Acta Biomaterialia 7 (2011) 2797–2803

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Preparation of stem cell aggregates with gelatin microspheres to enhance biological functions

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ARTICLE INFO

Article history:
Received 21 February 2011
Received in revised form 29 March 2011
Accepted 13 April 2011
Available online 20 April 2011

Keywords:
Mesenchymal stem cell
Aggregate
Gelatin microspheres
Proliferation
Osteogenic differentiation

ABSTRACT

The objective of this study is to improve the viability and osteogenic differentiation of cultured rat bone marrow-derived mesenchymal stem cells (MSC) by the use of gelatin hydrogel microspheres. Gelatin was dehydrothermally crosslinked at 140 °C for 48 h in a water in oil emulsion state. When cultured with the gelatin hydrogel microspheres in round, U-bottomed wells of 96-well plates coated with poly(vinyl alcohol) MSC formed aggregates homogeneously incorporating the microspheres. The viability of the cell aggregates was significantly higher compared with that of aggregates formed without microspheres. MSC proliferation in the aggregates depended on the number and diameter of the incorporated microspheres. Higher MSC proliferation was observed for aggregates incorporating a greater number of larger gelatin microspheres. When evaluated as a measure of aerobic glycolysis the ratio of L-lactic acid production/glucose consumption in MSC was significantly lower for MSC cultured with gelatin microspheres than those without microspheres. MSC production of alkaline phosphatase (ALP) and sulfated glycosaminaglycan (sGAG) was examined to evaluate their potential osteogenic and chondrogenic differentiation. The amount of ALP produced was significantly higher for MSC aggregates cultured with gelatin microspheres than that of MSC cultured without microspheres. On the other hand, the amount of sGAG produced was significantly lower for MSC aggregates containing microspheres. It is concluded that the incorporation of gelatin hydrogel microspheres prevents the aggregated MSC suffering from a lack of oxygen, resulting in enhanced MSC aggregation and cell proliferation and osteogenic differentiation.

1. Introduction

With the recent advent of stem cell biology various stem cells have become available and basic cell research has become more and more popular to clarify the molecular mechanisms of cell proliferation and differentiation. Most cells need to form an aggregate in which cell–cell interaction is physiologically promoted, enhancing cell survival and function [1]. For example, embryonic stem cells generally aggregate to form an embryoid body and consequently initiate differentiation along different cell lineages [2]. The aggregation of liver cells to form a spheroid is necessary to enhance metabolic activity [3]. Cell aggregates produce extracellular matrix proteins more efficiently than single cells [4]. Looking at the cell structure of body tissues such as liver and bone, cell aggregates function as the minimum unit of formation [5]. Taken together, aggregation can manipulate cell–cell interactions, resulting in enhanced biological formations of cells. Conventional cell culture has been performed in two-dimensional systems, which is very different from the local environment of cells in living tissues. Therefore, three-dimensional cell culture technologies have been developed [6–9]. However, when cell aggregates become too large the cells inside the aggregate tend to die due to a lack of oxygen and nutrients [10,11]. Such conditions often make it difficult in practice to perform vitro cell culture over a long time period, although long-term culture is required for basic research into cell differentiation and organization.

Gelatin is a biodegradable biomaterial which have been extensively used for medical, pharmaceutical, and cosmetic applications. Its biosafety has been proven through long-term practical usage [12]. Hydrogels of different shapes can be formulated and their feasibility as cell culture substrates [13–15] and cell scaffolds for tissue regeneration [16–20] or as carriers of growth factors and drugs [21–28] have been experimentally demonstrated. Gelatin microspheres are effective in enhancing growth factor-induced tissue regeneration [29–31]. Based on availability and the easiness of hydrogel formulation, gelatin was selected as the hydrogel material in this study.

The objective of this research was to obtain fundamental information on whether or not the incorporation of microspheres into cell aggregates can affect the viability and function of cells present in the aggregates. To this end, hydrogel microspheres were incorporated into aggregates of rat bone marrow-derived mesenchymal stem cells (MSC) to positively affect the supply of oxygen and

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doi:10.1016/j.actbio.2011.04.013

nutrients. The surface of a cell culture plate was coated with poly (vinyl alcohol) to prevent cell adhesion to the plate. Consequently, cells will naturally interact with each other to efficiently form aggregates. During cell culture gelatin microspheres were co-cultured to allow their incorporation into the cell aggregates to achieve a better supply of nutrients and oxygen to cells within the aggregates. The viability of MSC aggregates incorporating gelatin microspheres was evaluated and compared with that of cell aggregates formed without microspheres. We also examined the osteogenic and chondrogenic differentiation of MSC aggregates.

2. Materials and methods

2.1. Preparation of gelatin hydrogel microspheres

Gelatin microspheres were prepared by chemical cross-linking of gelatin in a water in oil emulsion state according to a method previously reported [32]. 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 200 or 400 r.p.m. for 10 min to prepare a water in oil emulsion. The emulsion temperature was decreased to 4 °C for natural gelation of the gelatin solution to obtain non-crosslinked microspheres. The resulting microspheres were washed three times with cold acetone in combination with centrifugation (5000 r.p.m., 4 °C, 5 min) to completely exclude residual oil. Then they were fractionated by size using sieves with apertures of 20, 32, and 53 μm (Iida Seisakusyo Ltd, Osaka, Japan) and air dried at 4 °C. The non-crosslinked and dried gelatin microspheres (200 mg) were treated in a vacuum oven at 140 °C and 0.1 Torr for dehydrothermal crosslinking of gelatin according to a previously reported method [24]. Photographs of gelatin hydrogel microspheres in the water swollen state were taken with a microscope (CKX41, Olympus, Tokyo, Japan). The size of 100 microspheres for each sample was measured using the computer program Image J (NIH, Bethesda, USA), from which the average diameter was calculated.

2.2. MSC preparation

MSC were prepared from the bone marrow of 3-week-old, male Fischer F344 rats (Shimizu Laboratory Supply, Kyoto, Japan) according to the conventional procedure [33]. Briefly, both ends of the rat femurs were cut away from the epiphysis and the bone marrow was flushed out using a syringe (21 gauge needle) with 1 ml of 10 mM phosphate-buffered saline (PBS), pH 7.4. The cell suspension was placed in two 25 cm^2 flasks (Iwaki Glass Ltd, Chiba, Japan) containing 4 ml of alpha minimum essential medium (αMEM) (Invitrogen, Carlsbad, USA) supplemented with 15 vol.% fetal calf serum (FCS) (Thermo Inc., Waltham, USA), penicillin (50 U ml^{-1}), and streptomycin (50 U ml^{-1}) (standard medium) and cultured at 37 °C in a 95% air, 5% carbon dioxide atmosphere. The medium was changed on day 3 and every 3 days thereafter. When the culture became sub-confluent, 7–10 days culture later, the cells were detached by treatment with PBS containing 0.25 wt.% trypsin and 0.02 wt.% ethylenediaminetetraacetic acid for 5 min at 37 °C. The cells were subcultured at a density of 2×10^4 cells cm^{-2}. When the cells had reached a sub-confluent state they were used for the following experiments. This was approved and performed according to the guidelines of the Institute for Frontier Medical Sciences, Kyoto University.

2.3. Preparation of the cell non-adhesion surface

Various types of poly(vinyl alcohol), kindly supplied from Unichika (Tokyo, Japan) (Table 1) were dissolved in PBS (1 wt.%). The solution (500 μl $well^{-1}$) was added to each well of a 24-well plate and incubated at 37 °C for 15 min. Then the solution was removed with an aspirator and the wells were washed twice with PBS (500 μl $well^{-1}$). The MSC suspension (4×10^4 cells ml^{-1}, 500 μl $well^{-1}$) was placed in the wells of the plate. After 4 h incubation, the number of cells present in the medium was determined by a fluorometric DNA assay according to a reported previously method [34]. Briefly, cells were carefully washed three times with PBS, collected, and stored at −30 °C until assay. After thawing the cells were digested in 30 mM sodium citrate-buffered saline solution (SCS), pH 7.4, containing proteinase K (250 μg ml^{-1}) and sodium dodecyl sulfate (0.2 mg ml^{-1}) at 65 °C for 12 h. After the cell sample solution (100 μl) was mixed with 100 μl of SCS containing 1 mg ml^{-1} Hoechst 33258 dye the fluorescence intensity of the mixed solution was measured in a fluorescence spectrometer (Sectra Max Gemini EM, Molecular Devices, Sunnyvale, USA) at excitation and emission wavelengths of 355 and 460 nm, respectively. Cell number was obtained by use of a calibration curve which had been prepared with suspensions containing determined numbers of cells. Experiments were performed on three wells for each sample unless otherwise mentioned. Then, the ratio of non-adherent cells to those adhering to the PVA-coated wells was calculated.

2.4. Preparation of MSC aggregates with or without gelatin microsphere incorporation

PVA with a 1800 degree of polymerization and 88 mol.% saponification was dissolved in PBS (1 wt.%). This solution (100 μl $well^{-1}$) was added to each well of a 96-well 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 $well^{-1}$). Gelatin microspheres of 17.6 ± 7.4, 47.9 ± 22.2, and 106.8 ± 17.8 μm diameter and MSC were separately suspended in standard medium. Suspensions of gelatin microspheres (0, 2×10^3, 2×10^4, 2×10^5, and 2×10^6, 50 μl $well^{-1}$) were added to the coated wells, followed by the MSC suspension (2×10^4 cells ml^{-1}, 50 μl $well^{-1}$). Experiments were performed on three wells for each sample unless mentioned otherwise.

2.5. Preparation of cell aggregate frozen sections

MSC (1×10^4 per well) were cultured with 1×10^4 gelatin microspheres (17.6 ± 7.4 μm diameter) to form the cell aggregates. After incubation for 7 days the aggregates were fixed with 4% paraformaldehyde at 4 °C for 1 h and embedded in optimal cutting temperature compound (Sakura Finetek Japan Co. Ltd, Tokyo, Japan) and frozen using liquid nitrogen. The frozen samples were sectioned using a cryotome (CM3050S, Leica Microsystems, Wetzlar, Germany) and stained with TO-PRO-3 (Molecular Probes, Eu-

Table 1
Physicochemical properties of PVA coated.

Degree of polymerization	Percentage of saponification (mol.%)
500	88
1000	88
1700	96.5
1800	88
1800	84.5

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