The American Journal of Pathology, Vol. 183, No. 1, July 2013

The American Journal of
PATHOLOGY
ajp.amjpathol.org

SHORT COMMUNICATION

Ultrasonography-Guided Intracardiac Injection

An Improvement for Quantitative Brain Colonization Assays

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Accepted for publication
March 26, 2013.

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Brain metastasis is a frequent occurrence in patients with cancer, with devastating consequences. The current animal models for brain metastasis are highly variable, leading to a need for improved *in vivo* models that recapitulate the clinical disease. Herein, we describe an experimental brain metastasis model that uses ultrasonographic guidance to perform intracardiac injections. This method is easy to perform, giving consistent and quantitative results. Demonstrating the utility of this method, we have assessed a variety of metastatic cell lines for their ability to develop into brain metastases. Those cell lines that were competent at brain colonization could be detected in the brain vasculature 4 hours after intracardiac injection, and a few adherent cells persisted until colonization occurred. In contrast, those cell lines that were deficient in brain colonization were infrequently found 4 hours after introduction into the arterial circulation and were not detected at later time points. All of these cells were capable of brain colonization after intraparenchymal injection. We propose that adherence to the brain vasculature may be the key limiting step that determines the ability of a cancer cell to form brain metastases successfully. Identifying brain endothelium-specific adhesion molecules may enable development of screening modalities to detect brain-colonizing cancer cells and therapies to prevent these metastatic cells from seeding the brain. *(Am J Pathol 2013, 183: 26–34; http://dx.doi.org/10.1016/j.ajpath.2013.03.003)*

The spread of primary cancer cells to form secondary brain metastases is a frequent occurrence in patients with cancer.[1,2] With improved treatments for primary cancers, there is an increased incidence in patients developing brain metastasis. Unfortunately, prognosis for patients with brain metastasis is grim regardless of the primary site, with a median survival of 2.3 months after detection.[3] Whole brain radiotherapy, fractionated radiation, and stereotactic radiosurgery are the mainstream therapies, but these modalities are more palliative than curative.[1]

Basic research into the mechanism of brain metastasis is limited, in part because of the dearth of animal models that consistently reproduce the clinical disease. Current methods for generation of brain colonization in experimental *in vivo* rodent models include injecting cancer cells directly into the brain parenchyma, the internal carotid artery, or the cardiac left ventricle.[4] Metastasis to the brain from primary tumors in rodent models is too infrequent to allow for experimental manipulation.[4,5] Direct injection of tumor cells into the brain bypasses adhesion and extravasation at the secondary site. Because brain metastases are generated from cells in the arterial circulation and the brain has no lymphatics, introduction of cancer cells into the arterial circulation via the carotid artery or the heart simulates the metastatic process once cells have left the primary site. Intracarotid injection delivers tumor cells precisely into the circulation, with all cells first seeding the brain. However, this approach is technically demanding, especially in mouse models, and carries a substantial risk of developing systemic inflammation and/or ischemia.[6] Tumors, not infrequently, also arise at the site of injection or at the head and neck.[6,7] The intracardiac route delivers a few injected cells to the brain because only 15% to 20% of cardiac output reaches the brain, further resulting in the potential for extracranial metastasis. The blind nature of intracardiac injections also results in many missed injections, delivering tumor cells to

Supported by Cancer Research UK (CRUK) studentship reference C5255/A9194 (L.B.) and CRUK reference C5255/A15935 and CRUK/EPSRC-funded Oxford Cancer Imaging Centre (J.S.B. and R.J.M.).

http://dx.doi.org/10.1016/j.ajpath.2013.03.003

sites other than the left cardiac ventricle.[8] However, cardiac injections are easier to perform and better tolerated.

Only a handful of cell lines actually result in brain colonization after intracarotid or intracardiac injection. The murine cancer cell lines 4T1, Lewis lung carcinoma (LLC), and B16 subclones are all well established in many different experimental models of metastasis, yet only 4T1 cells consistently form brain metastases.[9–14] 4T1 cells develop aggressive parenchymal brain metastases characterized by extensive microgliosis[12,15] analogous to the histological characteristics of human clinical samples.[16] The B16 melanoma cell lines, including the brain metastatic sublines, have been reported to grow primarily on the dura, meninges, or intravascularly after intracarotid injections[9,17–19] and only occasionally form intraparenchymal colonies.[20,21] LLC is used commonly as a lung metastasis model.[13,22] However, less commonly, spontaneous and experimental brain metastases have been reported.[7,13,23,24] Several human cell lines will form parenchymal brain colonies after intracardiac and intracarotid routes of injection, of which the human breast cancer MDA-MB-231 has been selected after cycles of brain colonization for variants with greater brain colonization capacity (MDA-MD-231-Br) and is extensively used in brain metastasis assays.[12,15,25,26]

To facilitate the study of brain metastasis, we have attempted to generate methods to improve the reliability and reproducibility of the brain colonization assays. Modification of the intracardiac injection method by using ultrasonographic guidance to visualize the site of injection (left cardiac ventricle) proved to yield more reliable distribution of cells and more reproducible development of brain colonies, with fewer adverse effects. Because ultrasonographic guidance enabled repeated injection of the same number of cells without error, we were able to compare quantitatively the ability of these cancer cells to adhere, survive, and extravasate. Our findings show that adhesion to and retention within the brain vasculature are key limiting steps determining the ability of disseminated cancer cells to form brain metastases and highlight an area for further development.

Materials and Methods

Cell Lines and Animals

4T1 murine breast carcinoma, 4T07 murine breast carcinoma, LLC, and B16 F10 melanoma cells were purchased from ATCC (Manassas, VA) and were stably transfected with pEGFP-C1 (Clonetech, St. Germain-en-Laye, France); 4T1 cells stably expressing luciferase (4T1-luc) were purchased from Caliper Life Sciences (Runcorn, UK). MC38 colon carcinoma cells expressing green fluorescent protein (GFP) were a generous gift from Prof. Carlos Arteaga (Vanderbilt University, Nashville, TN), and the MDA-MB-231 human breast cancer cells expressing GFP were a kind gift from Prof. Richard Treisman (CRUK London Research Institute, London, UK). All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% L-glutamine (Gibco-Invitrogen, Paisley, UK).

Female BALB/c mice (aged 8 to 10 weeks), severe combined immunodeficient (SCID) or C57BL/6 (Charles River, Margate, UK), were used, housed in individually ventilated cages (Techniplast, London, UK), and fed rodent diet and water ad libitum. All experiments were performed in accordance with UK Home Office licensing and University of Oxford (Oxford, UK) ethics committee.

Intracardiac Injections (Blind)

Mice were laid flat on their backs, with limbs secured, and the fur over the thorax was shaved. Determining the site of injection was followed as previously described.[27] Briefly, a $^3/_4$-inch 27-gauge inch needle was placed parallel to the sternum, under the rib cage, and injected angled 30° away from the sternum toward the heart. Repeated angling of the needle was necessary until a quick flashback of blood was observed in the syringe, indicating the needle was inserted into the heart. Holding the syringe steady with the other hand, 100 μL of cancer cells (10^5) was then injected in 20-μL increments.

Ultrasonography-Guided Intracardiac Injections

Mice were anesthetized and placed on their backs supine, slightly tilted toward the right side, with all four limbs secured on a heated stand. The fur covering the thorax was removed by shaving and by use of hair removal cream as any residual fur would interfere with the ultrasonographic image quality. A thick layer of ultrasonographic gel was placed on the bare thorax above the heart, and a stand-mounted 707B ultrasonographic probe (Visualsonics, Toronto, ON, Canada) was used to find the left ventricle of the heart. Tumor cells were resuspended using a 23-gauge needle, and 100 μL of the cell suspension was loaded into a 1-mL syringe. The syringe was fitted with a 3/4-inch-long 27-gauge needle and secured onto a needle holder, and its position was adjusted to be underneath the ultrasonographic probe until visualized in the ultrasonographic image. When the needle tract trajectory was deemed suitable, the needle was advanced through the intercostal space into the left ventricle. Cells were only injected after visual confirmation of the needle within the left ventricle of the heart, and a pulse of bright red blood was seen to flash back into the syringe. Tumor cells (10^5 in 100 μL) were injected slowly in 20-μL increments over 30 seconds.

Intracranial Tumor Injections

Mice were anesthetized, and heads were shaved and secured onto a stereotaxic frame. A microdrill was used to thin a 2- to 3-mm portion of the skull, allowing it to be peeled back without causing damage to the underlying dura, and 2000 cells in 1 μL of PBS were injected using a glass microcapillary