Examination of experimental pulmonary metastases

PhD thesis

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INTRODUCTION

Tumor-induced angiogenesis and host tissue affect tumor vascularization and connective tissue structure

In the 1970's, Judah Folkman presented his revolutionary theory that the growth of tumors requires the formation of new blood vessels, meaning tumors are angiogenesis-dependent. According to the angiogenic switch theory, tumors must activate new vessel formation to grow beyond 1–2 mm in size. This idea is still the basis of the anti-angiogenic therapy of tumors. As the structure of the vasculature and the connective tissue are different in various tissues and organs, the form of tumor vascularization is variable. There are two main forms of angiogenesis in primary tumors (mammary, intestinal, skin) with large amounts of connective tissues: sprouting and intussusceptive angiogenesis. Both processes result in increased vessel density accompanied with endothelial cell proliferation. However, in the main metastasic sites (liver, brain and lung) large amount of connective tissue is not present so the metastases utilize alternative vascularization mechanisms (glomeruloid angiogenesis, vessel co-option, postnatal vasculogenesis, vasculogenic mimicry) for blood supply.

Pezzella et al. showed in the mid-1990s that in case of primary and metastatic lung tumors the growing tumor mass is able to incorporate intact alveolar walls.

In liver metastases, it was shown that the differentiation grade of the tumor can also have an impact on the histologic structure of the metastases. Three different growth patterns were described in liver metastases of colorectal adenocarcinomas. In the replacement growth pattern (high grade), the structure of the liver is preserved. However, in desmoplastic and pushing growth patterns, the structure of the liver is disturbed. In the pushing growth pattern, the tissue structure of the liver is distorted. As a result, compressed liver parenchyma surrounds the metastases.

In the desmoplastic growth, a robust fibrous capsule separates the liver parenchyma from the tumor tissue. Previously we described the development of the vasculature in a “pushing-type” experimental colorectal carcinoma model (C38) in the liver. During the growth of metastases, smooth muscle actin (SMA)-positive cells appeared at the tumor-parenchyma interface, while hepatocytes disappeared from this region. This process resulted in the appearance of vascular lakes formed by the fusion of hepatic sinusoids at the border of the metastases. Fused sinusoids and collagenous matrix-producing SMA-positive myofibroblasts became incorporated into the growing tumor. The deepest part of the invagination was separated from the surrounding host tissue, and the process culminated in the formation of connective tissue columns with a centrally located, functional vessel.

In case of undifferentiated tumors, one possible way for vascularization of liver matestases is migration of isolated tumor cells along the basal membrane in space of Disse. During the process, tumor cells separate the endothelial cells from their own basal membranes, which then proliferate and form large, convoluted blood vessels in the metastasis. There was no connective tissue deposition in the peritumoral area in this type of tumor. It is important to note that there was no peritumoral endothelial proliferation in both pushing and invasive ("replacement" growth pattern) tumor growth, so angiogenesis is not taking place in this region.

The connective tissue structure of the tumors can also be determined by the presence of cells producing extracellular matrix components. In this context, connective tissue has not been observed in experimental brain metastases, since fibroblasts cannot be found in the brain parenchyma. As indicated above, metastases showing pushing or desmoplastic growth patterns are able to activate fibroblasts at higher rates than tumors showing invasive growth pattern.

Mouse lung adenocarcinoma cells are able to use the cellular side of the basal membrane as a substrate for their growth and spread during invasion of the peripheral nervous system, muscle and fat tissue. During this process, the host cells are separated from their own basal membrane by the tumor cells, the former become eventually degraded, but their basal membrane remains intact.

AIMS OF THE STUDY

Various methods of vascularization of primary tumors and metastases have been described in different tissues and organs, but the exact mechanism of the vascularization of pulmonary metastases has not yet been fully understood.

Therefore our objectives were:

1. Determination of vascularization mechanisms of experimental pulmonary metastases in tumors of different origin.

2. Investigation of the formation of connective tissue structure in pulmonary metastases of tumors with invasive and non-invasive growth patterns.

3. Comparison of the connective tissue structure and mechanism of vessel co-option of the C38 tumor in lung metastases and in subcutaneous tissue.

4. Determination of the origin of blood supply (bronchial/ pulmonary) of experimental pulmonary metastases and the effect of this on tumor cell proliferation.

MATERIALS AND METHODS

Cell lines

In vitro cultured HT1080 human fibrosarcoma, HT25 human colon adenocarcinoma, A2058 human melanoma, MAT-B-III rat mammary adenocarcinoma, B16 mouse melanoma, and C26 and C38 murine colon adenocarcinoma cell lines were used.

Animal models

For experiments, standard C57B1/6, Balb/c, SCID mice and Fischer 344 rats from the animal house of the 1st Pathology and Experimental Cancer Research Institute of Semmelweis University were used.

Animals were intravenously inoculated with tumor cells to generate pulmonary colonies/metastases.

C38 murine colon carcinoma cells prepared from subcutaneously growing tumors were unable to colonize the lungs. Therefore, a spontaneous lung metastasis model was used. Cell suspension from the subcutaneously growing tumors were injected into the footpads of the hind leg of C57Bl/6 mice. 18-28 days following tumor cell injection the leg was amputated to remove the primary tumor and to allow spontaneous lung metastases to form.

Vascular corrosion casting

To determine the size of the metastases and to investigate their origin of blood supply we used a two-color corrosion casting procedure. The MAT-B-III model was used for the investigation of blood supply of lung metastases. Due to the anastomoses between the pulmonary and bronchial system present in the normal rat lungs, the pulmonary system was first filled with a blue resin. Thus, we prevented the resin injected into the bronchial artery system from getting into the metastases through the pulmonary artery system. When the blue resin became solid, 1 ml of red casting medium was injected through the thoracic aorta.

Determination of the size of metastases and the ratio of metastases with arterial supply

Following injection of the blue and red resins into the pulmonary and bronchial systems, the lungs were removed and cut into lobes; right superior, right inferior and left lobes.

Using stereomicroscope images the diameters of the metastases were measured on the surface of uncorroded specimens. Once the resin was cured completely, the lobes were placed overnight in 30% KOH at 45^{o}C. Comparison of corroded and uncorroded specimens was used

to determine the origin of blood supply. Within the corroded specimens, metastases were designated “bronchial” if they were filled with any amount of the red resin acquired through a bronchial artery directly connected to the metastasis. Metastases not having a bronchial (i.e. arterial) blood supply appeared on the corroded specimens as holes. To determine the size of the metastases and the origin of blood supply, 218 metastases of the MAT-B-III mammary carcinoma line were analyzed.

Immunofluorescence analysis

In the frozen sections of different sites (lungs, subcutaneous), immunofluorescence techniques were performed using antibodies against CD31, αSMA, laminin, BrdU, connexin43, collagen I and podoplanin. Samples were analyzed by a confocal laser scanning microscope (Bio-Rad, MRC-1024, Munich, Germany).

Determination of tumor and endothelial cell proliferation rates of metastases of different blood supply

The prolfierating cells were identified by BrdU incorporation technique. In frozen sections, blood supply of the metastases was determined according to the presence of red resin in the intratumoral blood vessels, then the ratio of proliferating tumor cells was determined with the anti-BrdU staining. We studied 14 bronchial blood supply and 17 pulmonary blood supply metastases in 14 animals.

For the determination of intratumoral endothelial cell proliferation, anti-laminin and anti-BrdU labels were used, while peritumoral proliferation was studied with immune electron microscopy. Fourteen metastases supplied by the bronchial artery from 8 animals and 17 metastases supplied by the pulmonary artery from 6 animals were examined. 2300 cells were counted from each metastasis, while 329 endothelial cells of capillaries and venules were analyzed peritumorally.

Determination of oxygen dependence of proliferation of tumor cells *in vitro*

The proliferation capability of A2058 and MAT-B-III cells was tested by Alamar Blue *in vitro* under hypoxic (1% O_2) and normoxic (21% O_2) conditions.

Electron microscopy

Electron microscopic analysis of lung metastases was performed in case of the B16 and HT1080 tumor lines. The anesthetized animals were perfused via the left ventricle with 0.05 mol/L Na-cacodylate buffer containing 2% glutaraldehyde. 1-2 mm^3 pieces of tumor-containing tissues (lung, subcutaneous tissue) were fixed with glutaryaldehyde and 1% OsO_4 containing K-ferrocyanide. Ultrathin sections cut by an RMC MT-7 ultramicrotome were

contrasted with uranyl-acetate and lead nitrate and analyzed using a Philips CM10 electron microscope.

3D reconstruction

To illustrate the relationship between pulmonary parenchyma and tumor tissue, serial sectioning of frozen C38 pulmonary metastasis was performed, and after staining with anti-CD31 and laminin we reconstructed the tumor mass with the Biovis3D software.

Morphometric analysis of connective tissues of C38 tumors with different localization

Frozen sections of tumor samples from all locations were stained for CD31 and laminin. Sections were scanned using Pannoramic Scanner (3D-Histech Ltd., Budapest, Hungary), and a morphometric analysis was performed using Pannoramic Viewer software (3D-Histech Ltd.). Only the columns containing one individual vessel were used during measurements. The distance between the basement membrane (BM) of the central vessel and the laminin deposited by the tumor cells around the column was measured at two sides of the vessel. At least 5 mice/tumor location and three slides from each tumor were used. Ten to twenty vessels/slide were measured.

QRT-PCR analysis

Total RNA was isolated from 10^6 B16 mouse melanoma and $2.5x10^5$ C38 mouse colon carcinoma cells. RNA concentration was measured by a NanoDrop 1000 Spectrophotometer and 1 µg RNA per sample was converted into cDNA. A high capacity cDNA reverse transcription kit was used for cDNA synthesis. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control. All samples were run in triplicate in a 20 µL reaction volume. The results were obtained as threshold cycle (CT) values. Expression levels were calculated using the ΔCT method. The values were calculated as the mean values of three independent measurements, and the expression levels of mRNA in all samples were defined as ratios to GAPDH expression (%).

Statistical analysis

Data are represented as the mean ± SD of at least three independent experiments. The statistical significance of differences between groups was analyzed with Student's t test. Values of $p<0.05$ were considered statistically significant (GraphPad Software, La Jolla, CA).

RESULTS

Vascularization of experimental pulmonary metastases

Relationship between the alveolar structure of the lungs and the vascularization of metastases in the periphery of the tumor

We studied the process of tumor vascularization in six different models of experimental lung metastasis. The size of the metastatic lung nodules that we studied ranged from 100 microns up to several millimeters. Larger tumors (>5 mm in diameter) were only observed in rats injected with MAT-B-III. The mechanism of vascularization was independent of tumor size. We found that the tumors incorporated the pre-existing alveolar capillaries (i.e. vessel co-option). During the initial phase of vessel co-option, the incorporated capillaries were still sheathed by pneumocytes, but these incorporated vessels subsequently underwent different fates dependent on the model. Alveolar walls present within the tumor were also examined at the electron microscopy level. We found that the blood-gas barrier of the normal lung, which consists of an endothel cell layer, a basal membran and an epithelial layer, was preserved at the peripheral tumor areas. The lumens of the incorporated capillaries were free of tumor cells. These data indicate that the tumor cells gained access to a vascular supply by co-opting the normal alveolar capillaries.

Interaction between incorporated blood vessels and tumor cells inside the metastases

In all models examined except one (C38 colorectal cancer model, described later), ‘naked’ microvessels surrounded by tumor cells (i.e. blood capillaries not sheathed by a layer of pneumocytes) could be detected 100-200 microns inwards from the invasive edge of the metastases. The appearance of these pneumocyte-free blood capillaries occurred due to a unique process instigated by the tumor cells themselves. Tumor cells infiltrated into the alveolar walls and invaded in between the capillaries and the alveolar epithelium. This resulted in detachment of the pneumocytes from the underlying capillaries. We used electron microscopy to examine this process at higher resolution and observed tumor cells in the process of separating pneumocytes from endothel cells. During this process, the basal membrane of the blood-gas barrier was separated into an endothelial cell-associated basal membrane layer and an epithelial-associated basal membrane layer. After splitting occurred, the tumor cells attached firmly to both layers. We also examined the presence of pneumocytes in the metastases as a function of lesion size. Whilst the central area of smaller metastases contained pneumocytes, the central area of larger metastases was generally devoid of alveolar epithelial cells. It is apparent that, after they were detached from the microvessels, these

alveolar epithelial cells underwent fragmentation and disappeared. In contrast, the denuded and incorporated microvessels survived. These vessels appear to be functional, since extensive BrdU incorporation was observed in tumor cells surrounding these microvessels even in central tumor areas.

Peri- and intratumoral endothelial cell proliferation

Since the majority of metastases we studied in mouse models did not reach the critical size (1-2 mm, according to angiogenic switch theory), MAT-B-III rat mammary carcinoma tumors were used for further investigation. The size of these tumors exceeded the critical size: mean diameter was 3.36+2.23 mm (range: 0.3-14.8 mm; median: 2.8 mm). To determine whether these tumors activated new vessel formation, we examined the endothelial cell proliferation in these tumors. We compared the proliferation rate of endothelial cells in the peritumoral region (a band of normal lung tissue 100 μm wide that was directly adjacent to the metastases) versus intratumoral regions of metastases. Peritumoral endothelial cells showed a negligible proliferation rate (1.73+0.8% of endothelial cells), but the proliferation of endothelial cells was moderately increased intratumorally (12.8+3.2%). The difference in proliferation was statistically significant ($p<0.05$). However, we detected a slight increase in microvessel perimeters towards the tumor core compared to the periphery, suggesting that endothelial cell proliferation may result in vessel dilatation.

Distribution of connective tissue in the lung metastases of tumor lines with invasive growth pattern

Under pathological conditions, the collagen-containing matrix produced by activated fibroblasts can provide a micro-environment for tumor progression. However, the αSMA positive myofibroblasts, and the amount and distribution of connective tissue collagen showed differences in the experimental tumors. During the invasion of alveolar walls of each type of investigated tumors, fibroblasts were converted into activated myofibroblasts. These αSMA-positive myofibroblasts did become incorporated into the metastases in association with the alveolar capillaries and they were in close contact with the BM of the microvessels. The number of the αSMA-positive cells did not increase toward the center of the tumor in B16 melanoma and HT1080 fibrosarcoma, only a small amount of collagen deposition was observed around the enclosed veins. In contrast, the number of activated fibroblasts increased from peritumoral lung tissue toward the tumor center in MAT-B-III, C26 and especially in HT25 colon carcinoma metastases.

Investigation of connective tissue stucture and vascularization of C38 colon adenocarcinoma in lung and subcutaneous tissue

The C38 model of lung metastasis demonstrated different behavior compared to the other models. C38 tumor cells did not reinvade the alveolar walls and thus they could not detach the pneumocytes from the alveolar capillaries; instead, these tumors incorporated the alveolar walls 'as a whole' and induced a desmoplastic response in them. As a result, in C38 metastases, alveolar walls were continuously transformed into intratumoral tissue columns (centrally located capillaries embedded in connective tissue collagen and αSMA expressing activated fibroblasts surrounded by a BM). These structures developed gradually in the direction from periphery towards tumor center. First, in the walls of alveoli (of which lumens were filled with tumor mass), αSMA expressing activated fibroblasts appeared. The number of activated fibroblasts increased towards the tumor center. Consequently, the amount of deposited connective tissue collagen and, in turn, the space between the capillaries and the epithelium, also increased. Importantly, C38 cells did not invade this space (i.e. the tissue columns); they remained in the alveolar lumens. During the development of connective tissue columns, the epithelium facing the tumor became gradually fragmented. At the end of the process, connective tissue columns with the only central capillary develop toward the center of the tumor.

In order to determine the mechanism of incorporation more precisely, subcutaneous tissue was also examined for the formation of structure of blood vessels and that of connective tissue of C38 tumor. This model has the advantage over lung tissue that in the case of a tumor with a pushing type growth pattern, the boundary of the tumor and host tissue can be easily distinguished, so the phenomenon of incorporation can be well tested. As in the C38 lung metastasis, the activated fibroblasts (myofibroblasts) expressing αSMA and the accumulation of collagen can also be observed in the edges of C38 tumors growing in the subcutaneous tissue. Invaginations of different sizes were formed at the surface of the tumors containing vessels and perivascular connective tissue. Basal membrane deposited by the tumor delineated the invaginations. In early invaginations, the number of incorporated vessels and the amount of connective tissue were dependent on the size of the vessels and the invaginations. As invaginations with multiple capillaries moved deeper into the tumor tissue, tumor cells separated the microvessels from each other. This "maturation" process culminated in the appearance of connective tissue columns with a single central vessel. In detail, the cross-sectional view of the columns showed the following structural elements from inside out: endothelial layer, capillary basal membrane, αSMA-positive cells embedded in collagen-containing matrix, and BM of the tumor, which has the same structure as the connective tissue columns observed in the lungs.

Peritumoral fibroblasts are activated by tumor cells as evidenced by increased mRNA expression of the examined fibrotic factors (TGF-beta, PDGFB, FGF2, CTGF).

The origin of blood supply to lung metastases

Because the lung has a dual circulation system, we also analyzed the origin of blood supply of lung metastases. The anastomosis system between the bronchial and pulmonary circulation is quite similar to that observed previously by us in the mouse liver. Accordingly, we applied the same corrosion casting method. In accordance with other investigators, we could not identify bronchial arteries downstream from the main bronchi in mice. Therefore, we used the MAT-B-III rat mammary carcinoma system. In the rat lung, only the main arterial branches could be filled and thus it was not possible to decide whether segmental bronchi have arterial blood supply. Investigating the origin of blood supply in metastases with different size we found that 95% of the metastases with a diameter >5 mm had an arterial blood supply and 97% of the metastases with a diameter <3 mm had a pulmonary blood supply. 65% of the arterioles supplying the tumor cells were centrally positioned in the metastases. We also compared the proliferation rate of the metastatic cells: tumor cell labelling index of metastases with bronchial blood supply was significantly higher than those with pulmonary blood supply (43.5+6.1% vs. 35.3+2.5%, respectively; mean+SD; $p<0.05$). In *in vitro* experiments there was a significantly higher tumor cell proliferation rate at higher oxygen concentrations.

CONCLUSIONS

The major findings are the followings

1. The basic mode of tumor spread was the "flow" of the tumor mass from alveolus to alveolus for each tumor line studied. As a result, all of the investigated cell lines incorporated the pre-existing host tissue capillaries within the alveolar walls, without morphological signs of active peritumoral endothelial cell sprouting.

2. After the incorporation, invasive tumors infiltrated the alveolar wall and stripped the epithelium from the co-opted alveolar walls. During the process, the tumor cells seperate the basal membrane of the alveolar wall into an epithelial and an endothelial layer. However, the denuded vessels have remained functional.

3. The non-invasive C38 tumor triggered a desmoplastic reaction in the alveolar walls, resulting formation of connective tissue columns with central vessel in the metastasis that provided blood supply for the tumor tissue.

4. In the edges of growing C38 tumors in lung and subcutaneous tissue, fibroblast activation (myofibroblast) and collagen deposition induced by fibrogenic factors produced by tumor cells was observed. The final result of the incorporation process (which occurs in the subcutaneous tissue by formation of invaginations, while in the lungs by filling of the alveolar spaces and the pores of Kohn) was that in both tissues the same connective tissue structure and vascular system (connective tissue columns with central vascular) developed. The same structures were previously observed in liver metastases and in tumors growing in the cecal wall. Therefore, it can be stated, that in each localization connective tissue columns were formed (providing blood supply for the tumor tissue), where fibroblasts are present in the environment.

5. Rat mammary carcinoma lung metastases over 5 mm have blood supply through the bronchial artery. The higher oxygen concentration ensured by the bronchial artery provides growth advantage for these metastases compared to the ones supplied by the pulmonary artery. This was confirmed in *in vitro* experiments as well.

LIST OF PUBLICATIONS

Publications, related to the thesis

1. **Szabo V**, Bugyik E, Dezso K, Ecker N, Nagy P, Timar J, Tovari J, Laszlo V, Bridgeman VL, Wan E, Frentzas S, Vermeulen PB, Reynolds AR, Dome B, Paku S. Mechanism of tumour vascularization in experimental lung metastases. ***J Pathol.*** 2015 235(3): 384-396. IF.: 7,381.

2. Bugyik E, Renyi-Vamos F, **Szabo V**, Dezso K, Ecker N, Rokusz A, Nagy P, Dome B, Paku S. Mechanisms of vascularization in murine models of primary and metastatic tumor growth. ***Chin J Cancer.*** 2016 35: 19. Review. IF.: 4,111

3. **Szabó V**, Bugyik E, Dezsõ K, Tóvári J, Döme B, Paku S. Role of tumour cell invasion/migration in the vascularisation of experimental lung metastases. ***Magy Onkol.*** 2015 59(4): 319-323.

4. Bugyik E, **Szabó V**, Dezső K, Rókusz A, Szücs A, Nagy P, Tóvári J, László V, Döme B, Paku S. Role of (myo)fibroblasts in the development of vascular and connective tissue structure of the C38 colorectal cancer in mice. ***Cancer Commun.*** 2018 38(1):46. IF.: 3,822.

Publications, related to other topics

1. Papp V, Rókusz A, Dezső K, Bugyik E, **Szabó V**, Pávai Z, Paku S, Nagy P. Expansion of hepatic stem cell compartment boosts liver regeneration. ***Stem Cells Dev.*** 2014 23(1):56-65. IF.: 3,727

2. Rókusz A, Bugyik E, **Szabó V**, Szücs A, Paku S, Nagy P, Dezső K. Imatinib accelerates progenitor cell-mediated liver regeneration in choline-deficient ethionine-supplemented diet-fed mice. ***Int J Exp Pathol.*** 2016 97(5):389-396. IF.: 1,780

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