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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Breast cancer is the second most common cause of brain metastases, diagnosed in 10 to 15% of breast cancer patients and found at autopsy in 20 to 30%. Relatively little is known about how breast cancer cells metastasize to the brain, and what phenotypes characterize these cells. This is due in large part to the lack of suitable experimental models. The objective of the application is to develop experimental models to study the pathogenesis of breast cancer brain metastases, using intra-carotid artery injection of breast cancer cells into nude mice. This model will be used to test the hypothesis that brain metastases arise from the survival and growth of unique populations of cells. We found that a breast cancer variant, resulting from three cycles of injection and recovery of cells from brain metastases, has significantly greater potential for experimental metastasis in the brain of mice, following injection into the carotid artery. Also, the cells express elevated levels of two angiogenic factors, VEGF-A and IL-8. Variants isolated from lung metastases did not show increases in these factors. The selected variants will be used in continuing studies of the phenotype and angiogenic potential of breast cancer cells that metastasize to brain.

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The Biology of Breast Cancer Brain Metastasis

INTRODUCTION:

Breast cancer is the most common cancer of women in the United States of America. The prognosis for recurrence and death after treatment of the primary cancer is related to the disease stage and the presence of axillary lymph node metastases at the time of diagnosis. The majority of the deaths of women with breast cancer are the result of metastasis (1). In addition to the axillary lymph nodes, other sites where breast cancer metastases are found include the bone, liver, lungs and brain. Breast cancer is the second most common cause of brain metastases, after lung cancer. Brain metastases are diagnosed clinically in 10 to 15% of breast cancer patients, and found at autopsy in 20 to 30% (2). The survival after detection of brain metastases can be short, a matter of weeks, and the therapy currently available only offers the hope of surviving one year to 20% of patients (3). The median age of women developing breast cancer brain metastases is 51 years, which is several years younger than the age of women diagnosed with metastases to other organs. This may be a reflection of aggressive behavior of the breast cancers of relatively younger women. Another observation from reviews of clinical data is that the brain is not uncommonly the first site of relapse in patients who had previously received adjuvant systemic chemotherapy (3,3,4). This may be the result of the chemotherapeutic agents being unable to cross the blood-brain barrier and thus being ineffective against micrometastases. Experimental data from our previous studies suggest that the blood-brain barrier is intact around metastases of a certain size ($<0.2mm^2$), yet as the brain metastases enlarge the barrier becomes permeable (5). Although it is well known that breast cancers can metastasize to the brain, relatively little is known about how these metastases form, and what phenotypes are characteristic of breast cancers with brain-metastasizing potential. Without this information, the rational design of new therapies to prevent or control the growth of brain metastases is impossible. In large part, progress in understanding the biology of breast cancer brain metastasis has been limited by the lack of suitable cell lines and experimental models. The objective of this application is to develop experimental models to study the pathogenesis of breast cancer brain metastases, using intra-carotid artery injection of human breast cancer cells into nude mice. The repeated cycles of selection of cells from brain metastases and re-injection into mice will generate cell lines with enhanced brain-metastasizing potential. These will be compared with the original cells, and with variants from metastases in other organs of mice to determine the phenotypic characteristics of brain-metastasizing breast cancer cells. New therapeutic approaches need to be based upon the better understanding of the events involved in brain metastasis that may emerge from studies using this animal model.

BODY:

Objective: To isolate brain metastasizing variants of human breast cancer cells

i.) *In vivo* selection of variants:

MDA-MB-231 breast cancer cell line

Methods: The MDA-MB-231 human breast cancer cell line was transfected with an expression vector for enhanced green fluorescent protein (GFP), and a clone isolated with high expression of GFP (termed MDA-231 GFP). The cells were injected into the internal carotid artery of female nude mice (2×10^5 cells) using the technique described by Schakert et al. (6). The mice were observed daily, and killed when moribund. The survival time of each mouse was recorded. The mice were observed under a fluorescent dissecting microscope fitted with GFP filters, to

illuminate the brain metastases and help in the dissection of tumor tissue from the brain parenchyma. Samples of the tumor tissues were finely minced and placed in culture flasks with medium containing 800 μg/ml G418, the selection antibiotic used to isolate the GFP-expressing cells. Cultures were established from the brain metastases from three mice per group, and the cells combined to create the *in vivo*-selected variant line. The cells were then injected into the carotid artery of female nude mice, and the recovery of cells and tissue repeated. The *in vivo* selection was repeated three times, resulting in variant cell lines named MDA-231 BR1, MDA-231 BR2 and MDA-231 BR3. Samples of tissue from the brains of mice injected with the different variants were fixed in formalin or frozen in OCT for preparation of cryostat sections.

For a comparison with the brain-metastatic behavior of a variant of the MDA-MB-231 cell line from a different organ, a group of mice were injected via the carotid artery with MDA-231 LC3 cells. This variant was established from experimental lung metastases in mice injected via the tail vein, and the selection repeated to result in a variant that had been passaged three times *in vivo*.

Immunohistochemistry: Frozen sections of the brains with experimental metastases were hybridized with antibody against CD31/PECAM, to detect endothelial cells. Appropriate procedures for blocking endogenous peroxidase and non-specific hybridization were used. The sections were counterstained with hematoxylin. Sections of brain tissue from mice that were not injected with breast cancer cells were also stained, to assess the vessel density in the normal brain.

Quantitation of CD31-positive vessels: Ten random 0.159 mm^2 fields at x100 magnification were captured for each stained section (no hematoxylin counterstain on the imaged sections) using Optimas Image Analysis software (Bioscan, Edmond, WA). A structure was classified as a vessel on the basis of the criteria of Weidner *et al.* (7). The CD31-positive vessels were counted using Scion Image Beta 4.02 Win software (Frederick, MD).

Results:

Incidence of experimental brain metastases: Table 1 shows the results of injecting MDA-231 GFP cells and the brain metastasis-selected variants into the internal carotid artery of nude mice, expressed as the incidence of mice with brain metastases and the mean survival time. The mice injected with the metastasis-selected cells had significantly shorter mean survival than the mice injected with the original cell line (compared using the Student's t-test). In contrast, the animals that were injected with the MDA-231-LC3 cell line showed similar incidence of experimental brain metastases and survival times as the mice injected with the original cell line. The result suggests that different subpopulations of a heterogeneous cancer cell line can be isolated from the metastases that grow in different organs of nude mice.

Table 1: Incidence of experimental brain metastases and survival time of mice injected with MDA-231 GFP and brain metastasis selected variants

Cell line	Incidence of brain metastases	Mean survival time (days)	p value
MDA-231 GFP	82.4%	59.1 ± 2	
MDA-231 BR1	91.7%	51 ± 2.2	0.01
MDA-231 BR2	100%	45.9± 6	0.004
MDA-231 BR3	100%	41 ± 3.7	0.001
MDA-231 LC3	71.4%	59.3 ± 13	ns

When the growth rates of the different cell lines shown in Table 1 were compared *in vitro*, there were no differences in the doubling times, which were all approximately 27 h when the cells are grown in medium with 5% fetal bovine serum.

Visualization of the brain metastases of the MDA-231 GFP was facilitated by expression of the fluorescent protein. However, the expression was significantly reduced in the cells that had be successively cycled through the mice. Less than 5% of the MDA-231 BR2 and BR3 cells were found to express GFP, using flow cytometry to detect the fluorescence, although >96% of the original MDA-231 GFP cells were brightly fluorescent.

Counting microvessels in brain parenchyma and metastases: The growth of tumors has been shown to be dependent on the development of adequate blood supply through angiogenesis (7). The vascularization of the breast cancer brain metastases was assessed by counting the numbers of CD-31 positive stained vessels in sections with tumor lesions. The average number of vessels / 0.159mm^2 field at 100 x magnification was 40.5 ± 4 in sections with brain metastases of MDA-231 GFP, while in normal brain sections there were 24.8 ± 3.9 vessels /0.159mm^2 at this magnification (p = 0.045, Student's t-test). Qualitatively, the size of vessels in the brain metastases was greater than those in normal brain.

Table 2. Microvessel density in metastatic brain lesions of mice injected with MDA-231 GFP and brain metastasis-selected variants.

Cell Lines	Microvessel Density (MVD) ± SD
Normal Brain	24.77 ± 3.89
MDA-231 GFP	40.50 ± 4.06[a]
MDA-231 BR1	47.15 ± 7.13[b]
MDA-231 BR2	62.10 ± 3.97[c]
MDA-231 BR3	63.26 ± 2.53[d]

Average number of microvessels / 10 random field at x100 magnification.
a : $p = 0.04$ b : $p = 0.003$ c : $p = 0.001$ d : $p = 0.0001$

As shown in Table 2, the microvessel counts in the brain metastases of the selected variants of MDA-231 GFP were higher for the variants selected 2 or 3 times. A similar increase was not noted in the brain metastases formed by MDA-231 LC3 cells; the microvessel counts in these lesions were similar to those in the metastases of MDA-231 GFP.

MDA-MB-435 breast cancer cell line
Methods: As described above, the MDA-MB-435 cells were injected into the carotid artery of female nude mice, and the incidence of experimental brain metastases and survival times of the mice were recorded. Samples of tumor were dissected from the brains of three mice, and cultures established. These were pooled to derive the cell line MDA-435 KBR1. Additional variants of the MDA-MB-435 cell line used in comparison were: variants previously isolated from a brain metastasis (MDA-435 Br1) and lung metastases (MDA-435 Lung2) in a mouse with a mammary fatpad tumor (8); a line termed MDA-435 LVBr1, from a brain metastasis in a mouse injected via the left ventricle of the heart (Price, unpublished).

Results: Injection of the MDA-MB-435 cells into the carotid artery of mice resulted in brain metastases in 83% of mice, and mean survival time of 82 days. The line derived from brain metastases, MDA-435 KBR1 was injected into mice via the carotid artery, resulting in brain metastases in 86% of mice with a mean survival time of 54 days.

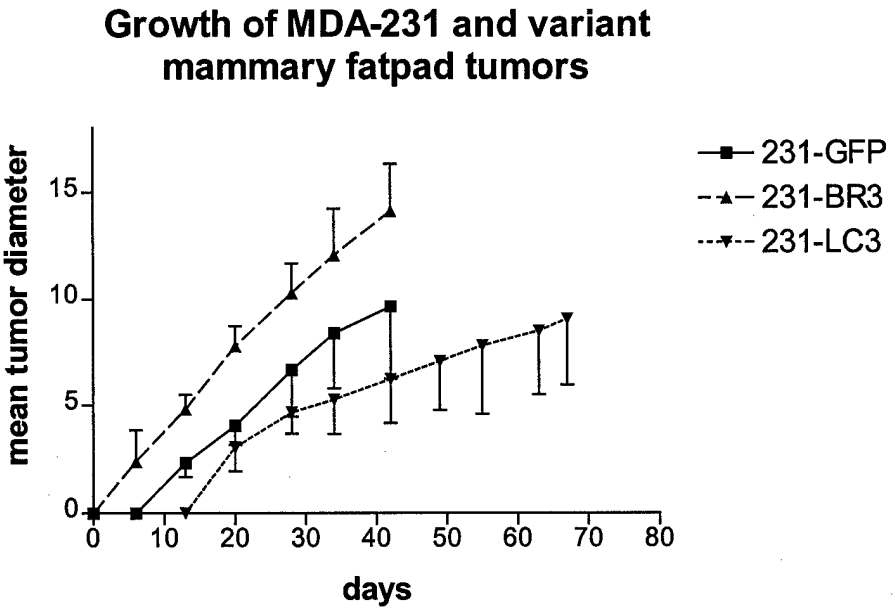
MDA-MB-361 breast cancer cell line
As described for the previous cell lines, the MDA-MB-361 cell line was injected into the carotid artery of female nude mice. The time for development of brain metastases was much longer than the other cell lines. We previously reported a median survival time of > 200 days for mice injected by this route with MDA-MB-361 (9). In the current studies, mice were killed from 150 days after injection, and attempts were made to isolate and culture breast cancer cell from the brain parenchyma. Although initial cultures of epithelial cells were achieved, we were unable to generate new variants of this breast cancer cell line.

Outcome: Variants of two human breast cancer cell lines were generated, with differing ability to colonize and grow in the brain of nude mice following injection into the carotid artery of mice.

ii) Comparison of tumorigenicity and metastatic capability following injection via different routes.
Methods: The breast cancer cells were injected into the mammary fatpad (mfp) of mice, as described previously (8), or into the lateral tail vein to assess the ability to form experimental lung metastases. The mfp tumors were measured at weekly intervals, and removed when mean tumor diameter was 1.5 cm. The mice were killed 4-6 weeks after tumor removal, and examined for distant metastasis. Mice injected in the tail vein were killed 60 days after injection.

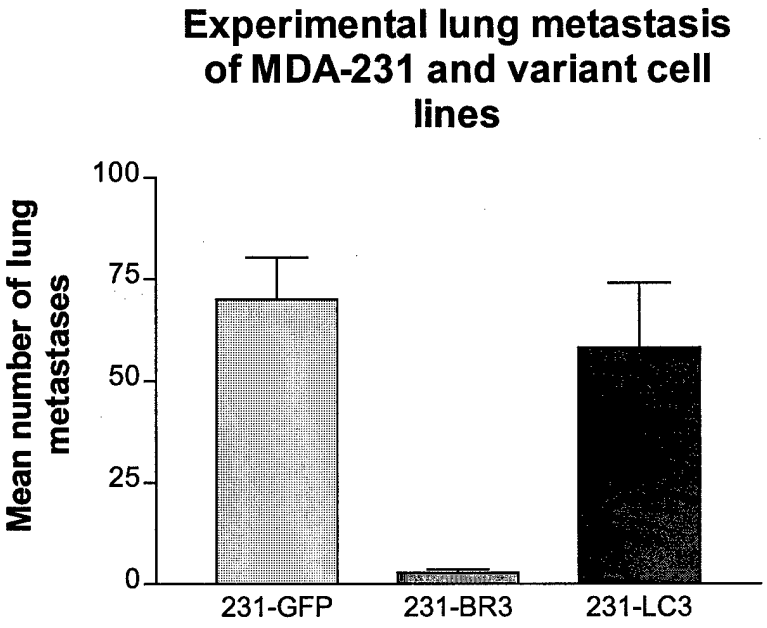
Results:
MDA-MB-231 variants; as shown in Fig. 1, the brain selected variant MDA-231 BR3 showed more rapid growth in the mfp than the orignal cell line and the lung metastasis selected line MDA-231 LC3

Figure 1:



No macroscopic metastases were found in any of the mice in this study. However, there was a difference in the ability of the variants to form lung metastase after i.v. injection of a bolus of 1 x 10^6 cells, as shown in Fig. 2

Figure 2:



This result was found in two replicate studies, with significantly fewer lung metastases in the mice injected with the MDA-231 BR3 cells, compared with either the MDA-231 GFP cells or the lung-selected variant MDA-231 LC3. In other studies in which the latter cell line was compared with the parental population, there was a modest increase in lung colony numbers, but no substantial increase in lung colonizing ability. This may suggest that the non-selected MDA-MB-231 cells are capable of efficiently surviving and growing in the lungs of nude mice.

MDA-MB-435 variants
The variants of this line showed only minor differences in the growth rate in the mammary fatpads of nude mice (Fig 3).

Figure 3:

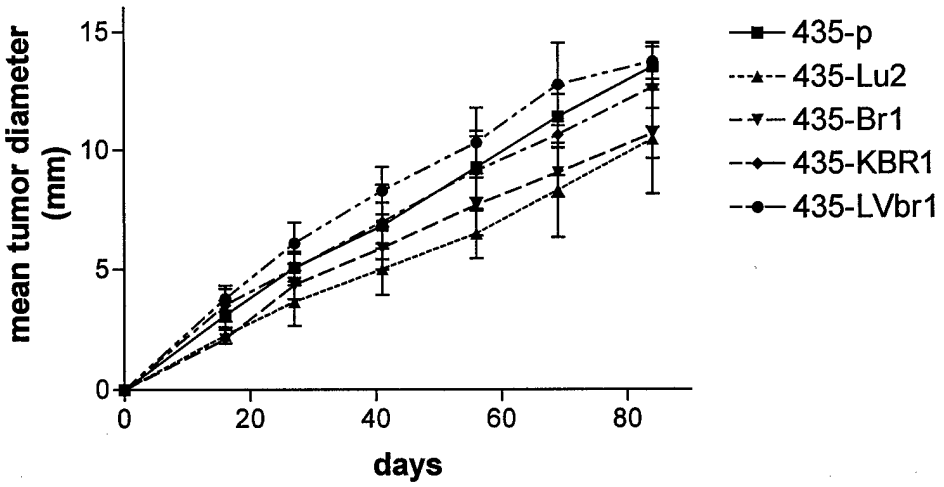


Table 3: Metastasis of MDA-MB-435 variants in mice with mammaryfatpad tumors

Cell line	tumor wt (g)	time to surgery	% macro mets	% micro mets
MDA-MB-435	1.96±0.32	96± 10	60%	70%
MDA-435Lung2	1.88±0.3	112	78%	78%
MDA-435Br1	1.83±0.5	94	11%	33%
MDA-435KBR1	1.9± 0.7	93.7± 21	50%	87.5%
MDA-435LVBR	2.12± 0.5	83.8 ± 12	90%	90%

Table 3 shows a comparison of the incidence of macroscopic and microscopic metastases to the lungs of the mice with the mammary fatpad tumors. Tumors were removed from the mice when at 1.5 cm mean diameter (mean time of surgery shown in table), and the mice killed up to 6 weeks later, or when moribund. The lungs were the predominant organ involved with distant metastases, with occasional metastases found in lymph nodes, chest wall, heart, and two animals with evidence of brain metastases. Thus, although several of the cell lines were isolated by selection from brain metastases, the new variants did not recapitulate the phenotype of metastasis to the brain from tumors in the mammary fatpad. The variants represent the metastatic heterogeneity of the MDA-MB-435 cell line, and show that the nude mouse can be used to isolate isogenic variants with differing metastatic ability. These will be used in further studies of the phenotype of metastatic breast cancer cells, comparing the variants with high lung metastatic ability (MDA-435 LVBR1, MDA-435Lung2) with those of lower metatatic ability (MDA-435Br1, MDA-435KBR1).

iii) Comparisons of phenotypes of the brain-metastasis selected breast cancer variants.

Methods:

ELISA for analysis of secreted angiogenic factors: The supernatants from cultures of the breast cancer cells (2 x 10^5 cells per well) in 12 well culture plates were collected after 72 hours incubation. The levels of VEGF and IL-8 protein in MDA-231 GFP and MDA-231 variant cells were determined by a VEGF and IL-8 ELISA reagents. The antibodies used for the VEGF detection were produced from immunization with recombinant human $VEGF_{165}$ (R&D Systems, Minneapolis, MN). Secreted VEGF and IL-8 in culture supernatant were quantitated by comparing the optical densities of the sample with a standard generated according to the manufacturer's instructions.

Total RNA preparation and Ribonuclease Protection Assays (RPA) for VEGF expression:

Total RNA was isolated from cultures of MDA-231GFP and MDA-231 BR3 using TriReagent (Sigma Chemical Co., St Louis, Mo). Antisense probe templates were used to generate biotinylated riboprobes following transcription by SP6 polymerase and using a BrightStar BIOTINscript nonisotopic transcription kit (Ambion, Inc., Austin, TX). The two probes used were; a 473-bp fragment of VEGF (from plasmid generously provided by Lee M. Ellis, M.D. Anderson Cancer Center) and a h-FGF-1 multi-probe template set (PharMingen International, San Jose, CA). The biotinylated probes were incubated for 16 hours with 35 μg of total cellular RNA at 45°C. The samples were digested with RNAse, and ethanol precipitated before separation on 5% acrylamide/8 M urea gels and transfer to nylon membrane. Protected fragments of RNA were detected using a BrightStar BioDetect kit (Ambion) following the manufacturer's recommended methods, and exposure to Kodak BioMax MR-2 film (Eastman Kodak Co., Rochester, NY). For the assessment of RNA half-life, cells were cultured in the presence of 5 μg/ml Actinomycin D (Sigma Chemical Co.), and RNA collected after 0, 1, 3 and 6 h incubation. The signals for VEGF and 18S RNA at each time point were quantified using densitometry, and the quantities of VEGF normalized to the amount of 18S for each sample.

Preparation of nuclear extracts and immunoblot analyses

Nuclear extracts for analysis of HIF-1α were prepared as described previously (10). Samples (30 μg / lane) were resolved by 7.5% SDS-PAGE, and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The nitrocellulose membranes were blocked with 5% powdered milk in TBST (150mM NaCl, 10mM Tris-HCl, 1% Triton X-

100, pH 8.0) for 16 h at 4°C, washed three times with TBST and incubated for 16 h at 4°C with a 1 : 1000 dilution of anti-HIF-1α antibody (BD Biosciences, San Diego, CA). Following incubation for 1 hour with a 1 : 5000 dilution of sheep anti-mouse-HRP conjugated antibody, the membranes were washed and developed with ECL detection reagents and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech). The same technique was applied for immunoblot analyses using antibodies to the epidermal growth factor receptor (EGFR), HER2/neu, MMP-9, MMP-2, TIMP-1, TIMP-2, E-cadherin and neuropilin, following manufacturers; recommended procedures, on whole cell-lysates.

Results:

Angiogenic factor expression

MDA-MB-231 variants: Variants selected from brain metastases of MDA-231 GFP released significantly more VEGF and IL-8 into culture supernatants that the original cell line (Fig 4a, 4b). VEGF levels in supernates from MDA-231 GFP and MDA-231 BR3 cultured in normoxic or hypoxic (0.5% O_2) for 16 h were compared. The hypoxic exposure did not impair the viability of the cells, as assessed by Trypan blue dye exclusion. VEGF concentrations were elevated in hypoxic cultures of both cell lines, but the increase was more marked in the MDA-231 GFP (~ 3-fold increase) than the MDA-231 BR3 supernatants (1.3-fold increase) (Fig 4 c). Analysis of VEGF-A RNA using RPA gave results consistent with the protein measurements, with higher levels expressed by MDA-231 BR3 cells, in normoxic or hypoxic conditions. VEGF-A was increased in both MDA-231 GFP and MDA-231 BR3 cells when cultured in 0.5% O_2

Figure 4 a: VEGF expression measured by ELISA

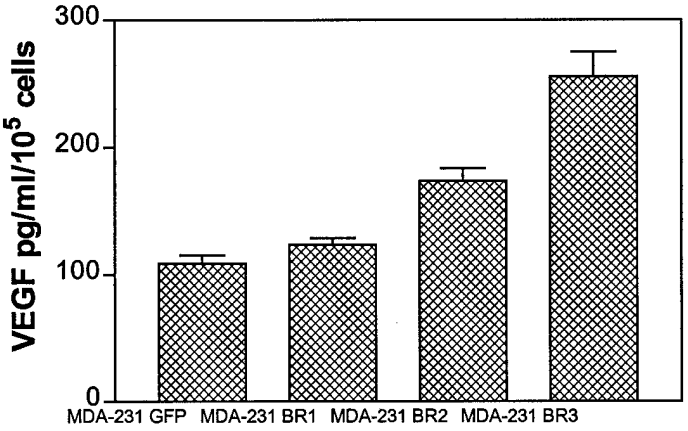


Figure 4 b: IL-8 expression measured by ELISA

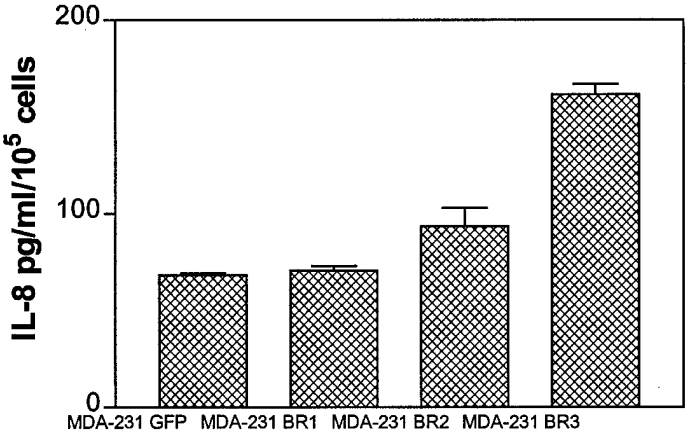
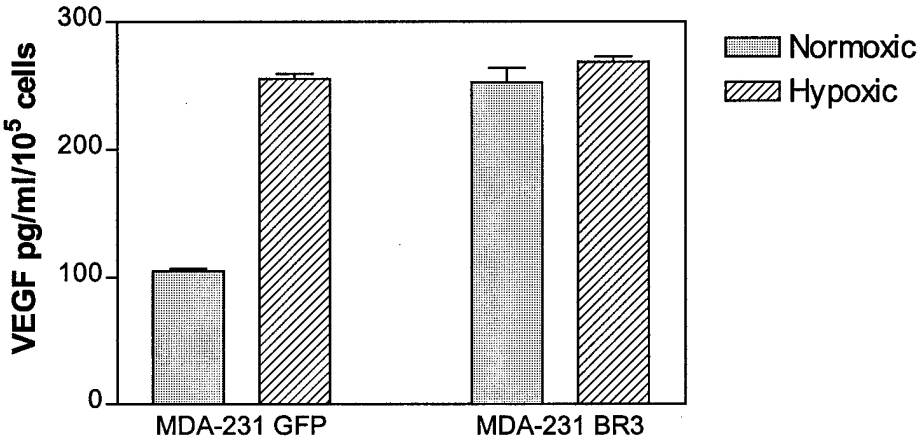


Figure 4 c: Hypoxic regulation of VEGF expression

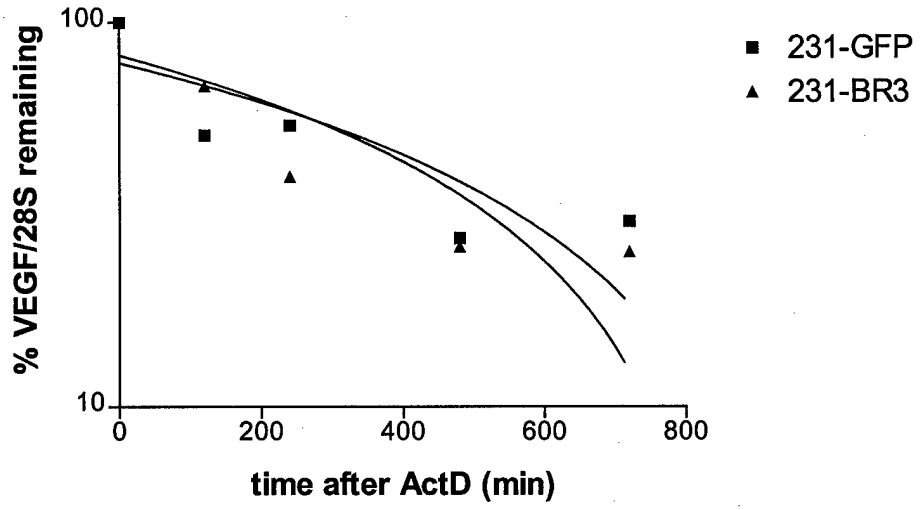


RPA assays for VEGF-A RNA showed similar differences in expression levels to the protein shown in these figures, in normoxic and hypoxic conditions. In contrast to the elevated levels of VEGF-A and IL-8 in the MDA-231 BR3 cells, the MDA-231 LC3 cells do not show increased expression compared with the original cell line, for either factor.

Hypoxia can upregulate the expression of VEGF-A, mediated in part by the transcription factor HIF-1. Protein levels of the HIF1α protein were detected in nuclear extracts prepared from MDA-231 GFP and MDA-231 BR3 cells cultured in normoxic and hypoxic conditions. Hypoxia for 2 – 16 h elevated HIF1α protein in both cell lines, yet there were no difference in regulation in the two variants to account for the higher VEGF-A expression in the brain

metastasis-selected cell line. Half-life determination showed that there was no apparent difference in the stability of the VEGF-A RNA in the two variant cell lines (Fig. 5). Continuing studies are in progress using reporter constructs to evaluate transcriptional regulation of VEGF-A and IL-8 expression in the cell lines, with the goal of identifying the mechanism underlying the constitutively elevated expression of these important angiogenic factors in the brain-metastasis derived variants.

Figure 5: Comparison of half-life of VEGF-A in MDA-231 variants



MDA-MB-435 variants:

VEGF-A and IL-8 were detected in the supernatants from cultures of all of the variants of MDA-MB-435. As shown in Fig. 6a, VEGF-A release showed regulation by hypoxia, with increased levels found in cultures from the stressed condition. In normoxic conditions, the brain metastasis derived lines (MDA-435 Br1, -KBR1 and LVBR) expressed higher amounts of VEGF than the parent, and MDA-435Lung 2 cell variant. When cultured with 1% O_2, the lung metastasis derived cells still had the lowest level of cytokine expression, while the non-selected parent showed 2.6-fold increase. The IL-8 expression by these variants, under normoxic and hypoxic conditions is shown in Fig.6b, illustrating the regulation by hypoxia, and also that, in contrast to the brain-metastasis selected variant of MDA-MB-231, the selected variants of MDA-MB-435 do not show increased expression of IL-8. The two sets of cell lines are being used in on-going studies of the regulation of the angiogenic factor expression, in cell lines with well-characterized tumorigenic and metastatic capabilities. One conclusion from the data is that the breast cancer cells that were isolated from lung metastase,and have high potential for growth in the lungs (from i.v. injection or spontaneous metastasis from mfp tumors) have the lowest levels of angiogenic factor expression, compared with the original lines, or those isolated from brain metastases.

Figure 6a: VEGF-A expression in MDA-MB-435 variants

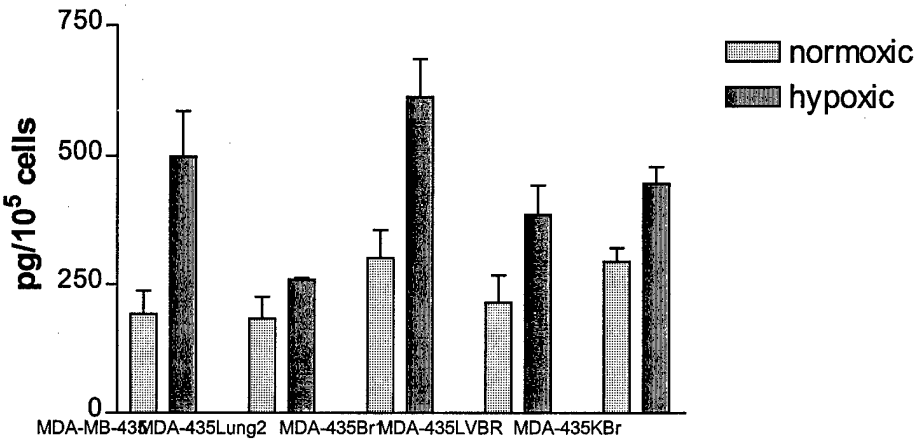
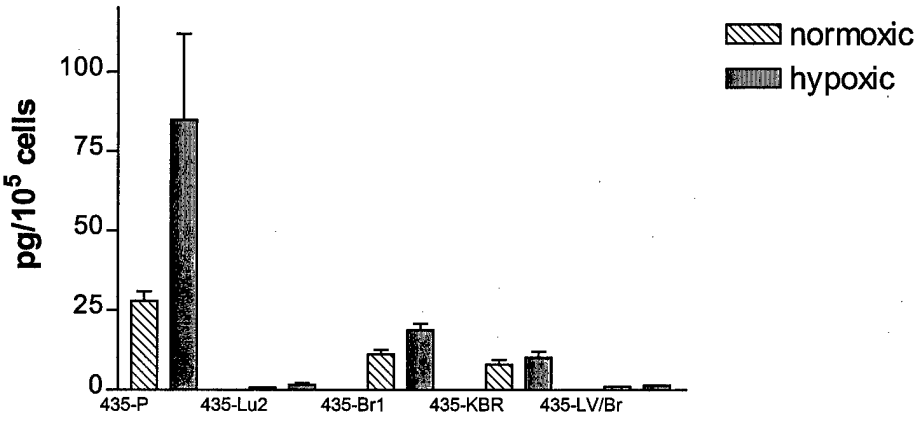


Figure 6b: IL-8 expression in MDA-MB-435 variants



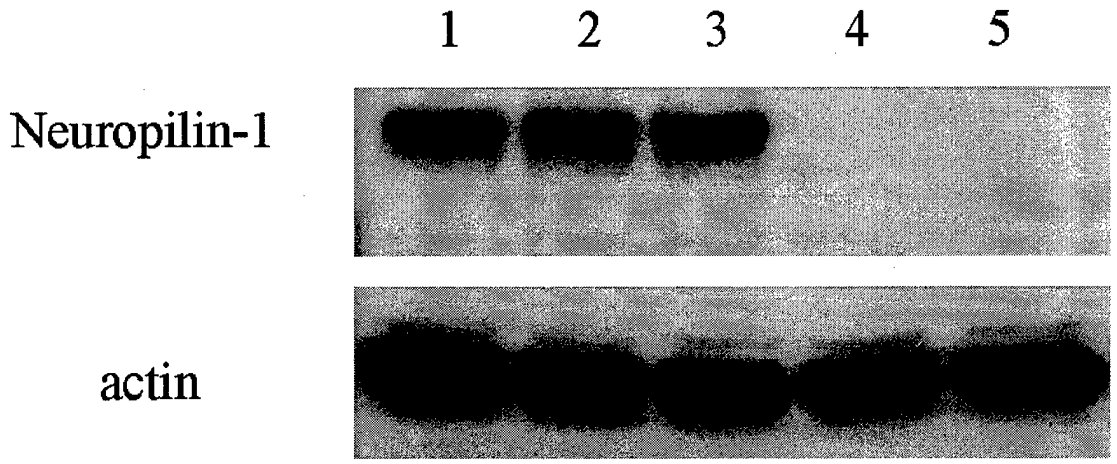


Figure 7: Immunoblot analysis of neuropilin-1 expression in breast cancer cells: Lane 1, MDA-231 GFP; lane 2, MDA-231 BR3; lane 3, MDA-231 LC3; lane 4, MDA-MB-435; lane 5, MDA-MB-468.

Some recent reports in the literature have suggested an autocrine role for VEGF-A in promoting the invasion, motility and survival of breast cancer cells (11) through mechanisms that remain to be elucidated. The potential role for the neuropilin receptor has been suggested, as this is a co-receptor for the 165-isoform of VEGF-A, and is abundantly expressed in the MDA-MB-231 cell line (12,13). Protein levels of neuropilin-1 were compared in various breast cancer cell lines, but no difference was found in the variants from different metastatic sites (Fig. 7). Ongoing studies with the cell lines are in progress to determine whether stimulation by VEGF-A can give the selected cells an advantage in a metastatic site, through stimulation of receptors that may associate with neuropilin (14). Measurement of phosphorylation of the VEGF-receptors VEGFR1 and VEGFR2 in MDA-MD-231 cells stimulated with VEGF failed to give evidence of autocrine stimulation via these receptors (data not shown).

Other phenotypic comparisons:

The expression of additional genes and/or proteins was compared in the different variants of the breast cancer cell lines that were isolated from brain metastases. No consistent or reproducible differences were noted in; E-cadherin, EGFR, HER2/neu, MMP-2, MMP-9 (immunoblots and by zymography of cell supernatants), TIMP-1 or TIMP-2. Analyses using RPA for IL-6 and IL6Rα using a multi-probe template kit showed no or barely detectable expression in any group. This study had been proposed following a previous report on the MDA-435 variants showing expression of IL-6 and IL-6R with a potential autocrine mechanism, as playing a role in survival of brain metastases (15). Ongoing studies, with improved detection methods will further explore this issue.

Discussion:

The goal of this study was to develop an experimental model of human breast cancer brain metastasis, to have an *in vivo* system for studying the biology and therapy of this most fatal aspect of the disease. Immunodeficient animals have been shown to be suitable for models of

metastatic disease, although there are relatively few human breast cancer cell line that can grow and metastasize in these animals (16). To facilitate the visualization of metastases, the human breast cancer cell line MDA-MB-231 was transfected with the EGFP gene, a variant of the wild-type GFP optimized for brighter fluorescence and high expression in mammalian cells (17). Similar approaches have been used by other investigators to localize metastases of a variety of human cancers (18-20). The fluorescence did aid in locating and recovering the breast cancer cells, in the first step of the selection process. Furthermore, we were able to follow the growth of intracranial lesions by examining live animals under UV illumination. However, we found that expression of the fluorescent marker was progressively lost through repeated cycles of injection and recovery. One explanation may be that the gene was expressed episomally, or was not stably integrated, and during the periods of *in vivo* growth in the absence of G418, there was no selective pressure to retain expression. Migliaccio *et al.*(21) reported that loss of GFP expression from K562 cells was due in part to loss of the gene, through unstable integration, and inpart to trancriptional silencing of the gene. There are also reports that GFP is cytotoxic for some cell types (22), although we did not observe any differences in proliferation of the MDA-231 GFP cells, which were as capable of forming tumors as the non-transfected MDA-MB-231 cells.

The cells recovered from the brain metastases after three cycles of injection and recovery of the metastases showed an increased ability to grow and survive in the brain parenchyma. This property was not shared by a variant line of MDA-MB-231 isolated from experimental lung metastases, suggesting that MDA-231 BR3 are a unique sub-population of cells derived from a heterogeneous breast cancer cell line. The MDA-231 BR3 cells have significantly reduced ability to form lung metastases, compared with the lung-selected cells when injected into the tail vein of nude mice. These two distinct variants of the same breast cancer line will be useful for distinguishing phenotypic differences important for metastatic colonization of different organs.

One factor that was differentially expressed by the brain-selected cells was the angiogenic and permeability inducing factor VEGF-A. This factor has been reported to play an important role in the development and growth of brain tumors and brain metastases (23-25). Our finding of increased VEGF-A expression by the brain metastasis-selected cells support these observations. One consequence of the increased VEGF-A expression was to promote the development of blood vessels in the brain metastases. Klusters *et al.* (26) showed that melanoma cells with low VEGF-A expression (*in vitro*) were capable of forming brain metastases. However, the enforced expression of $VEGF_{165}$ in the cells both accelerated and altered the pattern of growth of the brain metastases, and increased vascular permeability. The increased $VEGF_{165}$ lead to co-option of existing blood vessels, as opposed to branching or sprouting angiogenesis. Fidler *et al.* showed similar examples of vascular remodelling and dilation of vessels in brain metastases of different human cancers in nude mice (27). In the brain metastases of the breast cancer cells we found both increased vessel density and luminal enlargement. Breast cancer cells express a variety of angiogenic factors (28-30), and the increased release of IL-8 by the brain metastasis selected cells may be a contributing factor. IL-8 has been shown to be a mediator of angiogenesis and capable of inducing proliferation and sprouting by endothelial cells (31).

A recent report has described the $VEGF\text{-}_{165}$ isoform as an autocrine factor promoting the survival of MDA-MB-231 breast cancer cells, mediated by neuropilin-1 expression(13). Neuropilin-1 is a cell surface glycoprotein initially associated with axonal guidance of embryonic neurons, but also found to act as a coreceptor that can enhance the binding of $VEGF_{165}$ to the KDR/VEGFR-2. Neuropilin-1 is expressed by non-neuronal cells, including

endothelial cells and also some human cancer cell lines (12). The report of Bachelder et al suggested that neuropilin-1 may promote the survival of breast cancer cells in the presence of $VEGF_{165}$, by a mechanism that involves the PI3K pathway and activation of Akt. Preliminary studies comparing neuropilin-1 expression and Akt activation of the brain metastasis-derived variants with those of the original MDA-MB-231 cells and lung metastasis-derived cells did not reveal any clear differences that would implicate these molecules in the observed differences in metastastic properties.

A variety of stimuli have been reported to regulate the expression of VEGF-A in normal and malignant cells, including oncogenes, growth factors, cytokines and environmental stressors such as hypoxia and acidic pH (32-34). Hypoxia may impose a selective pressure resulting in the survival of the most aggressive and metastatic cells, which are the "fittest" for surviving in the environment. In hypoxic conditions, cells may adapt by increasing expression of genes involved in erythropoiesis and glycolysis in addition to angiogenic factors, including VEGF-A and IL-8 (35) The hypoxia inducible factor-1 (HIF-1), a heterodimeric transcription factor consisting of an inducible α and constitutive β subunits is a key factor in the upregulation of a VEGF-A (34,36). A balance between the angiogenic function of HIF proteins and inhibitory effects on tumor growth may be essential for the survival of cells in different environments (37). Increased levels of HIF-1_α are associated with increased VEGF and proliferation, and with increased pathologic stage in breast cancer specimens (38). In this study, the abundance and induction of HIF-1_α was not found to be different in the brain metastasis-selected cells. In normoxic conditions, higher levels of VEGF-A protein and RNA were maintained in these cells, suggesting that another mechanism(s) is responsible for increased angiogenic factor expression in the metastasis-selected cells. Signaling through the erbB family of tyrosine kinase receptors, and mitogen-activated protein kinase pathways have been implicated in regulation of angiogenic factors in different types of cancer cells (39-42). Studies are underway to investigate the contribution of growth factor- or cytokine-induced signaling pathways to the regulation of VEGF and IL-8 in the brain metastasis-selected breast cancer cells. As noted above, the *in vivo* selection process described in this report appeared to be organ specific, i.e. the cells that were isolated from lung metastases did not have increased ability to form brain metastases. The lung metastasis selected cells did not express higher levels of VEGF-A or IL-8, suggesting that whatever the mechanism underlying the increased expression of these factors, it was associated with the ability to grow in the microenvironment of the brain, but not the lungs. A similar lack of increased metastatic potential to the lungs was reported for human renal cell cancer cells transfected with VEGF (43). Metastasis-derived variants of another human breast cancer cell line, MDA-MB-435, have shown that the brain derived variants also express more VEGF-A than those isolated from lung metastases, supporting the observation in the MDA-MB-231 model.

In summary, we have developed a model for breast cancer brain metastasis, which was used to isolate variants with enhanced ability to form brain metastases. Two attributes of the brain-metastasizing cells were increased VEGF-A and IL-8 expression. This *in vivo* model and the selected variants can be used for further analyses of the phenotypes characterizing breast cancer cells that form brain metastases.

Key Research Accomplishments:

- Establishment of variants of human breast cancer cell lines from experimental brain metastases in nude mice, following intra-carotid artery injection of the cells.

- Demonstration that the brain metastasis-selected cell lines have enhanced ability to produce brain metastases in nude mice, compared with the original cell lines.
- Demonstration that the brain metastasis-selected cells have elevated expression of the angiogenic and permeability inducing factor VEGF-A, and of the angiogenic cytokine IL-8.

Reportable outcomes:

- Abstract in the Proceeding of the American Association for Cancer Research and presentation in a poster session at the Annual Meeting of the AACR, March 2001, "A Model of Human Breast Cancer Brain Metastasis", L.S. Kim and J.E. Price. (Proc. AACR, 41:A4913, 2001.)
- Abstract in the Proceeding of the American Association for Cancer Research and presentation in a poster session at the Annual Meeting of the AACR, April, 2002, Vascular endothelial growth factor promotes the growth of breast cancer brain metastases in nude mice. Kim, LS, Lu W, Price, JE. (Proc AACR, 43, A141, 2002).
- Abstract presented at "Era of Hope" Department of Defense Breast Cancer Research Program Meeting , September 2002. VEGF promotes the growth of breast cancer brain metastases in nude mice. Price, J.E., Kim, L.S., Lu, W., Cabioglu, N., Kiriakova, G. and Lev, D.C. (poster # p25-14).
- Kim, L.S., Huang, S., Lu, W. and J. E. Price;Vascular endothelial growth factor expression promotes the growth of breast cancer brain metastases in nude mice. Manuscript in preparation for submission to Cancer Research.
- Phenotypically distinct breast cancer variants available for further analyses on brain-metastatic ability, and on the regulation of key angiogenic factors.

List of personnel receiving pay from the research effort:

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Conclusions:

The hypothesis being tested in this project is that breast cancer cells in brain metastases represent specialized populations of cells endowed with the ability to survive and grow in the brain. Our results using a nude mouse model of experimental brain metastases to isolate selected populations suggest that these do differ from the original cell lines. The brain metastasis-derived cells have enhanced potential for metastasis in the brain. In addition, these selected cells show higher expression of VEGF, a factor that promotes angiogenesis and the permeability of blood vessels, and also of IL-8 another angiogenic factor. Significant points of these results are: 1) The selected cell lines can be used to identify phenotypes that may be unique to, or over-expressed in breast cancer cells with the ability to survive and grow in the brain. 2) The breast cancer cell lines injected into the carotid artery of nude mice is a reliable model of experimental brain metastases, which can be used for future studies testing novel approaches of therapy.

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Vascular endothelial growth factor promotes the growth of breast cancer brain metastases in nude mice,

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Breast cancer is the second most common cause of brain metastases, after lung cancer. The survival after detection of brain metastases can be short, a matter of weeks, and the therapy currently available only offers the hope of surviving one year to 20% of patients. However, relatively little is known about how these metastases form, and what phenotypes are characteristic of brain-metastasizing breast cancer cells Without this information, the rational design of new therapies to prevent or control the growth of brain metastases is impossible. MDA-MB 231 human breast cancer cells which had been transfected with a gene encoding green fluorescent protein (MDA-231 GFP) were injected into the internal carotid artery of nude mice, using the technique developed by Schackert et al in this department. The injection of $1x10^5$ cells resulted in experimental brain metastases in 82.4% of mice, by 90 days. The expression of GFP was used as a marker to aid in the dissection of metastatic cells from the brain parenchyma, which were expanded in tissue culture. Three rounds of injection and recovery of tissue resulted in three variants, MDA-231 BR1, -231 BR2, and -231 BR3. Mice injected with the MDA-231-BR3 cells developed a higher incidence of experimental brain metastases (100%), and were moribund earlier than mice injected with the original MDA-231 GFP cells (40 *vs* 59.2 days). Significantly more endothelial vessels/unit area (microvessel density, MVD) were counted in sections of brain metastases of the MDA-231 BR3 cells, compared with the MVD scored in metastases of MDA-231 GFP cells ($p=0.002$). Vascular endothelial growth factor (VEGF) is a key angiogenic factor in pathological situations that involve neovascularization as well as enhanced vascular permeability, with a high correlation between VEGF expression and peritumoral edema and vascularity of brain tumors. The brain metastasis-selected cells expressed more VEGF than the MDA-231 GFP cells, under normoxic ($p<0.001$) and hypoxic (0.5% O_2) conditions ($p=0.004$). Mice injected with MDA-231 BR3 cells were treated orally with an inhibitor of the VEGF receptor tyrosine kinase (PTK787). The MVD recorded in brain metastasis samples was significantly lower in mice treated with the inhibitor ($p=0.03$). The metastatic tumor burden was also reduced, and survival time increased ($p=0.08$), although the difference was not statistically significant. We conclude that elevated VEGF expression contributes to the ability of breast cancer cells to form brain metastases. Targeting endothelial cells with a VEGF-receptor specific tyrosine kinase inhibitor reduced angiogenesis and restricted the growth of the brain metastases.(Supported in part by DAMD17-00-1-0315)

VEGF PROMOTES THE GROWTH OF BREAST CANCER BRAIN METASTASES IN NUDE MICE

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Breast cancer is the second most common cause of brain metastases, after lung cancer. Relatively little is known about how the metastases form, and what phenotypes are characteristic of brain-metastasizing breast cancer cells. MDA-MB-231 human breast cancer cells were transfected with a gene encoding green fluorescent protein (MDA-231-GFP) and injected into the internal carotid artery of nude mice. The injection of 100,000 cells resulted in experimental brain metastases in 75% of mice, by 62 days. The expression of GFP was used as a marker to aid in the dissection of metastastic cells from the brain parenchyma, which were expanded in tissue culture, and then injected into the carotid artery. Three rounds of injection and recovery of tissue resulted in a variant termed 231-BR3. Mice injected with the 231-BR3 cells developed more experimental brain metastases, in a significantly shorter time than in mice injected with the original MDA-231-GFP cells. Significantly more endothelial vessels/unit area (microvessel density, MVD) were counted in sections of brain metastases of the 231-BR3 cells, compared with the MVD scored in metastases of MDA-231-GFP cells. Vascular endothelial growth factor (VEGF-A) is a key angiogenic factor in pathological situations that involve neovascularization as well as enhanced vascular permeability, with a high correlation between VEGF expression and peritumoral edema and vascularity of brain tumors. The brain metastasis-selected cells expressed significantly more VEGF-A than the original MDA-231-GFP cells when cultured in normoxic conditions. Mice injected with 231-BR3 cells were treated orally with an inhibitor of VEGF receptor tyrosine kinases (PTK787). The MVD recorded in brain metastasis samples was significantly lower in mice treated with the inhibitor. The metastatic brain tumor burden was also significantly reduced, and survival time increased, although the difference was not statistically significant. We conclude that elevated VEGF-A expression contributes to the ability of breast cancer cells to survive and grow as brain metastases. Targeting endothelial cells with a VEGF-receptor specific tyrosine kinase inhibitor reduced angiogenesis and restricted the growth of the brain metastases.

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