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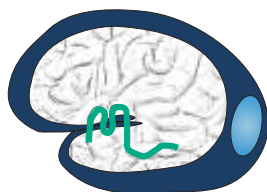


# **TRANSMIGRATION OF BREAST CANCER AND MELANOMA CELLS THROUGH THE BLOOD-BRAIN BARRIER: SIMILARITIES AND DIFFERENCES**

Ph.D. Thesis

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SZEGED  
2016

## INTRODUCTION AND AIMS

Brain metastases of malignant tumors have a very poor prognosis and are frequent complications for cancer patients. The most common cancers that spread to the brain are lung cancer (40-50% of brain metastases originate from lung carcinomas), breast cancer (15-25%) and melanoma (5-20%). The survival of patients with central nervous system (CNS) metastases is low: the median survival time is 4-6 months, and only 20-40% of patients are alive at 1 year after the diagnosis. Among all solid tumors, melanoma has the highest affinity to the brain, according to autopsy series 70-90% of these patients have brain metastases. Since the CNS parenchyma lacks a lymphatic circulation, the only possibility for cancer cells to reach the brain is via the blood stream, i.e. through the blood-brain barrier (BBB).

The main function of the BBB is to maintain the homeostasis of the CNS. The BBB is located at the level of cerebral capillaries in the forefront of the defense line of the CNS and restricts the free movement of solutes and cellular elements between the systemic circulation and neuronal tissue. The most important cellular elements of the BBB are cerebral endothelial cells (CECs), astrocytes and pericytes, which together with the extracellular matrix and neurones form the neurovascular unit. Endothelial cells lining brain capillaries are thin, flat cells interconnected by tight junctions (TJs), which are indispensable for the barrier function. Key components of the TJs are transmembrane proteins which are one of the most important elements of the paracellular permeability. The main transmembrane TJ proteins are occludin, claudins (mainly claudin-5), junctional adhesion molecules (JAMs) and other proteins. The most important peripheral proteins of TJs are zonula occludens (ZO) proteins: ZO-1 and ZO-2. Endothelial cells are also characterized by low number of vesicles and presence of different transporters and enzymes.

Metastatic tumor cells are able to cross this tight barrier, but the mechanisms involved in the migration of cancer cells through the BBB are largely uncharacterized. According to our previous results, during this process melanoma cells produce and release large amounts of gelatinolytic serine proteases, including seprase. These proteases facilitate the transendothelial migration of tumor cells. We have also shown that both breast cancer and melanoma cells are able to induce endothelial-mesenchymal transition of CECs, which results in loss of junctional integrity.

In our studies we aimed at further understanding the mechanisms of transmigration of tumor cells through the BBB. We focused on two tumor types

with high incidence of brain metastasis formation, i.e. melanoma and breast cancer. Comparison of the interaction of these two different tumor cell types with the cerebral endothelium might help in understanding whether the transendothelial migration step of metastasis formation has any role in the higher tropism of melanoma cells towards the CNS.

In addition, we investigated the signaling pathways involved in the diapedesis of melanoma and breast cancer cells into the brain. We have previously shown that inhibition of Rho/ROCK signaling and induction of the mesenchymal phenotype facilitated the transmigration of melanoma cells through the BBB. Besides mesenchymal migration, tumor cells can use the amoeboid one, which can be induced by Rac inhibition. Here we investigated the impact of Rac inhibition on the interaction of breast cancer or melanoma cells with the BBB. Moreover, we studied the role of PI3K signaling, since this pathway is frequently activated in different tumor cells.

Our investigations had five specific aims:

1. to compare the transmigration properties of melanoma and breast cancer cells through brain endothelial monolayers under static and dynamic conditions,
2. to compare the effects of melanoma and breast cancer cells on the tight junctions of confluent cerebral endothelial cells,
3. to understand the role of Rac signaling and of amoeboid vs. mesenchymal phenotype in the transmigration of melanoma and breast cancer cells through the BBB,
4. to investigate the impact of PI3K inhibition on the transmigration of tumor cells (melanoma and breast cancer cells) through the cerebral endothelium during brain metastasis formation,
5. to observe the effect of Rac and PI3K inhibitors on the barrier integrity of the brain endothelium.

Understanding the role of these mechanisms might lead to the elaboration of new preventive and treatment strategies in metastatic diseases of the brain.

## **MATERIALS AND METHODS**

### *Cell culture and treatments*

MDA-MB-231 and MCF-7 human breast cancer cells were kept in DMEM medium (Sigma) supplemented with 5% FBS (Lonza). A2058 human melanoma cells (obtained from the European Collection of Cell Cultures) were maintained in EMEM (Sigma) supplemented with 5% FBS (Sigma). A375 human melanoma cells were kept in DMEM medium (Sigma) supplemented with 10% FBS (Lonza). 4T1 mouse breast cancer cells were kept in RPMI medium (Lonza) supplemented with 5% FBS (Lonza). The hCMEC/D3 human microvascular cerebral endothelial cells (abbreviated as D3) were grown on rat tail collagen-coated dishes in EBM-2 medium (Lonza) supplemented with EGM-2 Bullet Kit (Lonza) and 2.5% FBS (Sigma). Rat brain endothelial cells (RBECs) were used for immunofluorescence experiments because of their superior barrier characteristics. Primary rat brain endothelial cells (RBECs) were isolated from 2-week old rats. Briefly, after removal of meninges cerebral cortices were cut into small pieces and digested with 1 mg/ml collagenase type 2 (Sigma). After separation of myelin by centrifugation in 20% bovine serum albumin (BSA), a second digestion was performed with 1 mg/ml collagenase/dispase (Roche). Microvessel fragments were collected after 10 min 1000•g centrifugation on Percoll (Sigma) gradient, and plated onto fibronectin/collagen-coated dishes. Endothelial cells growing out of the microvessels were cultured in DMEM/F12 (Life Technologies), 10% plasma-derived serum (First Link) and growth factors. In the first two days, 4 µg/ml puromycin was added to remove contaminating cells.

ROCK inhibitors (Y27632, Tocris and fasudil, Santa Cruz) were used in a final concentration of 10 µM. EHT1864 (Tocris), an inhibitor of the Rac family GTPases, was applied in a 20 µM concentration. LY294002 (Cell Signaling Technology) – a reversible and highly selective inhibitor of phosphatidylinositol 3 kinase (PI3K) – was used in a concentration of 25 µM.

### *Adhesion experiments*

Brain endothelial cells (D3) were grown until confluence in 24-well plates. Tumor cells (MDA-MB-231, MCF-7, A2058 or A375 cells) were fluorescently labeled using Oregon Green 488 carboxylic acid diacetate succinimidyl ester (Life Technologies) using the protocol supplied by the

manufacturer.  $5 \cdot 10^4$  tumor cells/well were plated onto the endothelial monolayer in serum-free medium and incubated for 90 min. Non-attached cells were washed and the remaining cells were fixed using ethanol/acetic acid (95/5) at  $-20^\circ\text{C}$  for 5 min. Tumor cells adhered to endothelial cells were photographed and counted using the Image-Pro Plus software (Media Cybernetics).

### ***Static transmigration experiments using time-lapse video imaging***

Human cerebral endothelial cells (D3) were cultured until confluence in 12-well plates.  $2 \cdot 10^4$  tumor cells/well were plated onto the endothelial monolayer in Leibovitz's L-15 medium (Sigma). Cells were monitored for 6 h using an Andor NEO sCMOS camera connected to a Nikon Eclipse Ti-E inverted microscope, equipped with a home built incubator set to  $37^\circ\text{C}$ . Phase-contrast images were made every 5 min from 5 optical fields/well and time-lapse videos were constructed. The movement of each tumor cell was evaluated and transmigrated cells were counted.

### ***Dynamic transmigration experiments using microfluidics***

To investigate the transmigration of tumor cells under low shear stress conditions we designed and constructed a biocompatible artificial capillary network. The microfluidic capillary device was fabricated from polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) using standard photolithography and soft lithography techniques. (Qin et al, 2010) Imprints of the microdevices were built by creating  $100 \mu\text{m}$  high SU8-2050 negative photoresist (MicroChem) layers on silicon wafers. The photoresist layers were exposed to UV light through a chromium mask (JD Photo-Tools), using a flood exposure source with mask aligner (500W Hg lamp, i-line, model 97435, Newport & Digital Exposure Controller model 68945, Newport). In order to prevent the attachment of PDMS to the SU8 molds, the molds were treated with tridecafluoro-1,1,2,2-tetrahydrooctyl-trichlorosilane (Gelest) under vacuum overnight. Positive replicas were fabricated by PDMS molding. The PDMS replicas were cured, inlet holes and bubble traps were punched and the devices were bound to PDMS-covered microscope glass slides using oxygen plasma treatment.

Prior to seeding of brain endothelial cells, the inner surface of the channels was coated with rat tail collagen. 106 D3 cells were collected in 100  $\mu$ l Leibovitz's L-15 media completed with 2.5% FBS (Sigma), growth factor mix (Lonza), hydrocortisone and gentamicin-amphotericin-B and injected into the microchannels. The microfluidic devices were placed in a home built incubator installed on a microscope stage set to 37°C. Cells were kept in "static conditions" for 24-36 h to reach a confluent layer. During this static state the medium was refreshed every 8 h. When the confluent endothelial layer fully developed, a continuous flow of 300  $\mu$ l/h rate was started and maintained for 24 h to mimic the blood circulation.

During transmigration experiments,  $3 \cdot 10^5$  tumor cells (A2058 or MDA-MB-231) were collected in 100  $\mu$ l media and injected manually (with syringe). After the injection, a continuous flow with 100  $\mu$ l/h rate was established and maintained for 6 h. Considering the physical parameters of the device (channel height of  $\sim 100 \mu$ m, channel width of  $\sim 240$ - $480 \mu$ m) and the used fluid flow rates (100-300  $\mu$ l/h), we can estimate the shear stress (based on (Song et al, 2005) acting on the endothelial cells in the microchannels. The applied fluid flow generated a low stress regime in our device, in which the shear stress was around  $\sim 0.3$ - $2 \text{ dyn/cm}^2$ .

Phase contrast microscopy images were taken during cell growth and transmigration phase, using an Andor NEO sCMOS camera and a Nikon Eclipse Ti-E microscope (Nikon), equipped with a 20 $\times$  Plan Fluor phase contrast objective and a Proscan II motorized microscope stage (Prior Scientific). We used the Nikon NIS Elements AR software (Nikon) to control the microscope setup during the recordings. Microscopy images were taken every 30 min during the endothelial cell attachment phase and every 5 min during the transmigration experiments.

### ***Immunofluorescence studies***

RBECs were cultured until confluence on collagen/fibronectin-coated filter inserts. Tumor cells (MDA-MB-231, A2058 or 4T1) were fluorescently labeled using CellTracker™ Red (Life Technologies) and plated onto the endothelial monolayer. After 5 h cells were washed and fixed with ethanol/acetic acid. After blocking with 3% BSA, filter inserts were incubated with anti-claudin-5 primary antibody (Life Technologies). The staining was visualized using Alexa488-conjugated secondary antibody (Jakson

Immunoresearch). Nuclei were stained with Hoechst 33342 (Sigma). Samples were mounted in FluoroMount-G (SouthernBiotech) and studied with a Nikon Eclipse TE2000U microscope connected to a digital camera (Spot RT KE, Diagnostic Instruments).

### ***Cell viability assay***

Viability of tumor cells and endothelial cells was quantified with the EZ4U non-radioactive cell proliferation and cytotoxicity assay (Biomedica). D3, MDA-MB-231 and MCF-7 cells were seeded in 96-well plates. Next day cells were treated for 5 h with 20  $\mu$ M EHT1864 or 25  $\mu$ M LY294002 in serum-free, phenol red-free DMEM (Life Technologies). After incubation with the EZ4U substrate for 45 min, the absorbance (OD at 450 nm) was detected using a BMG FLUOstar OPTIMA microplate reader.

### ***Wound healing assay***

Tumor cells (MDA-MB-231, MCF-7 or A2058) were seeded into 24-well plates. After attachment the cell layer was wounded by scratching with a pipette tip, washed with PBS, and exposed to treatments with 20  $\mu$ M EHT1864 or 25  $\mu$ M LY294002 in serum-free Leibovitz's L-15 medium. Cells were monitored over 24 h, and phase contrast images were taken every 30 min with an Andor NEO sCMOS camera connected to the Nikon Eclipse Ti-E inverted microscope equipped with a home-built incubator set to 37°C and a 20 $\times$  Nikon Plan Fluor objective, all placed onto a Prior Proscan II motorized stage (Prior Scientific Instruments). The wound healing effect was quantified by averaging the number of migrating cells counted in five wounded areas.

### ***Real-time impedance monitoring***

To monitor the effects of EHT1864 and LY294002 on D3 cells in real-time, we measured the electrical impedance using the xCELLigence system following the manufacturer's instructions (Acea Biosciences). Briefly, cells were seeded at a density of 104 cells/well into 100  $\mu$ l of media in an E-Plate® (i.e., 96-well tissue culture plates having micro-electrodes integrated on the bottom) and allowed to attach onto the electrode surface over time. The

electrical impedance was recorded every 15 min. When the impedance reached plateau (i.e. confluent monolayer with well-formed junctions), the cells were treated with 20  $\mu$ M EHT1864 or 25  $\mu$ M LY294002 for an additional 10 h. The cell impedance (which depends on cell number, degree of adhesion, spreading and proliferation of the cells and also on the tightness of the junctions), expressed in arbitrary units (cell index), was automatically calculated by the software of the instrument.

### ***Western-blot analysis***

Confluent D3 brain endothelial cells were treated with 20  $\mu$ M EHT1864 or 25  $\mu$ M LY294002 for 5 h. Cells were washed with PBS and scraped into ice-cold RIPA buffer (20 mM Tris, 150 mM NaCl, 0.5% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM sodium vanadate, 10 mM NaF, 1 mM EDTA/ethylenediaminetetraacetic acid, 1 mM Pefabloc®) and incubated on ice for 30 min. Lysates were clarified by centrifugation at 10,000•g for 10 min at 4°C. Proteins were electrophoresed and blotted onto nitrocellulose (Bio-Rad) membranes. Blocking was carried out at room temperature for 30 min in TBS-T containing 3% BSA. Anti-claudin-5 (Life Technologies), primary antibody was used. After washing the membranes in TBS-T (Tris-buffered saline and Tween 20), blots were incubated with the HRP (horseradish peroxidase)-conjugated secondary antibody (BD Transduction Laboratories) diluted in TBS-T. The immunoreaction was visualized using Clarity ECL Western-Blot Substrate kit (Bio-Rad) in a Bio-Rad ChemiDoc MP Imaging System.

## **RESULTS**

### ***Comparison of the adhesion and transmigration properties of breast cancer cells and melanoma cells in vitro***

The first step in the process of brain metastasis formation is the adhesion of tumor cells to CECs. To compare metastatic mammary carcinoma and melanoma cells' adhesion ability we applied static and dynamic in vitro models of the BBB. The time-lapse video experiments indicated that melanoma cells have increased ability to attach to the brain endothelium than breast cancer cells under static and dynamic conditions as well. We have also observed a



significant difference in the number of transmigrating melanoma and breast cancer cells under static and dynamic conditions as well. The number of cells not able to migrate through the brain endothelium was much higher in case of breast cancer cells than in case of melanoma cells. Moreover, invasive melanoma cells tended to complete the transmigration process much more rapidly than invasive breast cancer cells. The higher effectiveness of melanoma cells to migrate through the brain endothelium might be partly responsible for the higher propensity of melanoma cells to metastasize to the CNS.

### ***Effect of tumor cells on interendothelial junctions***

We have previously observed that melanoma cells are able to disrupt the TJs of CECs and use (at least partly) the paracellular way of migration. We were interested to understand whether breast cancer cells are also able to impair the junctional integrity of the cerebral endothelium. We have observed that melanoma cells could breach the junctions of RBECs as indicated by focal loss of claudin-5 staining, but this was not observed in case of breast cancer cells. These data suggest that differences in the transendothelial migration of mammary carcinoma and melanoma cells might be partly due to differences in their ability to impair interendothelial junctions.

### ***Effect of Rac or PI3K inhibition on the adhesion and transmigration of breast cancer cells and melanoma cells on the brain endothelium***

We aimed to understand which type of movement (amoeboid or mesenchymal type) is applied by breast cancer cells during transmigration through CECs. We observed that in case of breast cancer cells ROCK inhibitors were not able to influence the number of breast cancer cells adhering to the brain endothelium. On the other hand, the Rac inhibitor EHT1864 – which induces the amoeboid phenotype – hampered the adhesion and transmigration of both MDA-MB-231 and MCF-7 breast cancer cells. We also observed that the Rac inhibitor decreased the number of adherent and transmigrated melanoma cells as well. In addition, according to our results the PI3K inhibitor LY294002 significantly reduced the number of breast cancer and melanoma cells attaching to and transmigrating through the brain endothelium. Both Rac and PI3K inhibitors influenced the rapid transmigration of melanoma cells.

PI3K has been shown to regulate Rac through P-Rex1 in breast cancer cells. The morphology of PI3K-inhibited tumor cells was similar to that of Rac-inhibited cells, i.e. we could see a reduction in the number of elongated, flattened cells. This suggests that PI3K inhibition – similar to Rac inhibition – induces an amoeboid-like phenotype in both melanoma and breast cancer cells, and inhibits the transmigration of tumor cells.

We wanted to exclude the possibility that the inhibitory effect of EHT1864 and LY294002 on the adhesion and transmigration of breast cancer and melanoma cells was due to toxicity on tumor cells. Using the EZ4U assay no toxic effect of either EHT1864 or LY294002 on A2058, MDA-MB-231 and MCF-7 cells was observed. Moreover, the EZ4U assay did not show any toxicity of EHT1864 or LY294002 on D3 brain endothelial cells. Moreover, the wound healing assay indicated no change in the migratory properties of melanoma or breast cancer cells in response to EHT1864 or LY294002.

### ***Effect of Rac or PI3K inhibition on the junctional integrity of the brain endothelium***

We observed that EHT1864 induced a decrease in the impedance of D3 cells, as reflected by the cell index. After an initial drop induced by the medium change, the impedance of control and LY294002-treated D3 cells recovered rapidly. However, in case of EHT1864-treated cells the recovery was not complete, and after 5 h a significant drop in the impedance was seen. The cell impedance reflects changes in the cell number, viability and tightness of the junctions. Since no change in the viability of D3 cells was observed using the EZ4U assay, we next investigated the possible damaging effect of the Rac inhibitor on the TJs. We observed a significant down-regulation of claudin-5 protein in D3 cells in response to EHT1864. LY294002 did not significantly affect the amount of claudin-5 protein in D3 cells. This indicates that the Rac inhibitor may impair the barrier properties of the BBB.

## SUMMARY

Brain metastases are devastating complications of lung cancer, breast cancer, melanoma and other malignancies. CNS metastases have very limited therapeutic options and poor prognosis. One of the key steps in cerebral metastasis formation is transmigration of tumor cells through brain endothelial cells forming the BBB. However, little is known about the interactions between tumor and brain endothelial cells.

In order to understand the mechanisms of melanoma and breast cancer brain metastasis formation we applied static and dynamic in vitro models.

We demonstrated that melanoma cells have enhanced ability of adhesion to and transmigration through the brain endothelium than breast cancer cells both under static and dynamic conditions. Moreover, melanoma cells tended to complete the transmigration process more rapidly than invasive breast cancer cells.

Our experiments revealed that melanoma cells are more effective in breaking down the tight junctions of cerebral endothelial cells than breast cancer cells.

Our data indicated that inhibition of Rac impedes the adhesion and transmigration of melanoma and breast cancer cells as well.

We observed a reduction of melanoma and breast cancer cells able to attach to and to migrate through the brain endothelium in response to PI3K inhibition.

In addition, considering their potential therapeutic effects, we investigated the effect of Rac and PI3K inhibitors on the barrier integrity of cerebral endothelial cells. We have shown that the Rac inhibitor EHT1864 decreases the amount of junctional protein claudin-5, while the PI3K inhibitor LY294002 does not affect the integrity of the BBB. We suggest that targeting the PI3K/Akt pathway may represent a novel opportunity in preventing the formation of brain metastases of melanoma and breast cancer.

## **ACKNOWLEDGEMENTS**

I would like to express my deepest gratitude to my supervisors, Dr. István Krizbai and Dr. Imola Wilhelm, for scientific guidance and constant support throughout my PhD studies. I am very grateful to all my colleagues in the group and in the Biological Research Centre of the Hungarian Academy of Sciences.

I am especially grateful to the following colleagues: Dr. Csilla Fazakas, Dr. Péter Nagyősz, Ádám Nyúl-Tóth, János Haskó and Dr. Attila Farkas for all the time that we have spent together in the laboratory.

I wish to acknowledge all co-authors for enthusiastic participation in the preparation of experiments and manuscripts, especially Dr. Orsolya Sipos, Dr. Krisztina Nagy, Dr. Péter Galajda, Dr. Gergő Végh and Dr. György Váró.

I am very thankful to all the people who have supported me during my PhD work, especially to my friends. I am very grateful to my mother and Gábor, for their love, patience and support.

## LIST OF PUBLICATIONS

### *Full papers directly related to the subject of the thesis*

I. **Molnar, J.**, Fazakas, C., Hasko, J., Sipos, O., Nagy, K., Nyul-Toth, A., Farkas, A. E., Vegh, A. G., Varo, G., Galajda, P., Krizbai, I. A., Wilhelm, I. (2015). Transmigration characteristics of breast cancer and melanoma cells through the brain endothelium: role of Rac and PI3K. *Cell Adhesion and Migration* [E-pub ahead of print] (IF2014: 4.505)

II. Wilhelm, I., Fazakas, C., **Molnar, J.**, Hasko, J., Vegh, A. G., Cervenak, L., Nagyoszi, P., Nyul-Toth, A., Farkas, A. E., Bauer, H., Guillemin, G. J., Bauer, H. C., Varo G., and Krizbai, I. A. (2014). Role of Rho/ROCK signaling in the interaction of melanoma cells with the blood-brain barrier. *Pigment Cell and Melanoma Research* 27 (1):113-23. (IF2014: 4.619)

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