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Systemic dendrimer-drug treatment of ischemia-induced neonatal white matter injury

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ABSTRACT

Extreme prematurity is a major risk factor for perinatal and neonatal brain injury, and can lead to white matter injury that is a precursor for a number of neurological diseases, including cerebral palsy (CP) and autism. Neuroinflammation, mediated by activated microglia and astrocytes, is implicated in the pathogenesis of neonatal brain injury. Therefore, targeted drug delivery to attenuate neuroinflammation may greatly improve therapeutic outcomes in models of perinatal white matter injury. In this work, we use a mouse model of ischemia-induced neonatal white matter injury to study the biodistribution of generation 4, hydroxyl-functionalized polyamidoamine dendrimers. Following systemic administration of the Cy5-labeled dendrimer (D-Cy5), we demonstrate dendrimer uptake in cells involved in ischemic injury, and in ongoing inflammation, leading to secondary injury. The sub-acute response to injury is driven by astrocytes. Within five days of injury, microglial proliferation and migration occurs, along with limited differentiation of oligodendrocytes and oligodendrocyte death. From one day to five days after injury, a shift in dendrimer co-localization occurred. Initially, dendrimer predominantly co-localized with astrocytes, with a subsequent shift towards microglia. Co-localization with oligodendrocytes reduced over the same time period, demonstrating a region-specific uptake based on the progression of the injury. We further show that systemic administration of a single dose of dendrimer-N-acetyl cysteine conjugate (D-NAC) at either sub-acute or delayed time points after injury results in sustained attenuation of the 'detrimental' pro-inflammatory response up to 9 days after injury, while not impacting the 'favorable' anti-inflammatory response. The D-NAC therapy also led to improvement in myelination, suggesting reduced white matter injury. Demonstration of treatment efficacy at later time points in the postnatal period provides a greater understanding of how microglial activation and chronic inflammation can be targeted to treat neonatal brain injury. Importantly, it may also provide a longer therapeutic window.

1. Background

Extreme prematurity, defined as a gestational age of <28 weeks or a birth weight of <1500 g, affects up to 2% of all newborns in the United States. A high incidence of adverse neurological outcomes in this group calls for increased research in neuroprotective strategies [1,2]. Many of these infants (20%) develop cerebral palsy (CP), and about 50% will develop cognitive, behavioral, attention, or socialization deficits of variable degree [3–7]. Epidemiological studies suggest that neuroinflammation is associated with perinatal/neonatal white matter injury, the main underlying neuropathology in this patient population, and that active inflammatory processes may prevent regeneration and/or exacerbate brain damage in the premature infant [8]. This may sensitize the brain to further injury, due to long lasting microglial activation and increased expression of inflammatory cytokines [5–8]. Strategies to target and treat neuroinflammation, mediated by microglia and astrocytes, can potentially slow disease progression, and increase the therapeutic window while enabling normal development [9,10]. Therefore, activated microglia/macrophages and astrocytes are potent therapeutic targets. However, delivery of drugs for the treatment of diffuse brain injury in the neonate is a major challenge. Interestingly, since microglia/astrocytes are more phagocytic in this activated state [11], they may also be more amendable to selectively uptake 'small' nanoparticles like dendrimers, compared to other cells in the brain [12,13].

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Nanomedicine–based delivery strategies are emerging as promising candidates for the treatment of inflammatory disorders. Nanotechnologies can improve drug bioavailability and targeting, and provide sustained efficacy. Specifically, dendrimers are unique nanoparticles for targeted drug/gene delivery, including cancer and systemic inflammation [14–20]. The small size and high density of tailorable surface functional groups may provide significant advantages for therapeutic delivery to the brain [15,21]. PAMAM dendrimers are the mostly widely studied class of dendrimers due to their commercial availability, and have been shown to cross the impaired blood–brain-tumor [18] and blood–brain barriers (BBB) in animal models [17]. In a model of intrauterine inflammation, generation-4 hydroxyl (G4-OH) PAMAM dendrimers have recently been shown to accumulate significantly in the gut and brain of fetal mice following intra-amniotic administration [22]. In the presence of cardiac ischemia, G4 PAMAM dendrimer-small molecule conjugates have shown promise in reducing ischemia–reperfusion injury and improving recovery of cardiac function [23,24]. In the post-ischemic brain, Kim et al. have utilized PAMAM dendrimer mediated transfection of siRNA to primary neurons and glia to reduce infarct volume following a middle cerebral artery occlusion [25]. More recently, G4-OH dendrimer conjugated with N-acetyl cysteine (D-NAC) has been used in combination with dendrimer conjugated valproic acid (D-VPA) for the treatment of hypothermic circulatory arrest (HCA)-induced brain injury in a canine model [26]. Importantly, Kannan et al. have shown attenuation of the activated microglia response and improvement in the motor phenotype in a maternal inflammation-induced model of CP, following a single systemic administration of D-NAC in newborn kits with CP three days after the insult [17].

In each of these models, dendrimer uptake and localization is specific to the cells involved in ongoing injury and inflammation, at that time point. In the HCA-induced brain injury model, dendrimer accumulation was predominantly seen in dying and injured neurons, and activated microglia, when the dendrimer was administered immediately after the insult [26]. However, in the rabbit model of CP, when the dendrimer is administered three days after injury, at birth, dendrimer uptake was mostly associated with cells involved in ongoing inflammation in this disease (activated astrocytes and microglia), rather than in neurons [17]. To better design and appropriately time the administration of therapeutics, it is important to understand where the dendrimer ends up, and more specifically, what cells it localizes in. As seen in these previous models of brain injury, the cellular localization of dendrimer is dependent to some extent on the model, due to differences in disease etiology, disease progression, and also the timing of dendrimer administration in relation to the disease progression. Dendrimer distribution within the body, clearance, and brain uptake is also governed by dendrimer physicochemical properties [21]. Nanoparticle movement within the brain parenchyma and uptake by specific cells is impacted by nanoparticle size and surface functionality [27]. Comprehensive understanding of both global (whole body) and local (brain) biodistribution of a nanoparticle platform is crucial for better design and optimization of therapeutic platforms that can enhance efficacy, even in aggressive disease models [28].

In this study, we utilize a mouse model of ischemia-induced neonatal white matter injury to determine the biodistribution and brain uptake of the dendrimer at both sub-acute and delayed treatment time points. We chose this model since ischemia is thought to play a key role in neonatal white matter injury [29], and we have previously demonstrated post-ischemic neuroinflammatory response with microglial activation in this model [30]. Based on the temporal and spatial cellular localization of the dendrimer in cells involved in injury, we then performed a preliminary efficacy study to evaluate a potential therapeutic window for administering D-NAC therapy and to determine if similar efficacious results can be obtained later in the postnatal period in the presence of ongoing inflammation. This study provides a greater understanding of the therapeutic window for treatment of D-NAC in ischemic neonatal brain injury. This information will allow more rapid translation among similar models of disease, in this case neonatal white matter injury, and development of a more efficacious platform for clinical translation.

2. Materials and methods

2.1. Dendrimer-Cy5 and D-NAC preparation and characterization

We have previously studied the phosphate-buffered saline (PBS) and plasma stability and drug release mechanisms of both the D-NAC conjugates used here, and other dendrimer-drug conjugates [26]. At physiological conditions (PBS 7.4, 37 °C), and in plasma (37 °C), the D-disulfide-NAC conjugate was stable, and did not release NAC over a 24 h period. However, at intracellular GSH concentrations (2 and 10 mM), the conjugate released the drug readily within 3.5 h, indicating that use of a disulfide-linker enables rapid release of NAC from the conjugate only when it is exposed to an intracellular GSH-rich environment [31,32].

2.2. Mouse model of ischemia-induced neonatal white matter injury

Five-day old (P5) CD-1 mouse pups underwent permanent unilateral carotid artery ligation while kept mildly hypoxic under anesthesia for 15 min (transcutaneous oxygen saturation between 85–90%), followed by recovery in a 36 °C incubator for 30–60 min. These mice exhibit bilateral myelin pallor, ipsilateral ventriculomegaly, axonal injury, and astrogliosis, along with apoptosis of oligodendrocyte progenitor cells (PreOLs) and arrested maturation of PreOLs. This results in a decrease in mature oligodendrocyte numbers, as seen in patients [30,33].

2.3. Immunohistochemistry and image analysis for biodistribution study

Newborn mice that underwent unilateral carotid ligation on P5 (ligated group) and normal healthy age-matched controls, were administered 55 mg/kg of D-Cy5 intraperitoneal (i.p.) at P6 (sub-acute) or P10 (delayed) time points. Animals were euthanized at 24 h after D-Cy5 administration, and perfused with normal saline. Brain sections were stained for microglia/macrophages (goat anti-mouse Iba1 with donkey anti-goat IgG Alexa488), astrocytes (rat-anti-GFAP with donkey anti-rat IgG Alexa488), and oligodendrocytes (anti-CC1 with donkey anti-mouse IgG Alexa488). Confocal z-stack merged images, 20 μm thick, were obtained using a Zeiss LSM 710 microscope to detect co-localization.

2.4. Quantification of dendrimer uptake in the brain

Newborn mice that underwent unilateral carotid ligation on P5 (ligated group) and normal healthy age-matched controls were administered 55 mg/kg of D-Cy5 intraperitoneal (i.p.) at 'sub-acute' or 'delayed' time points. Animals were euthanized at 24 h after D-Cy5 administration, and perfused with normal saline ($n = 5$/group/time point). Ipsilateral and contralateral hemispheres were evaluated separately by HPLC. We have developed a sensitive method for tissue quantification with detection limits as low as 100 ng of D-Cy5/g of tissue (~0.001% of injected dose in brain, typically better than that for radiolabeled dendrimers). This method combines total fluorescence, fluorescence-HPLC chromatograms, and fluorescence/absorbance spectrum measurements, following appropriate tissue extraction, and prior calibration in each of the organs with known amounts of D-Cy5 [26,34].

2.5. NAC therapy for efficacy study

Newborn mice that underwent unilateral carotid ligation on P5 (ligated group) were systemically (i.p.) administered 10 mg/kg of