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Biodegradable gelatin microspheres enhance the neuroprotective potency of osteopontin via quick and sustained release in the post-ischemic brain

Yinchuan Jin [a,b], In-Yong Kim [c], Il-Doo Kim [a,b], Hye-Kyung Lee [a,b], Jin-Young Park [d], Pyung-Lim Han [d,e], Kyekyoon Kevin Kim [c,f,g,h,i], Hyungsoo Choi [c,f,*], Ja-Kyeong Lee [a,b,*]

[a] Department of Anatomy, Inha University School of Medicine, Inchon, Republic of Korea
[b] Inha Research Institute for Medical Sciences, Inha University School of Medicine, Inchon, Republic of Korea
[c] Department of Electrical and Computer Engineering, University of Illinois, Urbana, IL 61801, USA
[d] Department of Brain and Cognitive Sciences, Ewha Womans University, Seoul, Republic of Korea
[e] Department of Chemistry and Nano Science, Ewha Womans University, Seoul, Republic of Korea
[f] Micro and Nanotechnology Laboratory, University of Illinois, Urbana, IL 61801, USA
[g] Department of Bioengineering, University of Illinois, Urbana, IL 61801, USA
[h] Department of Material Science and Engineering, University of Illinois, Urbana, IL 61801, USA
[i] University of Illinois Neuroscience Program, Urbana, IL 61801, USA

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ABSTRACT

Gelatin microspheres (GMSs) are widely used as drug carriers owing to their excellent biocompatibilities and toxicologically safe degradation products. The drug release profile is easily tailored by controlling the cross-linking density and surface-to-volume ratio, i.e. size, of the GMS. In this study, we employed GMSs which are 25 μm in diameter and cross-linked with 0.03125% glutaraldehyde, to enable rapid initial and a subsequent sustained release. Therapeutic potency of human recombinant osteopontin (rhOPN) with or without encapsulation into GMSs was investigated after administrating them to rat stroke model (Sprague–Dawley; middle cerebral artery occlusion, MCAO). The administration of rhOPN/GMS (100 ng/100 μg) at 1 h post-MCAO reduced the mean infarct volume by 81.8% of that of the untreated MCAO control and extended the therapeutic window at least to 12 h post-MCAO, demonstrating a markedly enhanced therapeutic potency for the use of OPN in the post-ischemic brain. Scanning electron microscopy micrographs revealed that GMSs maintained the three-dimensional shape for more than 5 days in normal brain but were degraded rapidly in the post-ischemic brain, presumably due to high levels of gelatinase induction. After encapsulation with GMS, the duration of OPN release was markedly extended; from the period of 2 days to 5 days in normal brain, and from 2 days to 4 days in the post-ischemic brain; these encompass the critical period for recovery processes, such as vascularization, and controlling inflammation. Together, these results indicate that GMS-mediated drug delivery has huge potential when it was used in the hyperacute period in the post-ischemic brain.

1. Introduction

Owing to their excellent biocompatibilities and toxicologically safe degradation products [1,2], gelatin microspheres (GMSs) are widely accepted as drug carriers to deliver various growth factors, such as Bone Morphogenetic Protein 2 (BMP-2) [3,4], Vascular Endothelial Growth Factor (VEGF) [4] and Fibroblast Growth Factor (FGF) [5], stem cell [6] and plasmids [7] into tissues of the body to facilitate tissue regeneration and remodeling. Drug molecules bound to the gelatin matrix are released as gelatin degrades enzymatically, and therefore the release profile can be tailored by controlling the cross-linking density and surface-to-volume ratio, i.e. size, of the GMS [8,9]. However, this approach has been frustrated due to the polydispersity of the GMSs fabricated by conventional methods, such as emulsion or spray drying. To this end, the electric-field-assisted precision particle fabrication (E-PPF) method

* Corresponding authors at: Department of Electrical and Computer Engineering, University of Illinois, Urbana, IL 61801, USA. Tel.: +1 217 244 6345; fax: +1 217 265 0957 (H. Choi). Department of Anatomy, Inha University School of Medicine, Inchon, Republic of Korea. Tel.: +82 32 890 0913; fax: +82 32 884 2105 (J.-K. Lee).
E-mail addresses: hyungsoo@illinois.edu (H. Choi), jklee@inha.ac.kr (J.-K. Lee).

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has been developed, enabling the fabrication of hydrogel microspheres, including GMSs, of controlled size [10]. Recently, we reported the encapsulation of High Mobility Group Box 1 (HMGB1) A box in GMS fabricated via the E-PPF method, demonstrating that the GMS encapsulation markedly enhanced the neuroprotective effects of HMGB1 A box in the post-ischemic brain [11].

Osteopontin (OPN) is a phosphorylated glycoprotein, which is a soluble cytokine capable of stimulating signal transduction pathways in many different types of cells [12–14]. OPN is up-regulated in the post-ischemic brain [15,16] and plays a protective role due to its anti-inflammatory, neurogenic and nerve guidance effects [17–19]. OPN was induced in a delayed manner in stroke animal models [15,16], beginning from 24 h and peaking at 5 days; however, supplementing OPN during the early period substantially suppressed infarct formation and mitigated neuronal damage [20,21], indicating a more active role of OPN in a hyperacute period of post-ischemic brain.

To understand the optimal effects of GMS-mediated drug delivery in pathological brains, it is critical to ensure a pathology-reflected delivery of encapsulated protein in the pathological brains. This study was undertaken to examine the neuroprotective efficacy of recombinant human OPN encapsulated in GMSs in a rat model of focal cerebral ischemia. In addition, we presented the degradation profiles of GMSs in the normal and post-ischemic brain as determined using scanning electron micrographs, and the actual amounts of OPN released from GMSs into the parenchyma of normal and ischemic brain, demonstrating the usefulness of GMSs to elaborate the neuroprotective potency of OPN in the post-ischemic brain in a hyperacute period.

2. Materials and methods

2.1. Fabrication of GMS

Type A gelatin (300 g, bloom; Sigma Aldrich, St Louis, MO) was used to fabricate GMSs (diameter 25 μm) using the E-PPF method, as previously described [8,10,11]. Cross-linking was performed using 0.03125% (w/w) of glutaraldehyde (GA) (Sigma Aldrich, St Louis, MO) solution at 4 °C for 24 h, which was followed by quenching with glycine (Sigma Aldrich) at room temperature. The resulting GMSs were washed with deionized water and lyophilized. GMSs were characterized by scanning electron microscopy (SEM) (Hitachi S-4300SE, Hitachi, Japan).

2.2. Preparation of recombinant human OPN-loaded GMSs (rhOPN/GMS)

Recombinant human OPN (rhOPN, R&D Systems, Minneapolis, MN) was encapsulated in GMSs by adding an aqueous solution of rhOPN (0.25 mg ml^{-1}) to dry GMS, and then allowing the suspension to stand for 2 h at room temperature. Just prior to injection, sterile phosphate buffer solution (PBS, pH 7.4) was added to make the total volume of 2.5 μl for each rat. As a control, unloaded dry microspheres were hydrated by adding sterile PBS and the suspension was incubated for 2 h at room temperature and final injection volume was adjusted to 2.5 μl for each rat by adding PBS prior to injection. Encapsulation efficiency was determined by using a solid-phase sandwich ELISA kit (IBL, Fujioka, Japan). In brief, an aqueous solution of rhOPN (0.25 mg ml^{-1}) was added to dry GMS at various ratios (10, 50, 100, 200 ng rhOPN to 100 μg GMS) and then allowing the suspension to stand for 2 h at room temperature. Subsequently, the OPN/GMSs mixture was washed with distilled water (50 μl) two times to remove non-encapsulated rhOPN and supernatants were collected after each washing. After centrifuging at 1000 rpm for 1 min, the supernatant was withdrawn and amounts of rhOPN in the total collected supernatant were measured using the ELISA kit. The loading efficiency of rhOPN/GMSs was calculated using the following formula: Loading efficiency (%) = (total rhOPN − rhOPN in supernatant)/total rhOPN × 100.

2.3. Treatment with rhOPN or rhOPN/GMS

Rats were anesthetized with an intramuscular injection of a mixture of ketamine (3.75 mg/100 g body weight) and xylazine hydrochloride (0.5 mg/100 g body weight), and placed on a stereotaxic apparatus (Narishige Scientific Instrument Lab., Tokyo, Japan). The skull was leveled between bregma and lambda. rhOPN or rhOPN/GMS was injected stereotaxically into the striatum (bregma coordinates (mm): AP, −0.2; ML, −4.0; DV, −5.0) using a 26-G Hamilton microsyringe (80330, Hamilton Company, Reno, NV). For the dose test, 10, 50, 100 or 200 ng of rhOPN was administered at 1 h post-middle cerebral artery occlusion (MCAO). For the temporal test, naked rhOPN (100 ng) or rhOPN/GMS (100 ng/100 μg) was administered 24 h prior to or 1, 6 or 12 h post-MCAO. For the SEM study, a GMS (500 μg)/saline mixture (total 5 μl) was administered at 1 h post-MCAO and for ELISA, naked rhOPN (250 ng) or GMSs (100 μg) loaded with rhOPN (250 ng) was injected at 1 h post-MCAO. To examine long-term protective effects, naked rhOPN (100 ng) or rhOPN/GMS (100 ng/100 μg) was injected at 6 h post-MCAO.

2.4. Surgical procedure used for MCAO

Male Sprague–Dawley rats were housed under diurnal lighting conditions and allowed food and tap water ad libitum. All animal studies were carried out in strict accordance with the recommendations made in the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health and performed in accordance with the ARRIVE guidelines (http://www.nc3rs.org/ARRIVE). The animal protocol used in this study was reviewed and approved by the INHA University-Institutional Animal Care and Use Committee (INHA-IACUC) with respect to ethicality (Approval Number INHA-110321-81). MCAO was carried out as previously described [22]. In brief, male Sprague–Dawley rats (250–300 g) were anesthetized with 5% isoflurane in 30% oxygen/70% nitrous oxide and maintained using 0.5% isoflurane in the same gas mixture during surgery. Occlusion of the right middle carotid artery was induced for 1 h by advancing a nylon suture (4–0; AILEE, Busan, South Korea) with a heat-induced bulb at the tip (~0.3 mm in diameter) along the internal carotid artery for 20–22 mm from the bifurcation of the external carotid artery, which was followed by reperfusion for up to 14 days. The left femoral artery was cannulated for blood sampling to analyze pH, PaO_2, $PaCO_2$ and blood glucose concentration (I-STAT; Sensor Devises, Waukesha, WI). Regional cerebral blood flow (rCBF) was monitored using a laser Doppler flowmeter (Periflux System 5000; Perimed, Jarfalla, Sweden) and the success of occlusion was considered if ⩾70% reduction in cortical CBF occurred immediately after inserting an occluding suture. Animals were excluded if they failed to reduce CBF to less than 30% of the baseline during the MCAO or to restore the blood flow during the reperfusion. A thermoregulated heating pad and a heating lamp were used to maintain a rectal temperature of 37 ± 0.5 °C during surgery. Animals were randomly allocated to a sham (n = 12), MCAO (n = 63), MCAO + GMS-treated (n = 34), MCAO + rhOPN-treated (n = 56) or MCAO + rhOPN/GMS-treated (n = 35) group. Animals allocated to the sham group underwent identical procedures for skin incisions, a hole formation and stereotaxic manipulation but not MCA occlusion. In general, mortality was not observed during the surgery. However, the mortality after surgery is 7.4% (5 out of 68), which might be caused by a