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Remote spatiotemporally controlled and biologically selective permeabilization of blood-brain barrier

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ABSTRACT

The blood–brain barrier (BBB), comprised of brain endothelial cells with tight junctions (TJ) between them, regulates the extravasation of molecules and cells into and out of the central nervous system (CNS). Overcoming the difficulty of delivering therapeutic agents to specific regions of the brain presents a major challenge to treatment of a broad range of brain disorders. Current strategies for BBB opening are invasive, not specific, and lack precise control over the site and timing of BBB opening, which may limit their clinical translation. In the present report, we describe a novel approach based on a combination of stem cell delivery, heat-inducible gene expression and mild heating with high-intensity focused ultrasound (HIFU) under MRI guidance to remotely permeabilize BBB. The permeabilization of the BBB will be controlled with, and limited to where selected pro-inflammatory factors will be secreted secondary to HIFU activation, which is in the vicinity of the engineered stem cells and consequently both the primary and secondary disease foci. This therapeutic platform thus represents a non-invasive way for BBB opening with unprecedented spatiotemporal precision, and if properly and specifically modified, can be clinically translated to facilitate delivery of different diagnostic and therapeutic agents which can have great impact in treatment of various disease processes in the central nervous system.

1. Introduction

The blood–brain barrier (BBB), comprised of brain endothelial cells (BECs) with tight junctions (TJ) between them, regulates the extravasation of molecules and cells into and out of the central nervous system (CNS) [1,2]. Although the BBB serves to restrict the entry of potentially toxic substances into the CNS in its neuroprotective role, it constitutes a permeability barrier that hinders the delivery of many potentially important diagnostic and therapeutic agents to the brain [3]. Therapeutic molecules and antibodies that might otherwise be effective for diagnosis and therapy cannot cross the BBB in adequate amounts. It is believed that BBB excludes from the brain ~100% of large-molecule neurotherapeutics and more than 98% of all small-molecule drugs [4]. Overcoming the difficulty of delivering therapeutic agents to specific regions of the brain presents a major challenge to treatment of a broad range of brain disorders such as brain cancers, Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis, and neurological manifestations of acquired immune deficiency syndrome (AIDS) [5–7].

Several strategies have been developed to modulate BBB permeability to systemically administered therapeutic drugs. The available strategies for BBB opening may be broadly classified as chemical or physical-based BBB disruptions, or drug modifications that facilitate passage through an endogenous BBB. For example, osmotic agents can temporarily shrink brain endothelial cells and loosen the tight junctions to allow hydrophilic substances to diffuse into the brain [8,9]. Intracarotid infusion of mannitol has been clinically used to enhance chemotherapeutic drug penetration of the BBB in patients with malignant primary brain tumors or metastases [10]. Because of the technical challenges and risk of brain and ocular injury, this approach is used in only a few medical centers. The bradykinin agonist, RMP-7, has been extensively studied for increasing BBB permeability [11]. RMP-7 (combined with carboplatin) increased the delivery of carboplatin into intracranial

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brain tumors in mouse models [12]. However, it demonstrated unreliable curative effects for patients with glioma, and has not been approved for clinical therapy [13]. More recently, activation of adenosine receptor (AR) signaling in brain endothelial cells by adenosinergic drugs has been reported to increase BBB permeability, enabling both small and large molecules to enter the brain [14]. Physical disruption of the BBB is the oldest but most invasive method to open BBB. High-intensity focused ultrasound (HIFU) with or without microbubbles has been developed to use mechanical effects to disrupt the BBB [15–19]. Drug modification has also been extensively studied to increase BBB entry. To achieve this goal, drug molecules can be modified to be more lipophilic to increase the likelihood of BBB penetration [20], or can be packaged in a vector that crosses the BBB by receptor-mediated endocytosis [21]. However, drug modification is limited by chemical properties of individual drugs or receptor expression on endothelial cells in BBB.

Given the lack of a universally successful method of penetrating the BBB to deliver therapeutics, there is a great need to develop a translatable method which allows precise spatiotemporal control down to microscopic level to facilitate the entry of drugs across the BBB for treatment of neurological diseases. Recent reports have demonstrated remote activation of engineered cells in vivo by light [22–24], radio waves [22] or ultrasound [25] using inducible promoters to control transgene expression. Among them, spatial and temporal control of gene expression has been made with local heat deposition via focused ultrasound coupled with the use of thermosensitive promoter [25–27]. A recent study also demonstrated the feasibility to locally activate in vivo transgene expression of genetically engineered cells by focused ultrasound under MR-guidance (MR-HIFU) [25,26]. Furthermore, transcranial magnetic resonance-guided focused ultrasound (tcMRgFUS) technology has been used in clinical trial for the non-invasive treatment of various brain disorders such as essential tremor, and neuropathic pain proving that it is clinically possible to precisely deliver heat to target areas of the brain non-invasively [28–30].

Due to their capacity to migrate to disease sites, stem cells are emerging as feasible vehicles to therapeutically target different diseases in the central nervous systems and other sites [31–34]. One major challenge that limits the translational potential of stem cells as therapeutic vehicles is the fact that in addition to migrating towards the targeted diseased sites, stem cells are also attracted towards normal areas in the body that may be harmed if the stem cells non-selectively deliver or express highly toxic therapies [35].

We propose to use a combination of stem cell delivery, heat-inducible gene expression and mild heating with HIFU to remotely permeabilize BBB with unprecedented spatiotemporal precision. We propose to administer stem cells engineered to express pro-inflammatory factors that will permeabilize the BBB but only trigger their expression after heating with non-invasive image-guided HIFU. With this approach, the permeabilization of the BBB will be limited to where selected pro-inflammatory factors will be secreted secondary to HIFU activation, which is in the vicinity of the engineered stem cells and consequently both the primary and secondary disease foci. If successful, this degree of spatial and temporal precision in BBB permeabilization will be unprecedented. Our proposed approach can potentially be used as a remote controlled spatiotemporally precise platform technology for selectively permeabilizing the BBB which can be used to facilitate diagnosis and treatment of many CNS diseases.

2. Materials and methods

2.1. Animals

8-week old adult male athymic nude rats (~240 g, Harlan, Indianapolis, IN, USA) were used. All animal experiments were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) at the Wake Forest University School of Medicine.

2.2. Lentiviral plasmids

The 400 bp minimal human HSP70B promoter was a kind gift from Dr. Crit Moonen of Université Victor Ségalen, France [27]. The heat-inducible gene expression system was custom made from lentivrial plasmids by Gentarget (San Diego, CA). The lentivrial plasmid containing a rous sarcoma virus promoter (RSV) and dual fusion marker RFP-Blasticidin, was used as the backbone for cloning. The HSP70 sequence was ligated into the lentiviral plasmid, and the green fluorescence protein (GFP) or human TNFα codon sequence were cloned under HSP70 promoter to produce heat-inducible plasmids (Fig. 1A). The sequence verified constructs were packaged as lentiviral vectors in HEK293-TLV cells by using lentiviral packaging plasmids. The produced lentivector has a titer around 1×10^7 IFU/ml measured by ELISA assay.

2.3. Cell culture and in vitro studies

Human bone marrow mesenchymal stem cells (MSCs, Lonza, Walkersville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Jurkat cells and B16F10 cells were cultured in RPMI1640 medium supplemented with 10% FBS. Mouse neuron stem cells (NSCs) were cultured in the NeuroCult™ NS-A proliferation medium according to manufacturer's instruction (Stemcell Technologies Inc., Vancouver, Canada).

For gene transduction, 20 μl of lentiviral particles (mixed with polybrene at a 1:1 ratio) were placed in the media of cells in a 24-well plate and centrifuged (1200 RPM at 32 °C) for 60 min and subsequently placed overnight under normal cell culture conditions (37 °C/5% CO_2). After 24 h, the medium was replaced and cells were incubated at 37 °C for a further 48 h. Stable transfected cells were selected with 100 μg/ml blasticidin. For validation and optimization of heat-induced gene expression in vitro, stable transfected cells placed in Eppendorf tubes were precisely heated at 37 °C–45 °C and for different durations using a PCR cycler. The cells were then placed in 24-well plate in 6 replicates and incubated at 37 °C in a 5% CO_2 incubator for recovery. Luciferase activity was measured 14 h after heating induction by adding D-luciferin using a microplate reader (Biotek Instruments, USA). Bioluminescence imaging was also taken using a Xenogen IVIS 100 imaging system. To validate TNFα expression after heat induction, MSCs engineered with HSP70 (F-Luc-2A-TNFα) were induced at 43 °C for up to 20 min and incubated at 37 °C for 14 h. Cell culture supernatants were collected, centrifuged for 10 min at 3000 g to remove floating cells and debris, and stored at −80 °C. Supernatants were analyzed for TNFα by ELISA using a human TNFα ELISA kit according to the manufacturer's instructions (Thermo Scientific, IL, USA). The plates were read on a plate reader (Biotek Instruments, USA) at 450 nm (reference filter 550 nm) within 30 min. The luciferase activity was measured after adding D-luciferin. The minimum detectable concentration of TNFα was 2.0 pg/ml.

2.4. Intracranial stem cell implantation

Rats were anesthetized with an i.p. injection of ketamine (50 mg/ml) and xylazine (2.6 mg/ml), and placed in a small animal stereotaxic apparatus affixed with a microinjector unit (Harvard Apparatus, Holliston, MA). A 1-cm-long midline incision was made in the scalp, beginning midway between the eyes and terminating behind the lambda. A 3 mm burr hole was created with a surgical drill (Harvard Apparatus, Holliston, MA) 1.5–2 mm right of the midline and 0.5–1 mm posterior to the coronal suture through the scalp incision. For stem cell injections, a 10 μl syringe (Hamilton Co., Reno, NV) with a 30-gauge needle was mounted onto the injection pump and the needle positioned directly over the bregma. The x, y, and z axis coordinates were all set to zero. The needle was then positioned at the entry point, 0.5 mm posterior and 3.0 mm lateral of the bregma to the right. The needle was slowly inserted into the right ventricle to a depth of 4 mm below the surface of the skull, and the MSCs (1.0×10^6 cells in 5 μL of PBS) were injected