Regenerative Therapy 3 (2016) 58–62

Contents lists available at ScienceDirect

Regenerative Therapy

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

Original article

# Evaluation of bioactivity of octacalcium phosphate using osteoblastic cell aggregates on a spheroid culture device

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A R T I C L E  I N F O

*Article history:*
Received 30 November 2015
Received in revised form
27 January 2016
Accepted 10 February 2016

*Keywords:*
Spheroids
Calcium phosphate
Octacalcium phosphate
Oxygen-permeable chip
Osteoblastic differentiation

A B S T R A C T

Much attention has been paid to three-dimensional cell culture systems in the field of regenerative medicine, since three-dimensional cellular aggregates, or spheroids, are thought to better mimic the in vivo microenvironments compared to conventional monolayer cultured cells. Synthetic calcium phosphate (CaP) materials are widely used as bone substitute materials in orthopedic and dental surgeries. Here we have developed a technique for constructing a hybrid spheroid consisting of mesenchymal stem cells (MSCs) and synthetic CaP materials using a spheroid culture device. We found that the device is able to generate uniform-sized CaP/cell hybrid spheroids rapidly and easily. The results showed that the extent of osteoblastic differentiation from MSCs was different when cells were grown on octacalcium phosphate (OCP), hydroxyapatite (HA), or β-tricalcium phosphate (β-TCP). OCP showed the greatest ability to increase the alkaline phosphatase activity of the spheroid cells. The results suggest that the spheroids with incorporated OCP may be an effective implantable hybrid consisting of scaffold material and cells for bone regeneration. It is also possible that this CaP–cell spheroid system may be used as an in vitro method for assessing the osteogenic induction ability of CaP materials.

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## 1. Introduction

The development of three dimensional (3-D) cell culture models has attracted a great deal of attention in the field of tissue engineering, since 3-D cell cultures appear to better mimic the microenvironment around cells within the body as well as stimulate physiological responses compared to conventional two dimensional (2-D; monolayer) cultures. The creation of functional 3-D tissue with or without scaffold materials could be useful not only for tissue engineering, but also for helping to better understand basic mechanisms of cell–cell and/or cell–matrix interactions and tissue development.

One of the biggest problems of using 3-D cell aggregates in regenerative medicine is the development of hypoxia and subsequent cell death due to a lack of oxygen supply in the center of cell aggregates. To overcome this problem, we have developed an oxygen-permeable spheroid culture device [1]. As an alternative approach, other groups have reported the use of microspheres in cell aggregates in order to prevent a lack of oxygen and nutrients in the center of spheroids. In those studies, it was shown that the incorporation of microbeads made of gelatin [2] and alginate [3] into cell aggregates promoted cell activities.

Here we present a methodology to promote osteoblastic differentiation of mesenchymal stem cells by incorporating microparticles consisting of calcium phosphate (CaP) materials. We hypothesized that the presence of the CaP minerals may favor osteoblastic differentiation of cells in the cell aggregates. In this paper, we compare the formation of aggregates and the

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

http://dx.doi.org/10.1016/j.reth.2016.02.004
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osteoblastic differentiation of mesenchymal stem cell strain D1 with or without three types of CaP materials: hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), or octacalcium phosphate (OCP). HA and β-TCP are commonly used bone substitutes in clinical use. Our previous study showed that OCP promotes new bone formation compared to HA and β-TCP in bone defects of rat tibia [4]. In this study we present a novel 3-D cell culture system that could be an effective strategy for promoting osteoblastic differentiation of MSCs in vitro.

## 2. Materials and methods

### 2.1. Fabrication of spheroid culture chips

We prepared a spheroid culture chip as previously described [1]. Briefly, a polydimethylsiloxiane (PDMS) negative mold was replicated from a prototype culture device utilizing the thin PDMS membrane deformation by applying negative pressure [5]. A PDMS (Silpot 184, Dow Corning Toray, Co. Ltd., Tokyo, Japan) prepolymer was prepared by mixing the base and curing agent at a ratio of 10:1. The negative mold was immersed in 4% Pluronic F-127 (Sigma–Aldrich, St. Louis, MO, USA) solution for 24 h to facilitate wetting of the surface of the mold and to prevent PDMS-to-PDMS adhesion [1]. PDMS prepolymer was poured into the PDMS negative mold and cured at 70 °C for 1 h. The PDMS replica was peeled off from the mold and used in the cell culture in the present study (Oxy chip). The Oxy chip was designed to consist of multicavities (512 wells, 1.00 mm in diameter, 1.05 mm pitch, 1.06 mm in depth) in a triangular arrangement on a 25 × 25 mm section of the cell culture area.

### 2.2. Cell culture

Mouse bone marrow-derived mesenchymal stem cells (D1 ORL UVA [D1]) were obtained from ATCC (Rockville, MD, USA). The cells were maintained in minimum Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (PS, Invitrogen-Gibco, Carlsbad, CA) at 37 °C in a 5% carbon dioxide environment. The PDMS chips were sterilized in an oven (160 °C, 2 h). Before use, the PDMS chips were incubated with 2 ml of 4% Pluronic F-127 solution overnight. The polymer is adsorbed on the surface of the PDMS and prevents cell attachment [1,6]. The chips were then rinsed three times with DMEM to remove excess Pluronic F-127.

OCP was prepared by mixing calcium and phosphate solutions as previously described [7]. Commercially available sintered β-TCP (OSferion: OLIMPUS TERUMO BIOMATERIALS, Tokyo, Japan) and HA (APACERAM: PENTAX, Tokyo, Japan) were purchased. OCP, β-TCP, and HA granules were obtained by passing them through a standard testing sieve (270-mesh sieve and 53 μm). The sieved granules were sterilized by heating at 120 °C for 12 h. The average particle size of CaPs was measured using a SHIMAZDU SALD-2000J laser diffraction particle size analyzer. The analysis revealed that the average size of OCP, HA, and β-TCP were 30.5, 18.8, 41.9 μm, respectively.

D1 cells ($1.0 \times 10^6$ cells) were mixed with OCP granules (1.0 mg), β-TCP granules (2.0 mg), or HA granules (5.0 mg) in 3 ml of osteogenic differentiation medium (DMEM supplemented with 10% FBS, 1% PS, 50 μg/ml ascorbate 2-phosphate, 10 mM β-glycero phosphate, and 100 nM dexamethasone). Cells ($1.0 \times 10^6$ cells) without calcium phosphate granules were inoculated in the Oxy chip as a control group. All cells were cultured at 37 °C, 5% $CO_2$, and 95% air in humidified incubators. The culture medium was changed every two days.

### 2.3. Spheroid diameter measurement

To evaluate changes in spheroid diameter, spheroids were photographed with a photomicroscope (Leica DFC300 FX, Leica Microsystems Japan, Tokyo, Japan). Spheroid diameters were analyzed using an image analysis program for Windows (Image-Pro Plus 7.0, Media Cybernetics Inc., Bethesda, MD, USA). A minimum of 30 spheroids on each chip were photographed and diameters were measured. Spheroid diameter was defined as the average length of diameters measured at two-degree intervals joining two outline points and passing through the centroid.

### 2.4. Measurement of DNA content and alkaline phosphatase (ALP) of D1 cells

Cells on culture chips were rinsed three times with phosphate buffered saline (PBS). Spheroids were then retrieved from culture chips by washing them out with PBS using a plastic pipette. The collected spheroids were suspended in 0.5 ml of 0.2% Triton X-100 solution and sonicated in an ice bath. DNA concentration in cell lysate was measured using a Quant-iT™ PicoGreen® dsDNA kit (Invitrogen). ALP activity was measured using a commercially available kit (Wako Pure Chemical Industries, Ltd.). The ALP activity was normalized using DNA amounts as determined with the Pico Green kit.

### 2.5. Analysis by histochemistry

Cells with or without calcium phosphate materials were incubated in the culture chip for 7 days as described above. At day 7, cell culture chips were rinsed three times with PBS. The spheroids collected from the culture chips were fixed in 10% formalin for 24 h. Spheroids were then rinsed with PBS and embedded in 2.5% fibrin gel. Fibrin clot containing spheroids were fixed in 10% formalin for 24 h at 4 °C. Serial sections (3.5 μm) were mounted onto silane-coated slides and stained with hematoxylin-eosin (HE). Photographs were taken with a photomicroscope (Leica DFC300 FX, Leica Microsystems Japan, Tokyo, Japan).

### 2.6. Statistical analysis

Results were expressed as the mean ± standard deviation (SD). All experiments were performed at least three times and showed reliable reproducibility. Statistical differences among specimens were evaluated by Tukey–Kramer multiple comparison analysis. A value of $p < 0.05$ was regarded as statistically significant.

## 3. Results

### 3.1. Formation of D1 cell spheroids and CaP/cell spheroids

Fig. 1 shows light microscopic images of spheroid formation of D1 cells and formation of CaP/cell spheroids on the culture chips. Spheroids of only D1 cells formed on the chip within one day. The size of CaP/cell spheroids was always larger than that of spheroids consisting of only D1 cells during the culture period. An assembly of cells with OCP or HA was slower than that with β-TCP. However, all spheroids of CaP/cells became well-assembled at day 7. This result indicates that the efficiency of incorporation of CaP granules was very high with the Oxy chip without rotation or shaking regardless of the type of CaP material used.

Changes in spheroid diameter of each cell aggregate on the culture chips were measured and shown in Fig. 2. The initial mean diameter of OCP/cell, HA/cell, and β-TCP/cell spheroids was 300 μm, 383 μm, and 214 μm, respectively, and larger than D1 cells alone

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