Biomaterials 109 (2016) 1–11

Contents lists available at ScienceDirect

Biomaterials

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

Treatment of spinal cord injury by an advanced cell transplantation technology using brain-derived neurotrophic factor-transfected mesenchymal stem cell spheroids

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ARTICLE INFO

Article history:
Received 25 April 2016
Received in revised form
6 September 2016
Accepted 9 September 2016
Available online 10 September 2016

Keywords:
Spheroid
Cell transplantation
Non-viral gene transfection
Spinal cord injury
Biodegradable polycation

ABSTRACT

Curing spinal cord injury (SCI) is challenging because of the onset of multiple and irreversible pathological responses to such injury. To suppress the responses, we employed an advanced cell transplantation technology integrating three-dimensional spheroid cell transplantation with non-viral gene transfection using biodegradable polycations. Brain-derived neurotrophic factor (*BDNF*)-transfected mesenchymal stem cell (MSC) spheroids were transplanted at thoraces level (Th9) to SCI region in mice. *BDNF*-transfected MSC spheroid transplantation led to a significantly enhanced recovery of hindlimb motor function in acute phase of SCI with myelinated axons preserved at the SCI region, while use of either technology in isolation, BDNF transfection or spheroid culture, exerted only a limited therapeutic effect, demonstrating the importance of integrated approaches. Secretion of endogenous therapeutic proteins, such as anti-inflammatory factors, was greater in MSC spheroids than in monolayer culture MSCs, and these factors appeared to act synergistically alongside BDNF secretion in SCI treatment. This study forms a basis for cell therapy regulating complex pathophysiologic processes.

1. Introduction

Spinal cord injury (SCI) induces loss of sensorimotor and/or autonomic functions, and has a strong negative impact on the patient's quality of life [1]. After a primary disruption of spinal tissue by a direct mechanical force, a series of secondary events involving various pathological responses hamper the recovery from injury [2,3]. In the secondary phase, massive cell death is induced by oxidative enzymes and proinflammatory cytokines secreted by infiltrating immune cells, as well as by ischemia caused by disruption of a surrounding vascular structure [4–7]. In addition, excessive extracellular matrices produced by activated astrocytes and oligodendrocytes, called glial scarring, severely inhibits axonal regrowth [8,9]. Due to these multiple pathological responses, single approach treatment, such as steroid treatment, tends to have only a limited effect on SCI [10].

Cell transplantation therapy employing various cell sources, e.g. embryonic stem cells, induced pluripotent stem cells, neural stem cells, and mesenchymal stem cells (MSCs), is an attractive strategy to address the issues associated with SCI [11,12]. Little evidence exists that transplanted cells differentiate into neural elements directly at transplant sites. However, paracrine events induced by proteins and peptides secreted by the transplanted cells and specifically inhibiting secondary pathological response progression are considered to be the main therapeutic outcome [13–15]. It is

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http://dx.doi.org/10.1016/j.biomaterials.2016.09.007
0142-9612/

apparent that cell therapy is advantageous in the long-term and exerts stable therapeutic effects compared with drug administration. However, considering SCI pathogenesis, cell transplantation remains challenging because the transplanted cells are inevitably affected by the pathological environment of the host tissue, and their *in vivo* activity is significantly altered from that predicted *in vitro* [16].

Recently, we established a new system to potentially maximize the therapeutic effects of cell transplantation using a three-dimensional (3D) spheroid culture. Spheroid cell culture is a promising technique improving cell survival and function by preserving cell-to-cell interactions. Here, we used a system of micropatterned plates with 100-μm diameter cell adhesion areas regularly arrayed in a two-dimensional manner, surrounded by non-adhesive areas coated by polyethylene glycol (PEG) matrix (Cell-able™ multi-well plates; Toyo Gosei, Tokyo, Japan) [17]. After seeding cells onto these plates, a large number of cells assemble to the confined cell-adhesive areas, leading to the formation of 3D spheroids. The key feature of this system is that it allows generation of large amounts of spheroids with regulated diameter size (100 μm). While spheroids can be prepared by several other methods, such as hanging-drop methods and those using spinner flask, non-adhesive culture plate, U bottom plate and special scaffolds, precise control of spheroid size is difficult in these methods [18–21]. Previously, size-regulated spheroids prepared by our system sustainably secrete high amounts of albumin, demonstrating usefulness of the system in preparing cells secreting copious amounts of proteins [17]. For transplantation, the spheroids can be recovered in an injectable liquid suspension without disrupting their 3D structure by using thermo-responsive micropatterned plates [22] (Fig. S1).

To further augment the therapeutic effects of cell transplantation, we introduced an exogenous gene into the spheroid cells [23]. Introducing transgene(s) encoding secretory neurotrophic factor(s) into cells prior to transplantation can enhance protein secretion by these cells immediately after transplantation, over a prolonged period. This is beneficial for SCI treatment because it addresses the issue of the secondary pathologic processes. However, introduction of a gene into spheroid cells is not trivial. When using lipid-based transfection reagents, the spheroids tend to be disrupted due to membrane-disruptive quality of the reagents [23]. To overcome this difficulty, we developed an original cationic polymer, poly[*N*′-[*N*-(2-aminoethyl)-2-aminoethyl] aspartamide] [PAsp(DET)] [24–26]. This polymer selectively destabilizes plasma membrane under the acidic conditions of endo-/lysosomal compartment, without hampering membrane integrity at physiological pH. In fact, in pH 7.4 culture medium, cytotoxicity of PAsp(DET)/DNA polyplexes is significantly lower than that of lipid-based gene carriers. Once internalized via endocytosis, PAsp(DET) induces prompt destabilization of endosome membrane in response to the lower endosomal pH, facilitating transgene expression without disruption of the spheroid structure.

By integrating the micropatterned cell culture and gene transfection system technologies, we attempted SCI treatment using human MSC spheroids transfected with a gene encoding brain-derived neurotrophic factor (BDNF), a potent neurotrophic factor that exerts neuroprotective effects [27] (Fig. S1). Besides high anti-inflammatory and wound-healing potentials of MSCs, associated with paracrine secretion of therapeutic proteins and peptides [28,29], augmented BDNF secretion immediately after transplantation was expected to result in effective neuroprotection and nerve regeneration [27]. As described below, these efforts overcame severe pathological SCI processes, resulting in early recovery of motor function, with reduced damage of the spinal cord. We also investigated MSC spheroid secretion profiles of potential paracrine factors that might affect the recovery of neural function, emphasizing the benefit of spheroid transplantation.

2. Materials and methods

2.1. Preparation of spheroids from human MSCs

Thermo-responsive micropatterned plates were prepared by etching the micropatterned architecture onto thermo-responsive culture plates (Upcell, CellSeed, Tokyo, Japan), as previously described [22,23]. Briefly, cell adhesive area (100-μm diameter) coated with a thermo-responsive polymer was regularly aligned and surrounded by non-adhesive area coated with PEG. For *in vitro* analyses, a micropatterned plate with the same architecture but not thermo-responsive (Cell-able™, Toyo Gosei, Tokyo, Japan), was used. Human MSCs were purchased from Lonza (Allemdale, NJ, USA) and cultured with MSCGM-CD BulletKit MSC culture medium (Lonza, Basel, Switzerland). MSCs at passage five were seeded onto the micropatterned plates for spheroid culture, and onto polystyrene-coated plates for monolayer culture, 300,000 cells/well in 12-well plates and 30,000 cells/well in 96 well plates.

2.2. Non-viral transfection of pDNA

PAsp(DET) polymer with polymerization degree 52, as determined by ^{1}H-NMR, was synthesized as previous reported [24]. FuGENE HD was purchased from Promega (Madison, WI, USA). Secreted luciferase (Gaussia luciferase, Gluc) pDNA was constructed using pCMV-Gluc control plasmid (New England BioLab, Ipswich, MA, USA) [30]. Gene for non-secreted luciferase was from pGL4.13 (Promega). Gene for human brain-derived neurotrophic factor (BDNF) was from pUNO1-hBDNFa (InvivoGen, San Diego, CA, USA). Protein-encoding fragments of these plasmids were cloned into pCAG-GS plasmid (RIKEN, Tokyo, Japan) for gene expression under the control of CAG promoter/enhancer. For gene transfection, PAsp(DET) polymer and pDNA were separately suspended in 10 mM Hepes buffer (pH 7.3) and mixed at a total amino groups in polymer/total phosphate groups in pDNA ratio (N/P ratio) of 5. FuGENE HD was mixed with pDNA solution according to the manufacturer's protocols. The resulting polyplex, lipoplex, and naked pDNA were added to culture medium at pDNA dose 12 μg/well in 12-well plates, and 1.2 μg/well in 96-well plates. The transfection was performed 2 d after MSC seeding onto plates.

2.3. In vitro evaluation of luciferase expression

In vitro transfection efficiency was evaluated using Gluc, as a reporter [30]. Time-dependent profile of Gluc expression was evaluated by measuring Gluc activity in the culture medium using a Renilla Luciferase Assay System (Promega) and GloMax® 96 Microplate Luminometer (Promega), following the manufacturer's protocol. Cell culture medium was replaced with fresh medium precisely 24 h before each indicated time point, and Gluc expression during the last 24 h of every time point was shown (Fig. S3).

2.4. Spinal cord injury and MSC transplantation

C57BL6/J mice (female, 8 weeks old) were purchased from Charles River Laboratories (Yokohama, Japan). The mice were anesthetizing by intraperitoneal injection of pentobarbital (50 mg/kg) (Kyoritsu Seiyaku, Tokyo, Japan), and thoracic cord was surgically exposed. Contusion SCI was induced with Infinite Horizons Impactor (Precision Systems and Instrumentation LLC, Fairfax, VA, USA) with 50 kdyn (0.5 N) impact force at thoracic level Th 9. Immediately after SCI, MSCs were transplanted as described below.