

Examination of the vascularization of experimental brain metastases

PhD thesis

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TABLE OF CONTENTS

I. INTRODUCTION	3
I.1. Significance of tumor induced angiogenesis.....	3
I.2. Relationship of tumor structure and vascularity	3
II. AIMS	5
III. MATERIALS AND METHODS.....	6
III.1. Animal experiments	6
III.2. Sample processing	6
III.3. Molecular biological techniques.....	7
III.4. E-cadherin silencing in HT25 cell line using shRNA	7
III.5. Statistical analysis.....	8
IV. RESULTS.....	9
IV.1. Examination of experimental brain metastases	9
IV.2. Molecular biological examinations.....	10
IV.3. Wound healing	10
IV.4. shRNA E-cadherin silencing in the HT-25 cell line	11
V. CONCLUSIONS	12
VI. LIST OF PUBLICATIONS	13
VI.1. Publications, related to the thesis	13
VI.2. Publications, related to topics.....	13
VII. ACKNOWLEDGEMENTS.....	15

I. INTRODUCTION

I.1. Significance of tumor induced angiogenesis

The possibility that tumor growth requires formation of new vessels, was proposed in 1971. The evidences were defined by Judah Folkman in 1989. He observed that tumors grow slowly before vascularization, and after the appearance of vascular system they show exponential growth. He concluded that the growth of tumors is angiogenesis dependent and proposed that the inhibition of tumor growth can be achieved through inhibition of angiogenesis.

As the structure of different tissues and organs vary it is possible that the process of tumor induced angiogenesis takes place differently. Usually primary tumors contain large amount of connective tissue. Two forms of angiogenesis was described at these locations: sprouting and intussusceptive angiogenesis. Both processes result in the increase of vessel density and enhanced endothelial cell proliferation. Target organs of metastatic spread (liver, brain, lung) contain small amount of connective tissue but are highly capillarized.

Increasing amount of literature data show that instead of neoangiogenesis incorporation of host vessels may play an important role in the development of tumors. This process is known as vascular co-option. It was proposed for the very first time in 1987 that tumors can acquire their blood supply via incorporation of host vessels. This theory was neglected for years, but later, several research groups accepted this possibility. Pezzella et al. investigating human non-small cell lung cancer specimens revealed that tumors can grow without neovascularization in the lung. They divided the tumors into angiogenic and non-angiogenic types in which latter type the alveolar structure of the lung was preserved. The growth of tumors without neovascularization can particularly be true in case of the target organs of metastatic spread due to the dense capillary network and the low amount of connective tissue present in these organs.

I.2. Relationship of tumor structure and vascularity

Solid tumors are built up by tumor cells, extracellular matrix and cellular connective tissue elements. Proportion and spatial distribution of these components determines tumor structure. Vermeulen et al. defined three growth patterns in liver

metastases. These are replacement (the architecture of the liver is preserved slight increase in connective tissue deposition in the tumors is typical), pushing and desmoplastic (liver parenchyma is distorted). There is sharp tumor-parenchyma interface in case of pushing growth pattern and broad connective tissue separates the tumor tissue from the liver parenchyma in case of the desmoplastic growth pattern.

Tumor structure and growth pattern can be influenced by the differentiation rate of tumor cells too. Differentiated tumors show morphological features of the original tissue. According to morphological features, original tissue of undifferentiated tumors occasionally cannot be determined. During tumor progression the cells may undergo dedifferentiation. One of the most frequently investigated processes of this phenomenon is epithelial to mesenchymal transition (EMT). This process results in the loss of epithelial markers and appearance of mesenchymal markers. Regarding tumors, the process of EMT was brought in connection to invasion and metastasis. The regulation of EMT at molecular level is not completely understood, but one of its most important elements is the E-cadherin transmembrane glycoprotein which is a component of tight junctions.

Expression of E-cadherin influences the strength of the connection between tumor cells, thereby the structural cohesion of tumors. This supports the notion that there can be a strong relationship between the loss of E-cadherin and tumor progression.

According to the aforementioned, E-cadherin is also referred to as inhibitor of invasion and metastasis. The question, how E-cadherin exactly influences tumor progression remains to be elucidated.

II. AIMS OF THE STUDY

1. To determine the mechanism of vascularization of brain metastases in case of five different tumor cell lines.
2. Examination of the effect of growth pattern on the vascularization of brain metastases.
3. Analysis of the role of angiogenic factors and their receptors on RNA and protein level in the vascularization of brain metastases.
4. Examination of the effect of E-cadherin silencing on the growth pattern and vascularization of brain metastases.

III. MATERIALS AND METHODS

III.1. Animal experiments

Animals were obtained from the animal house of the Semmelweis University, Ist Department of Pathology and Experimental Cancer Research. During the experiments, 8 weeks old male C57Bl/6 and SCID mice were used.

Mouse C38 colorectal carcinoma, human HT25 colorectal carcinoma, HT-1080 fibrosarcoma, H-1650 lung adenocarcinoma and ZR-75-1 breast carcinoma cell lines were used.

To produce brain metastases and achieve appropriate tumor size, tumor cells were injected directly into the brain parenchyma of mice. The effects of direct injection were investigated using sham-operated mice (wound healing).

Mice were sacrificed 7-10 days after injection of C38 and HT-1080, 21-28 days after injection of HT25, H-1650 and ZR-75-1 cells. To examine the process of wound healing, mice were sacrificed after 2, 3, 4, 5, 7, 9, 14 and 21 days.

III.2. Sample processing

Mice received 200mg/kg BrdU (Bromodeoxyuridine) intraperitoneally 1 hour before termination. BrdU incorporated into the newly synthesized DNA was revealed using indirect immunohistochemical reaction. Besides BrdU, laminin labeling was performed. We used DAPI (diamidino-phenylindole) as cell nuclei marker. Specimens were investigated using Nikon TE300 fluorescent microscope (100x objective). Inside the field determined by the laminin (basal membrane of the vessels), BrdU labeled and unlabeled nuclei (endothelial cells and pericytes) were counted intratumorally and peritumorally (200µm from the edge of the tumor, 1 field of vision).

Micrographs captured by 10x objective of CD31 and laminin labeled sections were used for morphometric analysis. Vessel density (n/ mm²), vessel diameter (mm) and branching points were determined using Quick Photo Micro 2.2 software (Olympus).

Several specimens were prepared for electronmicroscopic analysis. Semi-thin (0,5µm, Reichert OmU2 Microtome) and ultra-thin (70-100nm, RMC MT-7

Ultramicrotome) sections were cut. Investigation of ultra-thin sections was performed using Philips CM10 electronmicroscope.

III.3. Molecular biological techniques

Protein lysates were prepared from all of the five tumor cell lines. Protein separation was performed using PAGE (polyacrylamide gel electrophoresis). After blotting onto PVDF (polyvinyl difluoride) membrane, expression of VEGF-A and β -actin was examined. ECL reagent was used for the development of signals. Pictures were captured using Kodak IS4000MM Digital Imaging System. Densitometric readings were performed using Kodak Molecular Imaging Software 4.0.3. Intensity values determined by the software were taken into consideration.

RNA was isolated from all of the in vitro maintained cell cultures using Trizol. Quality and quantity of RNA was measured by Nanodrop ND1000 spectrophotometer. Peritumoral and intratumoral regions were microdissected from 15 μ m thick sections of mice brains containing tumor tissue (PALM MicroBeam, Zeiss). RNA isolation was performed by using Ambion kit (RNAqueous-Micro Kit). cDNA synthesis was performed using cDNA High Capacity Archive kit (Applied Biosystems). Quantitative conversion of 1 μ g RNA (whole isolated RNA in case of laser microdissected samples) was performed in a volume of 100 μ l.

TaqMan Gene Expression AssayTM system was used for quantitative real-time PCR (QRT-PCR) analysis. GAPDH housekeeping gene was used as reference. All samples were run in triplicates, in 20 μ l reaction volume. The expression of VEGF-A, PDGF-B, ANG-1, VEGFR-2, PDGFR- β and TIE-2 was determined.

III.4. E-cadherin silencing in HT25 cell line using shRNA

Manufactured shRNA-coding plasmid was used for E-cadherin silencing (SureSilencing shRNA Plasmid for Human CDH1, SA Biosciences, Cat. No.: KH00135N). Chemical transfection (PEI, Polyethyleneimine, Sigma-Aldrich) was used to transfer plasmid DNA into cultured HT25 cells. G418 was used for cell selection from the second day (0,75mg/ml, Geneticin® Selective Antibiotic (G418 Sulfate), Invitrogen).

Immunocytochemical analysis was performed to check if the transfection was successful or not. Wild type and E-cadherin silenced cells were grown onto

cover slides and these were used for performing immunocytochemical reactions. Protein samples were prepared from the cells and in order to check the loss of E-cadherin, Western-blot analysis was performed besides immunocytochemistry.

Monoclonal E-cadherin silenced HT25 cell line was produced using 96 well plate. Cells were placed onto the plate and wells with just one single cell were marked and finally, according to morphological features 3 clones were selected for further culturing. From these, the third one was selected for the next experiments.

According to the immunocytochemistry and Western-blot, the proper cell line was cultured and injected into mice brains. HT25 wild type cells were injected as control.

Immunohistochemical and morphological analysis was performed on wild type and E-cadherin silenced tumors. We examined tumor growth pattern (panCK-FITC), intratumoral vessel density (CD31, laminin) and the proliferation rate of vascular cells (BrdU, laminin, DAPI).

III.5. Statistical analysis

We performed t-test to evaluate our data. We used a statistical significance level of 0,05.

IV. RESULTS

IV.1. Examination of experimental brain metastases

Brain metastases were classified into different groups according to their growth patterns observed on HE stained sections. Fluorescently labeled slides helped us to determine the relation of tumors to the elements and structures of the brain parenchyma. The two colorectal carcinomas (C38, HT25) and the HT-1080 fibrosarcoma showed pushing type growth pattern. Smooth tumor-parenchyma interface is the characteristic feature of this type of growth. H-1650 and ZR-75-1 tumors showed more invasive growth pattern. In case of H-1650, cohesive cell groups invaded the brain parenchyma, so this growth pattern was a transient (lobed edge) growth type between the pushing and invasive growth. In ZR-75-1 tumors, there were scattered round cells in the parenchyma at the periphery of the tumor.

Astrocytes were detached from the vessels by the tumor cells, so that, there were just a few GFAP positive cells intratumorally. The invasive ZR-75-1 breast carcinoma tumors contained the most astrocytes.

Desmin positive pericytes surrounded by basal membrane remained attached to the vessels. Tumors incorporated the vessels with pericytes which preserved their location between their own and the endothelial cell's basal membrane.

The brain parenchyma was displaced by the tumor cells, vessels were incorporated, thus vessels served as the only substrate for the tumor cells to attach to. As soon as tumor cells attached to the basal membrane of the vessels they deposited their own basal membrane onto the surface of the basal membrane of the vessels.

There was no difference between the vessel densities of peritumoral and control (tumor-free) areas. Intratumoral vessel density was significantly lower than peritumoral vessel density in all of the examined tumor types. There was no growth in vessel diameter in the peritumoral areas. There was one exception, the C38 colorectal carcinoma where the vessels touching the periphery of the tumor showed significant dilatation compared to the peritumoral values.

In all of the tumors, intratumoral vessel diameter was significantly higher than peritumoral vessel diameter. Vessels of the two colorectal carcinomas showed the highest diameter. This fact is in accordance with the observation that the proliferation rate of intratumoral vascular cells (determined after BrdU incorporation) increased notably only in the pushing type tumors. The proliferation rate in case of C38 was higher in the vessels touching the periphery of the tumors too. In the two invasive tumors (ZR-75-1, H-1650) proliferation rates of the vascular cells remained moderately low. Proliferation rate of the peritumoral vascular cells was negligibly low in all of the tumor types.

IV.2. Molecular biological examinations

VEGF-A, PDGF-B and ANG-1 relative expressions were determined in the 5 tumor cell lines and in microdissected intratumoral areas. mRNA expressions correlated well between cell lines and tumor samples. Interestingly, the C38 colorectal carcinoma and HT-1080 fibrosarcoma cells and tumors showed the lowest intratumoral angiogenic factor expressions in contrast their vascular cell proliferation rates were the highest. VEGF-A and ANG-1 expressions were highest in the HT-25 microdissected intratumoral sample. Regarding PDGF-B, ZR-75-1 intratumoral sample showed the highest mRNA expression level.

VEGFR-2, PDGFR- β and Tie-2 mRNA expression was determined in microdissected intra- and peritumoral samples. The expression of angiogenic factor receptors was higher in all intratumoral samples.

Protein expression level was determined in case of VEGF-A, using cell lysates of cultured cells. In contrast with mRNA levels, Western-blot showed just small differences between the cell lines.

Intratumoral angiogenic factor, angiogenic factor receptor and tumor cell VEGF-A protein expression levels did not correlate with the proliferation rate of intratumoral vessels.

IV.3. Wound healing

There was no increase in vessel density inside the wound or around it in case of all the examined time points compared to the normal brain parenchyma. Vessel density was significantly lower up to the 9th day then it reached the control value.

Vessel diameter corresponded to the control value. Proliferating cells could be identified between the 2nd and 5th day. Its maximum was at the 3rd day but this increase was negligible.

IV.4. shRNA E-cadherin silencing in the HT-25 cell line

After transfection and geneticin selection, E-cadherin silencing was monitored by immunocytochemical examinations. Monoclonal cell culture was started using 96 well plates. 3 clones were selected for further culturing. Differences between the cultured wild type and E-cadherin silenced cells could already be seen under phase-contrast microscope. Wild type HT25 cells grew in coherent groups E-cadherin silenced cells lost their cell-cell contacts cells were rounded and grew scattered. Immunocytochemistry showed that E-cadherin silencing was successful. According to the results we selected the 3rd clone for further experiments. We confirmed the silencing by Western-blot analysis as well. E-cadherin protein could not be detected in the silenced cell line.

The effect of silencing was examined also in vivo. Frozen sections of experimental brain metastases were used to determine the growth pattern of the silenced clone. The sections were stained by anti-panCK-FITC and anti-laminin to analyze the structure of the tumor and its relationship to the vessel of the brain parenchyma. The two variants of the HT25 cell line showed different growth patterns. Wild type cells showed the previously seen pushing growth pattern with smooth tumor-parenchyma interface. In contrast tumors of the E-cadherin silenced cells showed loosened tumor structure. Cells lost their contacts scattered rounded cells were characteristic at the periphery of the tumor.

Morphometric analysis showed that compared to the wild type tumors, vessel density increased and vessel diameter decreased significantly ($p \leq 0,05$) in tumors of E-cadherin silenced cells.

Proliferation rate of intratumoral vascular cells was significantly ($p \leq 0,05$) lower in E-cadherin silenced ($3,32 \pm 0,79\%$) than in wild type ($6,27 \pm 1,05\%$) tumors.

V. CONCLUSIONS

Major findings are the following:

1. Sprouting angiogenesis cannot be observed in the peritumoral brain parenchyma in all of the examined experimental brain metastases. Tumors acquired their blood supply by incorporation of the pre-existing vessels.
2. After examination of tumor structure, we divided the tumors in three types of growth patterns: 1. pushing (smooth tumor parenchyma interface), 2. transient (lobed edge), 3. invasive (scattered cells at the tumor periphery).
3. Relationship was revealed between the vessel density and growth pattern of experimental brain metastases. Differentiated tumors incorporated lower amount of vessels than loosely structured less differentiated ones. Diameter of intratumoral vessels and proliferation rate of vascular cells showed inverse correlation to vessel density.
4. There was no correlation between the proliferation rate of vascular cells and VEGF-A mRNA- or protein-, and PDGF-B, ANG-1 mRNA expression. The PDGFR- β , TIE-2 and VEGFR-2 mRNA expressions increased intratumorally in all of the tumors. This indicates that instead of new vessel formation, the examined angiogenic factors and their receptors rather have a role in stabilization of intratumoral vessels.
5. Differentiation status of tumors is in close relationship with the growth pattern. Lower differentiation state of the same tumor line results in tumors with higher vessel density. This is associated with lower proliferation rate of vascular cells and lower vessel diameter.

VI. LIST OF PUBLICATIONS

VI.1. Publications, related to the thesis

1. Dezsó K, Bugyik E, Papp V, László V, Döme B, Tóvári J, Tímár J, Nagy P, Paku S. (2009) Development of arterial blood supply in experimental liver metastases. *Am J Pathol*, 175:835-43.
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VI.2. Publications, related to other topics

1. Turányi E, Dezsó K, Bugyik E, Szurián K, Paku S, Nagy P. (2010) The primary mitogen (TCPOBOP)-induced hepatocyte proliferation is resistant to transforming growth factor- β -1 inhibition. *Liver Int*, 30:1505-10.
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