detail. In order to be able to analyze the relationship of the developing vasculature and the tumor colonies from the earliest stage, the tumor cells were labeled by mCherry. The vascularization of the MPM cell lines differed significantly. At the earliest time points (4–5 days following inoculation), a low number of tumor cells was located within small vascular proliferations (*Figure 3A,3B*). However, as they became larger, the SPC111 colonies pushed the vascular proliferations away and remained avascular for 2–4 weeks (*Figure 3C,3D*). In contrast, the P31 cells invaded and incorporated the network of the proliferating vessels and developed into well-vascularized tumors (*Figure 3E*).
To reveal the role of the vascular proliferations in the nutrition of the avascular SPC111 nodules, we determined the BrdU incorporation in the different regions of the tumor colonies (*Figure 3F*). According to the proliferation indexes, nutrients for the avascular tumor were provided at highest level by diffusion through the vasculature of the diaphragm. The BrdU counts of this area were followed by tumor areas next to the vascular plexuses located at the periphery of the tumor colonies. Interestingly, the lowest proliferation rate was detected at the area where the tumor faced the lungs (*Figure 3F*).

The first step of SPC111 tumor vascularization is a desmoplastic reaction at the base of the tumor nodule. Microvessels appeared in SPC111 tumors at a later stage of development, 5 weeks following inoculation (*Figure 4A*). Importantly, SPC111 vascularization was preceded by the development of desmoplastic connective tissue at the base of the tumor colonies, located predominantly at the center (*Figure 4B*). This desmoplastic matrix was subsequently invaded by the tumor evidenced by malignant cells appearing between the layers of the ECM (*Figure 4C,4D*). The process above resulted in the elevation and incorporation of an ECM network forming connective tissue paths in the avascular tumors (*Figure 4D,4E*). The matrix consisted of collagen I, fibronectin and myofibroblasts (*Figure 4A,4B*, Figure S1E-S1G). Using species-specific antibodies, we were able to demonstrate the mouse origin of this matrix (*Figure 4F*, Figure S3A). First, small capillaries originating from the vessels of the diaphragm appeared in the desmoplastic connective tissue beneath the colonies (*Figure 5A*). Later, these sprouted further inside the tumor within the connective tissue paths (*Figure 5B,5C*) and remained confined to this compartment (*Figure 4F*).
As for the ECM composition of P31 tumors, we observed large amounts of collagen I and fibronectin scattered among the tumor cells (*Figure 5D*, Figure S3B,S3C). However, in P31 tumors, the majority of collagen I was of human origin, accumulating preferentially in the tumor center (*Figure 5D*).

Collagen-1 mRNA expression is significantly higher in the P31 cell line. ECM deposition is an important process during tumor vascularization. By using immunofluorescent staining, we found significantly higher

collagen I accumulation in P31 nodules (*Figure 5D*). To validate the difference at the mRNA level, we examined the expression of the human COL1A1 gene with real-time PCR in both cell lines. We found that the relative expression level of the COL1A1 was significantly higher in the P31 compared to SPC111 (Figure S4).
In addition, we have also examined the relative expression of several other transcripts of interest concerning ECM, angiogenesis, ECM production, tumor cell invasiveness, cell-cell and cell-matrix adhesion, tumor suppression, and semaphorin signaling. Notably, the relative expression of the other examined ECM components (i.e., FN1 and LOX) was also higher in the P31 than in the SPC111 tumors (nevertheless, the differences were not statistically significant) (data not shown). No such trendlines were observed in case of the other genes examined.

***Interaction of MPM cells with ES in vitro*.** To study the influence of MPM cells on the formation of endothelial sprouts *in vitro*, we developed a co-culture assay consisting of sprout-forming aggregates of HUVEC cells and MPM spheroids of P31 or SPC111 cells. The spatial structure of multicellular HUVEC sprout arbors differed significantly in these co-cultures between the two MPM cell lines. To quantify our observations, we calculated anisotropy index values [0–1] where anisotropy of a symmetric sprout arbor is close to 0, whereas a heavily distorted arbor is characterized by an anisotropy index close to 1. SPC111 spheroids resulted in significantly higher endothelial sprout arbor anisotropy than P31 spheroids ($P<0.05$, *Figure 6A*). P31 spheroids permitted HUVEC sprout growth with minimal spatial distortion, whereas SPC111 spheroids repealed endothelial sprouts resulting in anisotropic sprout arborization (*Figure 6B* and Videos S1,S2)

***2D and 3D motility of MPM cells* in vitro.** To reveal if the difference in the vascularization of SPC111 and P31 tumors could be linked to differences in the migratory and invasive capacity of MPM cells, we also analyzed the motile and invasive activity of spheroid-forming MPM cells. In support of our *in vivo* findings, P31 cells showed significantly higher spreading (motility) on plastic and on fibronectin-coated plastic and, moreover, higher invasive capacity in collagenI and in collagenI/fibronectin containing gels (Figure 6C).

In summary, P31 cells exhibited significantly higher two-dimensional (2D) motility and three-dimensional (3D) invasion than SPC111 cells *in vitro*. In co-cultures of MPM and endothelial cells, P31 spheroids permitted endothelial sprouting (ES) with minimal spatial distortion, whereas SPC111 spheroids repealed endothelial sprouts. Both MPM lines induced the early onset of submesothelial microvascular plexuses covering large pleural areas including regions distant from tumor colonies. The development of these microvascular networks occurred due to both intussusceptive angiogenesis and endothelial sprouting and was accelerated by vascular endothelial growth factor A (VEGF-A)-overexpression. Notably, SPC111 colonies showed different behavior to P31 cells. P31 nodules incorporated tumor-induced capillary plexuses from the earliest stages of tumor formation. P31 cells deposited a collagenous matrix of human origin which provided “space” for further intratumoral angiogenesis. In contrast, SPC111 colonies pushed the capillary plexuses away and thus remained avascular for weeks. The key event in SPC111 vascularization was the development of a desmoplastic matrix of mouse origin. Continuously invaded by SPC111 cells, this matrix transformed into intratumoral connective tissue trunks, providing a route for ES from the diaphragm.
These data have been published in Kovacs I et al. *Transl Lung Cancer Res* 2022; 11:991-1008.

Additional mesothelioma-related works inspired and covered (in part) by the current grant

Importantly, the current grant also enabled our team to perform a multicenter study to explore the expression and prognostic impact of PD-L1 and PD-1 of tumor cells (TCs) and tumor-infiltrating lymphocytes (TILs) in mesothelioma. (Brcic L et al. Prognostic impact of PD-1 and PD-L1 expression in malignant pleural mesothelioma: an international multicenter study. *Transl Lung Cancer Res* 2021; 10:1594-1607.)

It is also important to mention that with the help of the current grant support we could review the role of exhaled biomarkers in various lung diseases including pleural mesotheliomas (Kiss et al. *Micromachines* 2023; 14:391. doi: 10.3390/mi14020391) and, moreover, we were able to summarize the recent developments in our understanding of different mechanisms contributing to oxidative stress in COPD. (Miklos et al. *Antioxidants* 2023; 12: 1196 doi: 10.3390/antiox12061196)

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