Non-invasive Lesion Imaging and Delivery of Nano-Therapeutics to the Ischemic Brain
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ABSTRACT
Ischemic brain microlesions are often asymptomatic, but can cause irreversible neurodegenerative damage if they remain undetected and untreated. Inflammatory processes, involving activated microglia, are triggered at and around ischemic lesion sites. Early detection of microlesions using fluorescent nanocrystals (quantum dots), together with therapeutic interventions, utilizing anti-inflammatory agents, may provide an effective nano-theranostic approach for ischemic stroke therapy.

Keywords: ischemic stroke, inflammation, quantum dot, minocycline, nimodipine

INTRODUCTION
“Silent strokes” or silent brain infarcts are now recognized as common cerebral lesions prevalent in the elderly population. Silent infarcts are asymptomatic, with no corresponding stroke episodes; however, these ischemic microlesions are shown to be associated with long-term, adverse health outcomes, often developing into strokes and causing neurodegenerative damage if they remain undetected and untreated [1]. At the ischemic core of the lesion, cells undergo rapid irreversible cell death, but viable cells in the penumbra (area bordering the ischemic core) can be protected and rescued with early interventions. Presently, there are no cures for stroke, and the only therapeutic option available is thrombolytic therapy (e.g. tissue plasminogen activator), which is only effective within hours of stroke onset. The lack of effective therapies is largely due to inadequate understanding of the neurodegenerative processes occurring at and around the lesion. Ongoing research suggest that multiple factors and cell types (e.g. astroglia, microglia) contribute to the neurotoxic changes in the ischemic core and penumbra regions; resulting in different modes of cell death concurrently [2]. Several classes of pharmacological agents are currently being investigated pre-clinically as potential therapeutics for stroke, including antioxidants, anti-apoptotic agents, glutamate receptor inhibitors, and anti-inflammatory agents [3].

Excessive inflammation is an early and key factor propagating neuronal death in a number of neurodegenerative diseases including ischemic stroke [4]. Inflammatory responses in the brain involve the activation of resident microglia, which have been recognized as the immune cells of the central nervous system, as they are described to express properties of monocytes [5]. In response to distressful stimuli or disturbances in the microenvironment, microglial cells become “activated” in an attempt to adapt to this change and to maintain the balance for the surrounding neurons. Activated microglia are known to have a dual role in mediating neuroprotective or neurotoxic signals depending on the extent of their activation. Once activated, microglia can produce trophic factors and exert neuroprotection to neighboring neural cells. On the other hand, in response to unrelenting stimuli, fully activated microglia can release a plethora of inflammatory factors (e.g. reactive oxygen species, nitric oxide, interleukin-1β and tumor necrosis factor-α), which in excess, can accumulate and aggravate the neural damage.

As inflammatory microglia play an important part in the pathogenesis of ischemia [6] and other neurodegenerative diseases, a number of therapeutic agents, targeting the microglia, are currently being investigated as potential treatments [4]. Of these anti-inflammatory candidates, minocycline and nimodipine are especially interesting due to the enhanced efficacy observed when used in combination (as a drug cocktail) to treat ALS [7] and ischemic stroke [8] in vivo.
Figure 2. Nano-therapeutics exert significant anti-inflammatory effects in LPS-induced microglia. Microglia were treated with LPS, and NO release was measured using the Griess Reagent (Sigma). (A) Cells treated concomitantly with minocycline as a free drug (free min) and incorporated into micelles (micelle-min) produced significantly less nitric oxide than cells treated with LPS alone. (B) Microglia treated with LPS and nimodipine as a free drug or incorporated into micelles also released less NO than LPS treatment alone. Mean values ± SEM are calculated based on triplicate measurements from independent experiments. *** indicates statistical significance of \( p < 0.001 \).

Minocycline is a second-generation tetracycline antibiotic routinely administered orally for the treatment of infectious and inflammatory diseases [9]. Recent studies also show that minocycline is an effective neuroprotective agent against hypoxic-ischemic stress in neonate brains [10, 11]. However, due to the serious systemic side effects (especially in the liver) observed at neuroprotective doses in vivo, it has been recommended to administer minocycline intravenously for brain delivery, despite the observed reduction in drug stability in the blood stream [12]. There is, thus, a need to use a drug carrier to improve the bioavailability of minocycline as a neuro-therapeutic agent.

Nimodipine, a dihydropyridine calcium channel blocker, has been shown to exert neuroprotection in cerebrovascular diseases including subarachnoid haemorrhage, traumatic brain injury and ischemic stroke [13–15]. Due to its lipophilic nature, nimodipine has a low bioavailability (<10% in the brain) when administered in the body, and undergoes extensive first pass metabolism in the liver. By enhancing the solubility of the drug using a carrier, its efficacy as a neuroprotective agent may also be greatly improved. We, therefore, propose to incorporate minocycline and nimodipine into polymeric micelles; (i) to overcome drug solubility and bioavailability issues in vivo, and (ii) to deliver these nano-therapeutics at effective doses to the ischemic brain.

Early detection of ischemic lesions is also essential for effective drug interventions [6]. Current methods for the detection of microlesions are mostly based on perfusion and hemorrhage imaging [16], and utilize high-end technology such as computed tomography (CT) scanning and magnetic resonance imaging (MRI); however these techniques are still limited in spatial resolution and the procedures are often very costly [17]. We propose here an alternative method for non-invasive imaging of lesions in the brain using fluorescent quantum dot (QD) nanocrystals.

Altogether, the objective of this study is to image brain microlesions with QDs and to deliver anti-inflammatory nano-therapeutics to the ischemic brain in vivo.

MATERIALS and METHODS

Near-infrared emitting CdSe/ZnS QDs (Qdota705, Invitrogen), surface-modified with polyethylene glycol (PEG), were selected for the imaging studies. Minocycline and nimodipine were incorporated into carboxymethyl-dextran-b-PEG micelles [18] and polycaprolactone-PEG2 miktoarm micelles [19] respectively (Figure 1). A hairless mouse model (SKH-1, Charles River) was selected for the imaging studies, and microlesions were induced mechanically by unilateral cortical devascularization. QDs were injected either intranasally or intravenously (tail vein). Animals were anaesthetized with isoflurane, and live imaging was done using the Optix MX3 system (Advanced Research Technologies). Immunohistochemical and biochemical analyses (i.e. cytokine release, nitric oxide release) were used to evaluate therapeutic effectiveness in: (i) an in vitro model of inflammation (e.g. lipopolysaccharide) using a microglia cell line (N9), (ii) an in vitro model of ischemia (e.g. oxygen glucose deprivation) using primary cortical cultures, and (iii) an in vivo animal model of ischemic brain lesion.

RESULTS and DISCUSSION

Microglial activation is often determined based on cellular morphological transformation (e.g. from ramified to amoeboid or even phagocytic), and the release of inflammatory mediators and cytokines [5]. Lipopolysaccharide (LPS) is a well-characterized inducer of microglial activation [20]. Binding of LPS to TLR4 receptors on the cell surface will trigger subsequent
intracellular signals including NF-κB activation, p38 kinase activation and increased expression of inducible nitric oxide synthase [21]. LPS was used to induce a microglial cell line (N9 cells) in vitro, and nitric oxide (NO) release was measured to determine the extent of inflammation (Figure 2). Cells treated concomitantly with LPS (10 μg/ml, 24h) and minocycline (50 μg/ml), either as a free drug or incorporated into CMD-micelles, produced significantly less NO when compared with cells treated with LPS alone (Figure 2A, p<0.001). As minocycline, in both forms, reduced the LPS-stimulated NO release to similar levels; this suggests that (i) the drug is released readily from the micelles, and (ii) drug activity is not altered due to micelle encapsulation, and can still exert potent anti-inflammatory effects [18].

Similarly, cells treated concomitantly with LPS and nimodipine (10 μM) released significantly less NO when compared to cells treated with LPS alone (Figure 2B, p<0.001); however, in this case, there is a distinct difference in the extent of NO reduction between the free nimodipine- and micellar nimodipine-treated cells, such that the free drug completely prevented the LPS-stimulated NO release. This data, later complemented by kinetics studies [19], suggests that nimodipine is perhaps released from the micelles at a slower rate than minocycline. Controlled nimodipine release is an important attribute, as micelle-nimodipine did not induce cytotoxic responses in microglia, in contrast to the free drug treatment [19].

In vivo, deep tissue fluorescence imaging is difficult to perform as the autofluorescence from animals (e.g. fur, biological fluids) is very high and often interferes with the signal from the fluorescent probe. Quantum dot nanocrystals, especially those emitting in the near-infrared (NIR) region, possess optimal photophysical properties (e.g. high photostability, high fluorescence intensity) that can overcome some of the limitations in live animal imaging [22]. Here we show that QDs administered in a non-invasive manner, via the intranasal cavity, can reach the brain rapidly, and QD fluorescence can be measured and quantified in live animals (Figure 3). Moreover, QD fluorescence may potentially be an indicator of the extent of lesion damage in the brain, as QD fluorescence is significantly enhanced in the lesioned brain compared to the normal brain. Lesioned animals treated with anti-inflammatory nano-therapeutics (i.e. minocycline- or nimodipine-containing micelles) have less QD fluorescence in the brain, suggesting potential therapeutic effects of the micellar drugs against ischemic lesions (Figure 4). Behavioral assessments complemented the preliminary findings from these imaging experiments.

Taken altogether, the results from these studies show that (i) QDs administered intranasally can localize in brain lesions rapidly; (ii) QD fluorescence can be used to quantify and correlate with the extent of the lesions, and (iii) micellar minocycline and nimodipine have significant anti-inflammatory effects both in vitro and in vivo.

**SIGNIFICANCE**

Small amounts of QDs can be used to detect cerebral microlesions non-invasively. Micellar anti-inflammatory drugs provide a novel and effective therapeutic alternative for ischemic injury. Results from this study point towards the development of a nano-anti-inflammatory theranostic tool based on fluorescent nanocrystals.

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REFERENCES


