Clostridium perfringens Enterotoxin as a Novel-Targeted Therapeutic for Brain Metastasis

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Abstract

Brain metastasis is the most commonly occurring intracranial tumor whose incidence seems to be increasing. With standard therapy, the average survival time of patients is ~8 months, and treatment often leads to neurologic dysfunction in long-term survivors, emphasizing the need for novel therapeutics. *Clostridium perfringens* enterotoxin (CPE) has recently been shown to rapidly and specifically destroy cancer cells expressing CPE receptors claudin-3 and claudin-4. Unfortunately, the utility of CPE is precluded by systemic toxicity because its receptors are expressed in numerous organs. Here, we provide the first preclinical evidence that CPE may be uniquely suited to the local treatment of brain metastasis. By immunohistochemical analysis, claudin-3 and claudin-4 were expressed frequently in metastases from breast (15 of 18), lung (15 of 20), and colon (12 of 14) carcinoma, and infrequently in metastases from renal cell carcinoma (2 of 16) and melanoma (2 of 16). In contrast, expression of claudin-3 and claudin-4 was absent in adjacent normal brain tissue. Further examination of the central nervous system (CNS) revealed low or undetectable levels of claudin-3 and claudin-4 in all regions tested by Western and immunohistochemical analysis. Treatment of breast cancer cell lines (MCF-7, MDA-MB-468, NT2.5-luc) and normal human astrocytes with CPE *in vitro* resulted in rapid and dose-dependent cytolysis exclusively in breast cancer cells, correlating with claudin-3 and claudin-4 expression. Moreover, intracranial CPE treatment significantly inhibited tumor growth and increased survival in two murine models of breast cancer brain metastasis, without any apparent local or systemic toxicity. These data suggest that CPE therapy may have efficacy against a wide variety of brain metastases without CNS toxicity. [Cancer Res 2007;67(17):7977–82]

Introduction

Metastases account for the majority of newly diagnosed brain tumors, with incidence rates roughly 10 times higher than that of primary neoplasms (1). Recent reports suggest that brain metastasis is diagnosed in 15% to 40% of cancer patients and is most commonly associated with cancers of the breast, lung, kidney, skin, and colon (2). Whereas improvements in cancer therapy have resulted in increased patient survival, the incidence of brain metastasis has also increased, likely due to the inability of therapeutics to cross the blood-brain-barrier (BBB; refs. 2, 3). With standard treatment, the average survival time for patients with brain metastasis is ~8 months, and long-term survivors often suffer damaging side effects from their treatment (4, 5). Therefore, the development of new and safer treatment options for brain metastasis is of immediate clinical importance.

Clostridium perfringens enterotoxin (CPE) has recently emerged as a potential cancer therapeutic. CPE has been shown to elicit rapid cytolysis of mammalian cells, occurring in as little as 5 min (6). Furthermore, studies suggest that CPE-induced cytolysis occurs specifically in cells expressing the tight junction proteins claudin-3 and/or claudin-4, which function as low-affinity and high-affinity receptors for CPE, respectively (7, 8). Studies in our laboratory and others have shown that claudin-3 and claudin-4 are expressed in numerous organs and overexpressed in several cancers, including breast, ovary, prostate, and pancreas, suggesting the potential of CPE in cancer therapy (9). Subsequent animal studies examining the antitumor effects of CPE after local delivery have shown some success; however, systemic toxicity precludes its use (10–12).

An innovative use of this toxin would be to treat carcinoma metastases to the brain. Recent studies show little to no claudin-3 or claudin-4 mRNA expression in whole-brain tissue, consistent with the fact that claudin-3 and claudin-4 are expressed solely in cells of epithelial origin, whereas most brain cells are of mesenchymal origin (11). This scenario may present a unique opportunity to treat and eliminate epithelial metastases to the brain without harming host brain tissue. Furthermore, the BBB may inhibit systemic exposure to CPE after local administration, thus preventing systemic toxicity. Here, we present, for the first time, evidence that claudin-3 and claudin-4 expression is undetectable in central nervous system (CNS) tissues with the exception of the ependymal cells of the choroid plexus, which display a low level of expression. Consistent with this observation, intracranial administration of CPE significantly inhibited tumor growth and increased survival in mice harboring breast tumors in the brain without any apparent toxicity to host CNS tissue. Taken together, these data suggest that CPE may be useful in the specific elimination of brain metastasis.

Materials and Methods

Cell lines and tissues. The breast cancer cell lines MDA-MB-468, MCF-7, and HS578T were obtained from American Type Culture Collection and cultured according to conditions specified. The murine mammary carcinoma cell line NT2.5 was kindly provided by Drs. R. Todd Reilly and Elizabeth M. Jaffee (Johns Hopkins University School of Medicine, Baltimore, MD). NT2.5 cells were maintained in RPMI supplemented with 20% fetal bovine serum (FBS), 10 mmol/L HEPES, 1% nonessential amino acids, 1 mmol/L sodium pyruvate, and 10 μg/mL insulin. For *in vivo*

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imaging, pEF1/Myc-His (Invitrogen) containing firefly luciferase (F-*luc*) was stably transfected into NT2.5 cells by electroporation using a nucleofector II device (Amaxa Biosystems). Individual stable clones were selected in the presence of 500 μg/mL G418 (Sigma) and maintained in 200 μg/mL G418 after expansion. Finite life-span normal human astrocytes were kindly provided by Dr. Carlos Pardo (Johns Hopkins University School of Medicine). Astrocytes were maintained in Eagle's minimal essential medium supplemented with 10% FBS and were not used beyond 10 passages. Paraffin blocks of cancer metastasis to the brain and cancer-free brain tissue were obtained from the Surgical Pathology Division of Johns Hopkins Hospital, observing institutional guidelines for acquisition of such specimens.

Cytotoxicity assay. Native CPE was isolated and purified as previously described (13). The biological activity of CPE per microgram was assessed by a cytotoxicity assay using our positive control cell line MCF-7. For the current preparation of CPE, 0.1 μg was identified as the minimum dose required to elicit complete cytolysis of MCF-7 cells. A single preparation of CPE was used for the completion of all *in vitro* and *in vivo* studies. For cytotoxicity assay, breast cancer cell lines (MDA-MB-468, NT2.5-luc, MCF-7, and HS578T) and finite–life span normal human astrocytes were plated in six-well plates and grown overnight to ~80% confluence in complete medium. Old medium was then removed and replaced with complete medium with or without CPE at concentrations ranging from 0.01 to 1 μg/mL. Cells were then incubated at 37°C for 60 min. Floating cells were collected and pooled with adherent cells removed by trypsinization. Total cells were then counted using a hemocytometer, and cell viability was determined by trypan blue dye (0.4%) exclusion. Differences in cytotoxicity between each experimental group were compared by unpaired Student's t test.

Immunohistochemistry. Paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded ethanol. Antigen retrieval was done by immersing sections in 0.01 mol/L sodium citrate (pH 6.0) and boiling by microwave for 20 min. Sections were then cooled to room temperature, and endogenous peroxidase activity was quenched by immersing in 0.3% hydrogen peroxide for 30 min. Blocking was then done by incubation in diluted normal goat (claudin-3) or horse (claudin-4) serum (Vectastain kit, Vector) as per the manufacturer's instructions. Sections were then incubated with rabbit polyclonal claudin-3 (Invitrogen) or mouse monoclonal claudin-4 (Invitrogen) at a 1:500 dilution for a period of 16 h. Diluted biotinylated antirabbit or antimouse IgG (Vectastain kit) was added

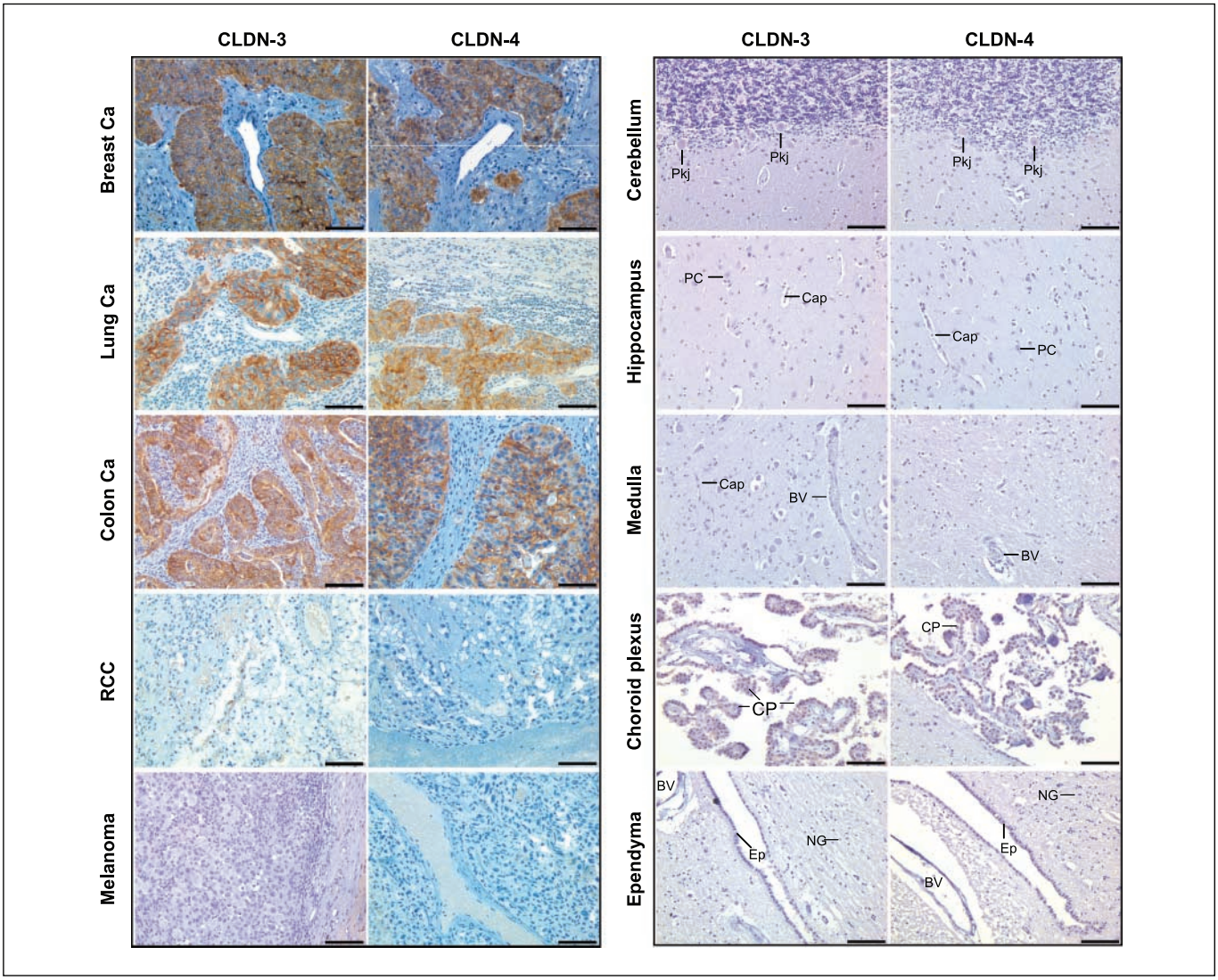

Figure 1. Expression of claudin-3 and claudin-4 proteins in brain metastases and CNS tissue. Immunohistochemical analysis was done on paraffin-embedded sections of human CNS tissue and brain metastases originating from primary breast carcinoma, lung carcinoma, colon carcinoma, RCC, and melanoma using claudin-3 and claudin-4 antibodies. Claudin-3 and claudin-4 proteins were visualized using DAB. Sections were counterstained with hematoxylin and visualized by light microscopy. *Scale bar*, 100 μm. *Pkj,* purkinje cells; *PC*, pyramidal cells; *Cap,* capillary; *BV,* blood vessel; *NG,* neuroglial cell; *Ep,* ependyma; *CP,* choroid plexus.

Table 1. Expression of claudin-3 and claudin-4 in carcinoma metastasis to the brain*

Origin of metastasis	Cases with claudin-3 expression/total cases (%)	Cases with claudin-4 expression/total cases (%)
Breast carcinoma	7/9 (77.8%)	8/9 (88.9%)
Lung carcinoma	7/10 (70%)	8/10 (80%)
Colon carcinoma	6/7 (86%)	6/7 (86%)
Renal cell carcinoma	0/8 (0%)	2/8 (25%)
Melanoma	1/8 (12.5%)	1/8 (12.5%)

NOTE: Data are compiled from immunohistochemical analysis of whole paraffin-embedded sections.

to the sections and incubated for 30 min. Vectastain ABC reagent was then added for 30 min. Claudin-3 and claudin-4 proteins were visualized using 3,3′-diaminobenzidine (DAB) as per the manufacturer's instructions (Vector). Sections were then counterstained in hematoxylin (Richard-Allan Scientific) for 10 s. Lastly, sections were dehydrated through graded ethanol, cleared in xylene, mounted, and cover slipped. Images were acquired by light microscopy.

Western blotting. Total protein was extracted from cell lines using lysis buffer consisting of 15% glycerol, 5% SDS, and 250 mmol/L Tris-HCl (pH 6.7). Total protein from human brain and spinal cord tissue was obtained from BioChain. Equal amounts of protein from cell and tissue lysates were resolved using 12% SDS-PAGE (Invitrogen). Protein was transferred to enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham). After Western transfer, membranes were probed with antihuman claudin-3 (Invitrogen), antihuman claudin-4 (Invitrogen), or β-actin (Amersham) antibody diluted 1:1,000 (claudin-3 and claudin-4) or 1:5,000 (β-actin). Horseradish peroxidase–conjugated antibody against rabbit or mouse IgG (Amersham) was used at 1:1,000, and binding was revealed using ECL (Amersham).

Animal studies. Female athymic nude mice were purchased from the animal production area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD), and female *neu*-N mice were kindly provided by Drs. R. Todd Reilly and Elizabeth Jaffee (Johns Hopkins University School of Medicine). Animals were maintained in specific pathogen-free barrier animal facilities approved by the American Association for Accreditation of Laboratory Animal Care. Institutional Animal Care and Use Committee approval was obtained for the project. Tumors were generated in 6-week-old to 8-week-old animals. On day 0, mice were given 2.5×10^5 MDA-MB-468 or 5×10^3 NT2.5-luc cells in complete media by intracranial injection. On day 5, animals were randomly assigned to two groups of 10 mice each and given either 0.5 μg CPE or PBS by intracranial injection. Treatment was then repeated on days 7 and 9. Intracranial injections were done as follows: mice were anesthetized, and a midline incision was made, followed by a 2 mm burr hole centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture. Once the dura was exposed, mice were transferred to a stereotactic frame, and a 26-gauge needle containing the material to be injected in a total volume of 5 μL was inserted into the brain to a depth of 3 mm and delivered over a 1-min period. The needle was then removed, the area was washed with sterile saline, and the incision was closed. Because tumor treatments were delivered through the same burr hole as that used for tumor cell administration, therapy was presumed to be centered on the tumor bed. All intracranial injections were done in a biosafety cabinet conforming to industry standards to maintain a sterile environment. For bioluminescent imaging, anesthetized mice were given 150 mg/kg D-luciferin i.p. Five minutes after injection, images were acquired using an IVIS 200 system (Xenogen). Bioluminescent signal was measured as photons per second per square centimeter in defined regions of interest using Living Image software (Xenogen). Mice were observed on a daily basis for any symptoms of tumor burden, including sluggishness, lack of grooming, hemiparesis, and weight loss. Mice were euthanized when moribund and brains were removed and fixed in 10% neutral buffered formalin for histologic examination by H&E staining. Differences in survival between experimental groups were analyzed using the log-rank test.

Results

Expression of claudin-3 and claudin-4 in brain metastasis and normal brain tissue. Several primary tumor types (e.g., breast carcinoma) have been reported to express claudin-3 and claudin-4, conferring sensitivity to CPE-mediated cytolysis (1). To determine whether claudin-3 and claudin-4 are expressed after metastasis to the brain, immunohistochemical analysis was done on cases of human brain metastasis originating from five common sources, including breast carcinoma, lung carcinoma, colon carcinoma, renal cell carcinoma (RCC), and melanoma (Fig. 1). Claudin-3 and claudin-4 were expressed in the majority of brain metastases from breast (≥77.8%), lung (≥70%), and colon (86%) carcinoma, while infrequently expressed in cases of RCC (≤25%) and melanoma (12.5%) brain metastasis (Table 1). Claudin-3 and claudin-4 expression were absent in adjacent normal brain tissue, consistent with the restriction of claudin-3 and claudin-4 expression to epithelial cells.

To more closely examine claudin-3 and claudin-4 expression throughout the CNS, immunohistochemical analysis was done on

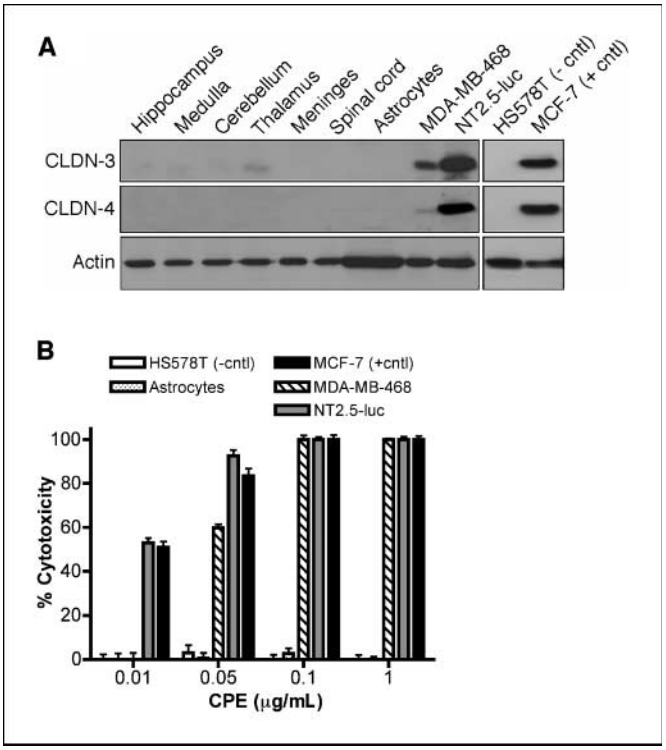

Figure 2. *A,* Western analysis was done on equal amounts of protein from total cell lysates using claudin-3 (*CLDN-3*), claudin-4 (*CLDN-4*), and actin antibodies. MCF-7 and HS578T breast cancer cells served as positive and negative controls (*cntl*), respectively. *B,* sensitivity of astrocytes, MDA-MB-468, and NT2.5-luc breast cancer cells to CPE-mediated cytolysis *in vitro*. MCF-7 and HS578T breast cancer cells served as positive and negative controls, respectively. Cells were incubated with or without CPE at concentrations of 0.01 to 1 μg/mL for 60 min. Total cells were pooled and counted using a hemacytometer, and cell viability was determined by trypan blue dye (0.4%) exclusion. *Columns,* percentage of cytotoxicity from representative experiments done in triplicate; *bars,* SD.

human tissues from a variety of regions of the brain and spinal cord, including hippocampus, medulla, cerebellum, choroid plexus, ependyma, thalamus, meninges, and spinal cord (Fig. 1). Claudin-3 and claudin-4 expression was absent in all CNS tissue with the exception of the modified ependymal cells that make up the epithelial lining of the choroid plexus. Although detectable, claudin-3 and claudin-4 expression in the choroid plexus seemed greatly reduced relative to that observed in brain metastases. Interestingly, claudin-3 and claudin-4 were not detectable in the ependymal cells comprising the adjacent ependyma. Consistent with immunohistochemical analysis, claudin-3 and claudin-4 expression was low or absent in all regions of CNS tested by Western analysis (Fig. 2*A*). Based on immunohistochemical analysis, the low expression of claudin-3 observed in hippocampus, medulla, cerebellum, and thalamus may be due to the presence of choroid plexus in these tissues. Taken together, these data indicate that claudin-3 and claudin-4 are frequently expressed in breast, lung, and colon carcinoma metastasis to the brain but absent in the majority of CNS tissue.

Susceptibility of brain cells to CPE-mediated cytolysis. Astrocytes are the most abundant glial cell and are believed to play a primary role in the formation of the BBB (2). By Western analysis, astrocytes lack detectable expression of claudin-3 and claudin-4 similar to the majority of brain cell types (Fig. 2*A*). Although various cell types lacking claudin-3 and claudin-4 have been shown to be resistant to CPE-mediated cytolysis (10, 14), the effect of CPE on brain cells has not been characterized. To determine whether brain cells are sensitive to CPE-mediated cytolysis, astrocytes and breast cancer cell lines with (MCF-7, MDA-MB-468, NT2.5-luc) and without (HS578T) detectable expression of claudin-3 and claudin-4 were treated with concentrations of CPE ranging from 0.01 to 1 μg/mL for a period of 60 min. After CPE treatment, all of the cells in the culture dish were counted, and percentage of cytotoxicity was determined by trypan blue dye exclusion (Fig. 2*B*). Astrocytes and HS578T cells were completely resistant to the cytotoxic effects of CPE, corresponding with the lack of claudin-3 and claudin-4 expression. Conversely, breast cancer cell lines expressing claudin-3 and claudin-4 (MCF-7, MDA-MB-468, NT2.5-luc) underwent rapid and virtually complete cytolysis in a dose-dependent fashion after CPE treatment. Furthermore, sensitivity to CPE-mediated cytolysis correlated with the level of claudin-3 and claudin-4 expression evidenced by increased cytolysis at lower concentrations of CPE in MCF-7 and NT2.5-luc cells relative to MDA-MB-468 cells. Thus, consistent with previous studies in cell lines from other tissues types (10–12), susceptibility to CPE-mediated cytolysis seems to be directly dependent on the expression of claudin-3 and claudin-4, rendering the majority of brain cells resistant to the cytotoxic effects of CPE.

Efficacy of CPE in the treatment of brain metastasis. To test the efficacy of CPE in the treatment of brain metastasis, two murine models of breast cancer brain metastasis were developed. In the first model, xenograft tumors were established in athymic nude mice using the human breast cancer cell line MDA-MB-468. In the second model, syngeneic tumors were established in *neu*-N mice using the murine mammary carcinoma cell line NT2.5, which was originally derived from a spontaneous mammary tumor in this strain (15). To allow noninvasive monitoring of tumor burden in this model by bioluminescent imaging, a NT2.5 stable clone expressing firefly luciferase (NT2.5-luc) was generated. Stereotactic administration of MDA-MB-468 or NT2.5-luc cells into the cerebral cortex of mice resulted in the formation of invasive tumors in all animals and yielded survival times ranging from 18 to 20 and 22 to 27 days, respectively. Although admittedly dissimilar from the clinical situation, these models allow intratumoral drug administration to

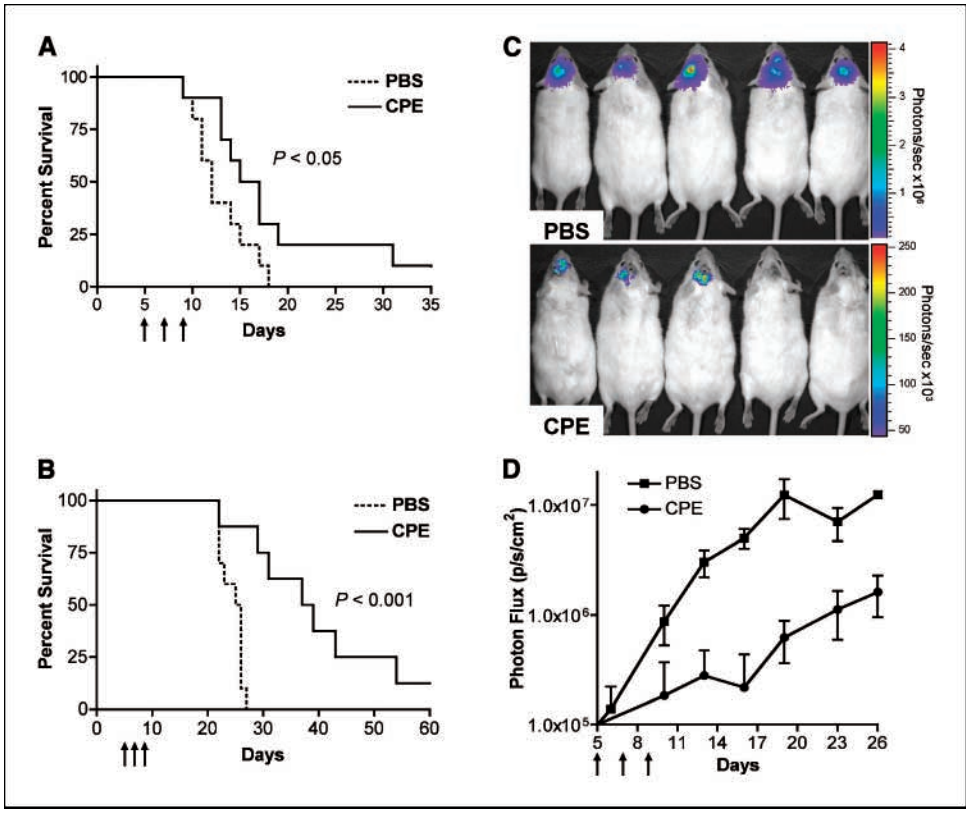

Figure 3. Efficacy of CPE in the treatment of breast cancer brain metastasis. *A* and *B,* brain tumors were established in mice using the human breast cancer cell line MDA-MB-468 and the murine breast cancer cell line NT2.5-luc. Tumors were treated by intracranial administration of 0.5 μg CPE versus PBS on days 5, 7, and 9. *C* and *D,* for the NT2.5-luc brain tumor model, noninvasive bioluminescent imaging was done twice per week beginning on day 4. Bioluminescent images from five representative mice are shown for each experimental group at day 19 (*C*). Photon flux was measured over the indicated time course as an indication of tumor growth (*D*). Differences in survival between experimental groups were analyzed using the log-rank test. *Arrows* (*A, B*, and *D*), dates of treatment.

be modeled accurately and rapidly, without the complication of tumor development in other organs. Furthermore, because the pattern of claudin-3 and claudin-4 expression in mice is similar to humans and confers equivalent sensitivity to CPE *in vitro*, these models allow the assessment of local and systemic toxicity of CPE.

To examine the efficacy of CPE in the treatment of breast cancer metastasis to the brain, MDA-MB-468 and NT2.5-luc brain tumors were established as described in Materials and Methods. Tumors were allowed to grow for 5 days, at which time microscopic tumor foci were evident by histologic analysis (data not shown). Mice were then treated with a total of three doses of either 0.5 μg CPE or PBS given every other day by stereotactic injection at the tumor inoculation site. This dose and schedule of CPE administration were found to be the maximum tolerated in mice. Elevated or more frequent dosing resulted in toxic shock within 1 to 2 h consistent with systemic exposure as reported in prior studies (16). After treatment, mice were observed daily over a period of 6 months and sacrificed when moribund. After sacrifice, brains were removed for histologic examination. CPE treatment resulted in a significant increase in survival time relative to animals treated with PBS, with one animal surviving the entire duration of each study (Fig. 3*A* and *B*). With CPE treatment, animals harboring MDA-MB-468 brain tumors showed a 33% increase in median survival time ($P < 0.05$) from 12 to 16 days, whereas animals harboring NT2.5-luc brain tumors showed a 49% increase ($P < 0.001$) from 25.5 to 38 days. The increased efficacy of CPE treatment in animals harboring NT2.5-luc versus MDA-MB-468 brain tumors correlated with the increased sensitivity of NT2.5-luc cells to CPE-mediated cytolysis relative to MDA-MB-468 cells observed *in vitro* (Fig. 2*B*). In addition, bioluminescent imaging of animals harboring NT2.5-luc brain tumors revealed a significant inhibition of tumor growth ($P < 0.01$) after CPE treatment as determined by quantification of bioluminescent signal (Fig. 3*C* and *D*), consistent with the increased survival observed in these animals (Fig. 3*B*).

Histologic examination of MDA-MB-468 and NT2.5-luc brain tumors revealed large areas of necrosis in all tumors treated with CPE compared with PBS-treated tumors, as evidenced by H&E staining of tumor sections (Supplementary Fig. S1*A* and *B*). Importantly, no toxicity was observed in normal brain tissue including the ependymal cells of the choroid plexus (Supplementary Fig. S1*C*), which displayed low but detectable expression of the CPE receptors claudin-3 and claudin-4 by immunohistochemical analysis (Fig. 1). The lack of sensitivity of choroid plexus to CPE-mediated cytolysis was not due to reduced susceptibility of murine cells to CPE because treatment of NT2.5-luc cells resulted in rapid and virtually complete cytolysis in a dose-dependent fashion, similar to that seen in human breast cancer cell lines (Fig. 2*B*). These results show that CPE treatment of breast cancer tumors in the brain significantly prolongs survival concordant with tumor necrosis, while causing no appreciable local or systemic toxicity.

Discussion

Successful treatment of brain metastasis is challenged by the ability to eliminate tumor cells without causing critical damage to the CNS. Tantamount to achieving this goal is the identification of molecular targets for therapy that are differentially expressed between tumor and normal tissue. Here, we have provided evidence that the CPE receptors claudin-3 and claudin-4 may represent such targets. Claudin-3 and claudin-4 were consistently expressed in breast, lung, and colon cancer brain metastasis while low or absent throughout all regions of the CNS tested. Furthermore, intracranial administration of CPE in mice harboring breast tumors in the brain significantly extended survival without any apparent damage to host CNS tissue.

Despite the potential of CPE in the treatment of brain metastasis, its clinical utility faces challenges common to that of all protein and chemotherapeutics. The primary issue is identifying an appropriate delivery method. Systemic administration of many therapeutics has proved ineffective in the treatment of brain metastasis due to the inability of drugs to effectively cross the BBB despite leakiness caused by tumor burden. Furthermore, due to expression of claudin-3 and claudin-4 in numerous organs, systemic administration of CPE would likely result in significant toxicity, as had been observed in mice (16). Opportunely, local administration into the brain may minimize this issue because the ability of CPE to cross the BBB should be limited based on its molecular weight of 35 kDa. Thus, the BBB could provide an advantage by inhibiting systemic exposure after intracranial CPE administration.

Although just three applications of CPE were sufficient to prolong survival in our study, a complete response was only achieved in 1 of 10 mice. Thus, to be effective and practical, CPE would likely need to be delivered in a fashion allowing continued treatment of brain metastasis while requiring minimal medical procedures. Local drug delivery systems, such as the Ommaya reservoir and Infusaid pump, are currently being used in the clinic to achieve this goal, although the efficacy of these systems can be limited by mechanical failure, obstruction of delivery, and infection. Another approach offering localized, sustained drug delivery without significant side effects is the use of biodegradable polymer wafers (e.g., Gliadel), which can be implanted after surgical resection (17). Where surgical intervention is not indicated, biodegradable polymer nanospheres, because of their small size (≥200 nm), could be used to deliver CPE to brain tumors by stereotactic or regional intraarterial injection (18). In the case of leptomeningeal metastasis, delivery of CPE into the cerebrospinal fluid by intrathecal administration may be appropriate. Although these drug delivery methods may be effective in the treatment of solitary metastasis, treatment of multiple brain metastases, which occurs in 60% to 85% of patients, will likely require novel strategies.

Although the clinical application of CPE faces several challenges, it also has several distinct advantages. The differential expression of claudin-3 and claudin-4 between brain metastasis and CNS tissue not only create a potential therapeutic window for CPE, but also present the opportunity for metastasis detection and treatment through antibody-based methods. In addition, the ability of CPE to down-regulate the tight junction barrier through binding to claudin-3 and claudin-4 (19) may potentiate standard chemotherapy by improving tumor penetration. In conclusion, these preliminary studies suggest that CPE therapy may be applicable to a wide variety of brain metastases without CNS toxicity, warranting further investigation into this treatment modality and setting the stage for the development of effective methodology for the slow, sustained delivery of CPE.

Acknowledgments

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