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Short communication

Neuro-differentiated Ntera2 cancer stem cells encapsulated in alginate beads: First evidence of biological functionality

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ABSTRACT

The present communication investigates an application of alginate encapsulation technology to the differentiation of the embryonic cancer stem NTera2 cells (NT2) into dopamine-producing cells. The encapsulation of cells in polymeric beads allows their immune isolation and makes them eligible for transplantation, thus representing a promising biotech tool for the delivery of biologically active compounds to the brain. The polysaccharide alginate is one of the most commonly used material for this procedure since it is well tolerated by various tissues, including the brain. Two different initial cell concentrations (i.e. $0.5 * 10^6$/ml and $1.0 * 10^6$/ml) were tested, in order to identify which one could better reflect the homogeneous cell distribution into the alginate beads and guarantee a good cell viability at different times of culture. As evidenced, the higher number of cells promoted the formation of clusters resulting in a better interaction among encapsulated cells and the subsequent promotion of mitotic activity. The distribution of alive/dead cells into the alginate beads was verified and followed at different time points through the fluorescein diacetate/propidium iodide (FDA/PI) staining, confirming the presence of living neuronal positive cells, as determined from fluorescence microscopy imaging. The functionality of the encapsulated NT2 cells was confirmed by their dopamine production capability as assessed by UV–Vis spectrophotometric analysis and by liquid chromatography–mass spectrometry (LC-MS). The NT2/microspheres system can be considered a groundbreaking experimental procedure, a functionally active platform, able to produce and release dopamine, and thus potentially exploitable for therapy in Parkinson's disease.

1. Introduction

During the last years the regenerative medicine has turned increasing attention to stem cells as attractive source to generate specifically targeted cell types for the treatment of different pathological conditions, thanks to their self-renew indefinitely in-vitro proliferation properties and multipotent differentiation capacity [1,2]. Transplantation of neural stem/precursor cells (NPCs) has been proposed as a promising therapeutic strategy in almost all neurological disorders characterized by the failure of central nervous system (CNS) and as endogenous repair mechanisms in restoring the damaged tissue and rescuing the lost function. However, the direct transplant of neural stem cells into the brain gives rise to a number of complications, including cell survival, immune rejections and teratomas that still preclude its clinical applicability [3]. Although their extensive therapeutic use is still held back by the need of more characterization, increasing amounts of experimental data constantly enrich the existing literature concerning stem cells. The embryonic stem cell line Ntera2/D1, derived from a human teratocarcinoma cell line, is committed towards the neural fate and can terminally differentiate in neurons (NT2-N) following exposure to retinoic acid (RA) [4–6]. Differentiated neurons resulting from this cell line may represent a potentially cell source to brain transplantation in neurodegenerative diseases [7]. Their therapeutic importance lies in the fact that, following RA treatment, NT2 cells undergo an irreversible commitment to terminally differentiate into stable postmitotic neurons, including the dopaminergic phenotype, even after transplantation in normal, ischemic or damaged murine brain [8]. These neurons were shown to form functional synapses and release of dopamine (DA) confirming the function as dopaminergic neurons [9,10]. Given these properties, the NT2 cell line has been reported to be a promising human cell source as a major player in studies of cell in vivo therapeutic application in many neurodegenerative

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diseases, particularly for Parkinson's disease (PD) [10]. Prior evidence has shown that during the development of the CNS, neuronal survival, growth, differentiation, innervation and maintenance of the dopaminergic phenotype are influenced by both the release of supporting/trophic factors and/or cell-cell interactions [11]. NT2 cells are able to replace effectively degenerated neurons because of their ability to integrate and adapt its phenotype to that target neurons, for example dopaminergic striatal neurons. They have been found to respond to environmental cues when transplanted into the excitotoxically lesioned striatum [12,13], sending out target-specific projections as well as expressing a site-specific phenotype. Although many pharmacological and surgical treatments for PD are currently available, none is able to provide a definitive cure and all of them show in the time different side effects [14,15]. Several regenerative and restorative approaches, including cell replacement therapy, have been proposed as an alternative method for treating PD patients. Accordingly, in order to overcome the limitations highlighted by the common practice of the direct cell transplantation, the strategy of cell encapsulation prior CNS transplantation has been developed for the creation of immunoprotected implants of cell-based therapy. The alginate, natural linear unbranched anionic polysaccharide, mainly extracted from brown algae, is the most frequently polymer used for cell encapsulation, allowing its hydrated 3D network cells to adhere, grow, migrate and interact each other [16], and it is currently being employed in different biomedical applications, including drug delivery and tissue engineering [17,18].

Alginate encapsulation consists in a very easy and versatile method, allowing to entrap and immunoisolate cells with high efficiency using mild fabrication conditions [19], due to the absence of organic solvents and acidic degradation products that could induce the denaturation and loss of biological activity. Alginate systems have shown great potential for the entrapment and transplantation of insulin-producing pancreatic Langerhans' islets [20,21], mesenchymal stem cells (MSCs) isolated from adult bone marrow and adipose tissues [22–28], genetically modified cells for gene and tumor therapy and other factor-secreting cells and tissues [29,30]. It has been also shown that the alginate encapsulation technique promotes better growth, differentiation, maturation or protein secretion of various cell types [31,32] allowing exchange of nutrients, oxygen and stimuli across the membrane, whereas the cells are protected from external threats such as antibodies from the host. In this communication, a first attempt to encapsulate NT2 cancer stem cells in alginate microcapsules is presented, in order to provide them a microenvironment which could mimic the stem cell niche and to use them as precursors of dopaminergic cells. This different approach of differentiation optimizes the differentiation into specific lineages as well as the tissue organization, providing a three-dimensional (3D) environment for growing cells. To assess the most suitable process parameters, alginate microcapsules of different permeability were produced by selecting gelling bath containing two different concentrations of $CaCl_2$. Further, different cell concentrations were also considered. The efficiency of the encapsulation procedure was validated through viability and growth tests of encapsulated cells by means of optical and fluorescence microscopies. Moreover, the presence of dopamine secreted by encapsulated cells was monitored on aliquots of the medium supernatants by means of UV–Vis spectroscopy and of LC-MS measurements.

2. Materials and methods

2.1. Cell culture

Ntera2/D1 (NT2) cells (ATCC, Manassas, VA) were grown at 37 °C, under 95% O_2/5% CO_2 atmosphere, in T75 flasks containing DMEM (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS), penicillin/streptomycin (10,000 UI/ml and 10 mg/ml, respectively) and 1% glutamine. The culture medium was changed twice a week, and subconfluent cultures (70–80%) were splitted 1:3 to 1:4 by scraping and dispensed into new flasks at a density of at least 5×10^6/ml viable cells. The cells were induced to differentiate in the presence of 10 mM retinoic acid (all-trans) (Sigma-Aldrich, St Louis, MO, USA) freshly prepared and added to the cultures twice weekly for 4 weeks, following a cell aggregation protocol [33], with minor modifications [6].

2.2. Cell encapsulation

The cell encapsulation was performed by means of extrusion-dripping method. For the NT2 encapsulation the cells were washed with phosphate buffered saline (PBS), detached using trypsin-EDTA and counted. Different concentrations of cells (1.0 and $0.5 * 10^6$/ml) were suspended in a 1% (wt/v) solution of sodium alginate of low molecular weight (Sigma Aldrich A0682, M_w 12–80 kDa, viscosity of 4–12 cps for 1% aqueous solution at 25 °C). The resulting cell suspensions were loaded in sterile plastic syringe (Terumo, 5 cm^3), equipped with a metallic needle (diameter size 27 G), and extruded, under sterile conditions, into anhydrous calcium chloride (Carlo Erba Reagents) gelling bath (0.1 M and 1.0 M) by means of an infusion pump (flow rate 10 ml/h) and setting a collecting distance between the dripping tip and gelation bath of 3 cm, in order to obtain beads via ionic cross-linking. The produced beads were left to harden in the gelling bath for 5–10 min, under continuous magnetic stirring, to allow the polymerization occurrence, and afterwards washed with sterile deionized water several times to remove the residual salt.

Two different $CaCl_2$ concentrations (i.e. 0.1 M and 1.0 M) of the gelling bath were selected in order to obtain beads with different permeability (HP (high permeability) and LP (low permeability) beads for $CaCl_2$ concentration of 0.1 M and 1.0 M, respectively). The encapsulated cells were maintained in DMEM medium in a humidified incubator (5% CO_2, humidity 95%) up to 30 days. The cell medium was refreshed every 2–3 days. As a reference, empty alginate beads were produced following the same procedure. The chosen designation of the different produced samples is summarised in Table 1.

The average bead diameter was determined from optical images by means of Image J (NIH) software, demonstrating that the set-up dripping process allowed to obtain very homogeneous beads, characterised by an average diameter size of around 1.5 ± 0.1 mm, immediately after their preparation.

2.3. UV–Vis measurement of dopamine secreted by NT2 cells at different differentiation times

Aliquots of NT2 supernatants (around 3 ml) harvested at different differentiation times, due to the exposition to RA for 3, 7, 10, 14 and 28 days, were submitted to UV–Vis measurements (Shimadzu 2450) in the range 220–1000 nm.

In order to stabilize the dopamine secreted into the cell culture medium, a solution of phosphoric acid (H_3PO_4, Sigma Aldrich, 7.5%) and sodium metabisulfite ($Na_2S_2O_5$, Carlo Erba Reagents, 23 mM) was added to all the supernatants to ensure immediate stabilization of dopamine [34].

The following UV–Vis spectra were also acquired as reference: DMEM, DMEM conditioned with H_3PO_4 and $Na_2S_2O_5$ solution, commercial dopamine hydrochloride ($C_8H_{11}NO_2$·HCl, 98%, Sigma Aldrich)

Table 1
Designation of the systems based on NT2 cells encapsulated within alginate beads in different conditions.

Sample	[NT2 cell] (10^6/ml)	[$CaCl_2$] gelling bath (M)
0.5NT2-HP	0.5	0.1
0.5NT2-LP	0.5	1.0
1.0NT2-HP	1.0	0.1
1.0NT2-LP	1.0	1.0