THE ROLE OF CALCIUM IONS IN MOUSE OSTEOBLASTS
DILEMMAS AMONG VIABILITY, PROLIFERATION, DIFFERENTIATION, AND CALCIFICATION

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Introduction

Our previous studies have shown that the alternate soaking technique is an effective strategy for depositing hydroxyapatite (HA) in collagen gels, and is potentially applicable to bone tissue-engineering [1:ORS paper last year]. Ca^{2+} concentration during the alternate soaking process is critical for achievement of appropriate HA formation in osteoblast-embedded gels, when gels are transplanted in the dorsal pockets of mice. Magnitude and velocity of HA formation in gels can be upregulated by increasing Ca^{2+} concentration. Conversely, from the perspective of osteoblast biology, Ca^{2+} concentration reportedly affects osteoblast survival *in vitro*, and Ca^{2+}concentrations above physiological levels have an adverse effects on osteoblasts in culture [1]. In fact, Ca^{2+} concentrations suitable for osteoblast proliferation, differentiation, and osteoblast-mediated bone formation have not been elucidated in the field of bone tissue-engineering. The present study aimed to determine optimal concentrations of Ca^{2+} under both monolayer and 3-dimensional (3D) culture conditions.

Methods

Mouse primary osteoblastic cells (mOB cells) were isolated from calvariae of 1-day-old Std-ddY mice by sequential enzymatic digestion. Cells were maintained in high glucose αMEM supplemented with 10% fetal calf serum and appropriate antibiotics. In 3D cultures of mOB cells, cells were dispersed in 1.25% type II collagen gel at a density of 1×10^7 cells/ml, followed by solidification using 1% PTE-10TGS crosslinker. Using various Ca^{2+} concentrations, cells were cultured under monolayer or 3D conditions for a designated period, and subjected to the following analyses:

1) Alkaline phosphatase (ALP) and von Kossa staining

ALP staining and von Kossa staining were performed after 7 days culture.

2) Cell viability

To assess cytotoxicity of Ca^{2+} ions, mOB cells were exposed to various concentrations of Ca^{2+} for 4 h with pH and osmotic pressure appropriately controlled. Viability of cells was measured using a Cell-Counting Kit (Wako Chemical Co., Japan) or MTT assay (Wako Chemical Co.) in monolayer or 3D culture, respectively.

3) Cell proliferation

Cellular proliferation at various time periods was measured using a Cell-Counting Kit or DNA assay using Hoechst 33258 in monolayer or 3D culture, respectively.

4) Ca^{2+} deposition

Cell layers were scraped off dishes with 0.5 N HCl in monolayer culture, and cell-embedded gels were dissolved with 1 N HCl in 3D culture. Samples were collected at 0, 1, 3, 7, 10, 14, 17, and 21 days after innoculation and stored at –80 °C. Accumulated Ca^{2+} within HCl extracts was measured using Sigma Diagnostic Kit 587 according to the cresolphthalein complexone method.

5) mRNA expression

Phenotypic characteristics of mOB cells were confirmed by measuring mRNA expression unique to osteoblasts. Total RNA was isolated from mOB cells at 7 days culture using an RNeasy Mini Kit (QIAGEN, Japan). RNA samples were treated with DNase to remove contaminating DNA. Aliquots of 2 μg total RNA were subjected to reverse transcription to cDNA using an Omniscript RT-Kit (QIAGEN). For mRNA quantification, cDNA samples were applied to real-time PCR, Light Cycler (Roche Co., Japan) using SYBER Green (QIAGEN). Expression of osteocalcin mRNA was determined and standardized by control mRNA of β-actin. Corresponding cDNA samples were electrophoresed in 2% agarose gel for semi-quantitive RT-PCR to ensure results of real-time PCR.

Results

Viability of mOB cells was maintained in the range of 2 to 6 mM Ca^{2+} for monolayer culture and in the range of 2.5 to 5 mM Ca for 3D culture (Fig. 1). Optimal Ca^{2+} concentration for cellular proliferation ranged from 4 to 6 mM for monolayer culture (Fig. 2), but a slightly lower concentration was suitable for 3D culture.

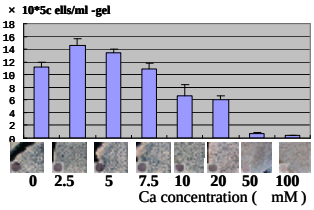


Figure 1: MTT assay in 3D culture. Dark-staining indicates viable cells.

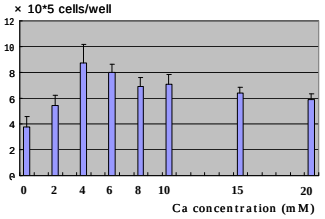


Figure 2: 2 days

Ca quantification and data from von Kossa staining indicated that Ca^{2+} deposition was prominent at 10 mM Ca^{2+}, and gradually decreased up to 20 mM Ca^{2+} in monolayer culture (Fig. 3). Conversely, in 3D culture, 6 mM Ca^{2+} yielded maximum deposition, which was maintained at >6 mM, irrespective of cell-embedding, suggesting that non-biological calcification operated in collagen gels (Fig. 4).

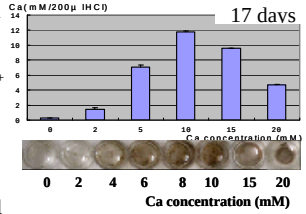
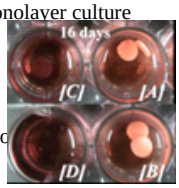


Figure 3: Ca deposition in monolayer culture

Data from ALP staining indicated that 0–6 mM Ca^{2+} stimulated ALP activity, whereas level of mRNA expression of final differentiation markers, namely osteocalcin, reached a maximum at 6–8 mM Ca^{2+} and declined with increasing Ca^{2+} concentration according to the results of quantitative RT-PCR (Fig. 5).



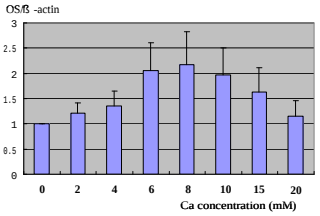


Figure 5. realtime PCR

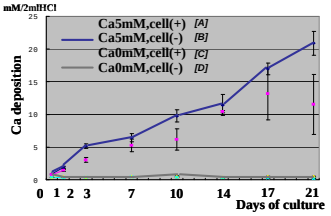


Figure 4. Ca deposition in gels

Discussion

Overall, the present results indicate that 2–6 mM Ca^{2+} is suitable for proliferation and survival of osteoblasts, whereas slightly higher Ca^{2+} (6–8 mM) favors osteoblast differentiation and matrix mineralization. Much higher Ca^{2+} (>10 mM) is cytotoxic. Although the alternate soaking technique is useful for bone tissue-engineering, a dilemma might be encountered in differential cellular responses to various Ca^{2+} concentrations. However, we believe that optimal Ca^{2+} concentration for osteoblasts embedded in a certain scaffold ranges within 2–10 mM. Ca^{2+} concentration reportedly exceeds physiological concentration at bone erosion sites and occasionally reaches a maximum of 40 mM [2]. In such situations, Ca^{2+} potentially plays a role as a coupling factors between osteoclasts and osteoblasts. This is explained by the fact that slightly elevated Ca^{2+} concentrations can depress osteoclast-mediated bone resorption via negative feedback loops [3], and can stimulate not only proliferation but also differentiation of osteoblasts, depending on the concentration. Consequently, the differential osteoblast reactivity to various Ca^{2+} concentrations shown in our study may account for bone homeostasis *in vivo* and provide the optimal Ca^{2+} concentration for bone tissue-engineering.

References

[1] Zhengmin Huang, et al. J. Biol. Chem. 276(24), 21351-21358, 2001.
[2] Silver IA, et al. Exp. Cell Res. 172: 266-276; 1988.
[3] Zaidi M, et al. J. Cell Physiol. 149: 422-427; 1991.