Opposing Effects of Pigment Epithelium–Derived Factor on Breast Cancer Cell versus Neuronal Survival: Implication for Brain Metastasis and Metastasis-Induced Brain Damage

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Abstract

Brain metastases are a significant cause of morbidity and mortality for patients with cancer, yet preventative and therapeutic options remain an unmet need. The cytokine pigment epithelium–derived factor (PEDF) is downregulated in resected human brain metastases of breast cancer compared with primary breast tumors, suggesting that restoring its expression might limit metastatic spread. Here, we show that outgrowth of large experimental brain metastases from human 231-BR or murine 4T1-BR breast cancer cells was suppressed by PEDF expression, as supported by in vitro analyses as well as direct intracranial implantation. Notably, the suppressive effects of PEDF were not only rapid but independent of the effects of this factor on angiogenesis. Paralleling its cytotoxic effects on breast cancer cells, PEDF also exerted a prosurvival effect on neurons that shielded the brain from tumor-induced damage, as indicated by a relative 3.5-fold reduction in the number of dying neurons adjacent to tumors expressing PEDF. Our findings establish PEDF as both a metastatic suppressor and a neuroprotectant in the brain, highlighting its role as a double agent in limiting brain metastasis and its local consequences. Cancer Res; 72(1); 144–53. ©2012 AACR.

Introduction

Brain metastasis, the most common cause of intracranial tumors in adults, occurs in approximately one quarter of adult patients with cancer, and represents a significant cause of morbidity. The incidence of brain metastasis appears to be increasing. One factor thought to be contributing to this increase is improvement in the systemic treatment of cancer leading to prolonged survival and thereby allowing the emergence of brain metastases (1–3). The brain is considered a "sanctuary site" because of restricted drug access caused by the blood–brain barrier (4) and the inflamed brain microenvironment (5). The prognosis for patients diagnosed with brain metastasis is poor. Palliative treatments include corticosteroids, surgery, whole brain radiation therapy, and stereotactic radiosurgery (SRS; refs. 1, 3).

While a metastasis to any site is devastating, metastases to the brain are particularly feared for their neural complications. The majority of patients with brain metastasis report impairment in neurocognitive function. Other symptoms include headache, focal weaknesses, seizures, ataxia, and speech deficits (1, 2, 6). Permanent, progressive cognitive deficits occur in some patients with brain metastasis, ranging from mild memory loss to severe dementia (2). These deficits result not only from progression of the metastatic disease but also as side effects of treatment, including both systemic chemotherapy ("chemobrain"; ref. 7) and whole brain radiation therapy (8). Profiling of resected breast metastases and the development of mouse models have greatly expanded our knowledge of acquisition of brain metastatic potential by cancer cells; however, almost nothing is known about the onset of neuronal damage that leads to neurologic symptoms.

A gene expression analysis of resected breast cancer specimens revealed the downregulation of pigment epithelium–derived factor (PEDF) expression in brain metastases by approximately 14-fold relative to primary tumors (9). Normal breast epithelial cells express high levels of PEDF protein. Loss of PEDF expression from these cells is correlated with cancer progression, and PEDF expression is inversely related with survival outcome in patients with breast cancer (10). The gene
expression trend suggested a further downregulation of PEDF in breast cancer cells associated with the acquisition of brain metastatic potential.

PEDF, a member of the serpin family of proteins, was discovered as a secreted factor from human retinal pigment epithelial cells with a potent neuronal differentiation activity (11). Numerous subsequent studies have shown a remarkable ability of PEDF to protect neurons from a variety of insults (12–16). PEDF shows a broad expression pattern, including high levels in the brain and in blood (17). In addition to its neuroprotective activity, PEDF has been implicated as a regulatory factor in numerous pathways, including lipid metabolism (18), stem cell renewal (19), modulation of the inflammatory response (20), antiangiogenesis (21), and tumor suppression (reviewed in refs. 22, 23). PEDF is under translational investigation for use in a variety of diseases including macular degeneration (24) and neurodegeneration (16).

Increasing lines of evidence point to multiple functional domains in the PEDF protein with either antiangiogenic or neuroprotective capacities (25–27), as well as multiple distinct cellular receptors (28–31).

The joint tumor-suppressive and neuroprotective activities of PEDF, combined with the observation that it is down-regulated in human brain metastases, suggested that it might have therapeutic value in the context of brain metastasis. Herein, we have used experimental brain metastasis and intracranial injection models to show that PEDF acts simultaneously as a prodeath molecule for cancer cells and as a prosurvival molecule for peritumoral neurons.

Materials and Methods

Cell lines

The human MDA-MB-231 brain-tropic cell line (231-BR) and murine 4T1-BR5 brain-tropic line were described previously (32, 33). The 231-BR line was obtained from Toshiyuki Yoneda, University of Texas, San Antonio, TX, and was authenticated by our laboratory to the American Type Culture Collection MDA-MB-231 line by short-tandem repeat profiling. The 4T1-BR5 line was obtained from Suyun Huang, MD Anderson Cancer Center, Houston, TX. R28 retinal progenitor cells were obtained from Gail Seigel, University of Buffalo, NY (34).

Subcloning and production of stable cell lines

The coding region for the human PEDF gene was a gift from the laboratory of Marta Garcia-Diez, Ciemat, Madrid, Spain. The coding region, with two tandem FLAG tags added to its C-terminus, was subcloned into the lentiviral expression vectors pCDH-CMV-MCS-EF1-Puro and pCDH-EF1-MCS-T2A-Puro (SBI). PEDF was also subcloned into the pJC13-1 vector (35), a gift from the laboratory of Gary Felsenfeld, NIDDK, Bethesda, MD. Additional details of DNA subcloning can be found in Supplementary Methods. Stable cell lines were generated in 231-BR cells by selection in 500 μg/mL of neomycin, and in 231-BR cells, by selection in 2 to 30 μg/mL of puromycin, for at least 2 weeks. Cells were subsequently maintained in the absence of selective agent and showed no loss of transgene expression.

Purification of PEDF, real-time label-free cellular analysis, and Western blot analysis

Recombinant human PEDF (or PEDF-FLAG) was purified as previously published (36). Cell impedance was measured using a real-time microelectronic sensor system (ACEA/Roche) as described previously (37). Cell viability was measured using a CellTiter-Glo viability assay kit (Promega) following the manufacturer's instructions. Data from two or more replicates were averaged. Western blot analyses were conducted according to standard protocols. The following antibodies were used: anti-FLAG (Sigma), anti-GAPDH (Cell Signaling Technology), mouse anti-human PEDF (Millipore), rabbit anti-PEDF (Bio-Products MD), and anti-α-tubulin (Calbiochem), diluted 1:1,000 to 1:5,000. Secondary antibodies (Santa Cruz) were diluted 1:2,000 to 1:5,000.

Hematogenous mouse models of brain metastasis

Experiments were carried out under an approved Animal Use Agreement with the NCI. Under isoflurane/O2 anesthesia, 5- to 7-week-old female NCr nu/nu mice (Charles River Laboratories) were inoculated in the left cardiac ventricle with 1.75 × 105 human 231-BR cells and immunocompetent BALB/c mice were injected with 5 × 106 murine 4T1-BR5 cells. Mice were euthanized under CO2 asphyxiation at time points correlated with the onset of weight loss, ataxia, and/or paralysis. Brains, bisected along the sagittal plane, were processed for cryosectioning as previously described (38). Ten-micrometer cryosections were prepared and processed for histology and immunofluorescence. To quantify the number of metastases, 10 sagittal sections, spaced every 300 μm, were cut through one hemisphere and hematoxylin and eosin (H&E) stained. Metastases were counted at 50× magnification using an ocular grid. Any metastasis that measured more than 300 μm in any direction was considered a large metastasis, as previously published (38).

Intracranial model of brain metastasis

Six- to eight-week-old female NCr nu/nu mice (Charles River Laboratories) were used. Experiments were carried out under an approved Animal Use Agreement with the NCI. Mice, divided into 3 groups to receive intracranial injections of PBS, vector control 231-BR/ins, or PEDF-231-BR/ins cells, were anesthetized with ketamine (83 mg/kg) and xylazine (8.3 mg/kg). Cells were injected into the right caudate nucleus as previously described (39). Mice, anesthetized as above, were perfusion fixed with approximately 10 mL of PBS followed by approximately 10 mL of 4% paraformaldehyde prior to extirpation of the brain 0, 1, or 3 days postimplantation. Whole brain was immediately frozen in optimal cutting temperature. Serial 10-μm cryosections were prepared along the horizontal plane starting from the dorsal surface of the cerebral cortex and progressing to a depth of 3 mm or above. H&E sections were prepared every 100 μm.

Histologic and immunofluorescence analysis

For Bielschowsky silver impregnation, 10-μm frozen sections were fixed in 10% phosphate-buffered formalin for 24 hours at 37°C or 48 hours at room temperature and stained using Sigma reagents. Fluorojade-B (Histochem) staining
was conducted similar to the published method (40), omitting sodium hydroxide treatment and staining in a final dye concentration of 0.001%. Immunofluorescence analyses were conducted as previously described (41). The following antibodies were used: anti-CD31 (BD Pharmingen), anti-cleaved caspase-3 (Cell Signaling Technology), anti-FLAG (Sigma), anti-human mitochondria (Thermo Scientific), anti-Ki67 (Vector Laboratories), and anti-human PEDF (Millipore). Secondary antibodies were conjugated to Alexa Fluor 488 and 568 (Molecular Probes). Nuclei were visualized with 200 μg/mL DAPI (4',6-diamidino-2-phenylindole, dilactate) stain (Molecular Probes). Numbers of cleaved caspase-3, FLAG, Fluorojade-B, Ki67, or PEDF-FLAG–positive cells or CD31-positive blood vessels were counted in images using DAPI to counterstain all nuclei and anti-human mitochondria to label human breast cancer cells. Ten sections, approximately 100 μm apart, were collected from each mouse for Fluorojade analysis. Five sections, approximately 200 μm apart, were collected for analysis of Ki67 and cleaved caspase-3 in the intracranial model.

**Statistical analysis**

A variety of ANOVAs were conducted on the raw or transformed data (as indicated by the Box–Cox transformation). Samples from experimental units were averaged and the square root of the sample size was used as a weight in subsequent analyses. One-way ANOVAs were used to analyze simple fixed-effect models. Two- or three-factor factorial ANOVAs were used to analyze more complex fixed-effect models. Mixed models were used as necessary to model random variation due to repeated experiments or other random effects. Analysis of covariance was used to account for important covariates. Linear regression was used to estimate growth rates of individual mice. Finally, for all ANOVAs, residuals were examined for normality and homogeneity and residuals were partitioned if found to be heterogeneous. The Wilcoxon rank-sum test was used to compare distributions if the data were not normally distributed and/or the sample sizes were very small. For pairwise comparisons, Holm method was used to adjust pairwise P values.

**Results**

**Effects of PEDF on cultured breast cancer cells and neural retinal precursors**

The effects of PEDF on two "triple-negative" breast cancer cell lines were examined in vitro. Cells were analyzed in real-time by measuring cell impedance and at endpoint by measuring biomarkers of live cells to confirm cell viability monitoring. At high cell density, 4T1-BR5 cells, a brain-tropic derivative of the 4T1 murine cell line, remained viable for several days in the absence of serum, but addition of increasing concentrations of purified recombinant human PEDF progressively decreased 4T1-BR5 cell viability (Fig. 1A; Supplementary Fig. S1A and S1B). Comparison of the 0, 10, and 100 nmol/L doses using the exact Jonckheere–Terpstra test showed a significant diminution of viability by PEDF (P = 0.022; Fig. 1B); comparison of vehicle versus PEDF for 4T1 cells (P = 0.022 by the same test; Supplementary Fig. S1A). Similar trends were also observed using 231-BR cells, a brain-tropic derivative of the MDA-MB-231 cell line (Fig. 1C and D).

We also tested the prosurvival activity of PEDF on neural progenitor cells. As illustrated in Fig. 1E, low-density rat retinal progenitor (R28) cells progressively died within a day of serum deprivation, but PEDF significantly increased R28 cell viability in a dose-dependent manner. Comparison of the 0, 10, and 100 nmol/L doses showed a significant protective effect of PEDF (P = 0.022; Fig. 1F). PEDF protein altered to include two-tandem FLAG-epitope tags on the C-terminus of the protein maintained its biologic activity, showing a statistical trend in reduced viability using the exact Jonckheere–Terpstra test (Supplementary Fig. S1C and S1D; P = 0.33 for PEDF, 0.067 for PEDF-FLAG). Thus, PEDF appears to have direct but opposing effects on the viability of breast cancer cells and normal neural retinal progenitor cells.

**Transient PEDF expression suppressed proliferation of brain metastases in two breast carcinoma models**

In agreement with the observation that PEDF expression is downregulated in human breast cancer brain metastases, neither of the brain metastatic breast cancer cell lines had detectable expression of PEDF protein (Fig. 2A, left column and data not shown). Human 231-BR cells were transduced with a human PEDF transgene to produce stable clones. The FLAG-tagged PEDF protein was efficiently secreted from the cancer cells and was detectable as a single band of 50 kD by Western blot analysis with both anti-PEDF (Fig. 2A) and anti-FLAG antibodies (not shown).

While PEDF exhibited negative effects on breast cancer cell viability in the absence of serum (Fig. 1), PEDF expression appeared well tolerated when cells were cultured in complete media, showing no decrease in anchorage-dependent growth when compared with vector control clones or untransfected 231-BR cells (Supplementary Fig. S2A). Nor did the PEDF-expressing cells show a decrease in migration across a collagen-coated membrane in a Boyden chamber assay (Supplementary Fig. S2B). PEDF protein expression appeared to be relatively uniform across cell populations as visualized by immunofluorescence and could be maintained in the absence of selection agent for multiple passages without noticeable changes in PEDF protein expression (data not shown).

To analyze the effect of PEDF on the brain metastatic potential of breast cancer cells, 3 vector control 231-BR clones and 3 PEDF-231-BR clones were injected into the left cardiac ventricle of immunocompromised mice. The number of brain metastases was quantified by histology of step sections through one brain hemisphere of each mouse 26 to 28 days later (Fig. 2B). PEDF expression had no significant effect on the number of micrometastases (<300 μm in a single direction) or leptomeningeal metastases. However, the number of large (>300 μm) parenchymal metastases formed from PEDF-231-BR cells (mean = 11.9; n = 60 mice for the three combined PEDF-expressing clones) was decreased to 66% of controls (mean = 18.1; n = 48 mice for the three combined control clones; P = 0.001).

Staining of tissue sections with anti-FLAG revealed that only 1% to 2% of tumor cells (352 of 22,092) retained PEDF
expression after 26 days in vivo. Metastases were costained for PEDF-FLAG and Ki67 (Fig. 2C), and the numbers of PEDF-FLAG, Ki67, and double-positive cells were quantified (Fig. 2D). As previously published (41), Ki67 staining was confined to metastatic tumor cells and absent in the surrounding brain cells (Fig. 2E). The majority (59%) of cells (7,812 of 13,317) were proliferating in control metastases (Fig. 2D, left column). In the mice inoculated with PEDF-231-BR cells, the proliferation rate among the subset of metastases in which no PEDF-expressing cells were visible was not significantly different from controls (57%; 8,387 of 14,732 cells). In contrast, the rate of proliferation (42%; 3,133 of 7,360 cells) was significantly reduced in the subset of metastases with mosaic PEDF expression (Fig. 2D, middle column). There was an even greater decrease in proliferation (12%; 42 of 352 cells) among the individual cells which maintained PEDF expression (PEDF-FLAG and Ki67 double-labeled cells; Fig. 2D, right column). The reductions in proliferation, both in PEDF-expressing cells themselves and in mosaic metastases containing a minority population of PEDF cells, were highly significant ($P = 0.002$ vs. control).

Vascular density was examined in control and PEDF brain metastases. Blood vessels were stained with anti-CD31, and the blood vessel density was counted both in brain metastases and in an adjacent region of brain devoid of tumor cells (Fig. 2F). In agreement with previously published results (42), brain metastases in the 231-BR model had a reduced vascular density relative to the surrounding normal brain ($P = 0.0005$). PEDF expression did not significantly alter the number of blood vessels in brain metastases ($P = 0.37, n = 6$ mice per group). This suggests that the observed reduction in proliferation and formation of large brain metastases is not due to an antiangiogenic effect of PEDF.

In an attempt to increase the fidelity of PEDF expression, a second expression construct was engineered. Transcription of PEDF was placed under control of the human EF1a promoter, and the transgene was flanked with duplicate transcriptional insulator elements derived from the chicken heat shock 4 locus.
This construct was used to express PEDF in the murine 4T1-BR5 brain metastatic cell line (Fig. 3A). PEDF expression appeared to be uniform and stable among clonal populations over many generations in vitro and had no effect on anchorage-dependent growth of these cells (Supplementary Fig. S2C). The expression construct is illustrated in Supplementary Fig. S2D.

Control and PEDF-expressing clones of 4T1-BR5 cells were inoculated into the left ventricle of syngeneic, A to C, representative sections of mouse brain (counterstained with DAPI, blue) 28 days postinjection of PEDF-231-BR cells. D, percentage of Ki67-positive cells in brain metastases 28 days postinjection was quantified. Left column, control 231-BR metastases; middle column, metastases from PEDF-231-BR cells containing at least one PEDF-expressing cell (mosaic); right column, PEDF-231-BR cells positive for both PEDF and Ki67. Results from 6 mice injected with control 231-BR cells and 6 mice injected with PEDF-231-BR cells pooled from 3 clones of each and from 2 experiments.
immunocompetent BALB/c mice. The number of brain metastases was quantified after 9 days (Fig. 3B). The insulator construct did not improve transgene stability in vivo. Similar to the results with 231-BR cells, PEDF expression was rapidly silenced in the syngeneic mouse model, and no PEDF-positive cells were visible at the endpoint (data not shown). However, transient PEDF expression in 4T1-BR5 cells lead to a significant reduction in micrometastases (median = 73) as compared with controls (median = 432, \( P = 0.018 \)) and a significant reduction in large brain metastases (median = 0 vs. 8, \( P = 0.005 \)).

To determine whether the tumor microenvironment influenced either the stability of PEDF expression or its effects on breast cancer cell proliferation, we implanted breast cancer cells into the mammary fat pad. The insulator construct was used to generate stable polyclonal (231-BRins) control and PEDF-expressing populations in 231-BR cells (Supplementary Fig. S2E). PEDF expression was relatively uniform across the populations in vitro (Supplementary Fig. S2F). PEDF expression significantly slowed mammary tumor growth rate (\( P = 0.005 \), two-sample \( t \) test; Supplementary Fig. S3A). Similar to the experimental brain metastasis models, very few cells at the endpoint maintained PEDF expression. However, the rare PEDF-expressing cells were Ki67 negative (Supplementary Fig. S3B). Blood vessel density was not significantly different (\( P = 0.55 \)) between the two groups, suggesting that the slower growth of PEDF tumors was not due to anti-angiogenesis (Supplementary Fig. S3C). Thus, using two models of breast cancer metastasis, and in two microenvironments, despite achieving only transient PEDF expression, a growth-suppressive effect of PEDF was repeatedly observed.

**PEDF expression rapidly inhibits proliferation and activates apoptosis of breast cancer cells in the mouse brain**

To study both the cancer-suppressive and the neuroprotective effects of PEDF simultaneously, metastatic breast cancer cells were directly implanted into mouse brains. A time course study was conducted to determine the stability of PEDF protein expression in vivo. Five hours postimplantation, PEDF-FLAG expression was visible in the majority of PEDF-231-BRins cells but was lost in many cells 24 hours postimplantation and was present in less than 1% of the cells by 3 days postimplantation (Fig. 4A).

To determine the effect of PEDF on the viability of the brain metastatic tumor cells, mice were necropsied 24 hours postimplantation of control or PEDF-expressing tumor cells. A third group of mice received an intracranial injection of saline to determine the effects of the stab wound on the mouse brain. Serial sections were prepared, starting from the needle entry site along the dorsal surface of the brain and progressing to the bottom of the lesion, at a depth of approximately 3 mm. Representative H&E-stained sections are shown in Fig. 4B. Five step sections, evenly spaced over a length of approximately 1 mm (which spanned most of the depth of the implanted tumor cells), were stained for either Ki67 (Fig. 4C and D) or cleaved caspase-3 (Fig. 4E and F). The majority (64%, 17,658 of 27,625) of the implanted 231-BRins cells in control lesions were Ki67 positive (proliferating; Fig. 4C, left). In PEDF-expressing lesions (Fig. 4C, right), proliferation was reduced to a minority population (37%, 5,278 of 14,186 cells, \( P < 0.0001 \)). In control 231-BRins tumors (Fig. 4E, left), apoptotic cells expressing cleaved caspase-3 were rare (4%, 1,286 of 28,763 cells). In contrast, more than a third (39%) of the cells in PEDF-231-BRins lesions (5,596 of 14,461 cells; \( P = 0.0001 \) vs. control) were undergoing apoptosis (Fig. 4E, right). Thus, while PEDF expression was well tolerated by breast cancer cells in vitro, it has a rapid and profound suppressive effect on tumor viability in the brain. Both control (Supplementary Fig. S4A and S4B) and PEDF tumors (Supplementary Fig. S4C and S4D) showed no alterations in their vasculature, suggesting that these effects are independent of the antiangiogenic effects of PEDF.

**PEDF protects neurons in the brain from damage induced by tumor cells**

In addition to the direct effect of PEDF on tumor cells, the intracranial model permitted an examination of the effects of secreted PEDF on the brain. Silver stain was used to visualize the morphology of brain neurites and was evaluated independently by two neuropathologists. Adjacent to
control 231-BR/ins lesions, the bundles of nerve fibers were frequently disorganized, with obvious thickened and thinned neurites indicative of neuronal damage (Fig. 5A, middle). A saline-only injection confirmed that neuronal damage outside of the lesion was due primarily to the tumor cells rather than the injection process (Fig. 5A, left). Far less damage was apparent adjacent to PEDF-231-BR/ins tumors, as compared with control tumors (Fig. 5A, right).

As the silver stains were only qualitative, a second stain for neuronal damage was used, Fluorojade-B (Fig. 5B). Fluorojade-B has been shown to label damaged brain neurons specifically. This dye appears to label neurons undergoing either necrotic or apoptotic death and has been used to analyze brain damage resulting from a variety of insults (40, 43–47). Fluorojade-B staining was not visible around breast cancer micrometastases but became apparent in advanced disease in the hematogenous brain metastasis model (Supplementary Fig. S5).

To examine the extent of damage in the intracranial model, 10 step sections spanning approximately 1 mm of the lesion were stained and counted to determine the number of injured neurons surrounding the lesions (Fig. 5C). In agreement with the silver stains, a small number of damaged neurons (200 per brain) were evident surrounding the stab wound in saline injection controls (Fig. 5B, left). There was a 15-fold increase in damaged neurons (2,950 per brain; \( P < 0.0001 \) vs. saline controls) when control 231-BR/ins cells were implanted (Fig. 5B, middle). The majority of this damage was suppressed by PEDF expression in the implanted cells (840 per brain; \( P = 0.008 \) vs. control tumor; Fig. 5B, right). Moreover, PEDF appeared to decrease the area of the brain impacted by the lesion (Fig. 5D). The farthest Fluorojade-positive neurons were only a few cell
lengths (mean = 86 μm) from the stab wound in saline-injected brain. Damage around 231-BRins tumors extended much further (mean = 269 μm; P = 0.0003 vs. saline controls). The radius of damage around PEDF-231-BRins tumors was significantly smaller than the damage around control tumors (mean = 145 μm; P = 0.009). These results indicate that PEDF can simultaneously act to promote survival in neurons while activating cell death in cancer cells in the brain.

Discussion

The effects of PEDF on breast cancer cells in the brain were analyzed using two brain-tropic cell lines, two expression constructs, and several routes of tumor cell inoculation. The outgrowth of breast cancer cells into large brain metastases was suppressed in both a xenograft model and in a syngeneic mouse model of experimental brain metastasis. PEDF also slowed the growth of tumors implanted directly into the mammary fat pad. These observations stand in agreement with the wide recognition of PEDF as being tumor suppressive (22, 23) and extends its activity to include breast cancer and brain metastasis.

Most of the previous studies which showed a tumor-suppressive activity of PEDF focused on its potent antiangiogenic properties. Our in vitro results show that PEDF was directly inhibitory to breast cancer cells. Importantly, this effect was reproduced in vivo. Transient expression of PEDF in brain metastases resulted in a reduction in proliferation without affecting the tumor vasculature. Moreover, when breast cancer cells were inoculated directly into the mouse brain, PEDF rapidly reduced cancer cell proliferation and also activated apoptosis. These effects occurred without an apparent change in tumor vasculature.
While PEDF transgene expression was stable in culture, expression was lost within hours in vivo. Loss of protein expression was observed with two different promoters and in both the presence and absence of transcriptional insulators. Because translational studies using adenoviral-driven PEDF expression are ongoing (24), it would be of interest to determine whether and how posttranscriptional regulation of PEDF protein expression occurs in vivo.

Cognitive decline is frequently observed in patients with brain metastases. There are at least three factors which contribute: progression of brain metastatic disease, "chemobrain" resulting from rounds of systemic chemotherapy, and radiation therapy used specifically to treat the brain metastases. It is currently difficult to determine the relative contributions of these factors to the observed cognitive decline. New models are needed to measure and differentiate between the negative effects of disease progression versus the negative effects of cancer therapies, on the brain. Our data show the use of Fluorojade-B staining as a quantitative measure of neuronal damage induced by brain metastases. The intracranial implantation model revealed that neuronal damage results not just from direct contact with tumor cells but extends to a concentric area around the brain metastasis. It is unknown whether this "collateral damage" results from the pressure of the expanding mass, edema, and/or toxic byproducts of tumor metabolism. Regardless, PEDF expression resulted in a 3.5-fold diminution in neuronal damage. Previous studies have established the neuroprotective activity of PEDF in vitro and in vivo (reviewed in ref. 48). This report, to the best of our knowledge, provides the first evidence that PEDF can protect brain neurons from cancer-induced damage.

The data prompt the exciting hypothesis that PEDF can prevent some of the neuronal and cognitive sequelae associated with the development of brain metastases, both by tumor-suppressive and neuroprotective effects. Further investigation will require that neurocognitive endpoints be validated in mouse experimental brain metastasis models. Moreover, the delivery of PEDF to the brain remains a significant obstacle. Functional subdomains of PEDF which recapitulate the neurotropic and antiangiogenic activity have been described (25–27). It will be important to determine which regions of PEDF are necessary for its direct effects on cancer cells. Delivery of the identified active peptide(s) could be attempted via an Omaya reservoir, but active blood–brain barrier transport may be needed such as Angiopep-2 conjugation (49).

There is evidence for multiple PEDF receptors at the cell surface (28–31). Differential expression of these receptors on neuronal, endothelial, and cancer cells may provide a partial explanation for the differential effects on these cell populations. Identification of which of these PEDF receptors are present on cancer cells, as well as further elucidation of signaling downstream of PEDF, could lead to the identification of new pharmacologic targets for both anti-cancer and neuronal survival therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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