Regenerative Therapy 3 (2016) 7–10

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original article

Induction of hepatic tissues in multicellular spheroids composed of murine fetal hepatic cells and embedded hydrogel beads

Wakako Motoyama [a], Kanae Sayo [a], Hirotaka Mihara [a], Shigehisa Aoki [b], Nobuhiko Kojima [a, *]

[a] Graduate School of Nanobioscience, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan
[b] Department of Pathology & Microbiology, Faculty of Medicine, Saga University, Japan

ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form
30 December 2015
Accepted 20 January 2016

Keywords:
Spheroid
Fetal hepatic cell
Alginate hydrogel bead
Cell polarity
Microstructure

ABSTRACT

Introduction: Three-dimensional (3D) multicellular spheroids are useful tools for simulation of cellular functions *in vitro*. However, it is difficult to culture certain epithelial cell types under 3D spheroid conditions because these cells cannot resist autonomous cell death, triggered by disordered cell polarity. The objective of this study was to find a method that enables spheroid culture of such epithelial cells utilizing hydrogel beads without cell death.
Methods: We used murine E14.5 fetal hepatic cells for the spheroid composition because they are sensitive to disorganized structures. Spheroids were formed by injecting 1-μl fresh medium containing 1000 fetal hepatic cells and the same number of alginate hydrogel beads (20 μm in diameter) into a 3% methylcellulose medium in the presence of dexamethasone and oncostatin M to induce hepatic differentiation. After 7 days of culture, microstructures were observed using hematoxylin and eosin staining and immunostaining using anti-CK8/18 antibody. Albumin secretion rate was determined by the enzyme-linked immunosorbent assay method. In addition, polarity-related proteins, E-cadherin, ezrin, and MRP2 were observed with immunostaining.
Results: Control spheroids without the use of alginate hydrogel beads showed extensive internal lack of epithelial hepatic cells. The spheroids containing alginate hydrogel beads exhibited sheet- or cord-like structures of epithelial hepatic cells, and it was clear that cell death of epithelial cells had been prevented. Albumin secretion data also supported the improvement of epithelial hepatic cell viability when alginate hydrogel beads were used. Localization of polarity-related proteins indicated the partial reconstitution of cell polarity in the spheroids using alginate hydrogel beads.
Conclusion: Based on these data, we concluded that the application of alginate hydrogel beads was effective in improving the epithelial hepatic cell culture of multicellular spheroids.

1. Introduction

Multicellular spheroids are important for cell-based assays because cellular functions are highly enhanced in the three-dimensional (3D) culture. However, necrosis in the central areas of the relatively larger multicellular spheroids can sometimes occur because of oxygen/nutrient limitation [1–6], and this necrosis causes a decline in the spheroid functions. In addition, epithelial cells can sometimes form “multicellular cysts” through the induction of apoptosis; this is because epithelial cells tend to form polarized monolayer structures instead of disorganized structures [7]. This event arising independently from oxygen/nutrient limitation is also at risk for diminishing of the spheroid functions. We previously published a method of embedding alginate hydrogel beads into spheroids to improve oxygen/nutrient supply [8]. In this study, we show that the method is also critical in preventing loss of epithelial cells when multicellular spheroids are formed with fetal hepatic cells. We attempt to clarify the formation of cell polarity in such spheroids by visualizing polarity-related proteins.

Abbreviations: 3D, three-dimensional; MC, methylcellulose; HE, hematoxylin and eosin; CK, cytokeratin; MRP, multidrug resistance-associated protein; ELISA, enzyme-linked immunosorbent assay.

* Corresponding author. Tel.: +81 45 787 2214; fax: +81 45 787 2413.
E-mail addresses: n155279f@yokohama-cu.ac.jp (W. Motoyama), n155264e@yokohama-cu.ac.jp (K. Sayo), i130613a@yokohama-cu.ac.jp (H. Mihara), aokis@cc.saga-u.ac.jp (S. Aoki), nobuhiko@yokohama-cu.ac.jp (N. Kojima).

Peer review under responsibility of the Japanese Society for Regenerative Medicine.

http://dx.doi.org/10.1016/j.reth.2016.01.007

2. Materials and methods

2.1. Fetal hepatic cells

Pregnant C57BL/6NCrSlc mice were obtained from Japan SLC (Hamamatsu, Japan), and fetal hepatic cells were isolated by collagenase digestion [9]. In brief, the E14.5 fetal livers were minced and enzymatically digested with Liver Digest Medium (17703-034, Waltham, MA Thermo Fisher Scientific). After the hemolysis of red blood cells, the hepatic cells were purified by centrifugation. Isolated cells were suspended in the Dulbecco's Modified Eagle's Medium (041-29775, Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Cellgro, 35-010-CV, CORNING, Corning, NY, USA), 2 mmol/l GlutaMAX (35050-061, Thermo Fisher Scientific), 1× MEM Non-Essential Amino Acid Solution (11140-050, Thermo Fisher Scientific), 1× Insulin-Transferrin-Selenium X Supplement (51500-056, Thermo Fisher Scientific), 50 μg/ml gentamycin (15710-064, Thermo Fisher Scientific).

2.2. Preparation of alginate hydrogel beads

Alginate hydrogel beads were obtained as reported in a previous study [8]. Tiny droplets of 1.5% w/v sodium alginate (Sigma–Aldrich, St. Louis, MO, USA) solution were discharged from an inkjet system (Cluster Technologies, Osaka, Japan) into a reservoir flask filled with 5% calcium chloride (Wako, Osaka Japan) solution. The droplets of the alginate solution change into a gel immediately after they have been dropped into a solution containing calcium ions. When a 25-μm diameter nozzle was used, the size of the droplet was approximately 20 μm. Alginate hydrogel beads were washed with phosphate-buffered saline and suspended in fresh culture medium.

2.3. Fabrication of hybrid spheroids

The aggregation method used to fabricate hybrid spheroids was previously reported [10]. To make the 3% methylcellulose (MC) medium, 3 g MC (viscosity, 4000 cP; M0512; Sigma–Aldrich) was sterilized by autoclaving in a bottle. To MC, 100 ml of the culture medium was added; it was dispersed by mixing with a magnetic stirrer on ice. The MC medium was poured into 35-mm petri dishes with a positive-displacement pipette (Microman; Gilson, Middleton, WI, USA). The cells and alginate hydrogel beads were suspended in the normal culture medium, and the density of cells and beads were both adjusted to 1×10^6 cells/ml. The injection of 1 μl of the normal medium with cells and beads into the MC medium resulted in the rapid aggregation of both cells and beads. Because the aggregates did not sink to the bottom of the petri dish, they were able to be cultured for 7 days in the MC medium without adhesion to the bottom. To prevent dessication, 250 μl of culture medium was added to the MC medium at day 3. Throughout the culture period of 7 days, 10^{-7}-M dexamethasone (041-18861, Wako) and 10 ng/ml-oncostatin M (495-MO-025, Bio-techne, Minneapolis, MN, USA) were also added to the MC medium for inducing hepatic maturation [9]. To remove spheroids from the MC medium, 5 U/ml-cellulase solution (Onozuka RS; Yakult Pharmaceutical Industry, Tokyo, Japan) was added to the MC medium, and the mixture was incubated for 30 min at 37 °C to digest MC. After this step, spheroids were easily collected in a test tube.

2.4. Staining of spheroid sections

Paraffin-embedded spheroids were sectioned and visualized by hematoxylin and eosin (HE) staining or immunohistochemistry. For the detection of specific proteins, several antibodies were used as a primary antibody that included the following: anti-cytokeratin (CK) 8/18 (GP11; PROGEN Biotechnik, Heidelberg, Germany); anti-E-cadherin (610181; Becton, Dickinson and Company, Franklin Lakes, NJ, USA); anti-multidrug resistance-associated protein (MRP) 2 (sc-5770; Santa Cruz Biotechnology, Dallas, TX, USA); and anti-ezrin (sc-58758; Santa Cruz).

2.5. Albumin secretion assay

After 7 days culture in the MC medium, multicellular spheroids with or without alginate hydrogel beads were removed from the MC medium by decreasing the viscosity of the medium and transferring spheroids to untreated 12 well plates with fresh culture medium. The spheroids were then incubated, and the medium was sampled at 0 h and 48 h. Albumin secreted into the medium was detected using an enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi, Gunma, Japan). At the end of the assay, genomic DNA was extracted from the spheroids and quantified using a fluorometer (Quantus; Promega, Madison, WI, USA) to normalize albumin secretion by cell number. All values were expressed as mean ± SD based on three samples. Student t-tests were used to compare the samples, and differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Prevention of loss of epithelial fetal hepatic cell in the multicellular spheroids

Primary fetal hepatic cells isolated from an E14.5 mouse were injected into the MC medium and cultured for 7 days in the presence of hepatic differentiation inducers, dexamethasone, and oncostatin M. Spheroids at day 7 were sectioned and observed by HE staining or immunohistochemistry. Fig. 1a shows that inside of the spheroid was healthy and there were cells on the surface of the spheroid showing darker pink color. Based on our previous study, fetal hepatic cells were mainly composed of fetal hepatocytes those are CK8/18, a marker for epithelial cells, positive [11]. However, Fig. 1c shows that CK8/18 positive cells were detected only the surface of the spheroid. In contrast, spheroids obtained by injecting

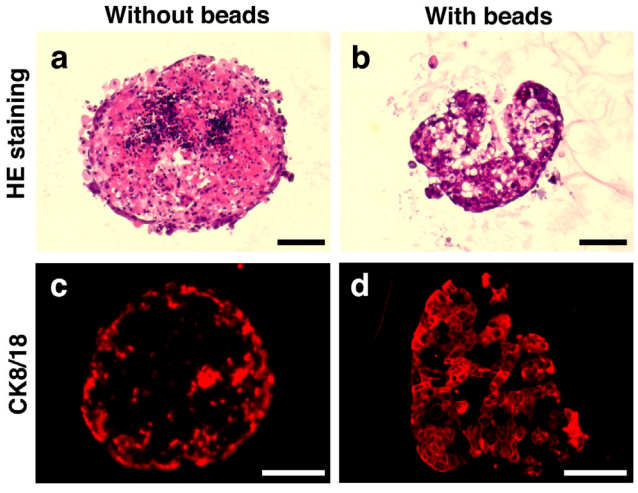

Fig. 1. Inner structure of multicellular spheroids comprising fetal hepatic cells. After 7 days culture, the spheroids in the MC medium were fixed, embedded in paraffin, and sectioned. (a and b) HE staining was performed to observe the inner structures. (c and d) CK8/18 was visualized by a fluorescent immunostaining method. a and c show cellular aggregates without beads, and b and d show cellular aggregates with beads. Bar: (a, b) 100 μm, (c, d) 50 μm.