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Original Article

Preparation of epithelial cell aggregates incorporating matrigel microspheres to enhance proliferation and differentiation of epithelial cells

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ABSTRACT

The objective of this study is to investigate the effect of matrigel microspheres (MM), gelatin hydrogel microspheres (GM), and matrigel-coated GM on the proliferated and biological functions of epithelial cells in cell aggregates incorporating the microspheres. The MM were prepared by a coacelvation method. When mammary epithelial EpH4 cells were cultured with the MM, GM, and matrigel-coated GM in round U-bottom wells of 96-multiwell culture plates which had been coated with poly (vinyl alcohol) (PVA) to suppress the cell adhesion, EpH4 cell aggregates with each microspheres homogeneously incorporated were formed. Higher EpH4 cells proliferation was observed for cell aggregates incorporating MM, GM, and matrigel-coated GM compared with the conventional 3-dimensional (3D) culture method. When examined to evaluate the epithelial differentiation of EpH4 cells, the β-casein expression was significantly higher for the cell aggregates incorporating MM than that of aggregates incorporating GM and matrigel-coated GM or the conventional 3D culture method. It is concluded that the proliferation and differentiation of mammary epithelial EpH4 cells were promoted by the incorporation of MM.

1. Introduction

Recently, cell researches have become more and more popular to clarify the molecular mechanisms of cell proliferation and differentiation. The epithelium is the first emerging tissue during ontogenesis, and epithelial cells play fundamental roles in embryo morphogenesis and organ development [1–5]. Epithelial cells have segregated apical and basolateral plasma membrane domains with asymmetric compositions of nutrient and fluid transporters which are required to carry out crucial vectorial transport functions and cytoplasmic polarity to generate different cell progenies for tissue morphogenesis [6,7]. However, there have been some problems by the culture of epithelial cells. In two-dimensional (2D) cell culture systems on a plastic plate, epithelial cells quickly lose their functions, and do not always proliferate as well as other types of cells. Because the local environment of epithelial cells is different from that of mesenchymal cells in living tissues [8]. As one tried to tackle this problems, epithelial cells are cultured with the feeder layer of fibroblasts for their proliferation, but their functions are biologically insufficient because of the lack of basement membrane components [9–11]. In three-dimensional (3D) cell culture systems, epithelial cells are often cultured with 3D basement membrane component-rich gels [12,13]. Cell aggregates are formed with a central lumen and polarized structures, but cells are not proliferated well, while cells in center of aggregates die by apoptosis [14–18]. We demonstrate that mouse preosteoblast MC3T3-E1 cells were cultured with gelatin hydrogel microspheres (GM) to form the MC3T3-E1 cell aggregates homogeneously incorporating GM for an enhanced cell proliferation and osteogenic differentiation [19]. The GM incorporation enabled cells to rescue the lack of oxygen in cell aggregates.

In the physiological condition, most cells are present in a 3D structure in which the cell–cell and cell–extracellular matrix interactions are naturally to allow cells to survive and biologically function [20]. This 3D structure of cells is important and essential to promote their functions. For example, embryonic stem cells generally aggregate to form an embryoid body, and consequently

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initiate their differentiation into different cell lineages [21]. The aggregation of liver cells to form a spheroid is necessary to enhance their metabolic activity [22]. Cell aggregates produce extracellular matrix proteins more efficiently than single cells [23]. Considering the cell structure of body tissues, such as liver and bone, cell aggregates biologically function as the minimum unit [24].

The objective of this study is to prepare a new 3D aggregates culture system of epithelial cells for an enhanced cell proliferation and differentiation. In this study, matrigel microspheres (MM) and matrigel-coated GM were prepared. Mouse mammary epithelial EpH4 cells were cultured with the microspheres to form cell aggregates homogeneously incorporating microspheres to evaluate the proliferation and differentiation in terms of the expression of differentiation markers. We examine the effect of MM, GM, and matrigel-coated GM on the cell behavior.

2. Materials and methods

2.1. Preparation of matrigel microspheres

Matrigel microspheres (MM) were prepared by a coacelvation method [25]. According to the coacelvation method, nanospheres or microspheres with narrow-size distribution and small size were prepared. Briefly, 1.0 ml of 10 vol% aqueous Becton, Dickinson and Company (BD) Matrigel™ Basement Membrane Matrix (BD Biosciences, Inc., Franklin Lakes, America) solution was prepared at 4 °C. Then, 4 ml of 2-butanol (Nacalai Tesque, Inc., Kyoto, Japan) was added to the matrigel solution at 4 °C. The resulting microspheres were gelationed for 1 h at 37 °C. Then, 2-butanol was removed by evaporation, and followed by centrifuged for 5 min at 14,000 rpm at 4 °C to obtain MM. The MM were stored at −30 °C until to use.

2.2. Preparation of gelatin hydrogel microspheres

Gelatin hydrogel microspheres (GM) were prepared by the chemical crosslinking of gelatin in a water-in-oil emulsion state according to the method previously reported [26]. Briefly, an aqueous solution (20 ml) of 10 wt% gelatin (isoelectric point 5.0, weight-averaged molecular weight 1,00,000, Nitta Gelatin Inc., Osaka, Japan) was preheated at 40 °C, and then added dropwise into 600 ml of olive oil (Wako Ltd, Osaka, Japan) at 40 °C, followed by stirring at 400 rpm for 10 min to prepare a water-in-oil emulsion. The emulsion temperature was decreased to 4 °C for the natural gelation of gelatin solution to obtain non-crosslinked hydrogel microspheres. The resulting microspheres were washed three times with cold acetone in combination with centrifugation (5000 rpm, 4 °C, 5 min) to completely exclude the residual oil. Then, they were fractionated by size using sieves with apertures of 20 μm (Iida Seisakusho Ltd, Osaka, Japan) and air dried at 4 °C. The non-crosslinked and dried gelatin hydrogel microspheres (200 mg) were treated in a vacuum oven at 140 °C and 0.1 Torr for the dehydrothermal crosslinking of gelatin for 24 h according to the method previously reported [19]. The pictures of gelatin hydrogel microspheres in the water swollen state were taken with a microscope (CKX41, Olympus Ltd, Tokyo, Japan). The size of 100 microspheres for each sample was measured using the computer program Image J (NIH Inc., Bethesda, USA) to calculate the average size.

2.3. Preparation of matrigel-coated gelatin hydrogel microspheres

The matrigel solution (20 μl) was dropped onto 2 mg of freeze-dried gelatin hydrogel microspheres (GM), followed by leaving at 4 °C for overnight to allow matrigel to absorb onto the microspheres. The matrigel solution was completely absorbed into the GM because the solution volume was much less than that theoretically required for the equilibrated swelling of microspheres. To evaluate the matrigel existence on the surface of matrigel-coated GM were incubated with an anti-laminin antibody (Abcam Inc., Cambridge, UK) for 60 min at 25 °C and subsequently Alexa Fluor® 488 Donkey anti-rabbit (Thermo Fisher Inc., Massachusetts, America) for 30 min at 25 °C, followed by fluorescent viewing with confocal laser scanning microscope (FV1000D, Olympus Ltd, Tokyo, Japan).

2.4. EpH4 cell culture

EpH4 cells of a mouse mammary epithelial cell line were transfected and clones selected as previously described [27]. EpH4 were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Thermo Inc., Waltham, USA) supplemented with 2 vol% fetal calf serum (FCS) (Thermo Inc., Waltham, USA), gentamicin, 3 μg/ml prolactin (Sigma–Aldrich Inc., St. Louis, America), 1 μg/ml hydrocortisone (Sigma–Aldrich Inc., St. Louis, America) and 5 μg/ml insulin (Sigma–Aldrich Inc., St. Louis, America) (standard medium) and cultured at 37 °C in a 95% air–5% CO_2 atmosphere. The culture medium was changed every 2 days and confluent cells were subcultured through trypsinisation. In the experiment of phosphotidylinositol 3-kinase (PI3K, Sigma–Aldrich Inc., St. Louis, America) inhibition experiment of EpH4 cells, the standard medium containing a PI3K inhibitor LY294002 (50 μM, Abcam Inc., Cambridge, British) was used and the cells were cultured for 72 h.

2.5. Preparation of cell aggregates incorporating MM, GM, and matrigel-coated GM

Poly(vinyl alcohol) (PVA, 1800 degree of polymerization and 88 mol% saponification) kindly supplied from Unichika (Tokyo, Japan) was dissolved in 1× Dulbecco's phosphate buffered saline (PBS, Nissui Ltd, Tokyo, Japan) to give a concentration of 1 wt%. The PVA solution (100 μl/well) was added to each well of 96-multiwell culture plate with either flat- or round-bottomed (U-bottomed) wells and incubated at 37 °C for 15 min. Then, the solution was removed by aspiration and the wells were washed twice with PBS (100 μl/well). MM, GM or matrigel-coated GM and EpH4 cells were separately suspended in the standard medium. The microspheres-free standard medium or the suspensions of MM, GM, and matrigel-coated GM (0, 2×10^3, 2×10^4, 2×10^5 microspheres/ml) (50 μl/well) were added to the PVA-coated wells, followed by the addition of EpH4 cells suspension (2×10^5 cells/ml, 50 μl/well). For the conventional 3D epithelial cells culture, 10 μl of matrigel solution was placed into the flat-bottomed wells of 96-well flat-bottomed culture plates at 4 °C, and then the culture plates were incubated at 37 °C for 15 min for matrigel polymerization. Next, EpH4 cells suspension (1×10^5 cells/ml, 100 μl/well) containing 2 vol% matrigel was added in the matrigel-treated wells. The pictures of cells 3D cultured with were taken with a microscope (CKX41, Olympus Ltd, Tokyo, Japan).

2.6. Evaluation of cells viability in cell aggregates incorporating MM, GM, and matrigel-coated GM

Live/dead assays were conducted using Live/Dead® Viability/Cytotoxicity assay (Invitrogen Inc., Carlsbad, UK) according to the manufacture's protocol. After 7 days culture, cell aggregates were rinsed with PBS, and then incubated with a solution containing 2 μM calcein AM and 4 μM EthD-1 in PBS for 30 min at 37 °C in the dark, followed by fluorescent viewing with the confocal laser scanning microscope.