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Functional hyaluronate collagen scaffolds induce NSCs differentiation into functional neurons in repairing the traumatic brain injury

Hongmei Duan [a,1], Xiaoguang Li [a,1], Cong Wang [a,1], Peng Hao [a,1], Wei Song [b,c,1], Manli Li [d], Wen Zhao [a], Yudan Gao [a], Zhaoyang Yang [a,*]

[a] Department of Neurobiology, School of Basic Medical Sciences, Captial Medical University, Beijing 100069, China
[b] School of Rehabilitation Medicine, Captial Medical University, Beijing 100068, China
[c] China Rehabilitation Research Center, Beijing 100068, China
[d] Department of Biomedical Engineering, School of Biological Science and Medical Engineering, Beihang University, Beijing 100191, China

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ABSTRACT

The traumatic brain injury (TBI) usually causes brain tissue defects, including neuronal death or loss, which ultimately results in dysfunction in some degree. The cell replacement therapy is now one of the most promising methods for such injury. There are currently various methods to induce the differentiation of stem cells into neurons, but all extremely complex, slow and unstable. Here we report that the sodium hyaluronate collagen scaffold loaded with bFGF (bFGF-controlled releasing system, bFGF-CRS) can induce neural stem cells (NSCs) to differentiate into multi-type and mature functional neurons at a high percentage of 82 ± 1.528% in two weeks. The quantitative real-time (QRT) PCR results reveal that a long-term activation of bFGF receptors could up-regulate ERK/MAPK signal pathways, thus facilitating the formation of presynaptic and postsynaptic structure among the induced neuronal cells (iN cells). The functional synaptic connections established among iN cells were detected by the planar multielectrode dish system. When jointly transplanting the bFGF-CRS and NSCs into the CA1 zone of the rat TBI area, the results suggested that bFGF-CRS provided an optimal microenvironment, which promoted survival, neuronal differentiation of transplanted NSCs and functional synapse formation not only among iN cells but also between iN cells and the host brain tissue in TBI rats, consequently leading to the cognitive function recovery of TBI rats. These findings *in vitro* and *in vivo* may lay a foundation for the application of bFGF-CRS and shed light on the delivery of exogenous cells or nutrients to the CNS injury or disease area.

Statement of Significance

A sodium hyaluronate collagen scaffold was specifically functionalized with nutrient-bFGF which can induce the differentiation of neural stem cells (NSCs) into multi-type and mature functional neurons at a high percentage in two week. When jointly transplanting the bFGF-CRS and NSCs into the CA1 zone of the traumatic brain injured area of adult rats, the bFGF-CRS could provide an optimal microenvironment, which promoted survival, migration and neuronal differentiation of transplanted NSCs and functional synapse formation among iN cells, as well as between iN cells and host brain tissue in TBI rats, consequently leading to the cognitive function recovery of TBI rats.

1. Introduction

The adult central nervous system (CNS) has a limited capacity to spontaneously regenerate following traumatic injury or disease [1]. To solve this problem, exogenous cells are transplanted after the adult CNS injury to substitute dead or injured tissues. This sounds attractive, but faces three problems: First, how to immobilize the transplanted exogenous cells at the local injured area to avoid their dispersion to other areas; second, the survival and activity of exogenous cells; third, integration of exogenous cells with host tissues [1]. When exogenous cells, together with saline or media, are injected into the CNS injured area, cell aggregation is almost inevitable, accompanied by a lower cell activity; after the injection, cells migrate dispersively or in cluster to other tissue

* Corresponding author.
E-mail address: wack_lily@163.com (Z. Yang).
[1] Five authors contributed equally to this work.

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areas, where they will be cleared up by immune cells and ultimately lose their biological functions [2]. At the injured area, the transplanted exogenous cells face a harsh micro-environment, while being threatened by the absence of cell adhesion and survival factors. Theoretically, the integration of transplanted cells with host tissues must be fast; actually, it is often hampered by physical and chemical barriers [1].

The published methods to induce the differentiation of various stem cells into neurons can not find immediate clinical applications due to a cascade of issues, such as the low iN productivity, long cycles, complicated protocols, and unstable results even under the same protocol [3–7].

Hyaluronate has been shown to play certain roles in cellular processes like cell proliferation, morphogenesis, inflammation and wound repair [2]. After modified specifically, the degradation speed of the hyaluronate can be slow and it can deliver growth factors towards the CNS [8–10]. In the present study, we designed a sodium hyaluronate collagen scaffold loaded with bFGF (a bFGF-controlled releasing system, bFGF-CRS for short). This system not only enabled the slow release of bFGF for up to five weeks under the physiological condition, but also served as a physical scaffold to facilitate the survival and adhesion of NSCs, and quickly induce (in two weeks) NSCs to differentiate at high percentages into mature neurons with strong synaptic formation capability. Meanwhile, the bFGF-CRS provided an optimal microenvironment for the functional synapse formation among iN cells, as well as between iN cells and the host brain tissue in TBI rats, consequently leading to the cognitive function recovery of TBI rats.

2. Materials and methods

2.1. Fabrication of the bFGF-CRS

Under sterile conditions, 20 mg sodium hyaluronate was dissolved in 50 ml of 0.5% sodium hydroxide aqueous solution, then stirred for 6–8 h at 25 °C; 200 μg collagen I block was added to 50 ml deionized water and allowed to swell for 6 h at 25 °C. Next, equal volumes of the sodium hyaluronate solution and collagen I solution were mixed. Finally, 10 ml vinylcyclohexane (5 wt%) was added into the above mixture solution, and stirred for 10 min at 25 °C. After 100-mesh sieve filtration, the sodium hyaluronate collagens solution was vacuum cooled, dried and prepared for incorporation with or without (control) bFGF protein.

After that, 10 mg sodium hyaluronate gelatinous particles described above and 20 ng bFGF were dissolved in 100 ml phosphate-buffered saline in 4 °C, the mixture of which was then vacuum cooled, dried and stored at 4 °C for use.

2.2. Characterization FTIR-ATR

The sodium hyaluronate collagen scaffolds with or without (control) bFGF protein were analyzed using a FTIR-7600 Spectrometer with Attenuated Total Reflectance (ATR). The FTIR-ATR spectra were carried out between 4000 cm^{-1} and 600 cm^{-1} with resolution of 2 cm^{-1} and 20 total scans.

2.3. Differential scanning calorimetry (DSC)

DSC measurements were carried out with a Mettler Toledo DSC 1 calorimeter using weighed samples of about 10 mg. The sodium hyaluronate collagen scaffolds with or without (control) bFGF protein were investigated. Samples were initially equilibrated at 20 °C for 10 min before heating, then were heated at 1 °C min^{-1} to 95 °C.

2.4. Isolation and culture of neural stem cells derived from the brain

Neural stem cells were isolated from the subventricular zone (SVZ) and the subgranular zone (SGZ) of newborn rats as previously described [11]. The dissociated cells in the defined medium were cultured and purified at a density of 50,000 cells/cm^2 in T25 culture flasks (Corning, NY, USA) and incubated at 37 °C in a 5% CO_2 humidified incubator. After 1–3 days of culture, cells were undergoing cell division and proliferating. Meanwhile, they were also forming neurospheres, which were suspended in the defined medium. Subsequently, adherent cells were discarded, and suspending neurospheres were collected by centrifugation, mechanically dissociated and subcultured as single cells in a new T25 culture flask at a density of 50,000 cells/cm^2 in a fresh defined medium. These cells again grew into new spheres in the subsequent 2–3 days. To obtain purified brain neural stem cells (Fig. 2A), the above subculture processes were repeated [12,13].

Then, the neurospheres were seeded in 24-well uncoated (no poly-D-lysine) tissue culture polystyrene plates (Corning, NY, USA) at 350 neurospheres/cm^2 with either: I, the defined medium plus the daily addition of 20 ng/ml soluble bFGF (sbFGF); II, the defined medium plus the control release system (CRS alone) (that is, the 10 mg/ml CRS without bFGF, added once to the medium only at the very beginning of the co-culture); III, the defined medium plus the bFGF-CRS (that is, the 10 mg/ml CRS incorporated with 20 ng bFGF, added once to the medium only at the very beginning of the co-culture). Half of the defined medium was changed every three days. At indicated time points (3 days, 1 week and 2 weeks after induction), the morphology of these neurospheres was observed under a phase contrast microscope (Olympus IX 71, Tokyo, Japan).

2.5. Determination of kinetics of bFGF release from the bFGF-CRS

The tests were performed as described previously [9,12,13]. Six independent equal volumes of medium-supernatants were collected from each group mentioned above at 0, 1, 3, 6, 12 h, 1–5 weeks after the initiation of the co-culture. An enzyme-linked immunosorbent assay was used to determine the kinetics of bFGF release from each group of the supernatant samples. The Emax ImmunoAssay System for the detection of bFGF from Promega was used according to the manufacturer's instructions. ELISA plates were coated with the Anti-Human bFGF polyclonal antibody and blocked with Block buffer for 1 h. After appropriately diluted samples were added, captured bFGF was bound by a second specific antibody, anti-bFGF mAb. Plates were washed, and incubated with anti-mouse IgG conjugated to horseradish peroxidase. Finally, the chromogenic substrate 3, 30, 5, 50-tetramethylbenzidine (TMB) was added, and the reaction was stopped by the addition of an acidic solution. Absorbance was read at 450 nm using an ELISA plate reader (Model 680, Bio-RAD, Japan). The amount of bFGF was determined from a calibration curve based on the known concentrations of bFGF.

2.6. Immunohistochemistry

The tests were performed as described previously [12,13]. The primary antibodies and their dilution rates are listed as follows: mouse anti-nestin monoclonal antibody (anti-nestin; 1:1000; Chemicon, CA), rabbit or mouse anti-β-tublin III (1:200; Chemicon, CA; Sigma, CA), rabbit or mouse anti-microtubule associated protein 2 polyclonalantibody (anti-MAP-2; 1:500; Chemicon, CA; Sigma, CA), rabbit anti-glial fibrillary acidic protein polyclonal antibody (anti-GFAP; 1:500; Chemicon, CA), rabbit anti-oligodendrocyte marker-Myelin basic protein monoclonal antibody (anti-MBP; 1:100; Zymed, CA), rabbit anti-Choline Acetyltransferase polyclonal