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Incorporation of gelatin microparticles on the formation of adipose-derived stem cell spheroids

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ABSTRACT

The three-dimensional (3D) cell culture method provides a physiological environment that is similar to the *in vivo* conditions. The spheroid culture method is a 3D cell-culture approach that facilitates aggregation and interaction of cells with other adjacent cells to establish a microenvironment *in vitro*. In this study, gelatin microparticles (GMPs) were introduced into spheroids to enhance stem cell proliferation and viability. GMPs with different sizes and numbers were prepared to investigate their corresponding effects on cell proliferation. To investigate the effect of surface charge on the properties of spheroids, positively and negatively charged GMPs were also prepared by conjugating poly-amino acids on the surfaces of the GMPs. GMPs were successfully incorporated into adipose-derived stem cells (ASCs) to form spheroids. The results revealed no cytotoxicity in all treatment groups. Spheroids combined with GMPs showed higher proliferation potential compared to cell spheroids alone, suggesting that GMP/cell spheroids can be used for efficient delivery of stem cells to defect sites without the need for surgery, thereby highlighting the potential use of GMP/cell spheroids for stem cell therapy.

1. Introduction

Stem cells are pluripotent, and thus, capable of constant self-renewal and differentiation into diverse cell types, making them useful for the treatment of diseases that require tissue regeneration [1]. In addition, stem cells modulate immunity to alleviate inflammatory responses [2,3]. In particular, mesenchymal stem cells (MSCs) have been widely studied for use in clinical applications because they are easy to obtain, ethical, and do not induce adverse immune reactions [4]. MSCs are often used in combination with bioactive macromolecules, such as growth factors or cytokines, to improve tissue regeneration [3,5,6]. In stem cell therapy and tissue engineering applications, MSCs are transplanted alone or incorporated into scaffolds to regenerate bone, cartilage, muscles, dermis, tendons/ligaments, and other connective tissues [7].

Recently, 3D cell culture has been widely investigated in the fields of biology, tissue engineering, and medicine because it provides an environment that is more physiologically similar to the human body than the conventional monolayer culture *in vitro* [8–10]. Spheroid culture is one of the well-known 3D culture methods that can produce cells at high densities. In spheroid cultures, the aggregated cells secrete extracellular matrix (ECM) and thus interact with each other in a microenvironment [11,12]. During spheroid formation, cells must minimize contact with the surface and establish frequent contacts with other cells [13–15]. Strategies to successfully induce cell contacts include the spinner flask, liquid overlay, hanging drop, concave well, and microfluidics methods [16–23]. The spheroids generated using stem cells have been studied to regenerate heart [24], liver [25], and cartilage [26] tissues.

However, oxygen and nutrients are often inaccessible to cells in the interior of the spheroid aggregates, which can lead to either cell death of the inner cells or differences in behavior between the outer and inner cells [27]. The proliferation of cells in spheroids is also limited by their sizes [28]. Several methods have been employed to overcome the lack of exchange of essential components in spheroids [28,29]. Larger spheroids have larger diffusion gradients; thus, researchers are currently developing methods to reduce spheroid size [30,31]. To overcome this problem, spinner flasks have been used to facilitate an even distribution of oxygen and nutrients throughout the medium and to maximize diffusion [32]. In the same manner, a method of flowing fluids in a microfluidic device to promote diffusion was also explored [33].

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In some cases, spheroids with biomaterials have been studied. Kim, Mihye, et al. chose heparin, which is abundant in the liver, as a hydrogel to encapsulate hepatocytes and showed spheroids were significantly more functional compared to single cell suspension in the heparin gel [34]. Fukuda, Junji, et al. generated controlled 3D co-culture systems using chitosan hydrogel. Human hepatoblastoma cells formed spheroids above patterned chitosan (glass exposed part) and NIH-3T3 fibroblasts attached on chitosan surface [35].

In our study, we employed gelatin microparticles to form spheroids with stem cells to investigate the mechanisms of cell proliferation. Gelatin contains arginine–glycine–aspartic acid (RGD) sequences on the surface to facilitate cell attachment and can be degraded by the enzymes produced by cells [36,37]. Thus, we hypothesized that incorporation of gelatin microparticles into stem cell spheroids could either increase cell viability or promote cell proliferation by providing a suitable surface for cell attachment and space for facile diffusion of nutrients [38,39]. In this study, gelatin microparticles (GMPs) with different sizes and surface charges were fabricated and used to form stem cell spheroids. The generated GMPs were then used to examine the effects of surface area and surface charges of GMPs on cell viability and proliferation to optimize the formation of GMP/cell spheroids.

2. Material and methods

2.1. Materials

Gelatin from porcine skin, paraffin oil, span 80, and 1-ethyl-(-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Glutaraldehyde (50%) and acetone were purchased from Daejung Chemicals and Metals (Seoul, South Korea). Adipose-derived stem cells (ASCs) were obtained from the CHA University. StemFit 3D® was purchased from Microfit (Gyeonggi, South Korea). All culture media were purchased from Hyclone (Chicago, IL, USA). Rabbit anti-porcine gelatin antibody was purchased from Mybiosource (San Diego, CA, USA). Alexa Fluor 488®, DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride), Rhodamine-Phalloidin, and a Live/Dead assay were purchased from Life Technologies, Thermo Scientific (Waltham, MA, USA). A cell counting kit-8 (CCK-8) assay was purchased from Dojindo (Kumamoto, Japan).

2.2. Gelatin microparticles

GMPs were prepared in accordance with previous studies using a water-in-oil method [40]. Briefly, 6 g of gelatin type A was dissolved in 40 ml of distilled water at 45 °C. Then, 100 ml of paraffin oil and 1.2 ml of span 80 were mixed, and the gelatin solution was added dropwise to the oil mixture. The mixture was stirred for 1 h at 60 °C at 200 rpm, after which the temperature was decreased to 10 °C. Then, 200 μl of 50% glutaraldehyde was added, and the mixture was stirred for 2 h. After washing and filtering with 500 ml of acetone, the excess acetone was removed using a desiccator. To visualize the effect of the GMP size on spheroid formation, GMPs were successively sieved using 5–20 μm, 20–38 μm, and 38–63 μm sieves (Cheonggye Commerce and Industry, Seoul, Korea). The prepared GMPs were subjected to EO sterilization (SE30, ALPS Corp., Gyeonggi, Korea) prior to use in cell experiments.

2.3. Surface charge modification of GMPs

The GMPs were modified with L-γ-glutamic acid, poly-L-arginine, and poly-L-lysine to facilitate the attachment of cells on the GMPs according to their surface charges. First, 50 mg of EDC and 1 mg of poly amino acids were dissolved in 10 ml of DPBS and incubated at room temperature for 20 min. Then, 3.89×10^5 GMPs were added to the solution, followed by incubation for 18 h. After washing to remove unreacted chemicals, the GMPs were sonicated for 30 min and freeze-dried for 48 h. The surface charges of the unmodified and modified GMPs were measured using a zetasizer (NANO ZS, Malvern instruments Ltd, Malvern, UK).

2.4. Gelatin microparticle morphology

Scanning electron microscopy (SEM) photographs were captured to confirm the morphologies of the GMPs. GMPs were pretreated with an ion sputter coater (E-1045, HITACHI, Tokyo, Japan) and observed using SEM (S-3400N, HITACHI, Tokyo, Japan).

2.5. Cell culture

ASCs were incubated at 37 °C in a 5% CO_2 incubator, and the culture medium was replaced every two days. The culture medium contained 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution in a low glucose Dulbecco's modified Eagle's medium (DMEM). Upon reaching 70% confluence, the ASCs were trypsinized for preparation of the GMP/cell spheroid aggregates.

2.6. GMP/cell spheroid formation

Seeding in StemFit 3D® was conducted according to the manual. Briefly, the microwells were washed with DPBS, after which 1 ml of 3% BSA solution was added, followed by incubation for 2 h. The GMPs were prepared in BSA solution at the following three concentrations: 97,250 MPs/ml (100 MPs/well), 194,500 MPs/ml (200 MPs/well), and 4,862,500 cells (1000 cells/well). The ASCs were also suspended in the BSA solution at 648,333/ml (1000 cells/well) and 1,296,666/ml (2000 cells/well). Next, 400 μl of the solution containing microparticles and 600 μl of cell suspension in the StemFit 3D® with 389 microwells were incubated in a CO_2 incubator for 1 h. After confirming spheroid formation using the microscope, the cells and gelatin particles remaining on the rim were removed. The culture medium was replaced with fresh medium every two days, and the spheroids were maintained in a CO_2 incubator. After washing with DPBS, the GMP/cell spheroids were harvested using a micropipette, centrifuged at 700 rpm for 1 min, and then characterized.

2.7. Immunofluorescence image

The cells and microparticles were immunofluorescently stained with different colors to observe the distribution and binding pattern of cells and microparticles in the spheroids. The cells were stained with DAPI in accordance with previous studies, and actin was stained with rhodamine-phalloidin [41]. Gelatin type A-specific antibodies with fluorescent dye were added at the same time as the rhodamine stain. The procedure for attachment of the fluorescent dye to the antibody was performed in accordance with the manufacturer's instructions. The stained GMP/cell spheroids were observed using a confocal laser microscope (SP8 X, Leica, Germany).

2.8. Cell viability test

A Live/Dead assay was used to confirm the morphological stability of spheroids in addition to cell viability. The GMP/cell spheroids were harvested from the StemFit 3D®, transferred to a 96-well plate, and reconstituted at the concentrations indicated in the manuals provided by Life Technologies. The spheroids were then placed in a CO_2 incubator for 30–60 min, and then, observed under a fluorescence microscope.

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