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Hydrogel delivery of erythropoietin to the brain for endogenous stem cell stimulation after stroke injury

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ABSTRACT

Drug delivery to the brain is challenging because systemic delivery requires high doses to achieve diffusion across the blood-brain barrier and often results in systemic toxicity. Intracerebroventricular implantation of a minipump/catheter system provides local delivery, yet results in brain tissue damage and can be prone to infection. An alternate local delivery strategy, epi-cortical delivery, releases the biomolecule directly to the brain while causing minimal tissue disruption. We pursued this strategy with a hyaluronan/methyl cellulose (HAMC) hydrogel for the local release of erythropoietin to induce endogenous neural stem and progenitor cells of the subventricular zone to promote repair after stroke injury in the mouse brain. Erythropoeitin promotes neurogenesis when delivered intraventricularly, thereby making it an ideal biomolecule with which to test this new epi-cortical delivery strategy. We investigated HAMC in terms of the host tissue response and the diffusion of erythropoeitin therefrom in the stroke-injured brain for neural repair. Erythropoietin delivered from HAMC at 4 and 11 days post-stroke resulted in attenuated inflammatory response, reduced stroke cavity size, increased number of both neurons in the peri-infarct region and migratory neuroblasts in the subventricular zone, and decreased apoptosis in both the subventricular zone and the injured cortex. We demonstrate that HAMC-mediated epi-cortical administration is promising for minimally invasive delivery of erythropoeitin to the brain.

1. Introduction

Stroke is the fourth leading cause of death in the world and causes 15 million debilitating injuries each year [1]. Stroke is caused by a disruption in blood supply to the brain, and is classified as either ischemic or hemorrhagic. In ischemic stroke, decreased blood flow results in an insufficient supply of nutrients to cells in the core, which leads to necrotic cell death. A secondary phase of injury subsequently occurs in the tissue surrounding the core (the penumbra), resulting in apoptosis [1].

There is currently no cure for stroke and the only clinically proven drug is tissue plasminogen activator, an anti-thrombolytic agent used to reduce the extent of injury [2]. However, this treatment does not afford tissue regeneration. A number of neuroregenerative strategies have shown improved functional recovery in animal models of stroke, including stem cell transplantation and endogenous stem cell stimulation. For the latter, exogenous factors are delivered to the brain to stimulate endogenous neural stem/progenitor cells (NSPCs) in the subventricular zone (SVZ) to promote tissue repair after injury [3]. Endogenous NSPC stimulation with erythropoietin (EPO) has resulted in NSPC proliferation, migration, and maturation, as well as promoting regeneration and replacement of cells and tissues lost following stroke injury [4,5].

EPO is a 30.4 kDa glycoprotein that has been shown to be both neuroprotective and neuroregenerative after injury to the central nervous system (CNS) [6,7], and it has been shown to reduce stroke cavity size clinically [8]. In the brain, EPO binds with the erythropoietin receptor (EPOr), which is expressed on multiple cell types including neurons, astrocytes, and NSPCs in the SVZ [7]. When bound to EPOr on NSPCs, EPO promotes their survival and differentiation into mature neurons [9].

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One limitation in using EPO, as well as a number of other growth factors, for stimulating endogenous brain repair after stroke is the lack of appropriate delivery systems. Systemic delivery of proteins, by intravenous or intranasal delivery, results in less than 1% crossing the blood-brain barrier BBB [10]. Moreover, intravenous delivery can lead to systemic toxicity at high concentrations [11,12] while chronic intranasal delivery is associated with systemic toxicity, patient discomfort, low patient compliance and thus sub-optimal therapeutic benefit [13,14]. Local drug delivery strategies typically involve the insertion of a cannula or drug delivery scaffold into the brain tissue, both of which are highly invasive [15,16]. The optimum paradigm involves delivering drugs from the cortical surface using a minimally invasive strategy, controlling the release and transport of the drugs such that they reach the SVZ, and maintaining the bioactivity of the drugs in order that they stimulate NSPCs upon reaching the SVZ.

With a view toward circumventing the blood-brain barrier and achieving delivery to the brain, we asked whether EPO could be delivered locally to the stroke-injured brain using an injectable hydrogel and what the brain host tissue response would be to this hydrogel. The physically cross-linked blend of hyaluronan (HA) and methyl cellulose (MC) (HAMC) is bioresorbable, injectable through a fine needle, and gels rapidly at physiological temperature [17]. HAMC spatially localizes the drug of interest at the site of delivery and facilitates short-term controlled release to the CNS [18,19].

One of the major limitations of delivering protein drugs to the brain is the fast rate of protein elimination and the consequent short penetration distance. Unmodified proteins often do not penetrate more than 1 mm in the uninjured brain, and penetration distance decreases significantly after brain injury because stroke injury results in the upregulation of many protein receptors in the brain [18,20]. Modification of proteins with poly(ethylene glycol), PEG, has been used to increase protein penetration distance [18,21–23].

Here we studied the diffusion of EPO from the HAMC hydrogel, delivered directly to the cortex, in the uninjured as well as stroke-injured mouse brain. In order to understand the kinetics of EPO penetration in the brain, EPOr expression was examined at the time points of EPO delivery: 4 and 11 days post-stroke. These two time points were selected because previous reports have delivered EPO at these time points, albeit using a highly invasive cannula/mini-pump system, and shown that in mice models of ischemia, cortical EPOr upregulation increases between 1 and 7 days post ischemia [24], and persists up to 28 days [25]. The host tissue response of EPO delivered from HAMC was examined in terms of: NSPC stimulation/migration and neurogenesis; and the inflammatory response by immunohistochemical staining for macrophages/microglia and astrocytes.

2. Materials and methods

2.1. Materials

Recombinant human erythropoietin (EPREX) was supplied by Ortho Biotech Canada (Toronto, ON, Canada). Sodium hyaluronan (HA, 1.4–1.8 $\times$ 10^6 g/mol) was purchased from NovaMatrix (Sandvika, Norway). Methyl cellulose (MC, 3.4 $\times$ 10^5 g/mol) was obtained from Shin Etsu (Chiyoda-ku, Tokyo, Japan). Mouse anti-human Ki-67 was purchased from BD biosciences (Mississauga, ON, Canada), mouse anti-rat NeuN and GFAP were obtained from Millipore Inc. (Billerica, MA, USA), rat anti-mouse $CD68^+$ and rabbit anti-mouse double-cortin were obtained from Abcam (Cambridge, MA, USA), and Vectashield with DAPI stain was purchased from Vectorlabs (Burlington, ON, Canada). Alexa 488 goat-anti-rat, Alexa 488 and 586 goat-anti-rabbit IgG, and Alexa 568 goat-anti-mouse IgG were obtained from Invitrogen Inc. (Burlington, ON, Canada). Sodium cyanoborohydride ($NaCNBH_3$), NaCl, $MgCl_2$, $CaCl_2$, $BaCl_2$, Na_2HPO_3, NaH_2PO_3, trehalose, and cresyl violet acetate were supplied by Sigma Aldrich (Oakville, ON, Canada). Triton X-100 was supplied by ACROS (NJ, U.S.A.). Artificial cerebrospinal fluid (aCSF [23]) and all buffers were prepared with distilled and deionized water prepared from a Millipore Milli-RO 10 Plus and Milli-Q UF Plus at 18 MΩ m resistivity (Millipore, Bedford, USA). Recombinant EPO ELISA kit was purchased from BD biosciences (Mississauga, ON, Canada).

2.2. Preparation of sterile HAMC hydrogel

HA and MC were dissolved separately in dH_2O at 4 °C overnight, sterile-filtered and lyophilized. The resulting sterile powders were kept at 4 °C until use. HAMC was prepared with 1.1% HA and 2.2% MC in sterile aCSF and mixed using a SpeedMixer (DAC 150 FVZ, Siemens). Immediately prior to injection, 100 μl of sterile EPO solution (10,000 U/ml) was added to 900 μL HAMC (yielding a final concentration of 1% HA and 2% MC), mixed and centrifuged to eliminate air bubbles.

2.3. In vitro release of EPO from HAMC

The time required for EPO to diffuse out of HAMC was determined in vitro. EPO was mixed into HAMC to yield a final concentration of 1000 U/ml, and 100 μl was injected into the bottom of a 2 ml eppendorf tube and gelled at 37 °C for 20 min. 900 μl of aCSF at 37 °C was added. The supernatant was completely replaced at each of the following time points and EPO concentrations determined using ELISA: 0, 30 min, 1, 2, 4, 6, 8, 12, 24 h.

2.4. Stroke surgeries and injection of drug delivery device

All animal work was carried out in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care) and approved by the Animal Care Committee at the University of Toronto. A total of 54 C57BL/6 mice (aged 9–11 weeks) were used in this study (Charles River, QC, Canada).

Stroke surgeries were carried out as described previously [26]. Mice were anesthetised with isoflurane, shaved and placed into a Kopf stereotaxic instrument. Scalps were cleaned and a midline incision made next, a small burr hole was made in the skull at the coordinates 2.25 lateral to the midline and 0.6 anterior to Bregema. Using a 26G needle, 1.0 μl of the vasoconstrictor endothelin-1 (400 pM, Calbiochem, Gibbstown, NJ, USA.) was injected 1.0 ventral to the brain surface at 0.1 μl/min. The needle was left in place for 10 min prior to removal. The incision was sutured, antibiotic ointment applied and animal recovered under a heat lamp.

The drug delivery system was injected at either day 4 or day 11 post-stroke (Supplementary Fig. S1). The sutures were removed to expose the stroke site and any tissue debris was removed. The drug delivery device was prepared as described previously [18] to spatially localize HAMC at the cortical surface. A disk with 2 mm opening was fixed over the burr hole with bone glue. 3 μl of either HAMC containing EPO or HAMC alone was injected into the hole such that the gel is in direct contact with the brain cortical surface. A second disk with no opening was fixed over the first spacer. The skin was sutured over the spacer system. For uninjured animals, the surgery was identical (without the stroke injury itself) to that of stroke-injured mice.

2.5. Analysis of in vivo protein penetration

Animals were sacrificed at 4 h, 1 day, and 2 days post injection and the drug delivery device containing HAMC was retrieved. The extracted device was placed into 0.5 ml 0.1% Tween 20 in PBS and agitated overnight at 4 °C overnight to extract any remaining EPO.

Brains were snap frozen with $CO_{2(s)}$ cooled isopentane and stored at −80 °C. A 3 mm coronal section around the injection site was prepared using the McIlwain tissue chopper (790744-11, Mickle laboratory engineering company, Surrey, UK). Dorsal-ventral sections (0.5 mm) were then cryosectioned from each coronal slice using a Leica CM3050S cryostat system. Each section was homogenized in 400 μl lysis buffer (40 mM trehalose, 1% Triton X-100 in dH_2O), and the homogenate supernatant was removed after centrifugation at 15,000 RPM for 15 min at 4 °C.

The amount of protein remaining in HAMC and in the brain homogenate at each time point was determined using a recombinant EPO ELISA kit (BD Biosciences) as per the manufacturer's instructions. The difference between the amounts of protein that remain in HAMC at each time was used to calculate the amount of protein released. We assume that no protein is lost from the entire system during the period of release and that all protein released has diffused into the brain tissue. The amount of EPO in the homogenate was used to generate tissue penetration profiles as well as the protein mass balance at each time point.

2.6. Brain tissue preparation for morphological analysis

At the appropriate time points animals were transcardially perfused with saline followed by 4% paraformaldehyde (PFA). Brains were extracted and fixed in 4% PFA at 4 °C overnight, followed by cryoprotection in 30% sucrose. Cryoprotected brains were snap frozen and cryosectioned to 10 μm.

2.7. Analysis of stroke cavity size

Sections were defatted in 50:50 chloroform:ethanol solution overnight, and rehydrated in 100%, 95%, and dH_2O for 2 min each. Cresyl violet acetate was dissolved at 0.1 w/v % in dH_2O and 0.3 ml of glacial acetic acid was added to 100 ml of