Cell Proliferation, Morphology and Differentiation of Transgenic-Cloned Pig Calvarial Osteoblasts on the Silicon-Substituted Hydorxyapatite Ceramics Fabricated via Ultrasonic Spray-Pyrolysis Technique

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Abstract
Hydroxyapatite (HAp) is one of the most well known materials used as a bone graft. Silicon (Si) ions were incorporated into the HAp lattice to increase the bioactivity of HAp, because Si appears to be beneficial to bone and connective tissue health. In the present study, to investigate the influence of Si on proliferation, morphology and differentiation of calvarial osteoblasts derived from transgenic-cloned pig, silicon substituted-hydroxyapatite (Si-HAp) ceramics were fabricated. As a result, a significant increment of cell proliferation and well stretching of actin bundles were observed on all samples. These data revealed that incorporation of Si did not affect the cell attachment, proliferation and morephology. In addition, substitution of Si accelerated osteogenic differentiation including increase of ALP activity and expression levels of bone-related genes. The results of the present study suggest that supplementation of Si would prove to be effective on bone formation and helpful in preventing osteoporosis.

Keywords: hydroxyapatite, silicon-substituted hydroxyapatite, ultrasonic spray-pyrolysis technique, osteogenic differentiation, Kusabira orange pig

Introduction:
Osteoporosis is a leading cause of morbidity and mortality in the elderly. Bisphosphonate drugs and estrogens by slow bone resorption could reduce the bone turnover; however, few drugs (rhPTH, strontium renelate and sodium fluoride being exceptions) can increase osteoblast activity and hence bone formation [1]. Therefore, most of the current therapies available for its treatment are limited to the prevention or slowing down of bone loss rather than enhancing bone formation. The maintenance of normal, healthy bone requires the coupling of bone formation to bone resorption. Hence, it's important to develop the new treatment that could enhance the bone formation.

Silicon, one of the potential elements for bone formation, appears to be beneficial to bone and connective tissue health. Previous studies showed strong positive associations between dietary Si intake and bone mineral density [2]. Based on these discoveries, silicon (Si) substitution to bioceramics was also attempted, and Si has been successfully incorporated into the hydroxyapatite (HAp) lattice while retaining the single-phase apatite structure. In our previous study, we have also succeeded to prepare Si-HAp powder with desired Si contents by ultrasonic spray-pyrolysis (USSP) technique and to fabricate the sintered Si-HAp ceramics [3]. This technique has an advantage that one can prepare stoichiometoric and homogenous compounds by spraying the solution with desired concentrations of metallic ions into the hot zone of an electric furnace. According to Botelho *et al.*, human osteoblasts on 0.8 wt % Si-HAp appear to respond by increasing protein production [4]. However, the exact biological roles of Si in bone health are still not clear. In the present study, to clarify the relationship between the levels of Si content in the HAp lattice

and increase of osteoblast activity, cellular responses were assessed on Si-HAp.

In addition, to detect the slight cellular responses to the several ceramics, calvarial osteoblasts isolated from neonatal transgenic-cloned pig expressing the red fluorescent protein, humanized Kusabira-Orange (huKO) [5] were used in this study. The pigs with systemic expression of huKO fluorescence are highly useful for observation of cellular morphology on various biomaterials.

Methods and Procedures:

Fabrication of Si-HAp by USSP

As previously reported [3], the Si-HAp powders with 0.8 mass% (Si-0.8) and 1.6 mass% (Si-1.6) of Si as a nominal composition were prepared by USSP technique. The resulting powders were uniaxially compressed at 100 MPa to form the compacts with the size of 10 mm in diameter and 1-2 mm in thickness. The dense ceramics were fabricated by firing the compacts at 1200ºC (HAp) and 1300 ºC (Si-HAp) for 5 h.

Cell culture and cell proliferation

Calvariae from neonatal transgenic-cloned pig [5] were cleaned, minced, and digested in 0.2% collagenase (SIGMA) and 0.2% dispase (Gibco) fifth for 10 min at 37°C. The digestion supernatants (first-third) were discarded and the next two digests were used to harvest cells. Thereafter, each supernatant containing cells was collected by centrifugation at 1000 rpm, pooled and resuspended in growth medium (α-MEM supplemented with 10% FBS, 100 units penicillin G and 100 μg/mL streptomycin). Cells were cultured in growth medium at 37°C, 5% CO_2, and 100% humidity and passages 2-4 were used. The number of proliferated cells was counted after the appropriate incubation period.

Morphological observation with epifluorescence microscope

Osteoblasts grown on several types of ceramics were fixed by 4% paraformaldehyde/PBS and double stained with Alexa Fluor®488-labeled phalloidin for actin and 4', 6-diamidino-2-phenylindole (DAPI) for nuclei. After washing with PBS, the specimens were examined with epifluorescence microscope (BX51, Olympus).

Measurement of Alkaline phoshatase (ALP) activity

After the cells were washed twice with PBS, the cellular proteins were solubilized with CelLytic™M (SIGMA) and centrifuged, and the supernatants were assayed for ALP activity according to manufacturer's instructions (Wako). One unit was defined as the activity producing 1 nmol of *p*-nitrophenol for 15 min. Protein concentrations were determined with a BCA protein assay kit (SIGMA).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

After the appropriate incubation period, total RNA was prepared from cells grown on ceramics. Single-stranded cDNA was synthesized by a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Expression of marker genes such as type I collagen (Col I), ALP, or osteocalcin (OC) for osteogenic differentiation was examined by semi-quantitative RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression was examined as an internal control. The obtained cDNA was amplified with primer sets as described in Table 1.

Table 1: Primer sequences used in this study

Name	Primer sequences (5'-3')
Col I	CCCTCCTGACGCACGGCCAA
	CGCTGGGACAGTTCTTGATT
ALP	CCTTGGTGCCAGAGAAAGAG
	GGGAAACTTGTCCATCTCCA
OC	ATGAGGCCCCTCACAC
	TGAGCTCACACACCTCCCT
GAPDH	ACCACAGTCCATGCCATCAC
	TCCACCACCCTGTTGCTGTA

Results and Discussion:

Comparison of cell attachment and proliferation on different types of Si-HAp ceramics

The interaction between cells and materials is mediated by cell-surface receptors and cell-adhesion proteins bound to the material surface. Since a surface must support cell attachment and following cell proliferation, we first investigated the use of ceramics as a cell culture substrate by comparing the adhesion of osteoblasts on HAp and Si-HAp (Si-0.8 or Si-1.6) ceramics. Cells were plated on these ceramics and allowed to attach for five hours. As a result, osteoblasts on Si-0.8 ceramics showed highest cellular adhesion among them, though there was no significant difference of cell attachment to these substrates respectively (data not shown). In this study, no obvious differences in the value of surface roughness, crystallinity and microstructure of ceramics were observed. Therefore, it is conceivable that other factors such as wettability and/or charge of these ceramics were influenced cell attachment. The hypothesis was supported by

previous study that material characteristics, wettability and charge, could be used to control cell behavior [6]. Next, in order to evaluate if the incorporation of Si could affect proliferative activity, we quantified cell proliferation on the use of ceramics by counting cell numbers. Cell proliferation indicated a significant increase in the growth of calvarial osteoblasts with culturing time on all samples (Fig. 1). The number of cells after 5 days of culture did not differ significantly in any of the substrates. These data suggest that cells reached confluent and started to differentiation. Especially, cells on Si-0.8 ceramics exhibited higher cell numbers at all time points compared to other materials. Those are consisted with the result of initial attachment. On the other hand, cell numbers of Si-1.6 ceramics were lower during culture period. Our data suggest that Si-0.8 ceramics provides suitable surface to calvarial osteoblasts and higher amount of Si affects cell proliferation. Therefore, proper content of Si was indispensable for culture conditions.

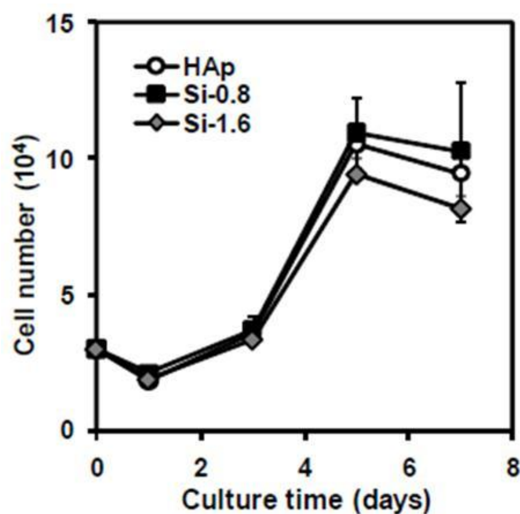

Fig. 1 Comparison of cell proliferation on HAp and Si-HAp ceramics (Si-0.8 or Si-1.6). Cell proliferation was assessed by counting of cell number at 1-7 days after plating on several types of ceramics.

Morphological observation of osteoblasts on different types of Si-HAp ceramics

The morphology of adherent osteoblasts on the different ceramics was visualized by fluorescence staining of cytoskeleton actin filaments and nuclei (Fig. 2). Actin (left panel) and nuclei were labeled with green and blue, respectively. Furthermore, calvarial osteoblasts isolated from neonatal transgenic-cloned pig expressed the red fluorescent protein were used in this study, we could easily observe the expression of huKO fluorescence (center panel) at the red wavelength.

The anchorage signal is transmitted through focal adhesions to the actin skeleton. In this way, cytoskeletal organization and, consequently cell morphology, are strictly dependent on interactions between integrin transmembrane receptors and extracellular sites of attachment. Even at a very early stage of cell spreading (5 h after seeding; initial attachment), the actin fibers of cells on HAp and Si-HAp ceramics were organized throughout the whole cell, with the thin stress fibers tracing the borders (data not shown). At 48 h, osteoblasts were spread and thick actin fibers could be observed on all substrates. The cells on Si-HAp ceramics (Si-0.8 and Si-1.6) exhibit F-actin stress fibers, which emanate from the cell periphery in a radial fashion toward the nucleus. However, no apparent difference in the cell morphology on any concentration of Si incorporation was found. These data suggest that incorporation of Si up to 1.6 mass% did not influence the cell spreading and morphology. These data were agreement with previous investigation [6].

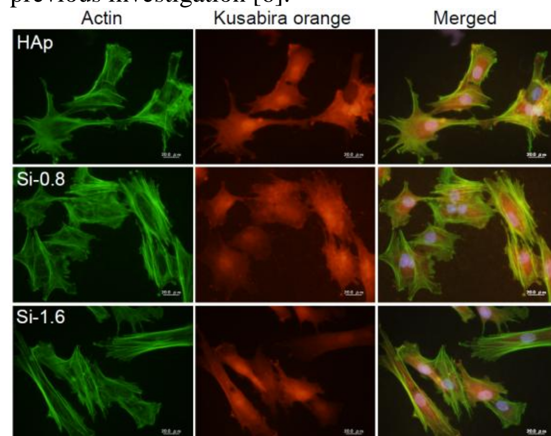

Fig. 2 Morphological observation of osteoblasts derived from huKO pig on HAp and Si-HAp ceramics at 48 h after plating. Cells were stained with phalloidin and DAPI. Images were merged with actin (green), Kusabira orange (red) and nuclei (blue).

Acceleration of osteogenic differentiation by incorporation of Si into HAp

When cell confluence is reached, osteoblasts will start to differentiate, and this phenomenon was observed on Si-0.8 at early time point (Fig. 1). ALP activity is an early marker of osteoblast differentiation *in vitro* which is known to increase during cell differentiation, marking the mature phenotype of bone-forming cells. We speculate that incorporation of Si stimulates the cellular activity.

In the present study, we evaluated the efficacy of Si incorporation into HAp on osteogenic differentiation in transgenic pig calvarial osteoblasts. We assessed the levels of differentiation by measuring ALP activity (Fig. 3), and gene expression levels of bone-related marker genes (Fig. 4). The ALP activity of cells cultured on both HAp and Si-HAp ceramics increased until the 21th day. In particular, cells cultured on Si-0.8 revealed higher ALP activity than others, indicated that incorporation of Si could enhance the ALP activity. These findings are agreement with previous studies [4, 7]. However, no significant differences between HAp and Si-HAp ceramics were observed, resulting more suitable amount of Si might stimulate ALP activity. Next, in order to compare the differential gene expression profile of calvarial osteoblasts between HAp and Si-HAp culture, we carried out semi-quantitative RT-PCR analyses of osteogenic marker genes (*type I collagen*; Col I, *ALP* or *osteocalcin*; OC) at the defined time points (7-21 days). The level of *GAPDH* mRNA was analyzed in the same sample as a house-keeping reference gene. When compared to the level of *GAPDH* mRNA, the relative levels of ColI, ALP and OC mRNA were changed on several types of ceramics (Fig. 4). Cells expressed ColI, an early phase marker, constantly abundantly until 21 days of culture. The expression level of Col I in the Si-1.6-cultured cells was slightly higher than others. As for ALP, increment of expression levels were detected all samples during culture periods. Among them, cells cultured on Si-0.8 ceramics showed notable increase at 21 days. These data were consistent with ALP activity detected by enzyme reaction. Finally, we investigated the expression of OC mRNA, which is a marker of late phase of differentiation. At 7 days of culture, a little expression of OC was detectable on HAp, though that was highly expressed on both Si-0.8 and Si-1.6 ceramics. As culture period passed, the urgent reduction of OC expression was observed on Si-0.8 ceramics, suggesting facilitation of osteogenic differentiation. These findings proposed that incorporation of Si is effective on the osteogenic differentiation, though more studies are integral to understand the optimum concentration of Si and its mechanisms.

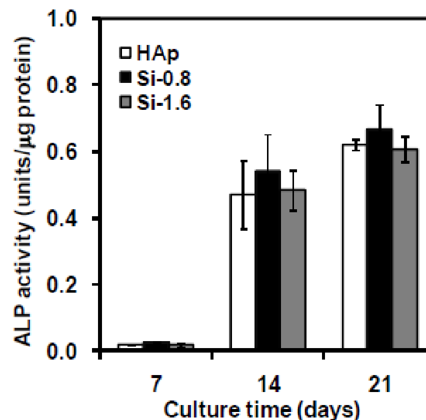

Fig. 3 Comparison of ALP activity on HAp and Si-HAp (Si-0.8 or Si-1.6) ceramics. ALP activity was assessed in cell lysates at 7-21 days after plating on several types of ceramics.

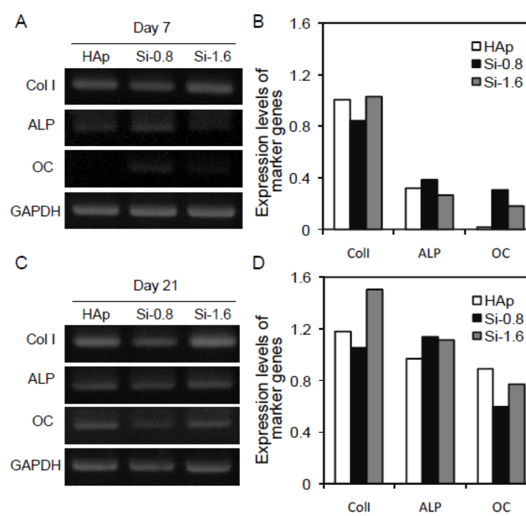

Fig. 4 Expression of osteogenic marker genes. mRNA expression of calvarial osteoblats for 7 or 21 days by cell cultured on HAp and Si-HAp ceramics (Si-0.8 or Si-1.6). PCR was used to analyze for gene characteristics by electrophoresis (A, C). Images of ethidium bromide-stained agarose gels were acquired and bands intensities were quantified (B, D).

Conclusions:

In the present study, to investigate the effectiveness of Si on cell proliferation, morphology and differentiation of pig calvarial osteoblasts, several types (0, 0.8, and1.6 mass% Si) of Si-HAp ceramics were fabricated by USSP technique. An increment of cell proliferation and no apparent changes of cell morphology on all samples reflect the suitability of incorporation of Si for cell attachment and proliferation. Furthermore, substitution of Si into HAp, especially 0.8 mass% Si, increased ALP activity and the expression levels of osteogenic marker genes. These results suggest that Si-HAp is

more bioactive than HAp prepared in this study and may be used as bone implant materials.

Additionally, calvarial osteoblasts derived from transgenic-cloned pig used in this study express the red fluorescent protein and their morphology could be easily observed without fixation and staining. These cells would be useful tools to evaluate biomaterials.

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