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Expression of MMP2, MMP9 and MMP3 in breast cancer brain metastasis in a rat model

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Abstract

In order to study the expression of MMP2, MMP3 and MMP9 in breast cancer brain metastasis, we used a syngeneic rat model of distant metastasis of ENU1564, a carcinogen-induced mammary adenocarcinoma cell line. At six weeks post inoculation we observed development of micro-metastasis in the brain. Immunohistochemistry and Western blotting analyses showed that MMP-2, -3 and -9 proteins expressions are significantly higher in neoplastic brain tissue compared to normal brain tissue. These results were confirmed by RT-PCR. *In situ* zymography revealed gelatinase activity within the brain metastasis. Gel zymography showed increase in MMP2 and MMP3 activity in brain metastasis. Furthermore, we were able to significantly decrease the development of breast cancer brain metastasis in animals by treatment with PD 166793, a selective synthetic MMP inhibitor. In addition, PD 166793 decreased the *in vitro* invasive cell behavior of ENU1546. Together our results suggest that MMP-2, -3 and -9 may be involved in the process of metastasis of breast cancer to the brain.

Abbreviations: BC – breast cancer; BD-IV – BerlinDurkrey; IHC – immunohistochemistry; MMP – matrix metalloproteinases; PBS – phosphate buffered saline; RT-PCR – reverse transcriptase polymerase chain reaction; TIMP – tissue inhibitors of metalloproteinases; WB – Western blotting

Introduction

The metastatic process of breast cancer (BC) has been the subject of intense scrutiny. The brain is one of the most common organs affected in the spread of BC that ultimately results in fatal development of the disease. Brain metastasis is an increasingly common complication in breast cancer patients [1]. Approximately 15–30% of breast cancer patients develop brain metastasis [2]. The exact role of the brain environment to the development of the metastatic process has yet to be clarified. A suitable specific environment is important to the development of tumor cells [3]. Many theories have been developed to study and understand metastatic behavior. Factors such as neoplastic cell molecular and genetic characteristics [4] and biological environment are thought to be determinant in the metastatic process.

Matrix metalloproteinases (MMPs) are a broad family of zinc-dependent proteinases that play a key role in extracellular matrix degradation, implicated in numerous pathogenic processes [5]. Tumor cells are thought to secrete these matrix-degrading enzymes and/or induce host cells to elaborate them [6]. MMPs have been associated with pathology within the central nervous system, in neoplastic disease, such as glioma and melanoma brain metastasis [7–9]; and non-neoplastic disease, such as trauma, ischemia and immune-mediated disease [10]. MMPs have also been extensively studied in the context of breast cancer prognosis. Most studies to date have been performed in human tissue collected from patients diagnosed with breast cancer or in breast cancer cell lines. Most reports suggest that increased expression of MMP-2, -3 and -9 proteins correlates with worse prognosis [11–14].

In this context, MMP-2, -3 and -9 are thought to play an important role in breast cancer invasion, metastasis and tumor angiogenesis [15]. MMP2 over-expression and activation have been associated with the invasive potential of human tumors. Active MMP2 and MMP9 were detected more frequently in malignant than benign breast carcinomas. MMP3 was observed in highly invasive breast cancer cell lines [15]. Some reports, however, do not correlate MMP2 and MMP9 immunohistochemical staining with the presence of metastases at the time of diagnosis or with disease outcome [15]. Absence of distinct positive immunoreaction for

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MMP-2, -3 and -9 has been observed in both invasive and non-invasive tumor cells without apparent differences in the staining intensity [15]. In this regard, additional *in vivo* studies that characterize MMP expression in metastasis are needed.

Few studies are available on the expression of MMPs within breast cancer metastasis [19, 20, 21, 22] and, to our knowledge, no one has characterized the expression and activity of these molecules in breast-to-brain cancer metastasis. It is important to determine if MMPs have different effects/roles in the development of metastasis in different organs because this may help to understand why BC cells metastasize to preferential organs. Here we focused on the metastatic process of BC to the brain in a rodent model. This understanding may be utilized in the development of the current therapeutic approach to metastatic cancer.

The metastatic rodent models for breast cancer described to date study mostly nodal, pulmonary and bone metastasis [21, 22]. In most, brain metastases occur as a non-predictable event and only sporadically. The use of animal models to study *in vivo* tumor progression and metastatic behavior is important to understand the mechanism of metastasis development. It is also an important tool for pharmacological evaluation of cancer therapy. Several synthetic MMP inhibitors are under investigation for clinical trials in patients with cancer. They are thought to inhibit both primary tumor invasion and metastasis [23, 24].

We here used an *in vivo* model that consistently produces brain metastasis [3] to evaluate the expression and activity of MMP-2, -3 and -9 in metastatic foci of BC in the brain. We found the levels of MMP-2, -3 and -9 mRNA and protein in BC brain metastasis are higher than the ones of normal brain tissue. Additionally, the activities of MMP2 and MMP3 in metastatic foci are higher than in non-affected brains. We also demonstrated that MMP inhibition by a specific MMP inhibitor decreases *in vitro* and *in vivo* cell invasive and metastatic behavior. To our knowledge, this is the first report of characterization of these molecules in brain metastasis of breast cancer in a rat model.

Materials and methods

Tumor cell line

The ENU1564 tumor cell line used in this study was developed in our laboratory and originated from an *N*-ethyl-*N* nitrosourea-induced mammary adenocarcinoma in a female Berlin–Druckrey IV (BD-IV) rat. This cell line is highly metastatic to brain and bone tissues [3].

Rat inoculation

Forty-day-old BD-IV rats were used. The animals were obtained from a colony maintained at Texas A&M University in accordance with institutional animal care guide-lines. The syngeneic animals were inoculated with $1{\times}10^4$ tumor cells in the left ventricle. Inoculation was performed on animals under Ketamine (87 mg/kg, intramuscular injection) anesthesia. The animals were euthanatized using Pentobarbital (150 mg/kg, intraperitoneal injection) when showing clinical signs of discomfort such as decreased response to stimulus. Complete necropsies were performed and tissues were sampled for histology evaluation.

Tumor collection

Brain samples were collected immediately after animal's death and placed on powdered dry ice until completely frozen. Samples were then kept at −80 °C. The samples from metastatic tissue were collected from frozen brain sections. The half of the brain that was frozen in powdered dry ice was sectioned using a cryostat in 12 μm sections and placed on gelatin-covered slides. Every fifth slide was stained with thionin stain prepared from a stock 1.3% thionin (wt/vol in H_2O). Metastatic foci were identified under light microscopy. This information was then used to dissect the metastatic tumor, on frozen brain tissue sections. Immediately after dissection another 12 μm section was stained in order to confirm the accuracy of the dissection.

Immunohistochemistry

Five-micron (5 μm) paraffin-embedded sections and 12 μm frozen sections were used. Deparaffinization, rehydration and antigen-retrieval were done by immersion of slides in DECLERE® (Cell Marque, Hot Springs, AR) commercially available buffer in moist heat (pressure cooker) for 15 min. Potential non-specific binding sites were blocked with 5% normal goat or rabbit serum in PBS. After blocking, the sections were incubated with primary antibodies purchased from Santa Cruz (Santa Cruz Biotech, Santa Cruz, CA), in dilutions of 1:200 for MMP2, and 1:25 for MMP3 and -9. After three five-minute washes in PBS, the sections were then incubated with either biotin-conjugated anti-rabbit or anti-goat IgG (Vector Laboratories, Burlingame, CA). A Vector-ABC streptavidin-peroxidase kit with a benzidine substrate was used for color development. Counter-staining was done with diluted hematoxilin. Sections that were not incubated with primary antibody served as negative control.

Western blotting

After microscopic dissection of frozen brain specimens, the tissue was homogenized in lysis buffer in a 1V: 10V dilution (50 mM Tris–HCl, pH 8.0, 300 mM NaCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, pH 8.0, and 0.1% SDS), supplemented with a mixture of protease inhibitors. Samples were run on a 9–12% SDS polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated one hour in

blocking buffer (20 mM Tris–HCl buffered saline containing 5% nonfat milk powder and 0.1% Tween 20). Blots were incubated at 4 °C overnight with anti-MMP2 (1:2000), MMP3 (1:500) and MMP9 (1:1000) (all antibodies from Santa Cruz Bitotech, Santa Cruz, CA), washed extensively and then incubated for 1h with a 1:5000 dilution of secondary anti-rabbit or anti-goat antibody. After additional washes, the blots were incubated with chemiluminescent substrate, according to the directions in the kit (SuperSignal® West Pico, Pierce, Rockford, IL).

Reverse transcription-PCR (RT-PCR)

We extracted total RNA from frozen specimens using Trizol reagent (Invitrogen, Gaithersburg, MD). First-strand cDNA was primed with oligo (dT), synthesized using RETROscript kit (Ambion, Austin, TX), and served as a template reverse transcription-PCR (RT-PCR). PCR primers were as follow:

- MMP2 primers (forward, GACCTTGACCAGAA CACCATCG; reverse, 5′-GCTGTATTCCCGACC GTTGAAC-3′);
- MMP3 primers (forward, 5′-CCTCTATGGACCTC CCACAGAATC-3′; reverse, 5′-GTGCCAATGCCT GGAAAGTTC-3′);
- MMP9 primers (forward, 5′-CCCCACTTACTTTG GAAACGC-3′; reverse, 5′-ACCCACGACGATAC AGATGCTG-3′).

Rat MMP-2, -3 and -9 cDNA sequences were obtained from the http://www.ncbi.nlm.nih.gov web site and MacVector® (version 7.0, Accelerys, San Diego, CA) software was used to design the primers. To demonstrate the integrity of the RNA samples used in the RT-PCR reactions, parallel amplifications with oligonucleotide primers for mouse β-actin cDNA (forward, 5′-ATGTACGTAAGCCAGGC-3′; reverse, 5′-AAGG AACTGGAAAAGAGC-3′) were performed.

Fluorescent-labeled substrate-based in situ *zymography*

Zymography is the choice method for evaluation of MMP2 and -9 activities [25, 26]. Because of the small size of the metastatic foci and the paucity of material collected, we opted for an *in situ* method [27]. The substrate was prepared by dissolving 0.1% fluorescent-labeled substrate (Molecular Probes, Eugene, OR) in gelatin according to manufacturer's instructions. Fifty microliter of substrate gel solution were pipetted onto the slide and evenly distributed on the slide surface. Frozen sections (10–15 μm) were placed on the coated slide. The slides were incubated in a moist box with Tris-buffer (pH 7.4) and the box was wrapped in foil to protect from light and placed at 37 °C. Results of *in situ* zymography were evaluated after 48 h of incubation. The samples were examined microscopically under UV light. Control samples were stained with thionin stain.

Gel zymography

Gelatin zymography was performed as described previously [28]. In brief, samples were electrophoresed on 10% (wt/vol) polyacrylamide gels containing 0.1% (wt/vol) gelatin and Ready Gel Zymogram Gel 12%, casein (Biorad, Hercules, CA). After electrophoresis, the gels were washed twice for 30 min each in 2.5% (vol/vol) Triton X-100 at room temperature and then incubated in substrate reaction buffer (50 mM Tris–HCl, 5 mM CaCl 2, 0.02% (wt/vol) NaN_3, pH 8.0) for 8–18 h at 37 °C with gentle shaking. The gels were then stained with Coomassie Blue R250 in 10% (vol/vol) acetic acid and 30% (vol/vol) methanol for 1–2 h and destained briefly in the same solution without dye. Proteolytic activities were detected by clear bands indicating the lysis of the substrate. Quantification of band density was carried out using Flour S MultiImager® technology from Biorad (Hercules, CA).

In vitro *and* in vivo *MMP inhibition assay*

PD 166793 (S-2-4′-bromobiphenil-4-sulfonylamino-3 methyl-butyric acid) was kindly provided by Dr J.T. Peterson (Cancer Molecular Sciences, Pfizer Global Research & Development, Ann Arbor, MI).

In vitro invasiveness was evaluated using the method previously described [29]. Boyden chambers were used for the invasion assay. Briefly, each Boyden chamber (Becton Dickinson Biosciences, USA) consists of a BD Falcon TC Companion Plate with Falcon Cell Culture inserts containing an 8μm pore PET membrane with a thin layer of Matrigel basement membrane matrix. First, the interior of the inserts was rehydrated for 2 h with warm (37 °C) bicarbonate based culture medium. The upper chambers were filled with 0.5 ml of cell suspension (1.25×10^5cells/ml) in Dulbecco's Modified Eagle Medium (Invitrogen Corporation, Carlsbad, CA). The same media including 10% fetal bovine serum was placed in the lower chambers as chemoattractant. Experiments using 10 μM PD166793 MMP inhibitor, in both chambers media, were conducted in parallel [30]. The chambers were incubated at 37 °C in a humidified atmosphere of 5% carbon dioxide for 24 h, non-migrating ENU1564 cells on the upper surface of the inserts were removed by wiping with a cotton swab, and the migrating cells on the lower surface were fixed and stained with Insta Stain 3 Step (S&K Reagent, Inc., Denver, CO). The invasive potential is quantified by counting the total number of cells on the lower surface of the inserts under a light microscope at 400× magnification. Three random visual fields were counted for each assay. Triplet was carried out in each invasion experiment.

To evaluate *in vivo* inhibition of MMP evaluation we conducted the following experiment. Ten BD-IV rats were inoculated with 1×10^4 ENU1564 via left ventricle. Five animals were selected randomly for the control group and for the drug treatment group. Animals from

the control group were daily inoculated via peritoneum with vehicle only. The five animals on the treatment group were treated daily with intraperitoneal injection of 5 mg/kg of PD 166793 as described previously [31]. The study had the duration of 24 days. All animals were sacrificed at the end of the study. Six sections of brain per animal were evaluated histologically, and foci of breast cancer brain metastasis were counted.

Statistical analysis

Paired Student's *t*-tests were performed with densitometry values obtained from Western blotting-autoradiographs analysis and by photograph zymography results using Flour S MultiImager® technology from Biorad (Hercules, CA). Differences were considered statistically significant when P was ≤0.05.

Results

Histological evaluation of brain metastatic foci

Six weeks after inoculation the animals began to show neurological signs, such as depression and/or head tilt, and were euthanatized. No macroscopic abnormalities were observed in the central nervous system upon necropsy evaluation. Histological evaluation of the brain revealed intra-cerebral metastatic neoplasia. Morphologically, small clusters of epithelial neoplastic cells resembling the cultured cell line could be observed. The neoplastic foci were scattered randomly throughout the brain, affecting more frequently the caudal aspect of the parietal lobes and cerebellum (Figure 1). The neoplastic foci varied in size from five to two hundred-micron. The estimated number of tumor foci per animal brain varied from five to fifty (Table 1). The tumor foci had absence of fibrous stroma, inflammatory cells or necrosis. Mild to marked astrocyte reactivity was observed around the neoplastic cells. Metastatic tumors in other organs, such as bone, lung and pancreas, were also observed (Table 2).

Table 1. Morphological characterization of brain metastatic foci.

	Tumor diameter (μm)	Number of tumor foci per brain section
Brain metastasis	43.3 ± 34.92	24 ± 15.44

Values are mean ± standard deviation.

Immunohistochemistry for MMP-2, -3 and -9 proteins in brain metastatic foci

Immunohistochemistry (IHC) was performed in order to characterize MMP-protein expression within the metastatic brain foci. Immunohistochemical staining for MMP2 showed immunolabeling with moderate intensity in the cytoplasm of neoplastic cells within the brain metastatic foci (Figures 2a and b). In addition astrocytes, microglial cells and endothelial cells also had mild staining. MMP3 staining was observed with strong intensity in the cytoplasm of neoplastic cells (Figures 2g and h). Mild staining was also observed in astrocytes. MMP9 staining was weak in the neoplastic cell cytoplasm and faint staining of glial cells was also observed (Figures 2d and e). Similar results were observed in neoplastic epithelial cells in the lung metastatic foci (data not shown).

Increased expression of MMP-2, -3 and -9 in brain metastatic foci

To confirm IHC results on MMP-2, -3 and -9 protein expression and in order to semi-quantify protein

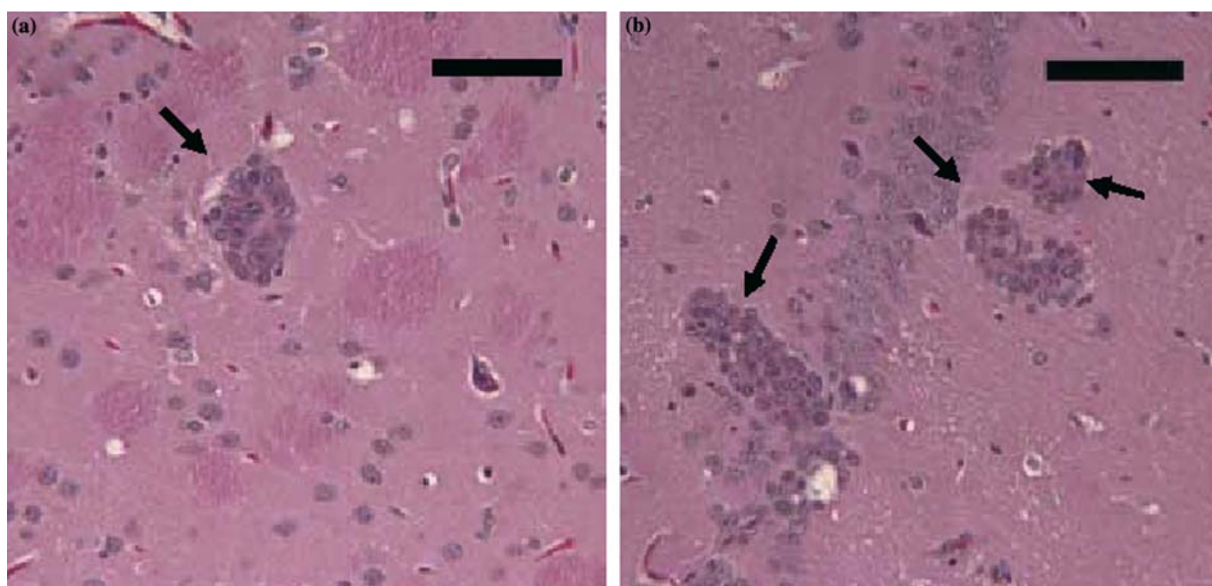

Figure 1. Histology of metastasis of breast cancer to the brain. The metastatic foci in the brain are observed as small clusters of epithelial neoplastic cells scattered randomly throughout the brain. Arrows indicate metastatic foci. Bar indicates 100 μm.

Table 2. Organ distribution of breast cancer metastatic foci.

Metastatic sites	Brain	Lung	Bone	Kidney	Pancreas
Numbers of animals affected/ total number of animals examined	6/10	10/10	4/5	3/5	1/5

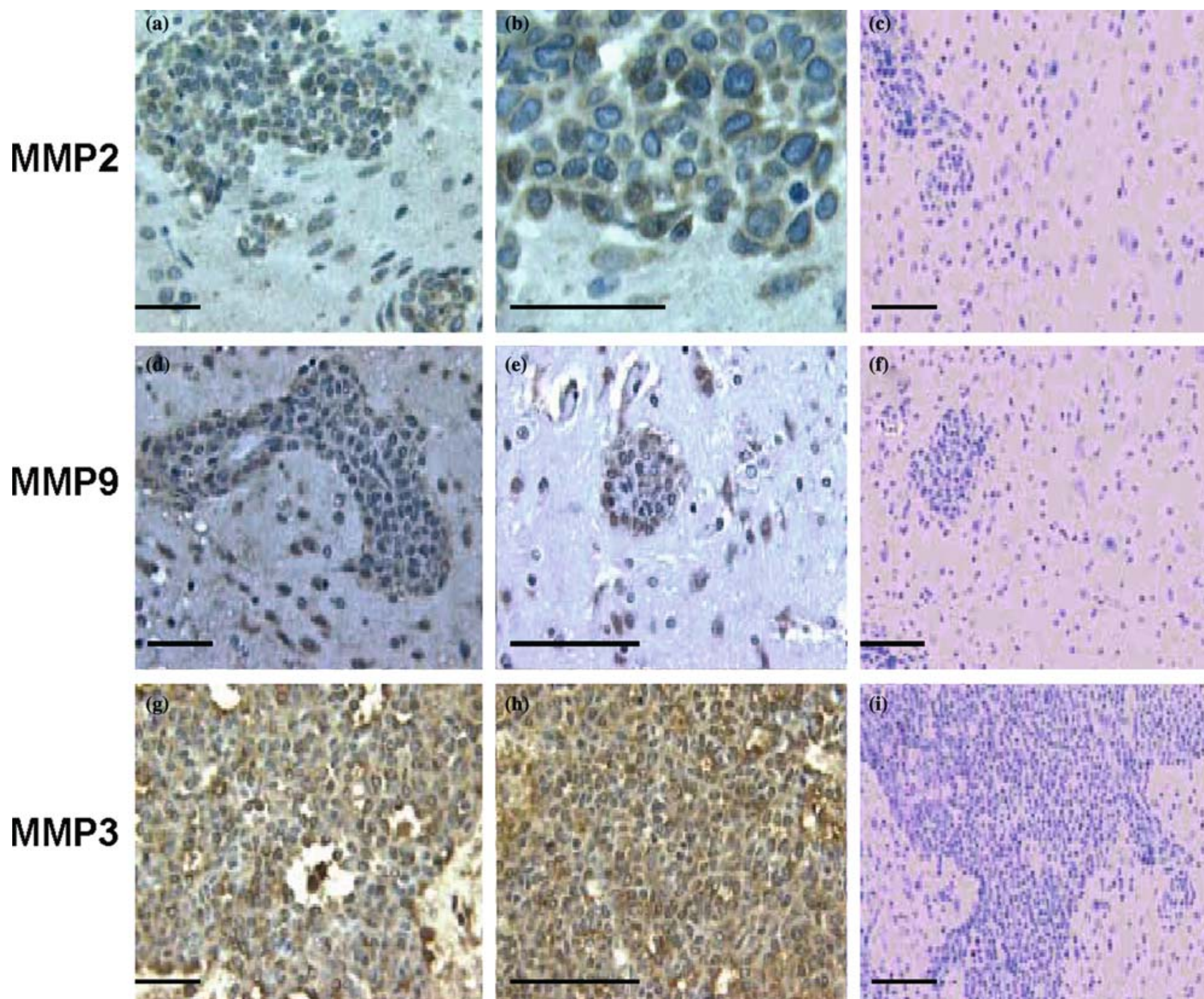

Figure 2. Localization of MMP-2, -3 and -9 in the brain metastatic foci. Immunohistochemical staining (brown) of MMP2 (a, b), MMP3 (g, h) and MMP9 (d, e) protein in the brain metastatic foci revealed positivity within neoplastic cell cytoplasm. Negative controls for MMP-2, -3 and -9 are respectively (c, i) and (f). Note that glial cells are also positive. Bars indicate 100 μm.

expression, we extracted protein from the metastatic neoplastic tissue. Evaluation of protein expression by Western blotting revealed that MMP3 expression was significantly higher in neoplastic brain metastasis tissue when compared with control tissue from brains of age-matched-non-inoculated rats ($P \leq 0.032$). Such difference was not observed in lung metastatic foci (data not shown). MMP2 was also more significantly expressed in brain metastasis of breast cancer when compared with normal brain control tissue ($P \leq 0.014$). MMP9 expression was also significantly higher in tumor tissue ($P \leq 0.049$) (Figure 3).

To confirm IHC and WB results on MMP-2, -3 and -9 protein expression, we extracted total RNA from frozen specimens. Semi-quantitative RT-PCR analysis of MMP2, -3, and -9 mRNA of brain metastatic foci of breast cancer was compared with mRNA obtained form age-matched-non-inoculated rats. The comparison revealed that the amounts of MMP2, -3, and -9 mRNAs of brain metastasis foci of breast cancer were higher than those of control tissues. This data is compatible with the WB results. MMP3 mRNA was more abundant in neoplastic tissue when compared with lower expression in controls. The same was observed from MMP2 and MMP9 (Figure 4).

Increased MMP2 and MMP3 activity in brain metastatic foci

To determine if the higher expression of MMPs was correlated with intra-tumoral increased enzyme activity, we performed zymography studies. *In situ* zymography

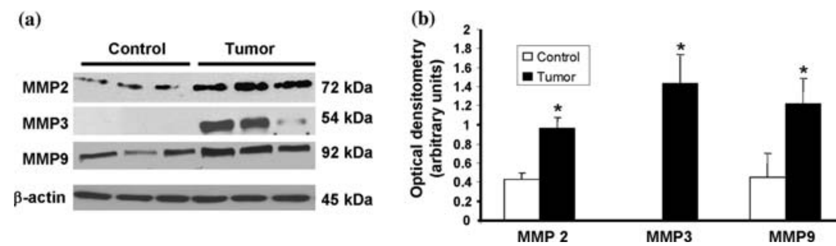

Figure 3. Increased expression of MMP-2, -3 and -9 protein in the metastatic brain foci. (a) Evaluation of protein expression by Western blotting. The membranes were stripped and re-probed with β-actin antibody to confirm equal loading. (b) Quantitative analysis of MMP-2, -3 and -9 expression was determined by densitometry. The results shown in the histogram are the mean ± standard deviation from three control and three tumor samples. (*) for statistically significant when P was ≤0.05).

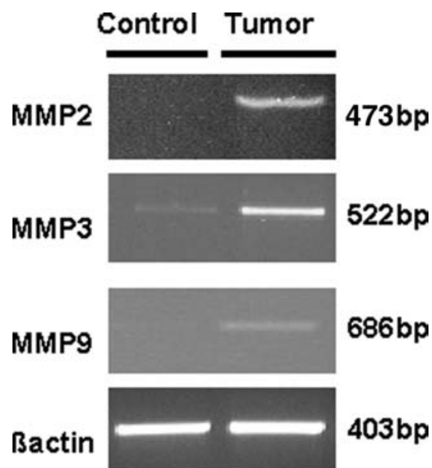

Figure 4. Increased expression of MMP-2, -3 and -9 mRNA in the metastatic brain foci. Semi-quantitative RT-PCR analysis was used to detect MMP2, -3, -9 and ß-actin in total RNAs from normal brain and metastatic brain foci. β-actin was used as an internal control.

revealed intra-tumoral gelatinase (MMP2 and/or MMP9) activity characterized by loss of fluorescence (Figure 5). Additionally gel zymography showed that there was a significant increase in both MMP3 and MMP2 activities ($P < 0.05$) (Figure 6). MMP9 activity was not detected on the samples evaluated.

Effect of MMP Inhibitor, PD 166793 on the in vitro *invasive potential of ENU1564 cells*

To determine whether the use of an MMP inhibitor (PD166739) has any influence on ENU1564 cells *in vitro* invasive potential a Matrigel-based invasion assay was performed. Boyden chamber chemoinvasion analysis showed that PD 166739 significantly reduced ($P \leq 0.001$) the number of ENU1564 cells that invaded the Matrigel when compared with non-treated control cells (Figure 7).

Effect of MMP inhibitor, PD166793 on brain metastasis of ENU1564 cells

To determine if MMPs play a role on breast cancer brain metastasis we inhibited MMPs' activities by treating animals with PD166793, a selective MMP inhibitor. The animals in the control group started to show neurological signs such as depression and obnubilation at day 24 post inoculation, and all animals were sacrificed at that time. Gross evaluation did not reveal any significant changes in the central nervous system. Upon histological evaluation three of the five animals in the control group had presence of brain metastasis. Brain metastases were not detected in the brains of animals treated with PD 166793 ($P \leq 0.03$) (Figure 7).

Discussion

In the present study, we found increased expression of protein and mRNA of MMP-2, -3 and -9 in BC brain metastasis, suggesting that these molecules may be relevant in the metastatic process of breast cancer to the brain in our rat model. We also determined that there is a correlation between MMP expression and enzymatic activity within the neoplastic foci, and that inhibition of MMPs' activities reduces the metastatic potential of breast cancer cells *in vitro* and *in vivo*.

We used brains of age-matched BD-IV rats as controls, assuming that differences in MMP expression would be attributable to the presence of tumors. Although ENU1564 expresses low levels of MMP2 and no detectable MMP3 or MMP9 *in vitro* (data not shown), our results show that MMP-2, -3 and -9 protein levels in metastatic foci had significantly higher expression than controls. This difference was especially marked for MMP3 with undetectable protein expression in the control tissue. Because brain cells are positive for MMP3 by IHC it is likely that the levels of MMP3 protein, although present, are too low to detect by the WB procedure performed. To ascertain whether increased expression of MMP-2, -3 and -9 proteins is correlated with their mRNA expression, cDNA was prepared from dissected tumor samples and RT-PCR was performed. Although this technique is semi-quantitative our results show that there are higher levels of MMP-2, -3 and -9 mRNA in metastatic neoplastic foci than in normal brain tissue.

In situ zymography results showed that there is a multifocal sharp increase in gelatinase activity and that it is morphologically associated with the neoplastic foci.

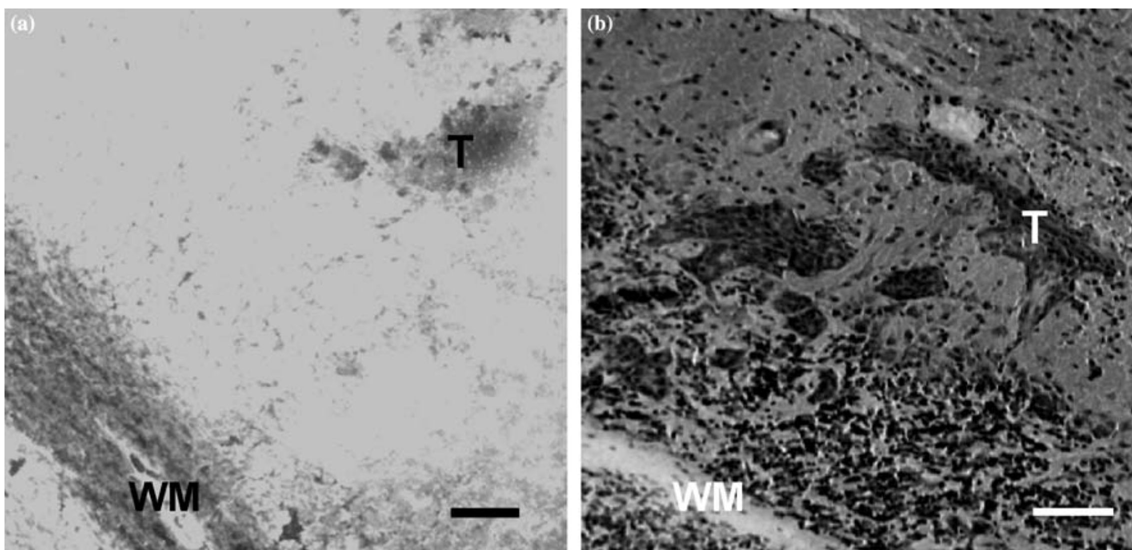

Figure 5. Evaluation of gelatinase activity in the brain foci by *in situ* zymography. (a) Frozen section observed under UV microscope. Marked gelatinase activity (loss of fluorescence) was observed within the tumor foci. (b) Thionin stain of the same area. WM represents white matter; T indicates tumor foci. Bars indicate 100 μm.

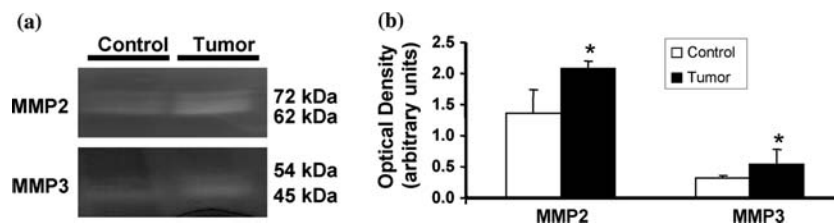

Figure 6. Increased MMP2 and MMP3 enzymatic activities in the metastatic brain foci. (a) Evaluation of MMP2 and MMP3 activities by gel zymography. (b) Quantitative analysis of MMP2, and MMP3 activity was determined by densitometry of respective active bands (62 and 45kDa). The results shown in the histogram are the mean ± standard deviation from three control and three tumor samples. (*) for statistically significant when $P \leq 0.05$.

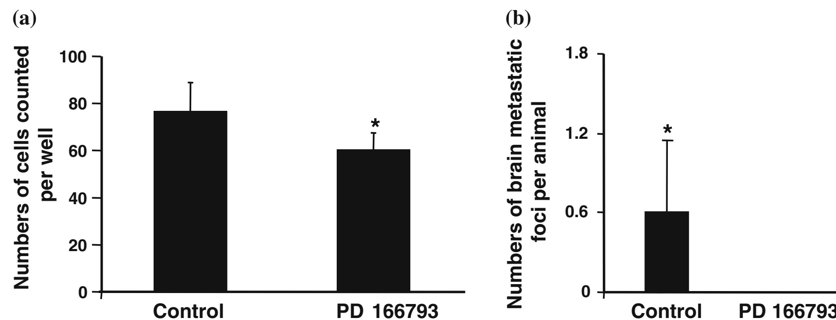

Figure 7. Decreased metastatic potential of ENU 1564 by PD 166793 a selective MMP inhibitor. (a) *In vitro* invasion chamber assay for ENU1564 cells was performed as described in material and methods. The results shown in the histogram are the mean ± standard deviation of two individual experiments run in triplicate. (b) Numbers of metastatic foci in the brain in control $(n=5)$ vs animals $(n=5)$ treated with PD 166973. (*) for statistically significant when $P \leq 0.05$.

Gel zymography results confirm the increase in gelatinase activity of MMP2 in tumor brain foci; however in spite of multiple technical variations we were unable to detect MMP9 activity in the samples evaluated. This result suggests that probably the active levels of MMP9 in brain samples are under the detection limits of our technique or that MMP9 may play a different role from active MMP2 in the early development of brain metastasis since we were able to detect MMP9 activity in under the same conditions in lung and mammary gland samples (data not shown). Additionally we observed a significant increase in MMP3 activity in the brain metastatic samples. Altogether these suggest that MMP2 and MMP3 play a role in the metastatic process.

Our results show that the levels of MMP2 and MMP3 protein and mRNA are increased in neoplastic foci, which corresponds to an increase in intra-tumoral enzymatic activity. These results are in accordance with

previous reports correlating MMP activity with metastatic and invasive behavior [5, 32]. Previous studies that describe MMP expression correlated with breast cancer metastasis reveal that MMPs may be important for the metastatic process. MMP2 is related to osteoclastic resorption in the metastatic process to the bone [20]. MMP2 and MMP9 latent forms are released in breast cancer cells in co-cultures with bone extracellular matrix [19]. Additionally, incidence of metastasis to the brain was increased in animals injected intracardiac with clones of breast cancer cells transfected with MMP2 [22]. Transfection with tissue inhibitor of metalloproteinases (TIMPs) has been reported to decrease cancer metastatic behavior [33, 34]. Conversely, TIMP evaluation in breast cancer patients has been associated with better prognosis [35]. This data correlates with the fact that MMPs have been associated with invasive and metastatic behavior of BC. High MMP2 serum levels are associated with adverse prognosis in node-positive BC, implying that this molecule is related with nodal metastasis [5]. Additionally, mostly MMP2 and MMP9 protein expression have been extensively correlated with poor breast cancer prognosis, and survival rates that are invariably associated with metastatic and invasive BC phenotypes [12, 14, 35].

The determination of what cell component of the tumor mass expresses MMPs is important in order to understand the role of these molecules in tumor development. Some studies have localized MMP2 to neoplastic epithelial cells. Others, however, associate them with different components of the tumor stroma [36] and/or angiogenic blood vessels [37]. MMP9 has been associated with neoplastic cell plasma membrane [16], non-neoplastic ducts and acini, stromal fibroblasts; endothelial cells, and tumor-infiltrating inflammatory cells including neutrophils, macrophages, and lymphocytes. Expression of MMP3 was observed in both tumor and stroma cells [38]. Normal breast epithelia reacted weakly positive for MMP3 mRNA [35].

In concurrence with previous reports [18], we observed MMP2 expression in epithelial cancer cells. However, the IHC staining was observed diffusely within the cytoplasm instead of the neoplastic cell plasma membrane [17]. Due to the absence of stroma in our tumor we were unable to determine if stromal cells or angiogenic blood vessels were positive. Moreover, as described in previous reports [22, 28], MMP3 and MMP9 staining was observed diffusely in the neoplastic cell cytoplasm with weaker staining for MMP9. Again, stroma evaluation was not possible. The variation in the intensity of staining may be due to different roles of these molecules in the metastatic process. However, IHC is not a good method to quantify expression and conclusions should be drawn very cautiously.

Stromal fibroblasts are thought to be important in stimulating the production of MMPs [39–42]. In our model, the extreme paucity of fibrous stroma, the absence of necrosis and inflammatory cell infiltrate (such as macrophage invasion) within the brain metastasis are highly suggestive of an alternate mode of MMP activation in this particular type of neoplasia within the central nervous system, and questions the need and role of inflammatory/macrophage infiltrate and fibroblast presence in the expression and activity of MMP molecules. We characterized morphologically the brain metastatic neoplasia together with MMP expression and activity, and concluded that it may be independent of the presence of inflammation and fibrous stroma-interaction.

More must be known in order to fully understand the mechanisms that regulate MMP activity within the central nervous system. MMP-2, -3 and -9 are expressed in normal brain tissue [43, 44], MMP-2, -3 and -9 are produced by neurons, astrocytes, glial cells and oligodendrocytes [45]. MMPs have also been associated with intra-cerebral tumor evolution, MMP2 with glioma *in situ* invasion, and MMP9 with intra-tumoral angiogenesis [46]. Astrocytes are thought to play a role in MMP9 activation and expression [43]. Astrocytic factors, such as TNFα and IL6 receptor, have been identified in cell cultures derived form metastasis of BC in the brain [4, 47]. Because of the growing evidence that astrocytes and/or glial cells have a role in the MMP cascade in the central nervous system, we analyzed how these cells react and express MMPs in our experimental conditions. Using Hematoxilin & Eosin histological stain we observed that there is marked astrocytic reactivity around neoplastic foci. Peritumoral astrocytic reactivity was confirmed using GFAP (glial fibrillary astrocytic protein) immunohistochemistry staining (data not shown). MMP-2, -3 and -9 staining of astrocytes and microglial cells was observed. The marked positivity of glial cells and astrocytes for MMPs, together with their presence around the tumor, suggest that these may indeed play a role within the MMP cascade [48]. Nonetheless, the presence of glial staining suggests that further studies are needed in order to characterize the role of those cells in MMP cascade in metastatic disease to the brain.

MMP inhibitors are being investigated as an important tool for cancer treatment. [23, 24]. In order to determine if MMPs play a role on breast cancer brain metastasis development we used PD 166739 (S-2-4′-bromobiphenil-4-sulfonylamino-3 methyl-butyric acid) as a selective MMP inhibitor that is known to decrease activities of MMP-2, -3 and -9 [49]. Unlike the first generation of MMP inhibitors, PD 166793 does not inhibit other metalloproteinases like TNF-α-convertase [30]. We observed slight but significant decrease in *in vitro* ENU 1564 invasion behavior when cells were in presence of PD 166793. Surprisingly, we observed a dramatic decrease in development of brain metastasis in animals treated with PD 166739. The disparity observed *in vivo* vs *in vitro* results may be associated with low levels of *in vitro* MMP expression; however these *in vivo* results are in concordance with our other *in vivo* results and strongly suggest that MMPs are important in the brain metastatic process of breast cancer.

In conclusion, we were able to use, for the first time, a rat model for distant breast cancer metastasis to the brain to successfully study expression and activity of MMP-2, -3 and -9. Our results indicate that MMPs are involved in breast cancer metastasis to the brain in our model.

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