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Role of the neural environment in the transmigration of melanoma cells through the blood-brain barrier

-Final report-

Introduction

Brain tumors are life threatening pathologies with limited therapeutic options, representing a major cause of death in cancer patients. The majority of the tumors of the central nervous system (CNS) are metastases, among which lung cancer, breast cancer and melanoma are the most common.

The first host cell types encountered by circulating cancer cells during brain metastasis formation are endothelial cells of the brain vasculature. Cerebral endothelial cells (CECs) have a highly differentiated phenotype, induced and maintained by the cross-talk with the surrounding cells of the neurovascular unit, including pericytes and astrocytes (reviewed in: **Wilhelm and Krizbai, 2012, Neuroglia 3rd edition, Chapter 33**). In this respect, CECs have special epithelial-like features, including the formation of a continuous belt of tight junctions between the cells and expression of a special set of transporters and enzymes, which result in the formation of the blood-brain barrier (BBB). Therefore, during extravasation from the blood stream into the brain parenchyma, melanoma cells have to migrate through the BBB. Nevertheless, interactions between melanoma cells and endothelium of the CNS are still largely uncharacterized (reviewed in: **Wilhelm et al., Int J Mol Sci 2013, 14:1383-411**).

Metastatic cells migrated through the capillary wall may find an ideal milieu for survival and growth. Large vessel density in the brain can be easily exploited by tumor cells as an alternative blood supply (vessel co-option) (reviewed in: **Wilhelm and Krizbai, 2015, Brain Mapping, Chapter 134**). Moreover, the BBB has a protective role against chemotherapeutics targeting tumor cells. Unfortunately, present treatment strategies of brain metastases are very ineffective; prognosis of the disease is extremely poor: the median survival of the patients is less than one year. Therefore, development of strategies aiming at preventing dissemination of metastatic cells into the CNS is urgently needed. This requires understanding of the mechanisms of brain metastasis formation, in particular, identification of key pathways and molecules involved in the interplay between tumor cells and cells of the CNS.

Aims

In the framework of the present OTKA proposal we aimed at understanding the mechanisms of transmigration of tumor cells through the BBB. Melanoma cells have an unexpectedly high affinity to the brain, the prevalence of brain metastases being 55-75% in melanoma patients. It is well documented that the neural environment plays a key role in the protection and growth of already formed melanoma brain metastases. However, it is not clear whether transmigration of melanoma cells through the BBB is influenced by cellular or soluble elements of the CNS. Therefore, our aim was to understand the role of the neural environment in the extravasation of melanoma cells into the brain.

In line with this, we have extended our experiments and compared the transendothelial diapedesis of melanoma cells and breast cancer cells. Breast cancer is the second most common cancer type giving brain metastases; however, in comparison to melanoma it has a much lower propensity to metastasize to the brain. Comparison of the interaction of these two different tumor cell types with the cerebral endothelium might also help in understanding whether the transendothelial migration step of metastasis formation has any role in the higher tropism of melanoma cells towards the CNS.

Results

Majority of our experiments were performed on relevant in vitro models of the BBB (reviewed in: **Wilhelm and Krizbai, Mol Pharm 2014, 11:1949-63**). We have completed the experimental plan described in the proposal. Moreover, in line with the obtained reults, we have performed additional experiments which are closely related to the project proposal.

Role of the neural environment in the formation of melanoma brain metastases

In order to understand the high incidence of melanoma brain metastases, we first tested the role of cellular and soluble factors of the neural environment on the transmigration of melanoma cells through the BBB.

In order to investigate the role of cellular elements of the brain in the transendothelial migration of melanoma cells, we isolated primary pericytes and astrocytes. We co-cultured them with brain endothelial cells and performed transmigration experiments using melanoma cells. Both pericytes and astrocytes strengthened the barrier function of brain endothelial cells, and as a consequence, reduced the number transmigrating tumor cells.

Astrocytes and pericytes are able to release different factors that influence barrier and/or other characteristics of the cerebral endothelial layer. Some of these factors may have direct effect on the transendothelial migration of melanoma cells. Among these factors are

neurotrophins which are secreted by all cell types of the neurovascular unit and have been shown to be key factors in the proliferation and angiogenesis of melanoma metastases in the brain. We tested the influence of neurotrophins on tumor cell migration through the brain endothelium by exposing the abluminal side of the BBB model to relevant concentrations of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT3 or NT4/5. NGF (in a concentration of 100 ng/ml) and BDNF (10 ng/ml) did not affect the rate of transmigration. On the other hand, presence of NT3 (10 ng/ml) or NT4/5 (10 ng/ml) increased the number of melanoma cells migrating through brain endothelial cells by 1.5- or 2-times, respectively. These results indicate that soluble elements of the CNS might induce migration of melanoma cells through the BBB, which might partially explain the high affinity of melanoma cells to the brain.

CB2 receptors of the cerebral endothelium were recently shown to reduce endothelial-immune cell interactions. Extravasation of tumor cells has several common aspects with diapedesis of immune cells; therefore, we wanted to test whether the cannabinoid system plays role in endothelial-melanoma interactions during brain metastasis formation. We found that both brain endothelial cells and melanoma cells express the CB2A transcriptional variant of the CB2 receptor, but not CB2B. We observed that activation of CB2 receptors using JWH-133 significantly reduced the number of melanoma cells able to attach to and to migrate through cerebral endothelial monolayers. Our results suggested that activation of CB2 receptors on both endothelial cells and melanoma cells contributed to the effect of JWH-133. Our data identify CB2 as a potential target in reducing the number of brain metastastes originating from melanoma.

These results were partly published in: **Haskó et al., Int J Mol Sci 2014, 15:8063-74**.

Signaling pathways involved in the transendothelial migration of melanoma and breast cancer cells during brain metastasis formation

In order to understand the role of the BBB in the high affinity of melanoma cells to the CNS, as a next step we characterized and compared the transendothelial migration properties of two brain metastatic tumor cell types with different tropism to the CNS.

Interaction of melanoma and breast cancer cells with brain endothelial cells was investigated by treating tumor cells with conditioned media of brain endothelial cells and by treating brain endothelial cells with melanoma- or breast cancer cell-conditioned media. Our real-time PCR results demonstrated that brain endothelial cell-derived factors induced an increase in the expression of of seprase, a membrane-bound serine protease in melanoma cells. We have previously shown that seprase has an important role in the transmigration of melanoma cells through the cerebral endothelium. We found that breast cancer cells do not express seprase, but express matriptase/ST-14, another membrane-bound serine protease, which, on the other hand, is not expressed by melanoma cells. Moreover, this protease was

not upregulated by brain endothelial cell-conditioned media. Our experiments have also demonstrated that inhibition of serine proteases is able to reduce transmigration of melanoma, but not of breast cancer cells through the brain endothelium.

Using static and dynamic in vitro approaches, we have also shown that melanoma cells have increased adhesion characteristics to the brain endothelium in comparison to breast cancer cells. Moreover, melanoma cells can transmigrate more rapidly and in higher number through brain endothelial monolayers than breast cancer cells. In addition, melanoma cells have increased ability to impair tight junctions of CECs. All these differences were not dependent on differences in the invasive and metastatic capacities between the melanoma and breast cancer cell lines used.

In conclusion, we found marked differences in the interaction of melanoma cells and breast cancer cells with the brain endothelium. Melanoma cells – the tumor cell type having the highest propensity to metastasize to the brain – proved to have higher ability to migrate through the brain endothelium than invasive mammary carcinoma cells. This seems to partly depend on proteolytic mechanisms and the increased ability of these cells to impair the junctional complex of CECs.

We have also assessed possible differences in signaling pathways involved in the transmigration of melanoma cells and breast cancer cells through the brain endothelium. ROCK inhibition (using Y27632) strengthened the adhesion force between melanoma and endothelial cells, and increased the number of melanoma cells migrating from the apical to the basolateral side of the brain endothelial monolayer. This effect could be reversed by the addition of the EHT1864 Rac inhibitor. In case of breast cancer cells, Y27632 could not increase the adhesion rate; however, EHT1864 significantly reduced the number of breast cancer cells attaching to and migrating through the brain endothelium. We have also shown that inhibition of PI3K impeded adhesion of both breast cancer cells and melanoma cells to the brain endothelium. In addition, inhibition of Rac (using EHT1864) or PI3K (using LY394002) inhibited the late phase of transmigration of breast cancer cells and the early phase of transmigration of melanoma cells.

On the other hand, the Rac inhibitor EHT1864 impaired the junctional integrity of the brain endothelium, while the PI3K inhibitor LY294002 had no damaging effect on interendothelial junctions. Altogether, our results suggest that targeting the PI3K/Akt pathway may represent a novel opportunity in preventing the formation of brain metastases of melanoma and breast cancer.

In addition, we were the first to describe that tumor cell-derived TGF-β induces endothelial-mesenchymal transition (EndMT) in CECs, characterized by loss of tight and adherens junction proteins, and induction of the expression of fibronectin, β1-integrin, calponin and α-smooth muscle actin (SMA). Activation of TGF-β in the conditioned media of both melanoma and breast cancer cells was able to induce EndMT of brain endothelial cells. Moreover,

activated conditioned melanoma media enhanced the number of melanoma cells adhering to and transmigrating through the endothelial layer, both in a TGF-β-dependent manner.

These results were partly published in: **Wilhelm et al., Pigment Cell Melanoma Res 2014, 27:113-23**; **Krizbai et al., PLoS One 2015, 10:e0123845**; **Molnár et al., Cell Adh Migr [Epub ahead of print]**.

Other results on melanoma brain metastasis formation

We have assessed the ability of Heliopsis helianthoides var. scabra-derived lignans to inhibit various steps involved in melanoma brain metastasis formation. Out of the six lignans tested, helioxanthin and (7E)-7,8-dehydroheliobuphthalmin proved to inhibit migration of both melanoma and brain endothelial cells, while helioxanthin also reduced adhesion of melanoma cells to the brain endothelium. Furthermore, helioxanthin and (7E)-7,8-dehydroheliobuphthalmin enhanced the barrier function of the BBB and expression of the tight junction protein ZO-1 at the junctions of CECs.

These findings suggest that helioxanthin and (7E)-7,8-dehydroheliobuphthalmin may have the potential to interfere with different steps of brain metastasis formation and to strengthen the BBB.

These results were published in: **Hajdu et al., J Nat Prod 2014, 77:2641-50**.

Other results

We have also acknowledged the support of the present OTKA grant in three research articles on different aspects of BBB function (**Kosson et al., Acta Neurobiol Exp (Wars) 2014, 74:26-32**; **Wilhelm et al., J Mol Neurosci 2014, 54:469-76**; **Nagyőszi et al., J Neurochem 2015, 135:551-64**) and a review paper on the functions of ZO-2, one of the plaque proteins of the tight junctions (**Traweger et al., Tissue Barriers 2013, 1:e25039**).