Brain, Behavior, and Immunity 62 (2017) 265–276

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Full-length Article

Maintaining unperturbed cerebral blood flow is key in the study of brain metastasis and its interactions with stress and inflammatory responses

Amit Benbenishty [a,b,c], Niva Segev-Amzaleg [b], Lee Shaashua [c], Rivka Melamed [c], Shamgar Ben-Eliyahu [a,c], Pablo Blinder [a,b,*]

[a] Sagol School of Neuroscience, Tel Aviv University, Israel
[b] Neurobiology Department, George S. Wise Faculty of Life Sciences, Tel Aviv University, Israel
[c] School of Psychological Sciences, Tel Aviv University, Israel

ARTICLE INFO

Article history:
Received 14 November 2016
Received in revised form 8 February 2017
Accepted 16 February 2017
Available online 20 February 2017

Keywords:
Brain
Metastasis
Blood flow
In vivo
Inflammation
Stress
Inoculation method

ABSTRACT

Blood-borne brain metastases are associated with poor prognosis, but little is known about the interplay between cerebral blood flow, surgical stress responses, and the metastatic process. The intra-carotid inoculation approach, traditionally used in animal studies, involves permanent occlusion of the common carotid artery (CCA). Herein we introduced a novel intra-carotid inoculation approach that avoids CCA ligation, namely – assisted external carotid artery inoculation (aECAi) – and compared it to the traditional approach in C57/BL6 mice, assessing cerebral blood flow; particle distribution; blood-brain barrier (BBB) integrity; stress, inflammatory and immune responses; and brain tumor retention and growth. Doppler flowmetry and two-photon imaging confirmed that only in the traditional approach regional and capillary cerebral blood flux were significantly reduced. Corticosterone and plasma IL-6 levels were higher in the traditional approach, splenic numbers of NK, CD3+, granulocytes, and dendritic cells were lower, and many of these indices were more profoundly affected by surgical stress in the traditional approach. BBB integrity was unaffected. Administration of spherical beads indicated that CCA ligation significantly limited brain distribution of injected particles, and inoculation of D122-LLC syngeneic tumor cells resulted in 10-fold lower brain tumor-cell retention in the traditional approach. Last, while most of the injected tumor cells were arrested in extra-cranial head areas, our method improved targeting of brain-tissue by 7-fold. This head versus brain distribution difference, commonly overlooked, cannot be detected using *in vivo* bioluminescent imaging. Overall, it is crucial to maintain unperturbed cerebral blood flow while studying brain metastasis and interactions with stress and inflammatory responses.

1. Introduction

Brain metastases are prevalent in various types of cancer, and are associated with poor prognosis (Kienast and Winkler, 2010; Preusser et al., 2012; Sperduto et al., 2010). To form brain metastases, malignant cells are subjected to physiological processes that are closely dependent on hemodynamics and cerebral vascular structure. Initially, tumor cells must be arrested in the capillary bed of the brain. This process is attributed to specific cell adhesion molecules (Hinton et al., 2010; Lee et al., 2004a,b) and size restrictions (Kienast et al., 2010; Lorger and Felding-Habermann, 2010), and takes place at locations where shear forces are reduced, such as bifurcations of small capillaries (Kienast et al., 2010). Thereafter, arrested cells need to cross the blood-brain barrier (BBB) and extravasate into the brain parenchyma to form brain metastases. Therefore, any experimentally-introduced alterations in cerebral blood flow dynamics, vascular structure, or BBB permeability, might directly hinder our ability to extrapolate findings obtained in such distorted physiological context to the "natural" process of brain metastasis.

Commonly, studies of brain metastasis aiming to study the various steps of the metastatic process, implement one of two intravascular inoculation approaches – (i) intracardiac and (ii) intra-carotid injection. The main disadvantages of the intracardiac injection (Conley, 1979), as reviewed by Daphu et al. (2013), are that (i) tumor cells are distributed from the heart in unknown and uncontrolled proportions to the brain, and (ii) malignant foci develop outside the brain as well. Both of these shortcomings pose

* Corresponding author at: Department of Neurobiology, George S. Wise Faculty of Life Sciences, Sherman Building, Room 419, Tel-Aviv University, Tel Aviv 69978, Israel.
E-mail address: pb@post.tau.ac.il (P. Blinder).

http://dx.doi.org/10.1016/j.bbi.2017.02.012
0889-1591/

substantial obstacles for interpretation of results, and for the study of various factors that may also affect cardiac blood distribution and blood pressure and flow, including surgical procedures (Kirnö et al., 1994), stress (Middlekauff et al., 1997), and a variety of health behaviors (Sullivan et al., 1989; Wilde et al., 2000). The intra-carotid artery (ICAi) inoculation was first described by Machinami (1973). In this approach, cancer cells are injected through the common carotid artery (CCA) into the internal carotid artery (ICA), which provides blood to the brain. However, a small puncture is cut in the artery for the insertion of the injection device, and to avoid consequent bleeding, this procedure necessitates permanent occlusion of the CCA, thus chronically alters blood flow. In more recent versions, the external carotid artery (ECA), which provides blood to peripheral head tissues (including the skull, face, and neck), is also occluded, in order to stream a larger proportion of tumor cells to the brain (Ushio et al., 1977). Due to the structure of the circle of Willis, blood provided by the contralateral ICA reaches both hemispheres of the brain, potentially avoiding severe ischemic conditions. However, acute and chronic changes in tissue oxygen supply may result in profound effects on systemic inflammation and immune activity (Melillo, 2011; Semenza, 2009), which are key mediators of cancer progression (Coussens and Werb, 2002; Dalgleish and O'Byrne, 2006; Grivennikov et al., 2010). We hypothesized that the permanent occlusion of the CCA may have major influences on the complex process of blood-borne brain metastasis and its study under different stress and inflammatory levels. To address this potential limitation, several methods which avoid ligation of the CCA have been recently developed (Chen et al., 2009; Chua et al., 2011; Do et al., 2014). Nevertheless, with respect to brain metastases, no study has been conducted to systematically assesse the significance of maintaining proper blood flow to the brain.

We herein present a novel carotid artery inoculation approach, where tumor cells are administered through the ECA to the ICA, namely – the assisted external carotid artery inoculation (aECAi) approach. The term assisted is derived from the use of a fine positioning device to access the ECA (~200 μm internal diameter), an otherwise a surgical challenge. The aECAi approach has all the advantages of the traditional ICAi method, while completely avoiding occlusion of the CCA. We compared the aECAi to the ICAi approach with respect to perturbations of cerebral blood flow, BBB permeability, stress, inflammatory, and immune responses, blood-borne synthetic particle distribution in the brain (Silasi et al., 2015), and metastatic efficacy and growth. We found our novel approach to avoid the marked distortions of key physiological parameters induced by the ICAi approach, and to result in markedly different outcomes and patterns of metastatic progression in the brain.

2. Materials and methods

2.1. Cell preparation

D122 Lewis Lung Carcinoma (LLC) cells were cultured in complete media (RPMI1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin; Biological Industries). Cells were double labeled with mCherry and Luc2 (pLNT/Sffv-MCS/ccdB plasmid was kindly provided by Prof. Vaskar Saha). For experiments assessing cancer-cell retention, D122 cells were incubated with ^{125}IUDR during the last 24 h of proliferation. Cells were washed and harvested (0.25% trypsin-EDTA; Invitrogen) at ~90% confluence, re-suspended in PBS supplemented with 0.1% BSA (Biological Industries), and kept on ice throughout the injection procedures, completed within 3 h of cell harvesting. More than 95% of cells were vital throughout the injection period.

2.2. Animals and anesthesia

All studies were approved by the institutional IRB committee for animal use and welfare. C57BL/6J male mice were used in all experiments (8–12 weeks old; age matched within experiment). Animals were housed under standard vivarium conditions (22 ± 1 °C, 12 h light/dark cycle, with ad libitum food and water). For anesthesia, mice were first anesthetized in 5% Isoflurane, and then maintained on 1.5–2% throughout the procedures. When anesthetized, core body temperature of animals was maintained at 37 °C.

2.3. Injection procedures

In experiments assessing blood flow and BBB permeability, no cancer cells were injected to avoid potential confounding effects. In experiments assessing organ tumor retention and histological outcomes, $1x10^5$ cells (100 μl) were injected in both methods.

2.3.1. Intra-carotid artery inoculation (ICAi)

The standard protocol was followed (Kienast et al., 2010; Schackert et al., 1989). Mice were anesthetized and restrained in a supine position under a dissecting microscope. The trachea was exposed, and muscles were separated to uncover the right CCA and ECA, which were then separated from the vagus nerve. A 6-0 silk ligature (659, Assut sutures) was tied around the ECA between the superior thyroid artery and the bifurcation of the ECA and CCA (Fig. 1b). A second ligature was loosely placed around the CCA just rostral to the ECA bifurcation, and another ligature was placed and tied further rostral on the CCA. The CCA was then nicked between the two CCA ligatures with a pair of micro-scissors (15000-03, FST), and a stretched polyethylene tube (PE10, Braintree Scientific) fitted on a 100 μl nano-fill syringe (NanoFil-100, WPI) was inserted into the artery lumen and threaded up to the bifurcation of the ligated ECA and the open ICA. Fluid containing the injected material (100 μl) was infused throughout 60sec. The tube was then removed, the loose CCA ligature immediately tightened, and the skin sutured.

2.3.2. Assisted external carotid artery inoculation (aECAi)

Mice were anesthetized and the trachea was exposed similarly to the ICAi method. Thereafter, the sternohyoid muscle was separated, and the ECA uncovered (Fig. 1c). A 6-0 silk-suture ligature was loosely placed around the ECA between the superior thyroid artery and the bifurcation of the ECA and CCA. A second ligature was tied on the ECA distal to the bifurcation, and the ECA was lightly stretched to allow smooth insertion of the needle. A 100 μl nano-fill syringe with a 34G beveled needle was mounted to a micromanipulator (M33, Sutter Inc.). The use of such a device drastically improves success rates and allows standardizing this procedure among laboratories. The needle was inserted slowly into the lumen of the ECA at an angle of ~15° and advanced to the point of bifurcation. The first ligature was tied around the needle, and 100 μl of the fluid containing the injected material was slowly infused throughout 60sec, exactly as in the traditional ICAi approach. The needle was then removed, the ligature quickly tied, and the skin sutured. Notably, use of a micromanipulator is key to the success of this procedure given the delicacy and size of the target vessel (~200 μm internal diameter).

2.4. Awake two-photon laser scanning microscopy

For two-photon microscopy measurements, mice were implanted with a polished and reinforced thin-skull (PoRTS) window, as previously described (Drew et al., 2010). Mice were then