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TITLE: Pathogenesis and Blood-Brain Barrier Heterogeneity of Breast Cancer Brain Metastasis

PRINCIPAL INVESTIGATOR: Weixin Lu, M.D., Ph.D.
Corazon D. Bucana
Alan J. Schroit

CONTRACTING ORGANIZATION: University of Texas, M.D. Anderson Cancer Center
Houston, Texas 77030

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Weixin Lu, M.D., Ph.D., Corazon D. Bucana, Alan J. Schroit

E-Mail: weixinlu@mdanderson.org

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14. ABSTRACT
The purpose of this study was to elucidate the pathogenesis of breast cancer brain metastasis and the role of the BBB in the process. Green fluorescent protein (GFP) expressing breast cancer cells were injected into the left ventricle of nude mice and the progression of brain metastases analyzed. Our data revealed that: i) 80% hematogenous metastatic cells homing in the brain extravasated and grew along blood vessels; ii) 20% metastatic cells attached to the microvessel wall did not extravasate immediately but proliferate within the vasculature, this leads to thrombosis-like complications such as infarction of brain parenchyma; iii) the cancerous thrombus can serve as a sustained releasing source of tumor cells to the downstream area through blood flow, making the spread of tumor cells extremely quick; iv) continuing intravascular tumor expansion led to disruption of blood vessels and BBB integrity; v) the overflowing metastatic cells proliferate and migrate along the host vasculature perivascularly to far distant sites and regain the protection of the BBB. vi) function of BBB is heterogeneous in different regions of tumor mass. These observations provide rationales for early diagnosis and treatment and improve our understanding of the role of BBB in chemotherapy of brain tumors.

15. SUBJECT TERMS
Breast Cancer, Brain Metastasis, Pathogenesis, Blood-Brain Barrier

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Introduction

Brain metastases are the most frequently occurring intracranial tumors, outnumbering primary brain tumors by a ratio of 10:1 (Shaffrey et al., 2004) and presenting the major causes of systemic cancer morbidity and mortality(Nathoo et al., 2005). Their incidence is increasing because of improved cancer therapy for the systemic disease (Shaffrey et al., 2004; Tosoni et al., 2004). Among patients with breast cancer, at least 10-15% of them develop brain metastases, making breast cancer the second most common cause of brain metastases(Boogerd, 1996; Ewend et al., 2001; Fenner and Possinger, 2002). Patients with brain metastases have a generally poor outcome with a median survival after diagnosis of approximately 4 months (Bradley and Mehta, 2004). It is predicted that brain metastases will become more problematic with the improved systemic control of metastatic diseases, because it is believed that most systemically administered agents have poor central nervous system penetration.

However, our knowledge about how brain metastases develop has, for the most part, been extrapolated from observation in other tissues. This has led to the concept that brain metastasis develops after extravasation of metastatic tumor cells into the surrounding tissue. Dogma also dictates that the blood-brain barrier (BBB) prevents the delivery of therapeutic agents through the BBB (Ewend et al., 2001; Landonio et al., 2001). We have developed a unique breast cancer brain metastasis model that allows us to visualize the development of brain metastasis and monitor BBB function with unprecedented high resolution. High expression of green fluorescent protein (GFP) allows us to trace the very few tumor cells and find the early tumor metastasis, and intravenous injection of rhodamine conjugated albumin facilitates monitoring the function of BBB under fluorescence microscope. Serendipitous observations indicate that our current concepts about pathogenesis and BBB function of brain metastasis (Fenner and Possinger, 2002; Walsh, 1996) might be incorrect. Accordingly, it is very important to further understand the mechanisms of the brain metastasis of cancer in order to design effective and specific therapeutic interventions for this devastating complication in cancer. Advances in this understanding of the pathobiology of brain metastasis may lead to novel targeted treatment paradigms and a better prognosis for patients with brain metastatic disease.

Based on this background, we proposed a new concept on the pathogenesis of breast cancer brain metastasis. The results obtained with financial support from DOD support most of what we hypothesized and tremendously broadened and enriched our understanding of the mechanisms of brain metastasis and the role of chemotherapy in brain tumors.

Body

Pathogenesis and blood-brain barrier heterogeneity of breast cancer brain metastasis

Background: Brain metastases are the most frequently occurring intracranial tumors, outnumbering primary brain tumors by a ratio of 10:1 (Shaffrey et al., 2004) and representing the major causes of systemic cancer morbidity and mortality(Nathoo et al., 2005). Their incidence is increasing because of improved cancer therapy for the systemic disease (Shaffrey et al., 2004; Tosoni et al., 2004). Among patients with breast cancer, at least 10-15% of them develop brain metastases, making breast cancer the second most common cause of brain metastases (Boogerd, 1996; Ewend et al., 2001; Fenner and Possinger, 2002). Most of these patients die within a few months of diagnosis since many systemic therapies are ineffective within the brain(Bradley and Mehta, 2004). Our knowledge about how brain metastases develop has, for the most part, been extrapolated from observation in other tissues. This has led to the concept that brain metastasis develops after extravasation of metastatic tumor cells into the surrounding tissue. Dogma also dictates that the blood-brain barrier (BBB) prevents the delivery of therapeutic agents through the BBB (Ewend et al., 2001; Landonio et al., 2001). We have developed a unique breast cancer brain metastasis model that allows us to visualize the development of brain metastasis and monitor BBB function with unprecedented high resolution. Serendipitous observations indicate that our current concepts about pathogenesis and BBB function of brain metastasis (Fenner and Possinger, 2002; Walsh, 1996) may be incorrect. The development of rational therapeutic strategies for the treatment of brain metastasis requires a complete understanding of the mechanism by which lesions develop.

Rationale/Purpose: We hypothesized that the anatomic structure of BBB prevents tumor cell extravasation. This results in local intravascular growth that embolizes and ultimately ruptures the vasculature. Once tumor ruptures the vessel wall, the characteristic perivascular growth spreads tumor cells far from ruptured site and then gains BBB protection. This model dictates that brain metastasis has a prolonged intravascular growth stage and the BBB is functionally heterogeneous in different tumor areas. Based on the unique pattern of brain metastasis proposed here, we assumed that the function of BBB in brain metastatic tumors should be temporally and spatially heterogeneous.

Objectives: The objectives of this proposal were to clarify the primary mechanism by which breast cancer develop brain metastasis with the emphasis on identifying the role of BBB in the process. The different models of breast cancer brain metastases developed in this laboratory allow us to visualize the whole procedure of brain metastasis development, interaction of tumor cells with BBB and the dynamic changes of the function of BBB with unprecedented clarity in direct *in vivo* natural tumor settings. By serial examination of the interactions between breast cancer brain metastasis and host vessels at different stages of brain metastasis growth, we would be able to provide a general framework of the mechanisms that underlie brain metastatic process. The proposal had the following specific aims:

1. Determine how breast cancer brain metastases initiate and grow with the particular emphasis on their interaction with the BBB.
2. Determine the changes in the function of the BBB in brain metastasis at different stages of tumor progression.

Original experimental design:

Analyses of the brain metastatic process and function of the BBB in brain metastasis at different stages of tumor progression.

Months 1-12: Nude mice will be injected with MDA-MB435GFP breast cancer cells into the left ventricle of the heart. At weekly intervals mice will be injected i.v. with rhodamine-albumin and sacrificed.

A) Superficial tumors will be localized by green fluorescent protein (GFP) using stereoscopic fluorescence microscopy. Tumor bearing regions will be further analyzed for supporting vasculature and intact/permeable BBB by red fluorescence (rhodamine-albumin) imaging. The areas will be surveyed, mapped and recorded.

B) Confocal microscopy – Thick slide frozen sections will be prepared and, where appropriate, fixed and stained with eosin-yellow CD31 antibodies to identify supporting endothelial cells. This will facilitate three-dimensional three color imaging of tumor (green fluorescence), blood vessel walls (yellow fluorescence) and patency of the BBB (red fluorescence).

Materials and Methods:

Mice. female athymic nude mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, Md.). The animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States department of Agriculture, Department of Health and Human Services, and National Institutes of Health. All the mice were used when they were 8- to 12- weeks old.

Establishment of GFP-expressing MDA-MB 435 (MDA-MB 435GFP) and 4T1 cell lines. The mammary carcinoma MDA-MB-435 (human) and 4T1 (mouse) cell lines were transfected with a plasmid, pEGFP-N1, that constitutively expresses an enhanced version of the GFP (Clontech Laboratories, Inc., Palo Alto, CA). FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) was used to aid the transfections. A protocol recommended by the manufacturer was followed. G418 selection at 500 μg/ml started 2 days after transfection. Two to 3 weeks later, colonies that emerged in Petri dish cultures were examined for GFP expression under a fluorescence microscope. Those colonies with robust GFP expression were picked, pooled, and expanded for further experiments.

Intracardiac injection of tumor cells. Female nu/nu mice were anesthetized by i.p. injection of Nembutal (45 mg/kg of diabutal (50 mg/ml) /saline/ethanol/propylene glycol 10/63/7/18). The anterior chest wall were scrubbed with 70% alcohol. A 30 gauge needle on a tuberculin syringe were inserted slowly into the second intercostal space 3 mm to the left of the sternum and aimed centrally. The spontaneous and continuous entrance of pulsating red blood into the transparent needle hub indicated proper positioning of the needle into the left ventricle of the heart. Gentle aspiration of the syringe were necessary to determine if the needle is in the left ventricle of the heart if red blood failed to enter the needle hub. 10^6 cells in 0.1ml saline were injected into the left ventricle of the heart over a 20-40 second period.

Selection of MDA-MB 435GFP cells preferentially metastasizing to brain. MDA-MB 435GFP cells were injected into the left cardiac ventricles of nude mice, brain metastases were harvested when mice became moribund, tumor cells were cultured, amplified and then injected back into the left heart of the nude mice to generate brain metastases again. By selecting brain

metastatic lesions for four cycles we established a unique human breast cancer cell line expressing green fluorescent protein that preferentially metastasizes to the brain. At least 80% of nude mice died of brain metastasis 2-3 months after the cell line was injected into their left cardiac ventricle.

Labeling of vasculature in vivo. Systemic labeling of vascular network was done by i.v. injection of 1 mg rhodamine conjugated albumin (Molecular Probes, Eugene, OR).

Examination of brain metastases and function alteration of BBB. At every time point or when the mice show signs of morbidity, the animals were injected i.v. with Rhodamine-albumin, 0.5-1 hr later the mice euthanized by inhalation of carbon dioxide in a closed chamber. The brains were removed and examined under fluorescence stereoscope ((Leica Model LZ12), equipped with narrow bandpass excitation and emission filters mounted in a selectable filter wheel (Ludl Electronic Products, Hawthorne, New York). Real-time images were directly captured with an Evolution MP camera (Media Cybernetics Inc., Silver Spring, Maryland or by frame capture from videotaped images.) or a Zeiss LSM 510 laser scanning confocal microscope (LSM510, Carl Zeiss Inc. Thornwood, New York) with krypton-argon and helium-neon lasers. The brain metastases were also harvested, frozen sections cut and fixed immediately in cold acetone for 5 min. Fluorescence microscopy for Rhodamine-albumin permeability in brain tissues was performed with an epifluorescence microscope equipped with a mercury vapor lamp and appropriate narrow bandpass excitation and emission filters (Ludl Electronic Products, Hawthorne, New York).

Immunostaining for CD31. Since the rhodamine labeled albumin was washed away and green fluorescence of GFP tumor cells quenched during the process of staining, so we failed to complete three-dimensional three color imaging of tumor (green fluorescence), We performed our routine CD31 staining and the results demonstrated the points all the same as we proposed. Briefly, acetone-fixed frozen sections were sequentially incubated with rat anti-mouse CD31 (PharMingen, San Diego, California), peroxidase-conjugated goat anti-rat antibodies (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) and diaminobenzidine.

Results:

Sequential observation of pathogenesis of brain metastasis using MDA-MB-435GFP and 4T1GFP revealed the complexity of mechanisms by which breast cancer develop brain metastases and functional heterogeneity of BBB in brain metastases.

Coexistence of different mechanisms in breast cancer brain metastases.

1) **Extravasation and extravascular growth.** At weeks 1 and 2, among 28 superficial MDA-MB-435GFP metastatic nodules consisting of cells cluster or a single cell, 22 of 28 were extravascular (Fig. 1), indicating that extravasation was the dominant mechanism of brain metastases. Perivascular nodular foci most located around capillaries and arterioles. The single cell outside vessels at week 1 indicated that some metastatic cells did not go proliferation immediately after tumor cell inoculation. After extravation, metastatic cells migrate and grow towards the adjacent blood vessels of the neighbor vessel network, and then proliferate along the vasculature perivascularly to distant sites (Fig. 2). For brain metastases at advanced stage tumor cells did not grow randomly at the tumor periphery, but aligned themselves longitudinally along the blood vessels. The longitudinal alignment could be continuous or discontinuous, indicating the migration of tumor cells along the

vasculature. And perivascular tumor growth could encompass the vascular perimeter completely or just partially (Fig. 2c). When cells divided and migrated along an existing vessel, they could spread almost as far as the whole hemisphere of brain without notable tumor nodule.

2) **Intravascular proliferation, vessel rupture, and extravascular expansion.** 6 of 28 metastatic nodules were intravascular at weeks 1 and 2. The intravascular nodules could also be found in brain at late stages and the nodular foci were larger. They were confined in arterioles or intermediate-size arteries and extended as strings in the shape of vessel, CD 31 staining showed that tumor cells were surrounded by CD 31 positive endothelial cells(Figs. 3). Cell division must have been occurring within the brain vasculature since strings of cells were never present at times earlier than 3 days. In our system, both vasculature and tumor cells were clearly visible under fluorescence microscope. The results suggest that the intravascular stage of brain metastasis be much more prolonged than has been predicated from other organ metastasis models. In addition to the direct observation of intravascular growth of metastatic cells, there were two additional evidences supporting the intravascular origin of brain metastasis. The first was the occlusion of the blood vessels (Fig. 4). This feature was quite apparent by the atrophy of local tissue under dissecting microscope and the necrosis of brain tissue during the early brain metastasis in H/E slides. The necrotic areas usually occurred in the deep parenchyma of the brain, and the size usually depended on the size of the blood vessel occluded. The second was the releasing of tumor emboli into the downstream area of brain. This phenomenon was clearly demonstrated in 4T1 cells model in Fig. 5. The shaded tumor cells from the intravascular metastatic colony could become the constant source of releasing tumor emboli to distant regions, causing the metastases of metastasis. Although the intravascular pattern of brain metastasis was not the dominant mechanism, it could easily lead to extensive tumor dissemination through the vasculature and damage the host tissues located in the downstream area by blocking blood flow. In the late stages (when the mice were moribund or 30 days later after tumor cell inoculation), continuous intravascular proliferation of tumor cells led to disruption of the surrounding blood walls (fig 6). After disruption of blood vessels, a completely different event took place. The overflowing (escaped) metastatic cells migrate and grow towards the adjacent blood vessels of the neighbor artery network, and then proliferate along the vasculature perivascularly to distant sites.

Heterogeneity of BBB function. At weeks 1-2, Rhodamine-albumin was not leaky if the tumor nodules were inside the vessels (Fig. 7). If the tumor cells were outside the vessels, the vessels could be leaky (Fig. 8) or not leaky (fig 1). When at the late stages of metastasis, the tumor size became bigger; the vessels became leaky, especially in the tumor center. But in the periphery of metastasis, the vessels were heterogeneous to the penetration of rhodamine-albumin, indicative of the heterogeneity of BBB function in brain tumors (Fig. 9).

Discussion:

High-resolution observation of GFP-labeled tumor cells metastases in isolated, perfused lung made Al-Mehdi at al conclude that hematogenous metastasis originates from the proliferation of attached intravascular tumor cells rather than extravasated ones (Al-Mehdi et al., 2000). This contradicts the traditional view that circulating metastatic cells must extravasate before proliferating into colonies (Fidler, 1990; Koop et al., 1995). This issue is extremely important for brain metastasis in that intravascular metastasis formation would offer us the opportunity to cure brain metastases by developing new approaches to early detection and giving intravascular drugs

despite the BBB existence. In fact, intravascular growth has been described in B16 melanoma brain metastases(Alterman and Stackpole, 1989; Raz and Hart, 1980), but it was termed as an exception because it has not been observed in other tumor systems and B16 melanoma brain metastasis almost always occurred in leptomeninges and the B16 tumor cells within the leptomeninges artery were enmeshed in large fibrin-like clots that disable tumor cells undergo active extravasation. Current concept dictates that brain metastases begin from malignant cells extravasation(Kawaguchi et al., 1982; Walsh, 1996). The view that tumor cells proliferate after their penetration of BBB is very discouraging and devastating, this makes the diagnosis and treatment of early brain metatstasis extremely difficult because of the specialty of BBB in hindering penetration of most systemically administered agents. Although the idea that tumor cells can affect BBB permeability(Kohn et al., 1989; Stewart et al., 1987; Zhang et al., 1992), the interpretation of this result in terms of the inefficacy of chemotherapy remains a fundamental problem.

The in vivo application of GFP-labeled tumor cells (Hoffman, 1998) allows us to observe pathogenesis of brain metastasis with unprecedented spatial resolutions. This greatly facilitates a broad investigation of development of brain metastasis. This is the first clear evidence that the same tumor cells can establish metastases by different mechanisms – extravasation and intravascular proliferation. The reasons why some brain metastases began intravascularly and some extravascularly, whether they were due to the difference of biological properties in tumor cells or the different vascular structure of various locations, remain unknown. The two deadly complications of intravascular growth of brain metastasis – the occlusion of blood vessel and the constant releasing of tumor cells into the downstream area- explain why brain metastasis is so calamitous and emphasize the necessity of chemotherapy. This will also explain why in some patients the imaging findings are of only a small tumor nidus but with a large amount of vasogenic edema or necrosis(Loeffler et al., 1997) . The existence of intravascular initiation of brain metastasis makes early detection and treatment clinically important and more practical. Though we hypothesized that the unique feature of richness of tight junctions among endothelia in BBB would prevent the metastatic cells from extravasation, it was obviously not the case, because in the very early stage single GFP tumor cell was quite often found outside the vessels. The function of the BBB is an important issue with regard to drug treatment of brain tumors. For the first time we demonstrated the temporal and spatial heterogeneity of the BBB function in the same brain metastatic setting, whether the difference in BBB function in small brain metastases in early stage was temporal, or due to the structural difference in BBB, or biologic properties in tumor cells remain important issues. Our results will not only explain the controversy between the evidence that BBB function is not intact and the lack of efficiency of chemotherapy but also suggest the necessity of drug therapy to prolong patient life.

More importantly, all the observations were made in the natural brain metastasis settings (with all the multiple cell types, the cytokines, etc. from the blood and the brain in place to interact with the metastatic cells) and from a large number of mice with different stages of brain metastases, the results should have more accurately reflected the clinical situations.

In conclusion, our results provide a framework about how breast cancer brain metastases initiate and progress and the heterogeneity of BBB function in brain metastases. They are important for us to better understand clinical presentations and develop new approaches to detecting and treatment of brain metastases.

Figure Legends

Fig. 1. Extravascular growth of breast cancer brain metastases. MDA-MB-435GFP breast cancer cells were injected into the left heart of female Nude mice. On day 14, mice were sacrificed and brain metastases examined with confocal microscope 1 hr after i.v. injection of rhodamine-albumin. The perivascular growth of metastatic cells indicates the completion of extravasation.

Fig 2. Perivascular distant migration of metastatic breast cancer cells. MDA-MB-435GFP cells were injected into the left heart of nude mice. Eight weeks later, the brain metastases were examined under fluorescence stereoscope. The micrographs show that tumor cells could travel far away from their primary sites (arrow) along the vascular network. Images were taken under fluorescence alone (a) or under dual fluorescence and bright illumination (b). c: H/E staining shows tumor cells completely or partially surrounded vessels.

Fig. 3. Intravascular growth of breast cancer brain metastasis at early stage. MDA-MB-435GFPBr cells were injected into the left heart of nude mice, one week later, the brain metastasis was examined under fluorescence stereoscope (a, b). The micrographs showed that the tumor cells were confined in vessels as strings. c shows immunostaining of the brain metastasis with CD31 antibody. Arrow indicates that tumor cells were wrapped by vascular wall.

Fig. 4. Intravascular proliferation of breast cancer brain metastasis and cerebral infarction. MDA-MB-435GFP cells were injected in the left heart of nude mice. Two weeks later, the brain metastases were examined by fluorescence stereoscope. Confinement of green tumor cells in the vessel under low (a) and high (b) magnification. c: H/E staining showed necrosis areas (indicated by white arrows) from cutting 1. d: Red arrow indicated tumor cells in vessel from cutting 2.

Fig. 5. Intravascular spread of brain metastasis under green fluorescence only (a) and with slight bright illumination (b). 4T1GFP cells were injected into the left heart of nude mice, two wks later, brain metastases were examined using fluorescence stereoscope. The arrow indicates the direction through which intravascular brain metastasis released tumor cells into downstream of vascular network.

Fig. 6. Vessel rupture by intravascular growth of brain metastasis. MDA-MB-435GFP breast cancer cells were injected into the left heart of female Nude mice. Four weeks later, mice were sacrificed and brain metastases examined with confocal microscope 1 hr after i.v. injection of rhodamine-albumin. The arrow points to the rupture site of vessel wall where tumor cells leaked out.

Fig. 7. Intact BBB in brain metastasis of early stage. MDA-MB-435GFP breast cancer cells were injected into the left heart of female Nude mice. One week later, mice were sacrificed and brain metastases examined with confocal microscope 1 hr after i.v. injection of rhodamine-albumin. The green tumor cells were confined in the vessels without the increase of BBB permeability.

Fig. 8. Increased permeability of BBB in brain metastasis of early stage. MDA-MB-435GFP breast cancer cells were injected into the left heart of female Nude mice. Two weeks later, mice were sacrificed and brain metastases examined with confocal microscope 1 hr after i.v. injection of rhodamine-albumin. The arrow points to the area of increased permeability for rhodamine-albumin.

Fig. 9. Heterogeneity of the BBB function induced by perivascular growth of brain metastasis. MDA-MB-435 GFP cells were injected into the left heart of nude mice, when the mice were moribund, their brain metastases were examined under fluorescence stereoscope 1 hrs after i.v. injection of Texas red-albumin. Note: the white arrow indicates increased vascular permeability but most vessels did not show increased permeability despite engulfed by tumor cells. a.GFP tumor. b. Rhodamine-albumin penetration.

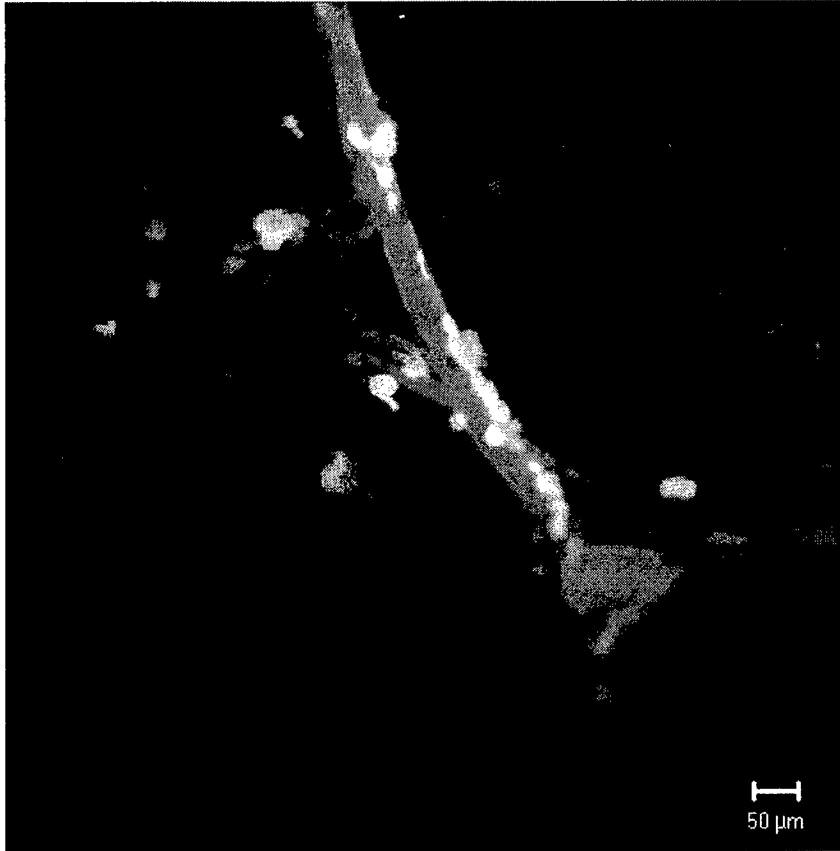

Fig. 1

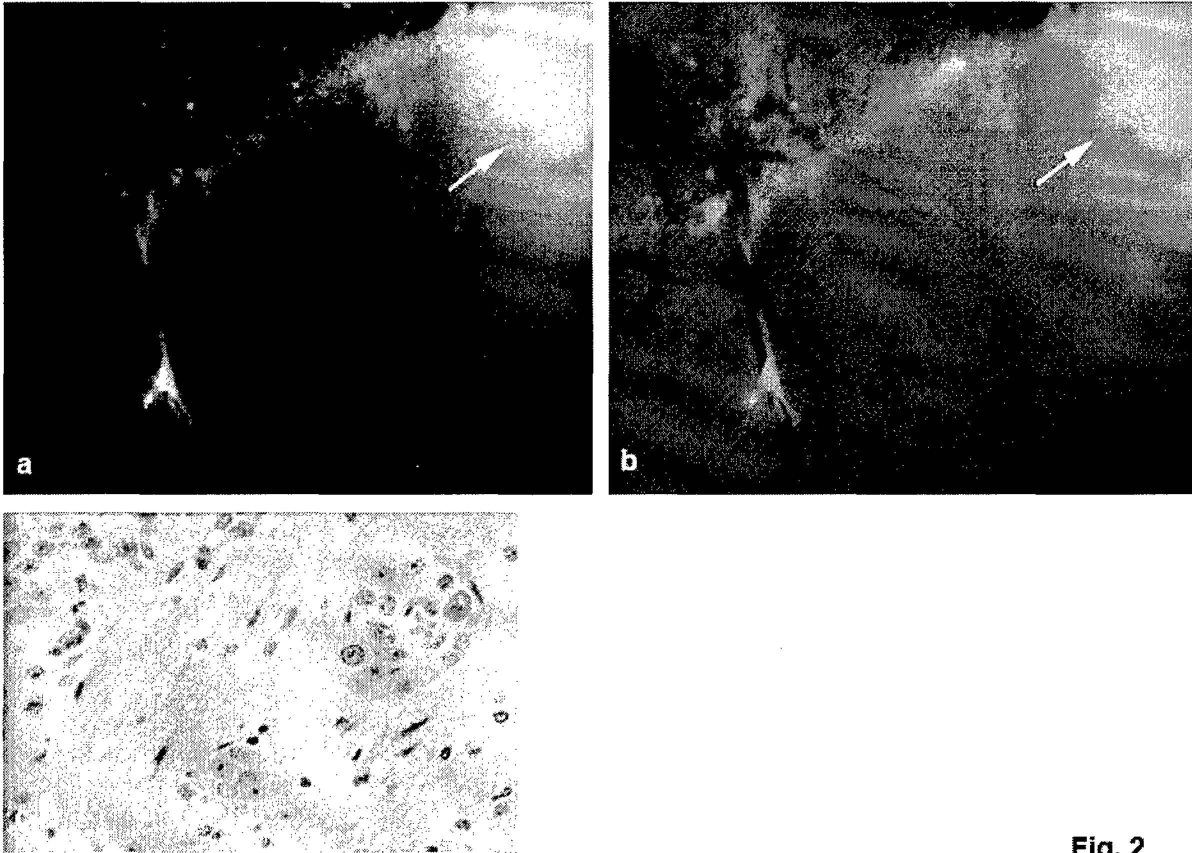

Fig. 2

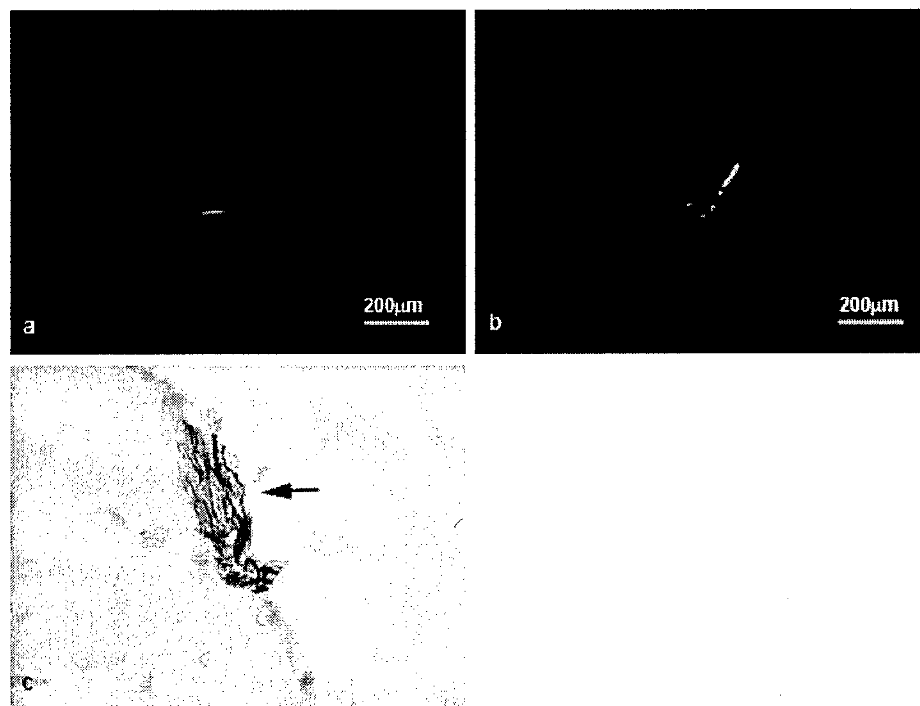

Fig. 3

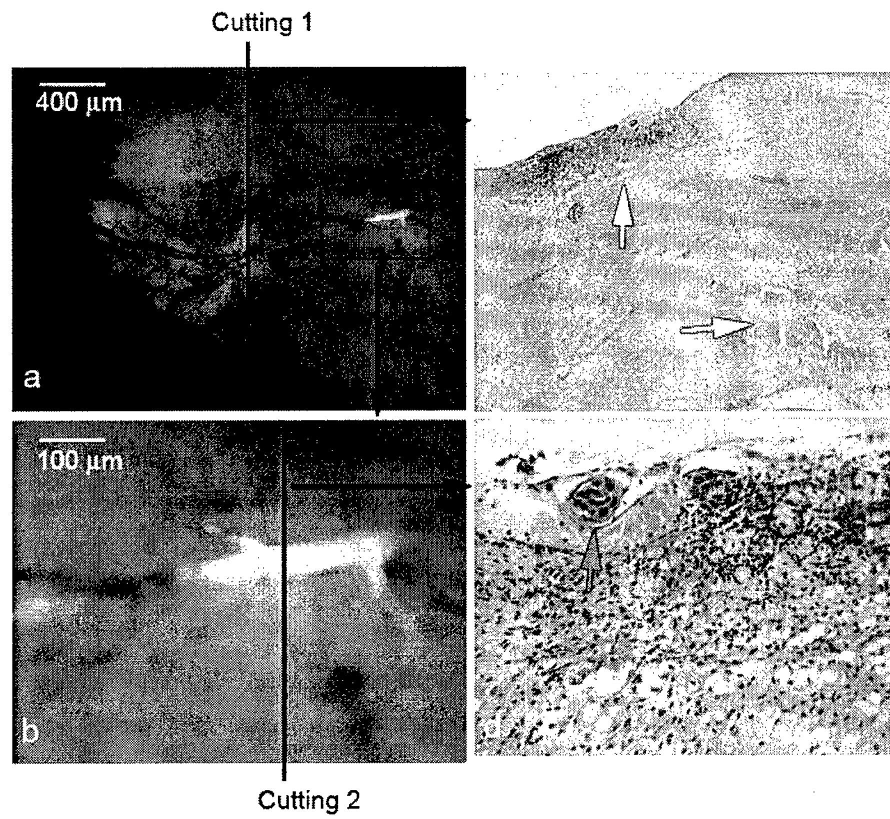

Fig 4

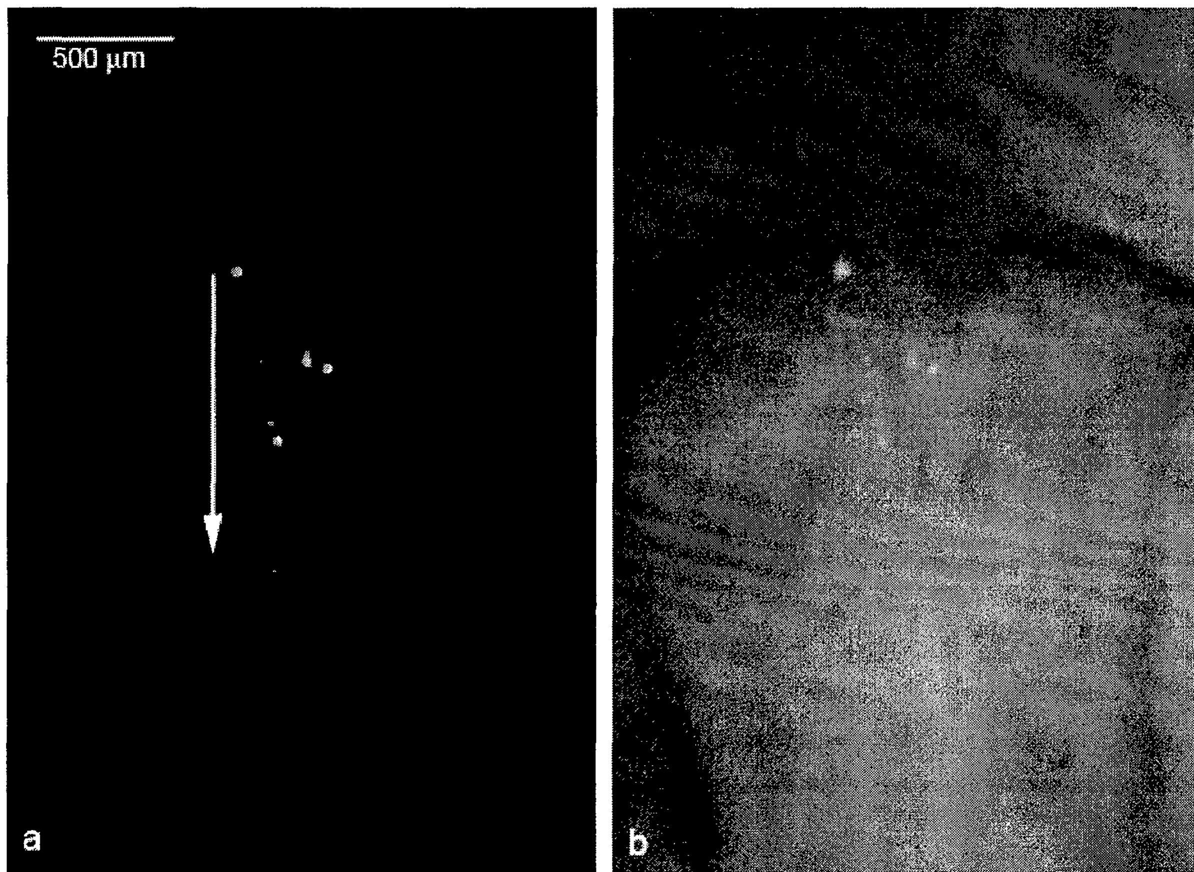

Fig. 5

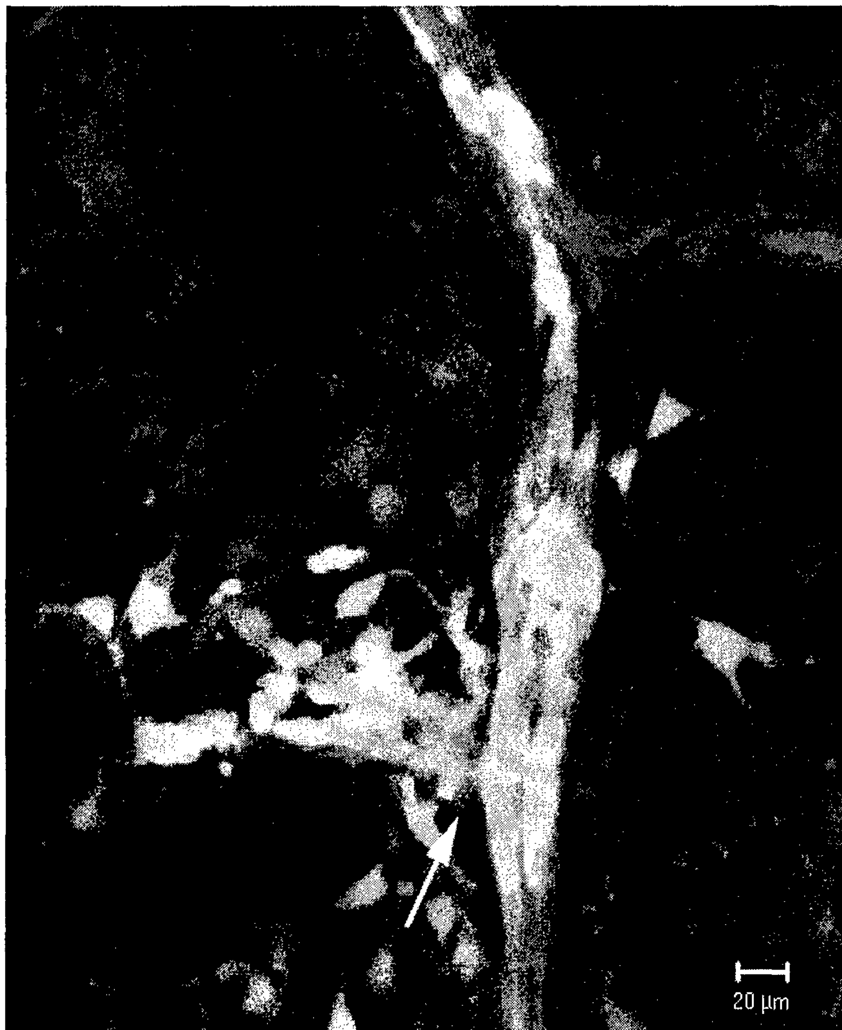

Fig. 6

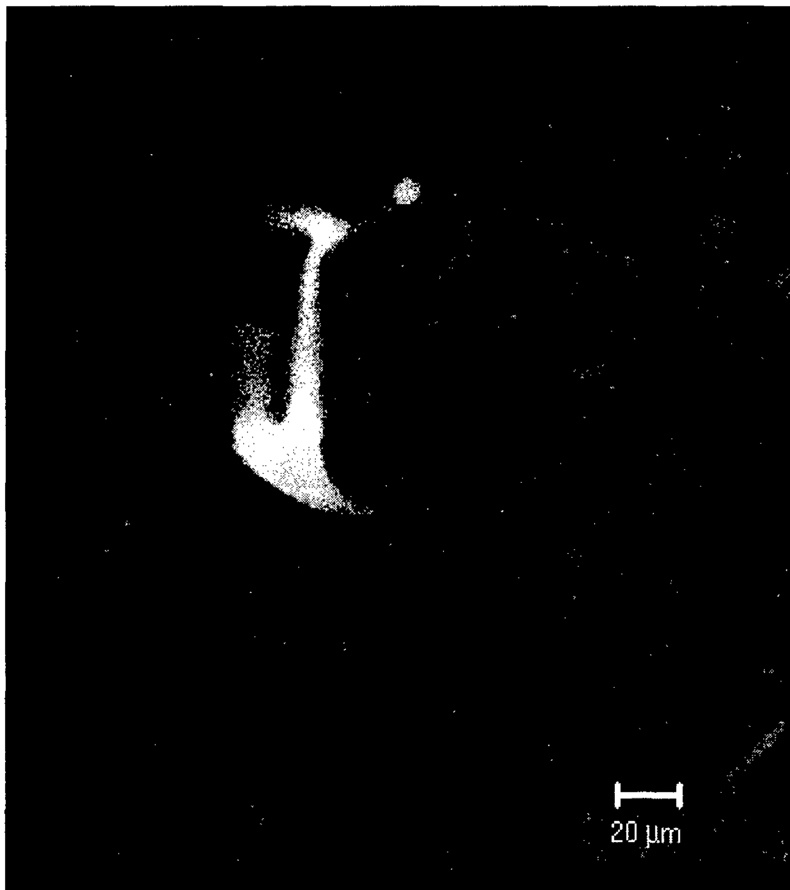

Fig. 7

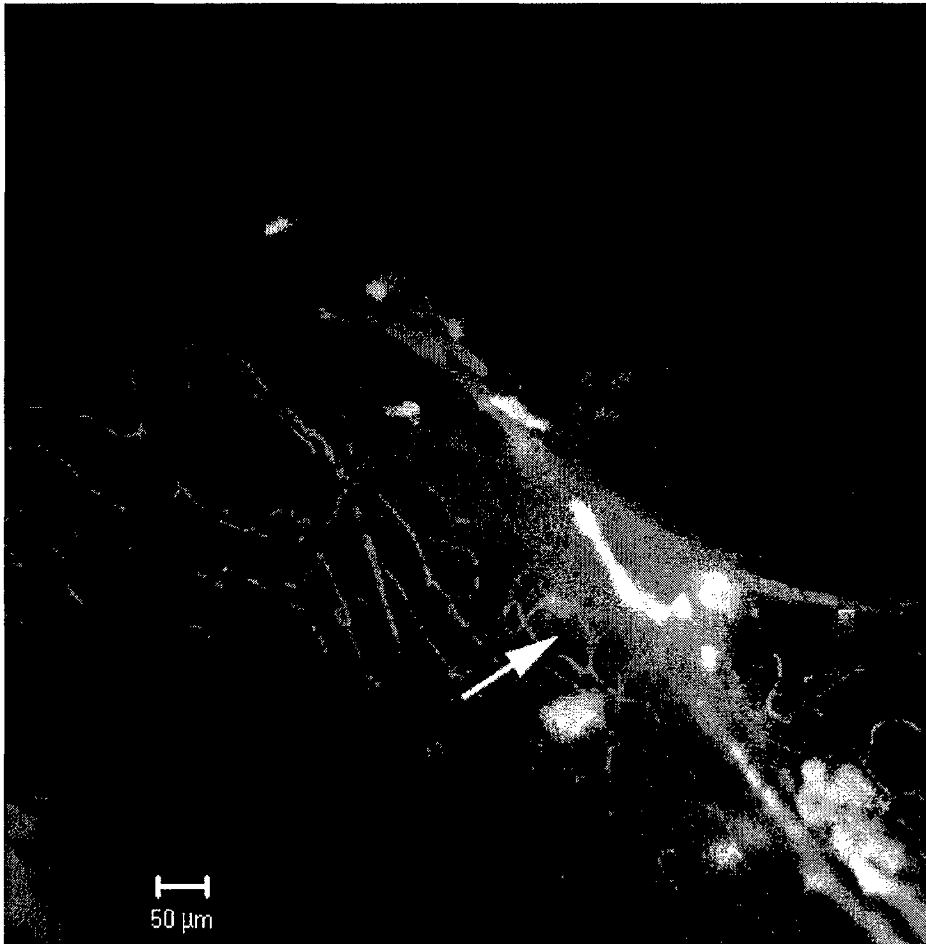

Fig. 8

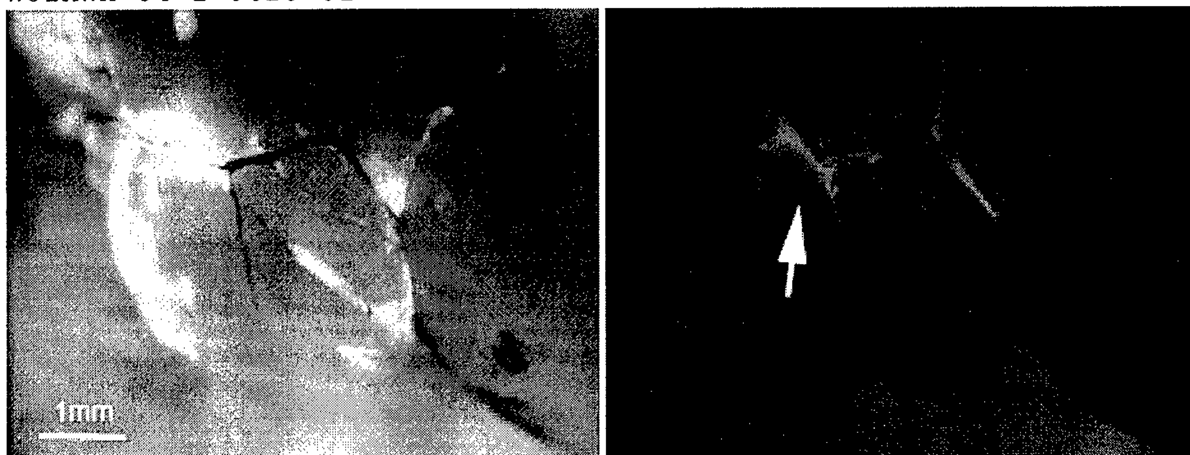

Fig. 9

Key Research Accomplishments:

- Abstract for Era of Hope, Breast Cancer Program Meeting 2005: Pathogenesis and blood-brain barrier heterogeneity of breast cancer brain metastasis
- Manuscript for publication: Pathogenesis and blood-brain barrier heterogeneity of breast cancer brain metastasis. In preparation

Reportable Outcomes:

The experiences obtained from the grant contributed to the application for the new idea award in DOD program: **Breast cancer growth and metastasis vascularize by tumor cell adaptive migration into and replacement of normal tissue.**

Conclusions:

The data from this proposal revealed the complexity of mechanisms by which brain metastases were developed. Breast cancer brain metastases could be established by tumor cells proliferation after extravasation or direct intravascular growth. The latter mechanism makes the brain metastasis extremely aggressive in that it serves as a consistent source to spread tumor cells to other areas of the brain and the block of blood supply by tumor growth in vessel damages brain tissue. The results showed the temporal and spatial heterogeneity of the BBB function in brain metastases. These results will significantly improve our understanding of the mechanisms behind the clinical presentation of patients with brain metastases, and help clinicians to optimize the therapeutic strategies addressing the complexities of the mechanisms and BBB function in brain metastases.

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Appendices:

between Rho, NHE, and polyFN. Identification of this critical signaling link may help us ultimately in the design of new anti-metastatic drugs directed at inhibiting the polyFN assembly on breast cancer cell surfaces, thereby preventing adhesion to lung endothelial DPPIV and reducing the risk of lung metastasis.

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P60-8: INVESTIGATION OF THE ROLE OF THE ARHGAP8 GENE IN REGULATION OF BREAST TUMOR CELL MIGRATION, INVASION, AND EPITHELIAL-TO-MESENCHYMAL TRANSITION

Cameron N. Johnstone,[1,2] Mark J. Bowser,[1,2] Laura M. Chang,[1,2] and Anil K. Rustgi[1,2,3]
[1]Gastroenterology Division, Department of Medicine, [2]Abramson Cancer Center and Family Cancer Research Institute, and [3]Department of Genetics, University of Pennsylvania, Philadelphia, PA
E-mail: cameronj@mail.med.upenn.edu

Recent work in our laboratory has focused on the identification of a putative tumor suppressor gene (TSG) within a 1 Megabase (Mb) interval on chromosome 22q13.31 in breast and colorectal cancers. One candidate TSG (ARHGAP8) was selected for further analysis on the basis of homology to the known TSGs NF1 and ARHGAP7/DLC-1. ARHGAP8 encodes a RHO GTPase activating protein (GAP) for RHOA, RAC1, and CDC42 in vitro, and was recently shown to physically interact with the Src homology-3 (SH3) domain of cortactin, the product of the EMS1 proto-oncogene.

Somatic mutation of ARHGAP8 was not observed in ninety-one breast and colorectal tumors examined. However, since ARHGAP8 contained a robust CpG-island in its proximal region, we examined ARHGAP8 mRNA expression levels in primary breast tumors and adjacent normal mammary gland, as well as in a panel of breast carcinoma cell lines. ARHGAP8 mRNA expression was reduced in approximately one third of primary breast tumors examined and was absent from three breast cancer cell lines (MDA-MB-231, MDA-MB-435, and BT549), and markedly downregulated in a fourth (Hs578T). Genomic Southern analyses demonstrated that the loss of expression was not due to ARHGAP8 homozygous deletion. Interestingly, these four cell lines also feature epithelial-to-mesenchymal transition (EMT), which includes a spindle-shaped morphology and loss of E-cadherin expression.

The involvement of DNA methylation and histone deacetylation in the silencing of ARHGAP8 expression in breast cancer cell lines was examined. Treatment with the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) robustly induced ARHGAP8 mRNA expression in MDA-MB-231 and BT549 cells but not in MDA-MB435 cells. Conversely, treatment with the demethylating agent 5'aza-2'deoxycytidine (5azadC) weakly induced ARHGAP8 mRNA expression in MDA-MB-231 and MDA-MB-435 cells, but not in BT549 cells. These data suggest that both histone deacetylation and DNA methylation may be involved in the silencing of ARHGAP8 expression in breast cancer.

To gain functional insights, N-terminal FLAG-tagged ARHGAP8 was reintroduced into MDA-MB-231, MDA-MB-435, and Hs578T cells, and also the immortalized mammary epithelial cell line MCF10A. The FLAG-ARHGAP8-transfected cells are being evaluated for alterations in cell cycle kinetics, adhesion to matrix, migration and invasion rates, as well as clonogenicity in soft agar, tumorigenicity and metastatic ability in vivo, and reversal of the EMT phenotype. A rabbit polyclonal antiserum recognizing ARHGAP8 is also being generated for immunohistochemical analysis of tissue sections and subcellular localization studies. This research has important implications for understanding how human breast tumors transition from a benign state to an aggressive state with potential for metastatic spread.

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P60-9: A NOVEL RAT MODEL OF SPORADIC, BLOOD-BORNE, VISUALIZABLE MICRO-METASTASES OF MAMMARY CARCINOMA IN THE BRAIN

Lois A. Lampson, Ph.D., Cara A. Tripp, and Dennis S. Meredith
Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA
E-mail: lampson@rics.bwh.harvard.edu

Brain metastases are increasingly important as a cause of fatality for breast cancer patients, as therapy at other sites improves. To develop new therapies, more appropriate animal models, especially for the earliest brain micro-metastases, are needed. Our aim is to develop a practical small animal model with an appropriate sporadic distribution of visualizable, blood-borne micro-metastases. We report simplified methods, making the model more generally useful, and describe early tumor entry to the brain.

Methods. The highly metastatic MATBIII variant of the well-studied 13762 rat mammary carcinoma cell line had been made to constitutively express the lacZ reporter gene product, E. coli-derived b-galactosidase (b-gal) (MATB/lacZ). The b-gal marker appears as a bright blue, cell-filling cytoplasmic stain after a simple histochemical stain in tissue sections. The smallest micro-metastases, even single tumor cells, are readily detected by ordinary light microscopy. Tumor is injected into the left common carotid artery, to favor delivery to the brain. To ask if ligating side-branches was necessary, we first compared the path of injected dye (1% methyl green), then the yield in brain vessels of inert polystyrene beads. One million tumor cells were then injected by the simplified method. Rats were sacrificed 0-7 days later by intra-cardiac perfusion with fixative, and 6u cryostat sections through the brain were stained histochemically (X-gal substrate) to reveal b-gal+ tumor cells.

Results: (1) Simplified methods. The injected dye was readily visualized in the intact rat, turning the ear and eye bright green on one side (as expected from the blood flow), simplifying the first comparison of different methods. Quantitative analysis of the yield of inert beads in the brain confirmed that the yield was not improved by ligating the side vessels. This greatly simplified training and the conduct of experiments. (2) Early tumor pattern. Microscopic tumor (1 or a few tumor cells) appeared in a sporadic pattern in the hemisphere of injection. Tumor was seen in the brain proper, meninges, and choroid plexus. The tumor was often clearly vessel-associated, sometimes appearing as emboli. The tumor cells were clustered together, and did not appear to invade the neuropil.

Conclusions: The model displayed the 3 major routes of blood-borne entry to the brain: vessels of the brain proper, meninges, and choroid plexus. Localization to the injection side is consistent with the blood flow. Emboli and clustering (not moving into the neuropil) are characteristic of human metastases. The ability to visualize the earliest micro-metastases, the varied and appropriate sites and forms of tumor entry, and the comparison between the affected and unaffected sides of the brain, will aid many kinds of studies of biology and therapy for metastatic breast cancer in the brain.

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P60-10: PATHOGENESIS AND BLOOD-BRAIN BARRIER HETEROGENEITY OF BREAST CANCER BRAIN METASTASIS

Weixin Lu and Alan J. Schroit
Department of Cancer Biology, University of Texas M.D. Anderson Cancer Center, Houston, TX
E-mail: weixunlu@mdanderson.org

A large number of young breast cancer patients died of brain metastasis. Our knowledge, which brain metastases develop after metastatic cells extravasate and localize and grow within brain tissue, has largely been extrapolated from observations in other tissues, and the existence of blood –brain barrier (BBB) has been the major reason for the ineffectiveness of current chemotherapeutic regimens. Development of effective therapies will depend on a better understanding of the pathogenesis of brain metastasis and the role of BBB in its development.

By using a selected, green fluorescent protein expressing breast cancer cell line (MDA-MB-435GFP) that preferentially metastasizes to the brain, we analyzed the progression of brain metastasis of MDA-MB-435GFP and its relationship to the BBB structure and function at different stages after injecting the cells into left heart of nude mice and intravenous injection of rhodamine labeled albumin before sacrificing mice. The strong GFP expression of the tumors and rhodamine-labeled vasculature allowed us to simultaneously localize small tumor lesions and to track the structure and permeability of the surrounding blood vessels. Histologic analysis of brain samples validated our gross observations.

The results up to the abstract submission were as follows: First, at the very early stage the hematogenous metastatic cells attached to the vessel endothelial cell do not immediately undergo extravasation but instead proliferate within the vasculature. Second, intravascular proliferation of tumor cells leads to thrombosis-like complications such as vasogenic edema and infarction of brain parenchyma, making it difficult to differentiate from other vascular diseases. Third, the cancerous thrombus can serve as a sustained releasing source of tumor cells to the downstream area through blood flow, making the spread of tumor cells extremely easy. Fourth, continuing intravascular tumor expansion disrupts the blood vessel, making the BBB not intact. Finally, the overflowing metastatic cells proliferate and migrate along the host vasculature perivascularly to far distant sites, bypassing the dependence of angiogenesis and regaining the protection of BBB.

We conclude that metastatic breast cancer cells proliferated in the vasculature with an intact BBB at the early stage, mechanic rupture of growing metastatic tumors disabled the function of BBB. The unique growth patterns of brain tumors at late stage made the

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BBB function heterogeneous. These rules of brain metastasis not only change previous views and improve the understanding of pathogenesis but also provide rationales for early diagnosis and treatment.

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P60-11: A MODEL OF COX-2 MEDIATED BONE METASTASIS IN HUMAN BREAST CANCER

Anthony Lucci, M.D., Balraj Singh, Ph.D., Jacob A. Berry, B.S., and Angela Shober, M.D.
University of Texas M.D. Anderson Cancer Center, Houston, TX
E-mail: alucci@mdanderson.org

Cyclooxygenase-2 (COX-2) is overexpressed in 40% of human invasive breast cancers. Breast cancers metastasize predominantly to the bone. The purpose of this study is to test the hypothesis that increased production of COX-2 in the breast cancer cells is responsible for initiating a series of events that facilitate the spread of breast cancer to the bone. Our experimental procedures include 1) the identification of genes affected by COX-2 overexpression in breast cancer cell lines, 2) to demonstrate that the gene expression alteration is relevent in an appropriate mouse model of breast cancer metastasis to bone, and 3) determine whether there is a correlation between COX-2 expression in primary human breast cancer and the presence of breast cancer cells in bone marrow. The spread of breast cancer cells to bone marrow, termed bone marrow micrometastasis (BMM), correlates with clinical bone metastasis.

We have identified several targets of COX-2 action in breast cancer cells, including urokinase plasminogen activator (uPA), interleukin-8 (IL-8), and IL-11. Using a mouse model in which we inject luciferase-transfected breast cancer cells into left ventricle and then image metastasis by whole-body imaging with the IVIS 200 system (Xenogen, Alameda, CA), we have evidence that COX-2 expression in breast cancer cells enhances bone metastasis and that MF-tricyclic, a COX-2 inhibitor, is able to inhibit bone metastasis in both prevention and treatment regimens. Analysis of the bone-seeking clones after isolating and culturing from the bone metastases shows that these cells produce significantly more prostaglandin E2 (PGE2), an important mediator of COX-2 effects, than the parental population of injected cells. In parallel with PGE2, the bone-seeking clones overexpressed uPA, IL-8, and IL-11, which have well established roles in invasion into basement membrane, metastasis, and osteolytic bone metastasis, respectively. At present we are at the early stage of collecting data from the human tumor samples (these studies were interrupted due to the change of grantee institution).

The data from the cell lines and the mouse model provide strong evidence for the role COX-2 in breast cancer metastasis to bone. Identification of uPA, IL-8, and IL-11 as downstream mediators of COX-2 in bone-seeking clones of breast cancer cells provides insights into the mechanisms through which COX-2 overexpression could facilitate tumor progression and enhance bone metastasis. Our results with the cell lines and a mouse model of bone metastasis indicate that COX-2 inhibitors may be useful for both prevention and treatment of breast cancer metastasis. This research will allow us to develop clinical trials aimed at preventing metastases using agents that inhibit COX-2.

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P60-12: CELL CLUSTERS OVERLYING FOCALLY DISRUPTED MYOEPITHELIAL CELL LAYERS CHANGE STATUS OF ESTROGEN RECEPTOR EXPRESSION DURING TUMOR INVASION

Yan-gao Man
Department of Gynecologic and Breast Pathology, Armed Forces Institute of Pathology and American Registry of Pathology, Washington, DC
E-mail: man@afip.osd.mil

Our previous studies revealed that a subset of estrogen receptor (ER) positive ductal carcinoma *in situ* (DCIS) contained focal myoepithelial (ME) cell layer disruptions, and over 80% of these disruptions were overlain by cell clusters with a total loss or marked reduction of ER expression, in contrast to the adjacent cells within the same duct, which were strongly ER positive (Man et al, Breast Cancer Res 5: R231-241, 2004). These cell clusters also showed a significantly higher frequency of genetic abnormalities and a higher expression of tumor invasion related genes (Man et al. Breast Cancer Res Treat, in press), suggesting that some of them might represent the direct precursor of the invasive lesions. This finding, however, is hard to reconcile with the factor that over 80% of the invasive breast tumors are ER positive. The current study attempted to identify the potential mechanism for our finding.

Consecutive sections were prepared from formalin-fixed and paraffin-embedded breast tissues of 15 cases of DCIS with micro-invasion. Sections were double immunostained for smooth muscle actin to identify focal ME cell layer disruptions, and for ER to locate ER negative cell clusters. Immunostained sections were examined, with special attention to the micro-invasive lesions that come out from focal ME cell layer disruptions. The ER expression status in cells at a different distance to focal ME cell layer disruptions were compared.

Each case contained at least 2 to over 10 ducts with focal ME layer disruptions. Over 80% of these disruptions were overlain by ER negative cell clusters, but the remaining non-disrupted portion was overlain by ER positive cells. Of these ER negative clusters, 12 from 9 cases were arranged as triangle-shaped edges, "puncturing" into the stroma. All the cells near the tumor core and focal disruptions were completely devoid of ER expression. A vast majority of the cells near the intersection between the basement membrane and the stroma were also ER negative, while ER positive cells were occasionally seen. The number of ER positive cells and intensity of ER immunostaining appeared to linearly increase as the cells invaded deeper into the stroma. A majority of the cells at the tip of the edge and adjacent microinvasive lesions were ER positive.

These findings suggest that most ER negative cells overlying focal ME cell layer disruption are likely to be undifferentiated or stem cells, which are not mature enough to express ER. They, however, could progressively gain the capability to express ER after a few cycles of divisions and differentiation.

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P60-13: TRAFFICKING OF BREAST CANCER METASTATIC CELLS IN BONE

Robyn J. Mercer,[1] Pushkar A. Phadke,[2] Jennifer L. Jewell,[3] Carol V. Gay,[1,5] Virginia Gilman,[1] D. R. Welch,[2,3,4,5] and Andrea M. Mastro[1,5]
[1]Department of Biochemistry and Molecular Biology, Penn State University, University Park, PA; [2]Department of Pathology, [3]Comprehensive Cancer Center, [4]Center for Metabolic Bone Disease, [5]NFCR-Center for Metastasis Research, University of Alabama at Birmingham, Birmingham, AL
E-mail: a36@psu.edu

Breast cancer cells frequently metastasize to bone where they cause osteolytic lesions. It is only after severe bone loss that the lesions become visible by X-ray. At that time the tumor masses are mostly visible in the ends of the long bones, areas of active hematopoiesis. However very little is known about the early trafficking patterns of the metastatic cells. The purpose of this study was to determine the movement of breast cancer cells in the long bones over time.

Method: Metastatic MDA-MB-435 human breast cancer cells, engineered to express green fluorescent protein (GFP) were inoculated into the left ventricle of the heart of athymic mice. The femurs were harvested at 1, 2, 4, 24, and 72 hr; and 1, 2, 4, and 6 wk. after the inoculation. Intact bones were examined by fluorescence microscopy. Some femurs were then fixed and decalcified for histochemistry or fluorescence microscopy and histomorphometry. At select times, other femurs were cut into proximal, distal and cortical shaft areas. Marrow was isolated from each section and analyzed by flow cytometric analysis to detect GFP or the DNA was extracted for detection of the presence of a human gene, HERV-K (Ac.No. M14123), by quantitative PCR.

Results: A few GFP expressing cancer cells could be clearly seen in the intact bones as early as one hour. Using fluorescence microscopy to examine 10-micron sections of the femurs, we detected isolated cancer cells at one week. By two weeks it was easy to detect clusters of GFP cells in the bone sections. Interestingly, the distal ends of the femur were populated by cancer cells first. Then the cancer appeared in the proximal ends. Almost never were cancer cells seen in the cortical shaft of the bone. These general patterns also were seen by flow cytometry and by presence of the cancer cell DNA. Based on previous in vitro data, we also measured apoptotic osteoblasts in the bone sections using a TUNEL assay. The numbers of apoptotic osteoblasts increased as the numbers of breast cancer cells increased. Moreover, apoptotic osteoblasts were in close proximity to breast cancer cells.

Conclusion: These results taken together suggest that once the cancer cells enter the bone marrow they traffic directly to the hematopoietic areas at the ends of the long bone. It is likely that factors localized in these active areas attract the cancer cells and provide a favorable environment for their growth. As the cancer cells multiply, local osteoblasts undergo apoptosis indicating that the bone will not easily be repaired even if the cancer cells are blocked. This information will be useful in planning strategies to prevent destructive bone metastasis early in the process.

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Curriculum Vitae

NAME: Weixin Lu, M.D. Ph.D.

PRESENT TITLE AND AFFILIATION

Instructor, Cancer Biology, MD Anderson Cancer Center, Houston, TX

CITIZENSHIP AND VISA STATUS

Chinese and Green Card

HOME ADDRESS (current)

5606 Bissonnet Street #132, Houston, TX 77081

OFFICE ADDRESS (current)

SRB1.265, 7777 Knight Road, Houston, TX 77054

EDUCATION

1983, 9-1989, 7: Department of Medicine, Shanghai Medical University, M.D.

1989, 9-1994, 7: Department of Internal Medicine, the Graduate School, Shanghai Medical University, Ph. D.

1994, 9-1997,2: Postdoctoral Fellow, Shanghai Institute of Cell Biology, Chinese Academy of Sciences

1997,2-Present: Postdoctoral Fellow, Research associate, Instructor, Cancer Biology, UT MD Anderson Cancer Center

CREDENTIALS

N/A

EXPERIENCE/SERVICE

N/A

HONORS AND AWARDS

N/A

RESEARCH

Patents Granted and Pending

Patents pending:

1. Therapy of cancer by insect cells containing recombinant baculovirus encoding genes. Isaiah J. Fodler, Zhongyun Dong, and Weixin Lu. International patent application No. PCT/US00/07200 and U.S. Serial No. 09/271,01
2. Adjuvant preparation for the induction of specific immunity. Isaiah J. Fidler, Zhongyun Dong and Weixin Lu. International patent application No. PCT/US01/17948 and U.S. serial No. 60/208,436
3. Active specific immunotherapy of brain metastasis. Isaiah J. Fidler, Zhongyun Dong and Weixin Lu. International patent application No. PCT/US03/33395 and U.S. serial No. 60/453,330
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EDITORIAL AND REVIEW ACTIVITIES

N/A

TEACHING

N/A

PROFESSIONAL MEMBERSHIPS/ACTIVITIES

Professional Society Activities, with Offices Held

Associate Member, AACR