FINAL REPORT

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Breast cancer is the second most frequent cause of brain metastases, formation of which is a multistep process yet poorly understood. To form a new colony in the brain the tumor cells must breach the tight blood-brain barrier (BBB), which plays a double role in this process. The tight barrier represents an impediment for circulating tumor cells, but it also has a protective role against chemotherapeutic drugs targeting brain metastatic cells. The metastatic tumor cells remain in close contact with endothelial cells and also interact with other brain residential cells e.g. pericytes, astrocytes. Thus adaptation of the tumor cells to the new microenvironment may ascertain the ability of metastasis formation *(Wilhelm et al., Foe or friend? Janus-faces of the neurovascular unit in the formation of brain metastases, DOI: 10.1177/0271678X17732025.)*

During the period of this project we successfully described several aspects of the formation of breast cancer brain metastases. The main steps of metastases formation investigated by us were the following: adhesion of tumor cells to brain endothelial cells, transmigration of tumor cells through the endothelial layer, interaction of tumor cells with the cellular components of the neurovascular unit. Among the risk factors of brain metastases, the triple negative (ER-, PR-, HER2-) breast cancer (TNBC) subtype has the worst outcome of mammary carcinoma subtypes. In our in vitro and in vivo experiments we have been using the mouse TNBC 4T1 and the human TNBC MDA-MB-231 cell lines.

The adhesion of tumor cells may depend on the tumor-derived extracellular vesicles (EVs), which are able to set the pre-metastatic niche for migrating tumor cells. In order to investigate the effect of EVs, we have pretreated brain endothelial cells with triple-negative breast cancer cell derived exosomes. The fluorescently labelled exosomes are taken up by endothelial cells and they accumulate in the cells in a time dependent manner. Through the immobilization of a living breast cancer cell to an atomic force microscope's cantilever, intercellular de-adhesions were directly measured by single cell force spectroscopy (SCFS) at quasi-physiological conditions. De-adhesion dynamics and strength was characterized by several different calculated parameters, involving aspects of both membrane and cell surface related factors. These experiments simulate the first contact of blood-circulating tumor cell with the brain endothelium. This first contact appeared to be weaker when endothelial cells were pre-treated with tumor cell derived exosomes. *(Fazakas et al., Breast adenocarcinoma-derived exosomes lower first-contact de-adhesion strength of adenocarcinoma cells to brain endothelial layer, DOI: 10.1016/j.colsurfb.2021.111810).*

Development of brain metastases may also depend on the expression of several proteases and their endogenous inhibitors, playing important role in the adaptation processes to the brain microenvironment.

Tumor cell-secreted proteolytic enzymes and their specific endogenous inhibitors contribute to different steps of cancer invasion and metastasis formation. Serine and cysteine proteases are key players in brain metastasis formation. Although the general role of proteolytic enzymes in

tumor progression and metastasis formation is well known, due to the presence of the BBB, metastatic processes of the brain have special characteristics. These specific aspects remain largely unexplored so far. The cell-cell interactions as well as the released factors might regulate the expression and function of proteases and their inhibitors in invading tumor cells as well as in host brain endothelial cells. We have previously shown that highly metastatic melanoma cells release seprase serine protease, and that the conditioned media of brain endothelial cell is able to induce the upregulation of seprase in melanoma cells. On the other hand, the conditioned media of tumor cells induced endothelial-mesenchymal transition, resulting in a facilitated transmigration of tumor cells through brain endothelial layer.

The main aim of the present proposal was to better understand the expression, regulation and function of serine proteases and their inhibitors in the formation of breast cancer brain metastases. The cellular cross-talk between brain endothelial cells and breast cancer cells resulted in the induction and increased release of serine proteases as revealed by zymography experiments. In order to identify these proteases, we examined the expression of several serine proteases in human (MDA MB 231, MCF 7 cell lines) and mouse (4T1) breast cancer cells. Seprase was not expressed by the human breast cancer cells; this gelatinolytic serine protease seems to be restricted to melanoma cells among brain metastatic cells. On the other hand, we found that breast cancer cells expressed uPA, matriptase (ST14), furin and TMPRSS4 and the endogenous inhibitors of these proteases, namely SERPINE1-2.

We hypothesized that these proteases and protease inhibitors might be upregulated or downregulated during brain metastasis formation. To investigate the effect of soluble factors released by the tumor microenvironment on the expression changes of these proteases and serpins, MDA-MB-231 breast cancer cells were treated with the conditioned media derived from brain endothelial cells or astrocytes. Interestingly, both conditioned media reduced the mRNA expression of uPA and furin, whereas the SERPINE2 and ST-14 mRNA expression levels decreased upon treatment with astrocyte-conditioned medium.

Matriptase (ST14) a type II transmembrane serine protease expressed by breast cancer cells has a controversial role in the progression of breast cancer. We have shown that matriptase is expressed at the mRNA and at the protein level as well by MDA-MB-231 cells. Applying the RNA interference method, we have successfully silenced the ST14 in MDA-MB-231 cells. However, in transmigration experiments the downregulation of matriptase has not influenced significantly the number of transmigrated breast cancer cells. The expression of ST14 showed a slight increase upon treatment of breast cancer cells with conditioned media of brain endothelial cells. We have observed that the HER2+ T47D breast cancer cells expressed ST14 in higher amount at the protein level in comparison to triple negative cell lines.

A different matriptase expression profile of the tumor cells has emerged between the step of transendothelial migration (when the tumor cells come in contact with brain endothelial cells) and the phase of intracerebral proliferation (contact with astrocytes).

The effect of brain endothelial cells on the expression proteases and protease inhibitors of breast cancer cells was assessed using an array study. Breast cancer cells were cultured for 24 hours

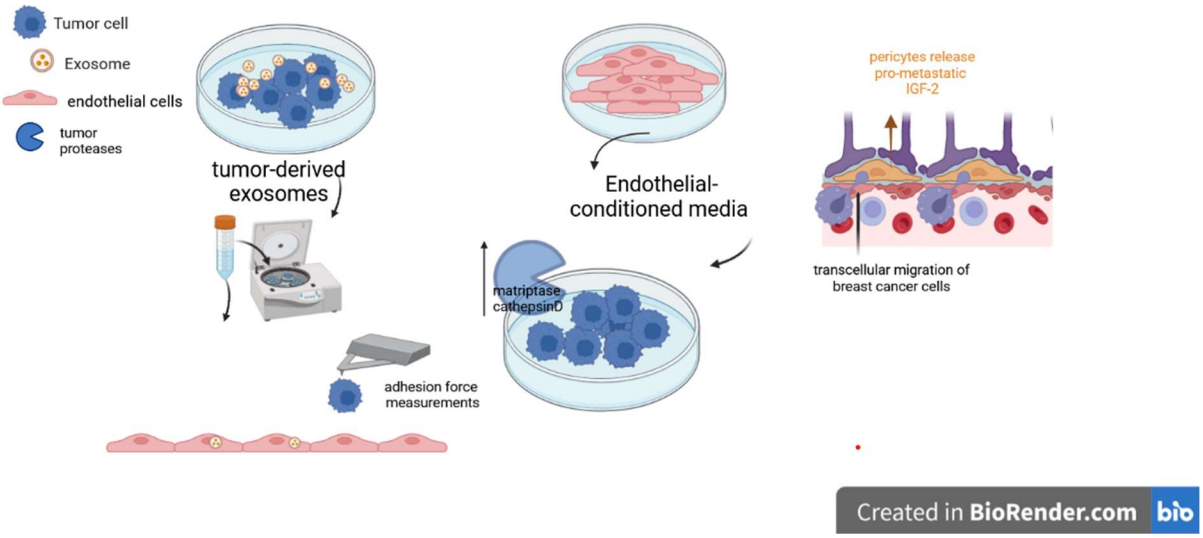
in endothelial cell conditioned media, followed by cell lysate preparation and the array study. The array detects simultaneously 32 different proteases and 32 protease inhibitors from the same samples. The soluble factors in endothelial conditioned media elevated the expression levels of cathepsin A, D, and X/Z/P and ADAM 9, and decreased the level of DPPIV (dipeptidyl dipeptidase 4). In the same time the expression of protease nexinII, cystatin A, B, C, EMMPRIN, HAI-1, HAI-2, TIMP-1 were decreased compared to control. The level of urokinase plasminogen activator (uPA) was very high in both control and endothelial conditioned media treated breast cancer cells.

According to literature data, the identified proteases and inhibitors are involved in tumor progression. Using western blot, we aimed to validate changes in the expression of cathepsin A, X/Z/P and D in tumor cells. We observed that endothelial conditioned media induced a significant increase in the relative amount of the processed (31kDa) form of cathepsin D. In the case of cathepsin A only a slight increase was detected using Western blot analysis, while the other cathepsin family members tested did not show any change in their expression.

Previously we have shown that in comparison to breast cancer cells melanoma cells have increased adhesion to and transmigration rate through brain endothelial cells. Metastatic melanoma cells disrupt the tight junctions of brain endothelial cells using the paracellular route in their migration to the brain parenchyma. Our new experiments revealed that breast cancer cells prefer the transcellular route in their extravasation. Using in vitro and in vivo methods we have demonstrated that breast cancer cells are able to incorporate themselves within the brain endothelium. During this process the cofilin is activated in endothelial cells involving the remodelling of the actin-myosin network. On the other hand, melanoma cells breach the BBB through the disruption of TJs and through the involvement of N-cadherin in the paracellular transmigration *(Herman, Fazakas et al. Paracellular and transcellular migration of metastatic cells through the cerebral endothelium, DOI:10.1111/jcmm.14156, rank Q1, shared-first author article).*

To decipher the changes induced by tumor cells arrest in circulation, transendothelial migration and survival in the new microenvironment we performed in vivo and in vitro experiments using FVB/Ant:TgCAG-yfp_sb #27 and Balb/C female adult mice and Tdtomato- or eGFP expressing 4T1 mouse cell lines. The morphological and functional changes of tumor cells and brain endothelial cells were followed using real-time in vivo (two-photon microscopy) and ex vivo microscopy. After inoculation tumor cells remained in the capillaries in a resting phase for the first 48 hours, and induced vasoconstriction and formation of endothelial plugs. We observed that tumor cells favored to adhere in vascular branching points and left the tight junctions intact in the first four days. The migration through the vessel walls occurred mainly on the 5th day, and was accompanied by blebbing of tumor and endothelial cells. During the extravasation and blebbing tumor cells released extracellular vesicles *(Hasko, Fazakas et al., Response of the neurovascular unit to brain metastatic breast cancer cells DOI: 10.1186/s40478-019-0788-1, rank Q1).*
Moreover, we have shown the importance of pericytes and pericyte-released insulin-like growth factor 2 (IGF2) in the formation of breast cancer brain metastases. Our in vivo and in vitro experiments unveiled that pericytes have a crucial role in the development of brain tumors.

Pericytes through the secreted extracellular matix components help the adhesion of tumor cells. Moreover, pericytes secreted insulin-like growth factor 2 (IGF2), which enhanced remarkably the proliferation of breast cancer cells *(Molnár et al., Pericyte-secreted IGF2 promotes breast cancer brain metastasis formation, DOI: 10.1002/1878-0261.12752)*.



Schematic representation of the main results.

We have also investigated the effects of potentially new therapeutic compounds derived from Juncus gerardii and Persicaria maculosa plants on tumor cell viability and proliferation. We tested the cytotoxicity of several phenanthrenes from Juncus gerardii as well as diarylheptanoid-type constituents from Persicaria maculosa in triple-negative breast cancer cells and in cerebral microvascular endothelial cells. Impedance measurements and viability tests showed that four dimeric compound gerardiins from Juncus gerardii were cytotoxic to breast cancer cells and endothelial cells *(Stefkó et al, Gerardiins A–L and Structurally Related Phenanthrenes from the Halophyte Plant Juncus gerardii and Their Cytotoxicity against Triple-Negative Breast Cancer Cells, DOI: 10.1021/acs.jnatprod.0c00631).*

The two chalcones, 2′-hydroxy-3′,4′,6′-trimethoxy chalcone and pashanone, from Persicaria maculosa decreased cell viability of tumor and non-tumor cells, while the other compounds did not show any potency. We have also shown that the 2′-hydroxy-3′,4′,6′-trimethoxy chalcone decreased cell viability in a concentration dependent manner. On the other hand, pashanone had toxic effect only in endothelial cells *(Vasas et al, Flavonoid, stilbene and diarylheptanoid constituents of Persicaria maculosa Gray and cytotoxic activity of the isolated compounds, DOI: 10.1016/j.fitote.2020.104610).*