A Morphological Study of Cell Aggregations on Mineralization

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Abstract— **The key criteria for assessing the success of bone tissue engineering is the quality and quantity of the mineralization within scaffolds. It has been demonstrated that stem cells and isolated primary bone cells can be induced to generate minerals in in vitro culture when using osteogenesis medium. The accumulation of calcium ion and inorganic phosphate from culture medium serves as nucleating agents for the formation of hydroxyapatite, which is the main inorganic component of bone. Bone nodule formation is one of the hallmarks of mineralization in such culture. In this project, we understook a morphological study in which human bone marrow –derived mesenchymal stem cells (hMSCs) and osteoblast cell line have been cultured into cell aggregates under various culture conditions. We investigated the effect of the culture substrates on the capability of the formation of cell aggregates in terms of size and numbers, and the capability of the aggregates expansion and mineralization. It has been revealed that the nature of substrate affected the size and numbers of the cell aggregates. The number of bone nodules and the minerals generated from cell aggregates were higher than in monolayer cell culture.**

I. Introduction

In the past decades, tissue engineering of bone is an intensive studying area. It has prospected that tissue engineering bone can become a highly potential therapy for bone diseases caused by trauma or aging-related degeneration, such as osteoporosis. Many researchers including us have reported that primary osteoblastic cells and stem cells are capable of mineralization up to certain levels in vitro [1-2]. Bone nodule formation is one of the hallmarks of mineralization in such culture. Interestingly, it has been reported that embryoid body aggregates can generate higher minerals quantity in comparison to embryonic stem cells [3]. In this project, we undertook a morphological study in which human bone marrow–derived mesenchymal stem cells (hMSCs) and osteoblast cell line have been cultured into cell aggregates under various culture conditions. We investigated the effect of the culture substrates on the capability of the formation of cell aggregates in terms of size and numbers, and the capability of the aggregates expansion and mineralization. The aim of this study is to demonstrate whether culturing osteoblast aggregates will enhance mineralization and nodule formation.

II. Materials And Methods

A. Cells Sources

Two representative cell groups, human bone marrow-derived mesenchymal stem cells (hMSCs) and mouse osteoblastic cell line MLO-A5 were used to generate cell aggregates. hMSCs were purchased from Lonza (Passage 2) and expanded in DMEM with 10% FCS, 1% Antibiotic-Antimycotic, 1% L-glutamine. MLO-A5 is kindly donated by Professor Lynda Bonewald, USA.

B. Treatment of Substrates Surface

Three types of substrates were used to generate cell aggregates. The first type of substrate was commercial suspension culture flask (Sarstedt). The type two and third substrates were PEO (F127 F127 Pluronic, BASF) coated Sarstedt 24-well suspension cell culture plate with different coating concentration. Two types of culture medium were used in the aggregate formation period. They were undifferentiated media, α-MEM supplemented with 5% SFBS + 5% BCS, and 1% antibiotic-antimycotic solution, and osteogenesis media in which α-MEM was supplemented with 10% FCS, 1% L-glutamine, 1% ascorbic acid, 1% dexamethasone, 1% glycerophosphate and 1% antibiotic-antimycotic solution for mouse cell culture.

C. Aggregate Formation

In the 24 well plates, 100,000 cells, either hMSC or MLA-O5 were seeded and cultured for 40 hours under 37°C and 5% CO_2. The variation of culture conditions and substrates are summarized in Table 1.

D. Bone Nodules Formation

After 40 hour growth in the suspension culture plates, the cell aggregates were transferred to normal 6-well cell culture plates (cell adhesive) for further cell proliferation and mineralization. The osteogenesis medium was used for the further culture.

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Table 1 The culture conditions of nodule formation

Group	Media	Sources of cell aggregates
1A	Normal	Non-adhesive suspension cell culture flask
1B	Osteogenic	Non-adhesive suspension cell culture flask
2A	Normal	PEO (Pluronic F 127) coating substrate 10 mg/ml
2B	Osteogenic	PEO (Pluronic F 127) coating substrate 10 mg/ml
3A	Normal	PEO (Pluronic F 127) coating substrate 20 mg/ml
3B	Osteogenic	PEO (Pluronic F 127) coating substrate 20 mg/ml

E. Characterisation of Aggregates and Minerals

The formation and outgrowth of aggregates were examined at given culture interval under a light microscope. After 10 days further culture, some cell wells were fixed with 10% formalin for Alizarin red staining and other quantitative and qualitative analysis for minerals including XRD and spectroscopy following Gregory method [4]. Some wells were trypinsed for cell counting.

III. Results

Both hMSC and osteoblasts cells have formed cell aggregates on both suspension and PEO coated culture plates. However, the numbers and size of the cell aggregates varied. Within 40 hour culture, hMSCs and osteoblasts generated high and homogeneous cell aggregates on commercial suspension plates with average aggregate size of 100 μm; whilst osteoblasts generated few but much bigger cell aggregates in PEO coated culture flasks regardless of original type of flask used for the coating. The average size of the big aggregates was 200-300 μm. With the same seeding density, the aggregates number generated in commercial suspension plate was about 50 to 100; whilst only average 5 big aggregates plus small number of aggregates found in PEO coated plates. The aggregates size increased with culture time (Figure 1). However, up to 60 hour or longer, the aggregates became unhealthy and dying at 100 hour (data not shown).

After transferring the cell aggregates into cell adhesive culture plate, all cell aggregates showed high rate of cell outgrowth. The cell outgrowth initiated from the aggregates' centre and displays a radial and symmetric cell population pattern (sun flower pattern). The proliferation of cells from aggregates continued along culture time, as demonstrated in Figure 2. Interestingly, after cell outgrowth, the aggregates remained thick and lump in the centre, which formed macroscopic visuable nodules.

The control specimens in which cells were seeded in cell adhesive plate (both hMSC and osteoblasts) straightly produced a homogenous cell growth with less visible nodule formation when the cells became confluent within the plates (Figure 3).

Alizarin red staining of both monolayer cell population and the cell population from aggregates demonstrated that the cell population from aggregates showed stronger red color than monolayer culture (Figure 4), indicating the higher mineral production. The preliminary XRD analysis of mouse cells after aggregates outgrowth confirms the presence of hydroxyapatite crystal and the minerals quantity was much higher in the specimens in which aggregates grew from PEO coated substrate in comparison to monolayer culture. A qualification analysis of minerals formation from hMSC cells from XRD is summarized in Table 2. The more quantitative analysis is being undertaken.

IV. Discussion And Conclusion

As far as we are aware that this is the first report on how hMSCs and osteoblasts can form cell aggregates and induce enhanced mineral formation. Our studies reveal that hMSCs and osteoblasts can form cell aggregates. These cell aggregates acted as embroyic body from embroyic stem cells and displayed higher potential for mineral formation. It is hypothesesed that the aggregates provide a more similar growth microenvironment as found in body, perhaps the aggregates contain high extrcellular matrix and can syntheses more organic matrix required for osteoid generation.

In conclusion, the two step cell culture process, i.e. formation of cell aggregates first and then mineralization through cell aggregates has a potential to enhance mineralization in the quantity and quality of minerals. Both hMSC and osteoblast cell line can generate high yield cell aggregates under appropriated substrate conditions.

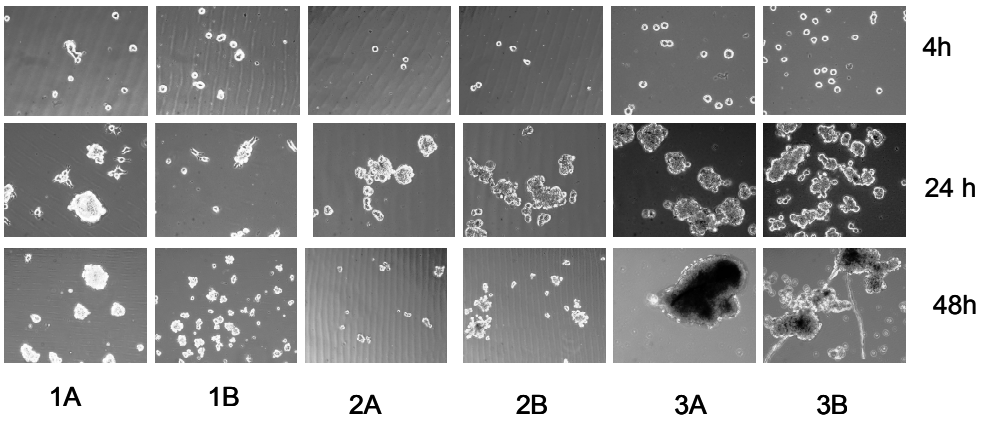

Fig. 1 The cell aggregates grew in different substrates and in different culture period. The original magnification is 100X

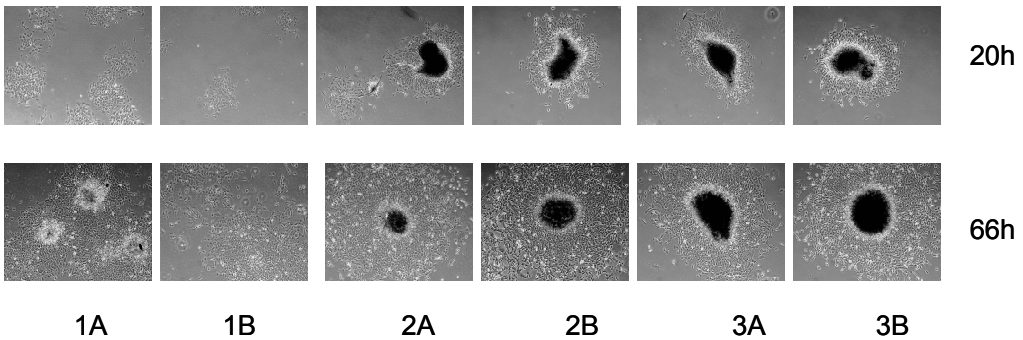

Fig. 2 The cell aggregates outgrew in cell adhesive culture plates under different culture period. The original magnification is 40X.

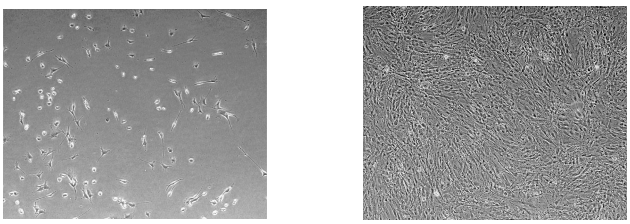

Fig. 3 The cells grew in adhesive culture plates: left: mouse cells; right: hMSCs

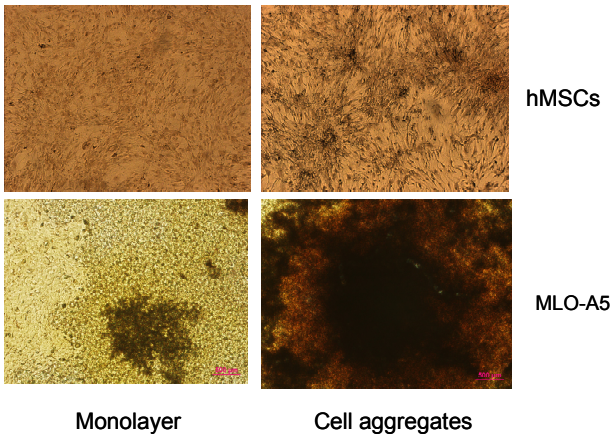

Fig. 4 Typical alizarin red staining after 10 days culture in hMSC and mouse cells

Table 2 The qualitative results from XRD analysis for the nodules

Group	XRD Evaluation
Control	No proper measurement due to insufficient specimen
1A	Amorphous phase is dominant. Crystallinity is better than 1B.
1B	Amorphous phase is dominant. Crystallinity is minimal.
2A	The best/highest crystalline phase Minimum amorphous structure
2B	Little amorphous structure Very high crystallinity ratio
3A	Crystallinity ratio high Less amorphous structure
3B	Crystallinity ratio high; less amorphous structure. Amorphous phase is less than 3A.

Acknowledgments

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