Biomaterials 63 (2015) 177–188

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Recapitulation of *in vivo*-like paracrine signals of human mesenchymal stem cells for functional neuronal differentiation of human neural stem cells in a 3D microfluidic system

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ARTICLE INFO

Article history:
Received 13 March 2015
Received in revised form
10 June 2015
Accepted 11 June 2015
Available online 14 June 2015

Keywords:
Microfluidic array
Human neural stem cell
Human mesenchymal stem cell
Glial cell-derived neurotrophic factor
Paracrine signal
Neuronal differentiation

ABSTRACT

Paracrine signals produced from stem cells influence tissue regeneration by inducing the differentiation of endogenous stem or progenitor cells. However, many recent studies that have investigated paracrine signaling of stem cells have relied on either two-dimensional transwell systems or conditioned medium culture, neither of which provide optimal culture microenvironments for elucidating the effects of paracrine signals *in vivo*. In this study, we recapitulated *in vivo*-like paracrine signaling of human mesenchymal stem cells (hMSCs) to enhance functional neuronal differentiation of human neural stem cells (hNSCs) in three-dimensional (3D) extracellular matrices (ECMs) within a microfluidic array platform. In order to amplify paracrine signaling, hMSCs were genetically engineered using cationic polymer nanoparticles to overexpress glial cell-derived neurotrophic factor (GDNF). hNSCs were cultured in 3D ECM hydrogel used to fill central channels of the microfluidic device, while GDNF-overexpressing hMSCs (GDNF-hMSCs) were cultured in channels located on both sides of the central channel. This setup allowed for mimicking of paracrine signaling between genetically engineered hMSCs and endogenous hNSCs in the brain. Co-culture of hNSCs with GDNF-hMSCs in the 3D microfluidic system yielded reduced glial differentiation of hNSCs while significantly enhancing differentiation into neuronal cells including dopaminergic neurons. Neuronal cells produced from hNSCs differentiating in the presence of GDNF-hMSCs exhibited functional neuron-like electrophysiological features. The enhanced paracrine ability of GDNF-hMSCs was finally confirmed using an animal model of hypoxic-ischemic brain injury. This study demonstrates the presented 3D microfluidic array device can provide an efficient co-culture platform and provide an environment for paracrine signals from transplanted stem cells to control endogenous neuronal behaviors *in vivo*.

1. Introduction

Stem cell paracrine signaling has been highlighted as an important mechanism underlying stem cell-mediated tissue regeneration. In particular, mesenchymal stem cells (MSCs) derived from bone marrow, cord blood, and adipose tissue have been known to secrete a wide spectrum of growth factors and cytokines that enhance angiogenesis, neurogenesis, and wound healing [1–4]. The production of a significant level of paracrine signals from transplanted MSCs can improve tissue regeneration and induce functional recovery of defective tissue via activation of endogenous cells in host tissue [5–7]. Accordingly, several studies have reported the potential use of MSCs as a therapeutic candidate for treatment

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http://dx.doi.org/10.1016/j.biomaterials.2015.06.011
0142-9612/

of neurological diseases [8,9]. Paracrine secretion of neurotrophic factors from transplanted MSCs has been proposed as one of the main contributions to neuronal regeneration [10,11]. Therefore, spatial and temporal patterning of these paracrine signals needs to be studied to enhance the paracrine effect of MSC therapy for survival, proliferation, and differentiation of endogenous neural stem cells (NSCs) or progenitor cells in the brain.

To date, most research studying paracrine signaling on neuronal differentiation have relied on either two-dimensional (2D) transwell-based co-culture systems or conditioned medium [12–15]. These conventional culture systems have been widely used for investigating the diffusional effects of soluble molecules secreted from one cell type on another without any direct contact. Indeed, several studies have reported that MSCs can direct NSC differentiation into neuronal lineage via secretion of soluble factors when both cell types are co-cultured in a transwell system [16,17]. Additionally, NSC differentiation has been shown to be affected when cells are incubated in medium conditioned with MSCs [12,13]. However, 2D transwell-based culture systems with an inserted protein-permeable membrane cannot provide a microenvironment that accurately reconstitutes the effects of paracrine signaling on NSC differentiation in a three-dimensional (3D) extracellular matrix (ECM) [18]. It is also difficult to offer mechanical and physiological properties of 3D stem cell niches in a transwell system. Culture systems that use conditioned medium containing secreted factors from specific cell types can be easily used to determine the effects of paracrine factors on NSC behavior [19,20]. However, the quality and quantity of growth factors in conditioned medium vary according to cell density and growth profile at harvest, and use of this method is dependent on following specific protocols for the collection and storage of conditioned medium [18]. This may lead to variability in growth factors contained in conditioned medium, making it difficult to certify the consistency of conditioned medium in experiments studying paracrine signaling. In addition, conditioned medium experiment cannot provide bi-directional signaling between the two cell types.

Microfluidic cell culture platforms can recapitulate stem cell paracrine signaling in *in vivo*-like microenvironments. Microfluidic devices have been used to create artificial microenvironments for stem cell niches and emulate *in vivo*-like distribution of cellular signals [18]. Our previous studies have reported that NSCs cultured in 3D microscale ECM hydrogel housed in a microfluidic device exhibit quite different cellular phenotypes and differentiation profiles compared to cells grown in 2D culture or 3D bulk hydrogels, illustrating the importance of reconstituting 3D microenvironments for studying NSC differentiation [21,22]. More recently, we examined the effect of brain endothelial cells (ECs) on NSC self-renewal and differentiation via co-culture of NSCs and brain ECs without direct contact in a microfluidic device [23]. In that study, we simulated the accumulation of factors secreted from brain ECs in a 3D microfluidic ECM that emulated physicochemical distribution and diffusion profiles of paracrine factors in a 3D microenvironment [23]. This environment cannot be recapitulated using a 2D monolayer culture nor a 3D culture with bulk ECM hydrogel. Therefore, microfluidic systems could be more appropriate for exploring the effects of MSC paracrine signals on NSC differentiation by providing spatial and temporal control of an *in vivo*-like microenvironment.

In this study, we report a 3D microfluidic system for recapitulating neurotrophic paracrine signals from human MSCs (hMSCs) for regulating the differentiation of human NSCs (hNSCs). To further increase the paracrine signaling capabilities of hMSCs, cells were genetically engineered to overexpress glial cell-derived neurotrophic factor (GDNF) using cationic polymer nanoparticles. GDNF, a member of the transforming growth factor-β superfamily, is a potent neurotrophic factor known to enhance the survival and neuronal differentiation of NSCs [10]. In a microfluidic array, hNSCs in a 3D collagen hydrogel were introduced into the device's central microchannels and then co-cultured with GDNF-overexpressing hMSCs (GDNF-hMSCs) housed in two channels on located on either side of the central channels. We performed a simulation analysis that predicted accumulation of secreted GDNF from hMSCs in the 3D microfluidic system, indicating that paracrine effects from GDNF-hMSCs can be enhanced through 3D culture in a microfluidic device. The presence of GDNF-hMSCs reduced glial differentiation of hNSCs, while promoting differentiation into neuronal lineage, particularly dopaminergic neurons. Additionally, the use of a 3D microenvironment to produce *in vivo*-like GDNF paracrine signaling from hMSCs yielded electrophysiologically functional differentiated hNSCs with neuronal phenotypes. The therapeutic effects from paracrine signals produced from GDNF-hMSCs were ultimately confirmed using an animal model of hypoxic-ischemic brain injury. The presented microfluidic array device could provide a microenvironment and co-culture platform that allows the effective prediction of MSC-induced paracrine effects on NSC differentiation.

2. Materials and methods

2.1. Fabrication of microfluidic array device

Microfluidic array devices were fabricated from poly(-dimethylsiloxane) (PDMS) using a conventional soft-lithography process as previously described [22]. The microfluidic array was designed to include eight channel units in a single device. Each unit contains a single central channel for 3D hNSC culture in ECM hydrogel, and one channel on each side of the central channel for culture of GDNF-hMSCs and supplementation with growth medium. Bonding of the PDMS microchannel component to a flat, ~80-μm thick PDMS membrane (Amed Co., Seoul, Korea) was modified to allow for easy disassembly of devices [21]. Briefly, both channel and flat membrane components were treated with oxygen plasma (CUTE; Femto Science, Seoul, Korea), exposed to air for several minutes, and assembled into a complete device. When this process was modified by allowing additional exposure to air after oxygen plasma treatment, it resulted in reversible bonding between the microchannel and flat membrane components. Bonded devices were cured in a drying oven for 12 h at 80 °C to allow PDMS surfaces to recover hydrophobic properties.

2.2. Poly(β-amino esters) (PBAE) synthesis

PBAE for genetic modification of hMSCs was synthesized as previously described [24]. Briefly, an acrylate-terminated C32 polymer (C32-Ac) was prepared by polymerizing 1,4-butanediol diacrylate (C) with 5-amino-1-pentanol (32) at a 1.2:1.0 M ratio of diacrylate to amine monomer at 90 °C for 24 h. Subsequently, amine end-modified C32 polymers were generated by reacting C32-Ac with 1,11-diamino-3,6,9-trioxaundecane (122) diamine monomer (Tokyo Chemical Industry Corporation, Tokyo, Japan) in anhydrous tetrahydrofuran. End-capping reactions were performed overnight at room temperature using a 1.6-fold molar excess of amine over acrylate end groups. The synthesized polymers (C32-122) were dissolved in anhydrous dimethyl sulfoxide at a concentration of 100 mg/mL and stored at −20 °C before use.

2.3. Stem cell culture

hMSCs derived from human adipose tissue were purchased from Invitrogen (Carlsbad, CA, USA) and cultured in Dulbecco's