SELECTIVE DISRUPTION OF THE BLOOD-BRAIN BARRIER BY PHOTOCHEMICAL INTERNALIZATION

Henry Hirschberg^{a, b} Michelle J. Zhang^b, Michael H Gach^c, Francisco A. Uzal^d, David Chighvinadze^b, and Steen J Madsen^{b,e}

 ^aBeckman. Laser Institute, University of California/Irvine, Irvine, CA
^bDept. of Health Physics and ^eUNLV Institute of Cellular and Molecular Medicine, University of Nevada, Las Vegas, Las Vegas, NV
^eNevada Cancer Institute Las Vegas NV
^dSchool of Veterinary Medicine, University of California, Davis, San Bernardino, CA

Abstract

Introduction: Failure to eradicate infiltrating glioma cells using conventional treatment regimens results in tumor recurrence and is responsible for the dismal prognosis of patients with glioblastoma multiforme (GBM). This is due to the fact that these migratory cells are protected by the blood-brain barrier (BBB) which prevents the delivery of most anti-cancer agents. We have evaluated the ability of photochemical internalization (PCI) to selectively disrupt the BBB in rats. This will permit access of anti-cancer drugs to effectively target the infiltrating tumor cells, and potentially improve the treatment effectiveness for malignant gliomas.

Materials and Methods: PCI treatment, coupling a macromolecule therapy of *Clostridium perfringens (Cl p)* epsilon prototoxin with $AlPcS_{2a}$ -PDT, was performed on non-tumor bearing inbred Fisher rats. T1-weighted post-contrast magnetic resonance imaging (MRI) scans were used to evaluate the extent of BBB disruption which can be inferred from the volume contrast enhancement.

Results: The synergistic effect of PCI to disrupt the BBB was observed at a fluence level of 1 J with an intraperitoneal injection of Cl p prototoxin. At the fluence level of 2.5J, the extent of BBB opening induced by PCI was similar to the result of PDT suggesting no synergistic effect evoked under these conditions.

Conclusion: PCI was found to be highly effective and efficient for inducing selective and localized disruption of the BBB. The extent of BBB opening peaked on day 3 and the BBB was completed restored by day 18 post treatment

Photonic Therapeutics and Diagnostics V, edited by Nikiforos Kollias, Bernard Choi, Haishan Zeng, Reza S. Malek, Brian Jet-Fei Wong, Justus F. R. Ilgner, Kenton W. Gregory, Guillermo J. Tearney, Laura Marcu, Henry Hirschberg, Steen J. Madsen, Proc. of SPIE Vol. 7161, 716136 · © 2009 SPIE · CCC code: 1605-7422/09/\$18 · doi: 10.1117/12.810552

Introduction

Gliomas represent 40% of all primary brain tumors, contributing up to 78% of all malignant brain tumor cases. The continued poor prognosis for these patients is mainly due to the aggressive infiltrating nature of these tumors. In most cases glioma cells have already infiltrated 2-3 cm into the surrounding normal brain at the time of bulk tumor resection [1]. These infiltrative tumor cells are protected by the blood-brain barrier (BBB) which normally prevents harmful substances from entering the brain [2,3]. Unfortunatly few anti-cancer drugs can effectively cross this barrier to target the infiltrating tumor cells [4]. Failure to eradicate infiltrating glioma cells inevitably results in tumor recurrence and further treatments are usually palliative in scope. Therefore, destruction of infiltrating tumor cells is a crucial step for curing malignant gliomas. This cannot be accomplished until methods are developed to: (1) deliver drugs across the BBB at a selected site, or (2) selectively disrupt in a site specific manner this protective barrier. The BBB is formed by tightly connected brain capillary endothelial cells. The impermeability of the BBB is the result of a number of unique features of this barrier. Firstly, the physical restriction imposed by tight junctions between endothelial cells greatly reduces paracellular permeability. Additionally, the transport system regulation of endothelial cells limits the number and types of molecules that undergo transcellular transport. Lastly, the metabolic activity of endothelial cells, with powerful enzymes metabolizing many potentially harmful substances, adds to the difficulties faced by molecules trying to penetrate the BBB.

Clostridium perfringens (Cl p) is a rod-shaped, gram-positive bacterium which produces four major protein toxins consisting of alpha, beta, epsilon and iota toxins In particular, the Cl p epsilon toxin and its prototoxin are of interest in this study, since they are known for their ability to cause widespread opening of the BBB [5-8]. Several studies support the existence of an epsilon toxin receptor in the brain capillary endothelial membrane. Through binding to specific receptor sites on the brain endothelial membranes, epsilon toxin causes changes and damage to the vascular endothelium resulting in BBB disruption.

Photochemical internalization (PCI) is a novel technology that is under development for utilizing the properties of PDT to enhance the drug delivery of macromolecules in a site-specific manner. PCI as a drug delivery technology has many advantages [9]. There are no restrictions on the size of the molecules that can be effectively delivered, making PCI highly suitable for a wide variety of molecules. 2) PCI also exhibits high site-specificity, which limits the biological effect to only illuminated areas and lowers the potential systemic side effects of the delivered drug. 3) PCI is a method that increases the therapeutic efficacy of a wide range of macromolecules allowing for the possibility of using lower drug doses to minimize morbidity. 4) PCI is well suited for combination with other modalities or strategies for targeted drug delivery, thus increasing the potential for further therapeutic improvements The efficacy of PCI-mediated delivery of Cl p epsilon

prototoxin for selective opening of the BBB in inbred Fischer rats has been investigated in this study. Since the overall objective of the proposed work is to use PCI to produce localized opening of the BBB, only low concentrations of Cl p were administered, i.e., concentrations sufficiently below the threshold for BBB opening. Disruption of the BBB was achieved by combining sub-threshold doses of Cl p with sub-threshold PDT light fluences, i.e., the so-called PCI effect. Magnetic resonance imaging was used to infer the extent of BBB disruption and to track BBB dynamics following each treatment procedure. Specifically, gadolinium contrast enhancement was used as a marker for BBB disruption.

Materials and methods

Inbred male Fischer rats (Simonsen Laboratories, Inc, Gilroy, CA) weighting about 350 g were used in this study. The animal care and protocol were in accordance with institutional guidelines. For the surgical procedures, the animals were anaesthetized with Pentobarbital (25 mg/kg i.p.). Buprenorphin (0.08 mg/kg s.c.) as a post-operative analgesic was administered to animals following surgery and twice per day for three days thereafter. All animals were euthanized at the end of the study or at the first signs of distress. The euthanasia procedure was accomplished with an overdose of Pentobarbital (100 mg/kg i.p.).

PCI Treatment Protocol

Animal was injected with the photosensitizer $AlPcS_{2a}$ (1 mg/kg, i.p.). 48 hr later, *Cl p* prototoxin was administered intraperitoneally at a concentration determined to be non-toxic. At the time of light treatment, anaesthetized rats were fixed in a stereotactic frame. A skin incision was made exposing the skull and an optical fiber was placed in contact with the surface of the skull 1mm posterior from the bregma and 2mm to the right of the midline. Surface light irradiation was given approximately 60 minutes after *Cl p* administration at light fluence levals of 0.5,1.0 and 2.5 J at a fluence rate of 10mW. Following treatment the wound was closed with sutures and the rats freed from the frame. PDT controls consisted of photosensitizer and light treatment as described above but in the absence of Clp.

MR Imaging

Animals were imaged in a 7.0 T animal MR scanner (Bruker) acquired on days 1, 3, 5, 8 and 18 post treatments. Animal was anesthetized using Isoflurane. A small surface coil was placed on top of the target area and 0.8 ml of a gadolinium-based contrast agent (Multihance), was injected i.p. T1-weighted (TR = 700 ms; TE = 14 ms) post contrast MR image were taken 15 – 20 min after contrast injection. Since Multihance, with a molecular weight of 1058.2, is too large to cross the intact BBB, any contrast enhancement evident on T1-weighted images was taken as direct evidence of BBB disruption induced by the corresponding treatment.

Data analysis

Contrast volume and intensity was analyzed using OsiriXVP software on a Mac OS platform. Contrast content was manually outlined on each T1 contrast slice. Contrast volume was calculated according to the following equation: $V=\Sigma(Si _ 0.15) \text{ cm3}$ where Si represents the area calculated on each 1.5 mm thick slice where contrast could be discerned.

Histological preparation

Animals were sacrificed 21 days following PDT treatment and their brains extracted. The brains were sectioned in two at the position of light delivery and fixed by immersion in 10 % buffered (pH 7.2) formalin prior to paraffin embedding. Four micrometer thick coronal sections were obtained from the original cut surface representing the position of light application. The sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope by an independent pathologist blinded to the treatment modes.

Results

The effects of increasing light fluence levels for Clp/ PCI on the BBB was performed using light fluences of 0.5,1 and 2.5J and a Cl p prototoxin concentration of 1:100 (Figure 1). The T1-weighted post contrast MRI scans of a typical response to treatment is shown in the figure. As can be seen, increasing fluence levels resulted in an increasing contrast volume. PCI induced contrast volume was significantly greater than that resulting from PDT alone (ie no Clp). Although the PDT effect at 0.5 and 1J was negligible this was not the case at 2.5J where a clear albeit smaller BBB disruption was apparent compared to that seen in the precence of Clp.



Fig 1 T1-weighted post contrast MRI scans after PCI treatment for 3 different fluence levels. All T1 post contrast images were taken 15 minutes following i.p. contrast injection.

Fig 2 shows the time course of BBB disruption at a fluence of 1J for both PDT (light + photosensitizer but no Clp) and Clp-PCI. BBB disruption peaked at day 3 and was generally gone by day18.



Figure 2. Comparison of T1-weighted post contrast MRI scans after PDT or PCI treatment. All animals received a light fluence of 1 J; the PCI treated animals in addition to light received an i.p. injection of Cl p at a concentration of 1:100. All animals were scanned on days 1, 3, 5 and 18 after treatment. T1 MR images were taken 15 minutes following i.p. contrast injection. The n values listed above each data set indicate the number of animals used in each group.

Histological analysis In areas exposed to either PDT or Clp-PCI, in the proximity of light application no significant pathology was observed in coronal sections obtained from animals subjected to fluences of 0.5J or 1J. At higher fluence levels of 2.5 J, areas with clear tissue damage, large numbers of infiltrating gitter cells and gliosis was observed. Edema and chronic hemorrhage with hemosiderin loaded macrophages were also present as well as blood vessels showing hyperplastic endothelial cells (data not shown).

Discussion

The inherent tendency of glial tumors to infiltrate into normal brain tissue has set a limit to the effects of conventional treatment. Although surgical resection reduces the pressure effects of the bulk tumor, it is the diffusely invading tumor cells, well beyond the resection margin, that continue to grow and migrate causing damage to normal brain parenchyma. Most importantly, migratory cells are protected to varying degrees by the blood brain barrier (BBB) which acts as a formidable barrier against systemic delivery of chemotherapeutic agents. The drug levels observed in gliomas are likely due to passive diffusion across a compromised BBB found in these tumors. In contrast, the concentration of drugs or photosensitizer in infiltrating cells is contingent on the drugs molecule's ability to cross the BBB and selectively accumulate in these cells [12,13].

The results of the experiments described in this work demonstrated that PCI of Clp was more effective in opening the BBB in a limited region of the brain than PDT at low fluence levels (fig 2).

The data presented in Figure 1 show that increasing light fluences (0.5-2.5 J) resulted in increasing BBB disruption. In a recent study we have shown that ALA mediated PDT at relativly high fluence levels (9-26J) could also result in BBB degradation [14]. In this case the BBB opened after 2hours but was essentially closed 72hours later. In contrast the time course for PCI of Clp BBB opening was much more protracted (fig2) peaking at day three or four and closing by day 14. This would provide an effective long interval for drug delivery.

PCI is a multi-step procedure and, in addition to light fluence, this therapeutic modality requires effective timing for optimal effects. First, the timing between photosensitizer administration and prototoxin delivery is important: a sufficient amount of $AlPcS_{2a}$ must be present in endothelial cells of the target region prior to Cl p injection. If the time interval is too short, the concentration of photosensitizer in the plasma membranes may be insufficient. Similarly, long time intervals may result in the clearance of photosensitizer prior to prototoxin administration. Previous animal studies suggest that a 48 h interval is sufficient. Second, the timing between prototoxin administration and light irradiation is also important. If the interval is too short, the number of endosomeencapsulated Cl p molecules will be insufficient for the PCI effect. Conversely, a long time interval increases the likelihood that Cl p-laden endosomes will be captured by lysosomes resulting in degradation of the prototoxin. The results of other investigators suggest that time intervals between 1 and 4 hours are optimum[15].

In comparison with osmotic opening of the BBB disruption, the biggest advantage of the PCI effect on the BBB is its site selectivity. The osmotic opening method involves the administration of a hypertonic solution (e.g. mannitol) to cause shrinkage of the endothelial cells resulting in the disruption of tight junctions and non-selective opening of the BBB [16]. This global disruption of the BBB is problematic because it allows passage of a wide variety of molecules into the brain. Even though an i.p. injection of Cl p prototoxin was used in the PCI procedure, and this injection method can lead to a global distribution of prototoxin, the low concentration of prototoxin used (1:100 dilution) was unable to cause BBB disruption in the absence of light treatment. The effect of Cl p on the BBB is therefore limited to regions of the brain exposed to adequate light fluences. The results of the present study suggest that, under the appropriate conditions, PCI is a safe and efficient method for the selective disruption of the BBB in rats. Due to Cl p

toxicitiy, it is highly unlikely that *Cl p*-based PCI approaches will be used in human clinical protocols. Nevertheless, the results of the present study provide the basis for further PCI studies using non-toxic vasoactive compounds including bradykinin, leukotrienes and histamine[17].

Acknowledgment

The authors are grateful for the support of the Nevada Cancer Institute which sponsored this research through the NVCI Collaborative. Henry Hirschberg is grateful for the support of the Norwegian Radiumhospital Research Foundation.. Portions of this work were made possible, inpart, through access to the Laser Microbeam and Medical Program (LAMMP) and the Chao Cancer Center Optical Biology Shared Resource at the University of California, Irvine. These facilities are supported by the National Institutes of Health under grants RR-01192 and CA-62203, respectively.

References

- 1. Wallner, KE, Galicich, JH, Krol, G, Arbit, E, Malkin, MG. Patterns of failure following treatment for glioblastoma multiforme and anaplastic astrocytoma. Int. J. Radiat. Oncol. Biol. Phys. 1989; 16:1405-1409.
- Ballabh P, Braun A and Nedergaard M. The blood-brain barrier: an overview: Structure, regulation, and clinical implications. Neurobiology of Disease 2004; 16:1-13.
- 3. Huber J, Egleton R and Davis T. Molecular physiology and pathophysiology of tight junctions in the blood–brain barrier. Trends in Neurosciences 2001; 24:719-725.
- 4. Abbott N. Physiology of the blood-brain barrier and its consequences for drug transport to the brain. International Congress Series 2005; 1277:3-18.
- 5. Worthington R and Mulders M. The effect of Clostridium perfringens epsilon toxin on the blood brain barrier of mice. Onderstepoort Journal of Veterinary Research 1975; 42:25-28.
- 6. Nagahama M and Sakurai J. Distribution of labeled Clostridium perfringens epsilon toxin in mice. Toxicon 1991; 29:211-217.
- Zhu C, Ghabriel M, Blumbergs P, Reilly P, Manavis J, Youssef J, Hatami S and Finnie J. Clostridium perfringens Prototoxin-Induced Alteration of Endothelial Barrier Antigen (EBA) Immunoreactivity at the Blood–Brain Barrier (BBB). Experimental Neurology 2001; 169:72–82.

- Dorca-Arévalo J, Soler-Jover A, Gibert M, Popoff M, Martín-Satué M and Blasi J. Binding of epsilon-toxin from Clostridium perfringens in the nervous system. Veterinary Microbiology 2008; 131:14-25.
- 9. Berg K, Bommer J and Moan J. Evaluation of sulfonated aluminum phthalocyanines for use in photochemotherapy. Cellular uptake studies. Cancer Letters 1989; 44:7-15.
- Prasmickaite L, Høgset A, Selbo P, Engesæter B, Hellum M and Berg K. Photochemical disruption of endocytic vesicles before delivery of drugs: a new strategy for cancer therapy. British Journal of Cancer 2002; 86:652-657.
- Berg K, Høgset A, Prasmickaite L, Weyergang A, Bonsted A, Dietze A, Lou P, Bown P, Norum O, Mali H, Møllergård T and Selbo PK. Photochemical internalization (PCI): A novel technology for activation of endocytosed therapeutic agents. Medical Laser Application 2006; 21:239-250.
- 12. Pardridge W. The Blood-Brain Barrier: Bottleneck in Brain Drug Development. NeuroRX 2005; 2:3-14;
- 13. Abbott N and Romero I. Transporting therapeutics across the blood-brain barrier. Molecular Medicine Today 1996; 2:106-113.
- Hirschberg H, Uzal FA, Chighvinadze D, Zhang MJ, Peng Q, Madsen SJ. Disruption of the blood-brain barrier following ALA-mediated photodynamic therapy. Lasers Surg Med. 2008 Oct;40(8):535-42
- 15. Høgset A, Prasmickaite L, Selbo P, Hellum M, Engesæter B, Bonsted A and Berg K. Photochemical internalisation in drug and gene delivery. Advanced Drug Delivery Reviews 2004; 56:95-115.
- 16. Doolittle N.D, Miner M.E, Hall W.A, Siegal T, Jerome E, Osztie E, McAllister L.D, Bubalo J.S, Kraemer D.F, Fortin D, Nixon R, Muldoon L.L, Neuwelt E.A: Safety and efficacy of a multicenter study using intra-arterial chemotherapy in conjunction with osmotic opening of the blood-brain barrier for the treatment of patients with malignant brain tumors. Cancer. 88(3):637-47, (2000
- 17. Matsukado K, Sugita M and Black K. Intracarotid low dose bradykinin infusion selectively increases tumor permeability through activation of bradykinin B2 receptors in malignant gliomas. Brain Research 1998; 792:10-15.