Experimental Cell Research 343 (2016) 218–222

Contents lists available at ScienceDirect

Experimental Cell Research

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

Research Article

Decreased proliferative, migrative and neuro-differentiative potential of postnatal rat enteric neural crest-derived cells during culture *in vitro*

Hui Yu [a,b], Wei-Kang Pan [a], Bai-Jun Zheng [a], Huai-Jie Wang [a], Xin-Lin Chen [b], Yong Liu [b], Ya Gao [a,*]

[a] Department of Pediatric Surgery, the Second Affiliated Hospital, Xi'an Jiaotong University, No 157, Xi Wu Road, Xi'an 710004, Shaanxi, China
[b] Institute of Neurobiology, Environment and Genes Related to Diseases Key Laboratory of Chinese Ministry of Education, Xi'an Jiaotong University, No 96, Yan Ta Xi Road, Xi'an 710061, Shaanxi, China

ARTICLE INFO

Article history:
Received 28 November 2015
Received in revised form
11 March 2016
Accepted 7 April 2016
Available online 8 April 2016

Keywords:
Phenotypic analysis
Proliferation
Migration
Apoptosis
Differentiation

ABSTRACT

A growing body of evidence supports the potential use of enteric neural crest-derived cells (ENCCs) as a cell replacement therapy for Hirschsprung's disease. Based on previous observations of robust propagation of primary ENCCs, as opposed to their progeny, it is suggested that their therapeutic potential after *in vitro* expansion may be restricted. We therefore examined the growth and differentiation activities and phenotypic characteristics of continuous ENCC cultures. ENCCs were isolated from the intestines of postnatal rats and were identified using an immunocytochemical approach. During continuous ENCC culture expansion, proliferation, migration, apoptosis, and differentiation potentials were monitored. The Cell Counting Kit-8 was used for assessment of ENCC vitality, Transwell inserts for cell migration, immunocytochemistry for cell counts and identification, and flow cytometry for apoptosis. Over six continuous generations, ENCC proliferation potency was reduced and with prolonged culture, the ratio of migratory ENCCs was decreased. The percentage of apoptosis showed an upward trend with prolonged intragenerational culture, but showed a downward trend with prolonged culture of combined generations. Furthermore, the percentage of peripherin$^+$ cells decreased whilst the percentage of GFAP$^+$ cells increased with age. The results demonstrated that alterations in ENCC growth characteristics occur with increased culture time, which may partially account for the poor results of proposed cell therapies.

1. Introduction

The enteric nervous system (ENS) gives rise to enteric neural crest-derived cells (ENCCs), and is a complex network that controls motility and secretion of the gastrointestinal tract [17]. During the course of embryogenesis, defects in proliferation [27], migration [7] and differentiation [2] of ENCCs results in failure to form the ENS, which is distinctive of Hirschsprung's disease (HD) [24]. Previous studies [3,6] have shown that ENCCs rapidly propagate and form characteristic neurospheres composed of a mixture of progenitors and differentiated progeny cells including neurons and glia [12]. Some ENCCs in the gut remain in an immature state [8], displaying both self-renewal and multipotent activities throughout the adult life [1,23]. Consequently, studies [25,28,29] in stem cell-based therapy have guided their efforts towards ENCCs, raising the possibility of using these cells as a potential resource. In one study; however, the transplanted ENCCs remained undifferentiated 8 weeks after transplantation [29]. In fact, the method for *in vitro* expansion of undifferentiated ENCCs is by primary isolation from fetal or newborn guts, followed by culture and propagation, indicating that ENCCs could be a great resource to provide platforms for investigating and modeling cell-based therapeutic approaches to address neurocristopathies.

However, development of the ENS is spatiotemporally controlled by interplay of the ENCCs, as well as cues from the surrounding gut mesenchyme and other signals or pathways [9,10,11]. Additionally, different culture conditions *in vivo* or *in vitro* may result in different biological and phenotypic characteristics in ENCCs. Any understanding of ENCC biology and phenotype would further increase our knowledge of HD pathogenesis and may advance potential treatments, including cell-based transplantation approaches. To date, little information on the biology and phenotype of ENCCs *in vitro* has been obtained prior to cell transplantation. In this study, we aimed to investigate whether ENCCs retain the stable developmental potency of counterpart ENCCs *in vitro*.

With expanding knowledge of postnatal rat ENCCs *in vitro*, the

* Corresponding author.
E-mail address: ygao@mail.xjtu.edu.cn (Y. Gao).

http://dx.doi.org/10.1016/j.yexcr.2016.04.005
0014-4827/

purpose of this study was to investigate the potential of ENCCs and explore their proliferation, migration, apoptosis, and neuroglia differentiation characteristics in ENS-derived neurospheres to better define their *in vitro* behavior.

2. Materials and methods

2.1. Animal preparation

Experimental protocols followed the guidelines established by the National Institutes of Health Guide for Care and Use of Laboratory Animals. Neonatal Sprague-Dawley (SD) rats were supplied by the Center of Experimental Animals, College of Medicine, and Xi'an Jiaotong University. All protocols were approved by the Animal Care and Use Committee at Xi'an Jiaotong University.

2.2. ENCC isolation and cell culture

Cultures were prepared under aseptic conditions using the procedures described in our previous study [29]. Briefly, SD rats were euthanized and sacrificed by quick cervical dislocation. The outer layers of the guts were dissected under a stereomicroscope and dissociated by trituration and trypsinization into single-cell suspensions. These suspensions were then plated in Dulbecco's Modified Eagle Medium (DMEM)/F12 1:1 (Life Sciences, USA, #SH30023.01B) supplemented with N2 (0.5%, Gibco, USA, #17502-048), B27 (1.0%, Gibco, USA, #12587-010), recombinant fibroblast growth factor-basic (bFGF, 10 ng/ml, Gibco, USA, #PHG0021), recombinant epidermal growth factor (EGF, 10 ng/ml, Gibco, USA, #PHG0311), 2-mercaptoethanol (50 μmol/L, Gibco, USA, #M3148), and penicillin and streptomycin (100 U/ml, gibco, USA, #15140148), and were maintained at 37 °C in a 95% air/5% CO_2 water-saturated atmosphere. Cells were seeded into culture flasks at a density of 5.0×10^5 cells/ml and were cultured for 7 days as floating multicellular aggregates in preparation for sub-culture at an identical seeding density. After dissociated into single cells, ENCCs were routinely counted with trypan blue staining and a hemocytometer under a light microscope.

2.3. Counting the percentage of $p75^{NTR}$-positive cells in the neurospheres

After being dissociated from the neurospheres, single-suspension ENCCs were transferred to slides as cell drops, which were double stained with 4′,6-diamidino-2-phenylindole (DAPI) and $p75^{NTR}$ antibody using an immunocytochemical method. $DAPI^+$ and $p75^{NTR+}$ENCCs in the same field of view were respectively imaged by fluorescence microscopy. The double-labeled $p75^{NTR+}$/$DAPI^+$ and $DAPI^+$ only cells were counted after image composition using DP manager software, and the percentage of $p75^{NTR}$ positive cell ($p75^{NTR+}DAPI^+$ cell number/$DAPI^+$ cell number) in the neurospheres was determined.

2.4. ENCC sub-culture and differentiation

Neurospheres derived from ENCCs were mechanically dislodged from the plates, dissociated using trypsin/EDTA (Life Sciences, USA, #SH30042.01) and replated at a density of 5.0×10^5 cells/ml for subsequent experimentation. To further differentiate them into neuronal and glial cells, single-cell suspensions from neurospheres were plated onto polylysine-coated plates in N2B27 medium comprising 1.0% fetal calf serum (FCS, Gibco, USA, #10099-141) in the absence of bFGF and EGF, and cultured for 3 days.

2.5. Flow cytometry (FCM) analysis of apoptotic cells

The ENCCs were dissociated into single-cell suspensions and collected by centrifugation according to the manufacturer's instructions. To assess apoptosis, the Annexin V-FITC/PI Apoptosis Detection Kit (YEASEN, CHN, #40302ES20) was employed. Briefly, after suspending the cells in 500 μl of Binding Buffer, 1.0×10^5 cells were mixed with 5 μl Annexin-FITC and 5 μl propidium iodide and incubated for 10 min at room temperature. Apoptosis was measured by FACSCalibur within 1 h.

2.6. Hanging cell culture inserts analysis of the migratory cells

Cell migration was determined using Millicell Hanging Cell Culture Inserts (8.0-μm pore size, PET track-etched membrane, BD Biosciences) in a 24-well plates as described previously [26]. Briefly, 500 μl of ENCC single-cell suspension (1.0×105 cells/ml) was plated in serum-free DMEM medium in the top chamber, while 750 μl of DMEM medium with 1.0% FCS was added to the bottom chamber to act as a chemoattractant. After 72-h incubation, ENCCs remaining on the upper surface of the filter were wiped off, while the ENCCs that had migrated through the filter were fixed with methanol, stained with hematoxylin and counted under a light microscope in five predetermined fields. Assays were performed in triplicate.

2.7. Immunocytochemistry analysis

Single cells were washed and rinsed with Hank's Balance Salt Solution, fixed with 4% paraformaldehyde for 5 min at room temperature, incubated with primary antibodies overnight at 4 °C, and then with secondary antibodies for 1 h at 25 °C. Before immunocytostaining, 1% triton X100 (Sigma-Aldrich) was applied. The primary antibodies used were $p75^{NTR}$, peripherin, and glial fibrillary acidic protein (GFAP). The cells were then stained with DAPI and the appropriate secondary antibodies (Cy3-conjugated goat anti-rabbit IgG, Abbkine, USA; Cy3-goat anti-mouse IgG, Proteintech Group, USA). Photographs were taken using fluorescence microscopy equipped with a DP70 digital camera and DP Manager software was used for analysis. All counts were performed by counting the number of immunopositive cells for a given antibody and the results were shown as numbers of positive cells in five random fields.

2.8. Statistical analysis

Statistics were carried out using either a two-tailed Student's *t-test* or one-way analysis of variance (ANOVA). A P value < 0.05 was considered statistically significant.

3. Results

ENCCs proliferated rapidly to form neurospheres. After 2 days, however, the majority of seeded cells had died, whilst the surviving cells began to proliferate to formed irregular neurospheres at day 2 (Fig. 1A), lobular-bodies at day 5 (Fig. 1B), and later characteristic neurospheres with a more compact appearance at day 7 (Fig. 1C). Furthermore, most of the adherent neurospheres detached from the culture plate surface at day 3 and continued growing as free-floating neurospheres at day 5. ENCC numbers at primary, third and sixth passage are shown in Fig. 1D. Positive identification of ENCCs in neurospheres using $p75^{NTR}$ immunocytochemistry is shown in Fig. 1E. After dissociation of the primary neurospheres, sub-cultured neurospheres were successfully generated. The above result suggested that prolonged culture