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Neural stem cells encapsulated in a functionalized self-assembling peptide hydrogel for brain tissue engineering

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ABSTRACT

Brain injury is almost irreparable due to the poor regenerative capability of neural tissue. Nowadays, new therapeutic strategies have been focused on stem cell therapy and supplying an appropriate three dimensional (3D) matrix for the repair of injured brain tissue. In this study, we specifically linked laminin-derived IKVAV motif on the C-terminal to enrich self-assembling peptide $RADA_{16}$ as a functional peptide-based scaffold. Our purpose is providing a functional self-assembling peptide 3D hydrogel with encapsulated neural stem cells to enhance the reconstruction of the injured brain. The physiochemical properties reported that $RADA_{16}$-IKVAV can self-assemble into nanofibrous morphology with bilayer β−sheet structure and become gelationed hydrogel with mechanical stiffness similar to brain tissue. The in *vitro* results showed that the extended IKVAV sequence can serve as a signal or guiding cue to direct the encapsulated neural stem cells (NSCs) adhesion and then towards neuronal differentiation. Animal study was conducted in a rat brain surgery model to demonstrate the damage in cerebral neocortex/neopallium loss. The results showed that the injected peptide solution immediately *in situ* formed the 3D hydrogel filling up the cavity and bridging the gaps. The histological analyses revealed the $RADA_{16}$-IKVAV self-assembling peptide hydrogel not only enhanced survival of encapsulated NSCs but also reduced the formation of glial astrocytes. The peptide hydrogel with IKVAV extended motifs also showed the support of encapsulated NSCs in neuronal differentiation and the improvement in brain tissue regeneration after 6 weeks post-transplantation.

1. Introduction

Traumatic brain injury (TBI) occurs when a sudden trauma creates damage to the brain. The severe brain injury may cause extensive tissue loss of cerebral parenchyma which results in cavities formation due to the primary destruction and secondary injuries such as ischemia and inflammation [1]. Although there are many studies for the investigation of molecular and cellular mechanisms of central nervous system regeneration in the past decades, brain injury is almost irreparable due to the poor regenerative capability of neural tissue [2]. By cell/tissue transplantation and preventing tissue loss from primary destruction and secondary pathophysiological injuries, reconstruction of extracellular matrix in brain tissue may be achieved [3,4]. However, it has been restricted by a critical shortage of donors and the high risk of graft rejection.

Given the limitations above, tissue engineering approach may provide an opportunity for injured neural tissue reconstruction. It is necessary to supply an appropriate three dimensional (3D) matrix, or scaffold, for the repair of injured neural tissue especially in brain [5,6]. A promising tissue-engineered scaffold for reconstruction of brain tissue is able to bridge structural gaps, re-knit the injured brain, provide a substrate for neurite outgrowth, and serve as a guiding pathway for endogenous cells migration and axonal elongation [7]. In addition, cells encapsulated in 3D scaffold are often chosen to create a biomimic microenvironment for cell growth so that cells serve as active role participating in regeneration process by secreting their own extracellular matrix and regulated

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cytokines and growth factors [8]. Although there are some endogenous progenitor neural cells presenting in multiple locations in the adult brain, they have restricted ability to generate new functional neurons in response to injury due to limited cell numbers and lack of appropriate guiding cues and niche for homing these cells to injury site [9,10]. Neural stem cells possessing the potential of unlimited self-renewal and the ability to differentiate into neurons, astrocytes and oligodendrocytes are much favored and as cell source for neural regeneration. In consequence, cellular transplantation from stem cells is one option to repair the injured central nervous system by replacing the lost function of cells resulting from the brain injury.

The self-assembling of bio-molecules is a common phenomenon in biology, including DNA self-assembly to double helix, protein aggregation, cellular lipid membrane development, and polysaccharide interaction. In recent years, several new biomaterials have been developed and designed through the concept of self-assembly of bio-molecules and grown in importance for uses in tissue engineering, drug delivery and microfluidics [11,12]. The use of self-assembling peptides (SAPs) with nanofibrous network structure which mimics native ECM may therefore be a promising option. With the property of sol–gel transition in physiological condition and numerous advantages from peptide-based material, SAP has been regarded as a promising alternative for 3D hydrogel scaffold or cell/gene (drug) carrier for the applications in tissue engineering and drug delivery system. The additional functional motifs with various modification ratios and multi-extended diversity can be conjugated and extended at the terminal residue sequence of specific-designed SAPs to enrich SAP possessing therapeutic functionality for different biopharmaceutical applications and to serve as medical disease treatment modality. Here, we specifically enriched $RADA_{16}$, one of the SAPs developed by Zhang S. et al. [13], with IKVAV motif and evaluated its medical applications in brain neural tissue repair of the central nervous system. IKVAV, the motif from the α1 chain of laminin-1, could promote neuronal cell attachment, migration, and neurite outgrowth [14]. In addition, several studies reported that IKVAV sequence modulates neural cell behaviors to promote neuronal differentiation at an injury site while potentially limiting the effects of reactive gliosis and glial scarring, which are ubiquitous neuropathological disease processes [15–17]. Those studies demonstrated that the cell behavior, especially the neural differentiation, was guided by the presence of IKVAV motifs.

In this study, we established a SAP 3D scaffold with conjugated IKVAV motif to improve survival of transplanted neural stem cells and further enhance neuronal differentiation, neurite proliferation and outgrowth. The objective of this study is to supply a functionalized self-assembling peptide scaffold with encapsulated neural stem cells to enhance the reconstruction of the injured brain.

2. Materials and methods

2.1. Peptide solution

$RADA_{16}$ (AcN-RADARADARADARADA-CONH2) and $RADA_{16}$-IKVAV (AcN-RADARADARADARADAIKVAV-CONH2) (purity > 85%) were purchased from Biopharmaceutical firms (Chinese Peptide Company, Hangzhou, China) and used without further purification. Purity and identities of the peptides were confirmed by analytical high performance liquid chromatography (HPLC) and mass spectrometry (MS), respectively. All aqueous peptide solutions were prepared by using Milli-Q water (18.2 MΩ), stored at 4 °C and sonicated for 30 min before use.

2.2. Physiochemical properties analyses

The secondary structure of peptides was studied using circular dichroism (CD) measurements of 0.01% (w/v) peptide solutions at pH = 4. The data were collected on an Aviv model 202 spectrophotometer (Aviv Instrument Inc., US) with a 1 mm path length at room temperature.

The morphology of peptide scaffolds was analyzed using atomic force microscopy (AFM). In brief, 10 μl of 0.01% and 1.0% (w/v) peptide solution was placed on the surface of clean cut silicon wafer. Each sample was set on the mica for 30 min, washed it with 100 μl water three times and then dried by blowing anhydrous nitrogen. AFM images were collected with a Scanning Probe Microscope (DI Dimension 3100, Veeco Instruments Inc, US) using the dynamic force mode in ambient air. Mickromasch NSC15 micro-cantilevers were chosen with tip radius of 10 nm, spring constant of 40 N/m and frequency of 325 kHz. The images were gathered with 256 × 256 pixel resolution. The main parameters of the AFM measurements were as follow: I gain/P gain (integral gain/proportional gain) 0.20/0.30, amplitude setpoint 0.370 V, and scanning rate 0.8–1.0 Hz. The AFM scan of each sample was repeated more than three times.

RheoStress 600 (Thermo Scientific Instrument Inc., US) equipped with a cone and plate geometry system (cone with diameter: 35 mm, angle: 2°) was used for rheology and gelation behavior analysis. 200 μl of 1% (w/v) peptide solution was loaded on the plate for measurement. For the gelation behavior study, additional 100 μl of pure water, normal saline (0.9% NaCl) or culture medium was added around the peptide sample to trigger the peptide solutions assembling into hydrogel. During all measurements, frequency sweeps ranging from 100 rad/s to 0.1 rad/s were performed at a gap height of 1.05 mm and a constant shear stress of 1 Pa at room temperature.

2.3. Culture of neural stem cells and cell seeding in 3D SAP hydrogel

Rat neural stem cells (HCN-A94-2, gift from Dr. Fred Gage, the Salk Institute, La Jolla, CA) were used in this study and encapsulated in hydrogel. Cells were cultured and expanded as spheres in neural basal medium (Gibco) containing 1% penicillin-streptomycin (Gibco), 2 mM Gluta Max (Gibco), 2 ng/ml bFGF (Gibco), and 1× B27 supplement without vitamin A (Gibco). In the case of cell viability and proliferation investigation, expansion medium as described above was used. As for the *in vitro* differentiation study, cells were cultured in neural basal medium containing 1% penicillin-streptomycin (Gibco), 2 mM Gluta Max (Gibco), 1× B27 supplement without vitamin A (Gibco), 1% FBS (Gibco), and 30 μM retinoic acid (Sigma).

For cell viability and proliferation evaluation, total number of 10^4 NSCs was seeded in hydrogel per well. As for qPCR and cell differentiation analysis, total number of 10^5 NSCs was used. The procedures were as described below. Briefly, 10 μl NSCs suspension was mixed with 50 μl $RADA_{16}$ or $RADA_{16}$-IKVAV peptide and added into 96-well plate (BD Falcon) followed by gentle addition of 30 μl/well medium. Allowed it to undergo self-assembling at 37 °C for 5 min, and then 100 μl medium was added. All samples were incubated in 37 °C and the culture medium was changed every 3 days.

In order to trace the transplanted neural stem cells (NSCs), the NSCs used for specific selected groups were especially labeled in advanced with green florescence protein (GFP) by BacMam GFP transduction kit (Invitrogen).

2.4. Cell viability and proliferation assay

Viability of NSCs encapsulated in 3D hydrogel scaffold was determined by LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probe). Staining procedures were conducted by the manual instruction. In brief, the Live/Dead stain solution was prepared with 2 μM calcein AM and 4 μM EthD-1 in PBS. At various pre-determined time interval, the culture medium was discarded and each sample was incubated in 100 μl stain solution for 45min in 37°°C. The stain solution was then removed and washed with 100 μl PBS.

To investigate the proliferation rate of NSCs seeded in various hydrogel scaffolds, MTS quantitative assay (Promega) was performed. After cultured for various pre-determined time points, the culture medium was discarded and peptide hydrogel were destroyed through vigorously pipetting. Each sample was incubated in 20% MTS solution for 3 h in 37 °C. Afterward, 100ul of supernatant was aspirated by centrifuge at 2000 rpm for 5min followed by measurement of absorbance by a microplate reader at 490 nm and reference wavelength at 660 nm. Supernatant from hydrogel without encapsulated cells was prepared as described above and used as blank solution.

2.5. In vitro immunocytochemistry

To analyze neuronal or glial differentiation of seeded NSCs in 3D SAP hydrogel, the immunocytochemistry was performed. In brief, the culture medium was discarded and 3D hydrogel was rinsed with PBS, fixed with 4% formaldehyde in 0.1 M PBS (pH 7.0) for 1hr at room temperature followed by the double staining procedure. Primary antibodies and secondary antibodies were applied sequentially for 1 h at room temperature. Primary antibodies: mouse monoclonal anti-Nestin antibody (Millipore), mouse monoclonal anti-Glial Fibrillary Acidic Protein (GFAP; Millipore), rabbit monoclonal anti-β-Tubulin, class III C-term (Millipore), rabbit polyclonal anti-Microtubule Associated Protein 2 (MAP2; Millipore), mouse monoclonal anti-Neurofilament (NF-H; Millipore), rabbit polyclonal anti-Synapsin I (Millipore) were applied. Rhodamine conjugated goat anti-mouse and FITC conjugated goat anti-rabbit secondary antibody were used for detection. Stained cells were examined under confocal laser scanning microscopy.