The Effects of SiC Foams on Cell Proliferation and Differentiation in Primary Osteoblasts

Lin Wu1), Yue Yuan1), Fengyu Hao1), Zhenming Yang2), Jinsong Zhang2) and Meng Yu3)

1) Department of Prosthodontics, School of Stomatology, China Medical University, Shenyang, China
2) Materials Fabrication and Processing Division, Institute of Metal Research, Chinese Academy of Sciences, Shenyang, China
3) Key Laboratory of Transgenetic Animal Research, Liaoning Province, Department of Laboratory Animal of China Medical University, China Medical University, Shenyang, China

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Abstract: The objective of this study was to investigate the possibility of silicon carbide (SiC) foam as an alternative material for porous hydroxyapatite (HA). The characters of two materials were evaluated and compared by a series of in vitro biological tests. The result of the morphology showed that SiC foam provided beneficial structure for cell migration. On the level of primary-osteoblasts proliferation measured by MTT assay and cell cycle analysis showed that the proliferation rate increased in the early stage on HA but in the advanced stage on SiC foam. There was no significant difference between the two materials on the level of cell differentiation detected by alkaline phosphatase (ALP) assay and real-time PCR. All of the results indicated that SiC foam was comparable to HA in terms of biocompatibility and bone conductivity.

Key words: Biocompatibility, Osteoblasts, Porous hydroxyapatite, Silicon carbide foam

Introduction

Facing the problems of large-area or irregular bony defects, the search for bone substitutes with satisfactory compatibility and sufficient strength became one of the major hotspots in bone tissue engineering1,2). Degradability is one of the requirements of the ideal scaffold for bone tissue engineering. However, it is still unclear that whether degradation speed and bone formation rate is harmonious or not, and whether the degradation products have potential damage to human body or not3,4). Being one of the common-type calcium-phosphate ceramics that have similarity of their composition and structure compared with the mineral phase of bone, hydroxyapatite (HA) has been frequently used for bone augmentation, as it is a bioactive and biocompatible material with excellent osteoconductive properties5,6). However, Low strength and high brittleness of porous HA made it difficult to use in the large area bone defects and process into special shapes for irregular bone defects7).

Due to the existence of the above problems, it is necessary to produce a new biological ceramic. ChongTian et al. adopt new techniques to prepare silicon carbide (SiC) foams, which possess high strength, appropriate elastic modulus and good machinable properties8,9). SiC foam has attracted much more research interest in the field of biomaterial in the past decade10-13). Previous experimental results already suggest that this new material has good biological safety14). The purpose of this research was to compare the biocompatibility of SiC foam and porous HA by a series of in vitro tests.

Materials and Methods

Preparation of ceramic discs

SiC foams presented in this paper were supplied by the Institute of Metal Research, Chinese Academy of Sciences. During fabrication, slurry mixed with carbon powder, bakelite resin and alcohol was poured onto the polyurethane foams with structures of open cells. The excess slurry was then removed and a slurry layer was deposited on the surface of the polyurethane foam. The foam was then dried and the process was repeated several times in order to obtain a sufficiently thick layer, corresponding to the apparent density needed for the silicon carbide foams. Then the green materials were heated in 99.99 % nitrogen atmosphere by a pyrogenation process to obtain carbon foams. The excess slurry was then removed and a slurry layer was deposited on the surface of the polyurethane foam. The foam was then dried and the process was repeated for several times. Finally, the foams were sintered at 1700 °C for 0.5 hours in a vacuum furnace to form the SiC foams. The sintered
SiC material is p-type semiconductor by Hot-Probe method. In the experimental group, the porosity of SiC foam is 70–80%, pore size is 800–1000 μm, elastic modulus is 20 GPa, and the compressive strength of it isn’t less than 30 MPa. The X-ray diffraction (XRD, D/max-2500PC, Rigaku) pattern from SiC sample is shown in Fig.1. In the control group, porous hydroxyapatite (HA), bioactive ceramic, was provided by Engineering Research Center of Sichuan University. The porosity of HA is 70–80%, pore size is 200–500 μm, and the compressive strength of it isn’t less than 1.5 MPa.

In vitro experiments, the size of the two materials is Φ13mm×2mm. Before the experiment, all of the materials were cleaned by ultrasonic vibration and high pressure steam sterilization (121 °C, 0.21 MPa).

Cell culture and seeding
Osteoblasts isolated via sequential digestions of neonatal rat cranium were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in 5% CO2 and 100% humidity. The medium was changed every two days. The characters of the passage 3 osteoblasts were identified using alkaline phosphatase (ALP) staining, collagen immunofluorescence staining and alizarin red staining. Passage 3 to 5 osteoblasts were seeded onto the surface of prewetted SiC foam or porous HA in the 24-well plates at a density of 1×10⁵ cells per disc. The experimental procedures of this study were conducted under the principles of the care and application of laboratory animals (No:398, approved by Ministry of Science and Technology of the People’s Republic of China on Sept. 30, 2006).

Scanning electron microscopy
The co-cultures of osteoblasts and discs were detected for the cells attachment and distribution at 1, 3, 5 and 7 days postcell seeding using scanning electron microscopy. The samples were rinsed three times with PBS solution and then fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.0), followed by 1% osmium tetroxide in acetone. The samples were dehydrated, dried, gold coated and viewed under scanning electron microscope (SEM, FEI Quanta 600, Hillsboro, Oregon, USA).

MTT assay
The cytotoxicity caused by different materials to osteoblasts was estimated using the 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, USA) assay. The co-cultures of osteoblasts and discs were incubated for 1, 3, 5 and 7 days before the replacement of the medium with phenol-red-free DMEM containing 1 mg/ml MTT. The samples were further cultured with the MTT solution for 5h and the formazan produced by normal mitochondrial activity was soluble in dimethyl sulfoxide (DMSO, Sigma, USA). Then the liquid was transferred to a 96-well plate. Optical density (OD) was measured at a wavelength of 490 nm.

Flow cytometric analysis
Flow Cytometer was applied to test the cell cycle. At 1, 3, 5 and 7 days, the osteoblasts on the discs were detached using trypsin and fixed with ice-cold ethanol 70% (v/v) overnight. The fixed cells were stained for 30 min at 4 °C by propidium iodide solution (with 0.05‰ propidium iodide, 0.02‰ Rnase, 0.01M TritonX-100, 0.1% sodium citrate) away from light. The cell cycles were detected by Flow Cytometry (FACS Calibur FlowCytometry, BD company, USA). Proliferation index (PI) was calculated according to manufacture’s instrument.

ALP activity assay
The osteoblasts on the discs were detached using trypsin at 1, 3, 5 and 7 days. The cell suspension was lysed by ultrasonic cell disruptor. ALP detection kit (Hou-bio life technology Campany, Hongkong, China) was used to quantify the amount of p-nitrophenol produced by osteoblasts on the discs. Optical intensity was measured at 405 nm with microplate reader (Spectra Max M5, Molecular Devices Corporation, CA, company, USA). Then the content of ALP was calculated according to manuals provided by kit manufacture’s instrument.

Real-time quantitative PCR
Total RNA was extracted by RNAsimple Total RNA kit (TIANGEN, BEIJING, China). The expression levels of two kinds of ossification gene by osteoblasts on the different discs were assessed by real-time PCR using Brilliant SYBR Green QPCR Master Mix (TIANGEN) with a Light Cycler apparatus (Exicycler 96, BIONEER, Korea). The PCR cycling consisted of 40 cycles of amplification of the template DNA with primer annealing at 68 °C. The relative level of expression of each target gene was then calculated using the 2^{-△△Ct} method. The amplification efficiencies of primer pairs were validated to enable quantitative comparison of gene expression. All primer sequences were designed using primer 5.0 software. The primers of osteocalcin (OCN) were as followed: forward 5'-GGCGGCAGTAAGGTGGTGAATAG-3', and reverse 5'-ATGCGTCCTGGGAAGCCAATGTTG-3'.

Figure 1. XRD pattern of SiC form.
primers of core binding factor α1 (Cbfa1) were as follows: forward 5'-CTACTCTGCGAGCTACGAAA T-3', and reverse 5'-GGAGGATTTGTGAAGACCGTT-3'. Each real-time PCR was performed with at least 3 parallel experimental samples; and representative results are displayed as target gene expression normalized to reference gene. Error bars reflect one standard deviation from the mean of technical replicates.
Statistical analysis
All statistics were performed using SPSS 16.0. Differences between two groups were analyzed by the paired-samples t-test. Statistical significance was defined as \( p < 0.05 \).

Results
Surface topography of ceramic discs
The surface morphologies of these two materials were quite different under SEM. SiC form had smooth, even struts with microporous surface and good pore interconnectivity (Fig. 2a). The surface of porous HA showed a bowl-like shape and irregularly (Fig. 2b).

Identification of primary osteoblasts
The cultured cells had typical morphological characteristics of osteoblasts (Fig. 3a), and ALP staining, alizarin red staining and collagen I immunofluorescence staining showed positive (Fig. 3b, c and d). The photos of the ALP staining showed that the proportion of the stained cells was 92.64% ± 2.26% with IPP6.0 software (Media Cybernetics, Inc., USA) analysis.

Morphologic comparison
Like the porous HA, osteoblasts formed long spindles or flat polygons and connected with each other via pseudopodia on the surface of the SiC foam at each time point (Fig. 4). But on the 7th...
day, osteoblasts could be observed in the relatively deep inside and even at the back side of SiC foam, while these can not be observed on porous HA. The results suggested that both the materials had good ability for cells attachment. However, the good pore interconnectivity of SiC foam provided beneficial structure for cell migration and showed preferable osteoconduction than bowl-shape porous HA.

**Cell proliferation**

MTT assay (Fig. 5a) showed that there were no statistically significant differences between the number of the live osteoblasts exposed to SiC foam and that to porous HA after a culture period of 1, 3 and 5 days. But on the 7th day, OD value of SiC foam was significantly higher than that of porous HA, which indicated that the viability of cells on SiC foam were superior to those of porous HA in a long run. PI represents the proliferation state of the cell mass, which is the sum of the percentages about the DNA synthesis phase (S phase) and the RNA synthesis phase (G2/M phase) cells. The distribution of cell cycle between the two groups was similar and there was no significant difference in the PI value (Fig. 5b), which indicated the two materials had a comparable effect on the cell cycle.

**Cell differentiation**

ALP is an early biochemical marker of the mature extracellular matrix and is critical in the process of matrix calcification. The results of this experiment were that the content of ALP was in a positive correlation with time during the observation period and there was no statistical difference between the two materials(Fig. 6a). The results of real-time PCR demonstrated that the relative expression of OCN and Cbfa1 mRNA in the samples of both materials increased with the culture time initially, and decreased except for OCN of SiC on the 10th day (Fig. 6b, c). There were differences on the 1st and 5th day, but no difference indicating a similar differentiation between the two materials on the 10th day. This indicated that the two materials had similar ability for osteogenic differentiation of primary osteoblasts, especially in the latish stage.

**Discussion**

Porous HA is a typical bioactive ceramics, which is safe and non-poisonous when implanted into human body. Porous HA also have the capability of osteoconduction. Therefore, the present study was designed with porous HA as the control group to evaluate the performance of SiC foam. The results showed that SiC foam and porous HA had comparable influences or long-term effects on the adhesion, proliferation and differentiation capacity of primary osteobasts.

It has been proved by many experiments that a trace of biodegradation of porous HA may occur in nutrient solutions, releasing ions including Ca\(^{2+}\), HPO\(_4\)\(^{2-}\), PO\(_4\)\(^{3-}\), which can benefit the mineralization of matrix, improve the material’s surfactivity, and facilitate the adhesion, proliferation and differentiation of osteoblasts. From the comparable results of SiC foam and porous HA in this study, it can be considered that SiC foam probably induces cellular physiological responses on the interface, which can be attributed to the electrochemistry character of SiC. SiC is a kind of semiconductor. SiC used in this study is a p-type semiconductor with excess holes and vacancies which carries a positive charge at the interface\(^{16}\). SiC foam can facilitate the adsorption of proteins and promote the cellular adherence because that -COOH groups of protein molecules such as serium albumin in the media could be electrostatically attracted to the positive surfaces of SiC. With respect to cellular responses to charged surfaces, a trend was observed by Kizuki et al., who found increased osteoblast-like (MC3T3-E1) cell numbers on positively charged HA compared to a negatively charged HA surface two days after seeding\(^{17}\). And the same trend was found in in vivo study\(^{18, 19}\). The authors attributed the increase in bone formation on the positively charged surfaces to the electrostatic attraction to -COOH groups of protein molecules such as fibrin, fibronectin, osteocalcin, and bone morphogenetic proteins (BMPs), improving platelet adhesion and osteoblast migration\(^{20}\). Further experiments remain to be done to confirm the conjecture.

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References