Mechanical Variation and Proliferation Behavior in Hydroxyapatite Based Scaffolds with Mesenchymal Stem Cells

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Abstract— **Calcium phosphate bioceramics such as hydroxyapatite (HA) have widely been applied as scaffolds in bone tissue engineering because of high osteo-conductivity and biocompatibility. In the present study, continuous porous HA scaffold was fabricated using the template method. Human mesenchymal stem cells (hMSC) were then seeded into the HA scaffold up to four weeks to observe the proliferation behavior and variation of the compressive mechanical properties. The scaffold with hMSCs was also characterized by scanning electron microscopy (SEM). It was found that the compressive strength and elastic modulus tend to increase with increasing culture time due to proliferation and attachment of the cells. The HA scaffold was also found to be suitable for hMSC adhesion, spreading and proliferation. Moreover, improvement of cellular adhesion was achieved by introducing RGD (Arg-Gly-Asp) peptide into the HA scaffold. We can conclude that the HA scaffold provides good environmental conditions for hMSCs as an artificial extracellular matrix. It is also important to note that the cellular adhesion can be effectively improved by RGD.**

Keywords— **Bone tissue engineering, Calcium phosphate. RGD, Compressive property, Stem cell**

I. Introduction

Tissue engineering has been considered as one of the key technology to cure damaged organs and tissues instead of doing pharmacological treatment, transplantation, or implantation of artificial organs or tissues [1]. Tissue engineering is an interdisciplinary field that applies the principles of engineering and life science to the development of biological substitutes which are able to restore, maintain, or improve function of tissues. In bone tissue engineering, scaffold plays an important role for regenerating artificial bone tissues in vitro as the matrix for tissue formation and is required to have three-dimensional porous structure with high porosity, pore interconnectivity, uniform pore distribution, surface properties permitting cell adhesion, differentiation, non-cytotoxicity and osteoconductivity [2-5]. In order to develop porous structural scaffolds, different kinds of fabrication method such as use of organic porosifiers [6,7] and sponge template [8] have been applied.

Hydroxyapatite (HA) is one of the popular bioceramics used for bone tissue engineering because it possesses chemical structure very similar to carbonate apatite that is the major inorganic component of bone [9,10] and is also known to have good osteoconductivity and biocompatibility [11,12]. Many studies have been performed to understand the fundamental properties of HA scaffolds. For example, Werner et al. [11] studied the flexural strength of HA scaffolds at different temperature and pore size with different size of porosifiers. Physico-mechanical and biological evaluations of porous HA scaffold were conducted [13] and also in vitro study of cells growth on porous HA ceramic was also performed [14]. Moreover, in vitro and in vivo studies on the biocompatibility and cells activities of HA based composites were conducted [15-17]. However, variational behavior of mechanical properties of HA scaffold with human mesenchymal stem cells (hMSCs) has not been clarified yet.

In the present study, therefore, variation of compressive mechanical properties of continuous porous HA scaffold was investigated for 4 weeks after hMSCs were seeded into the scaffold. In addition, effect of RGD peptide on the adhesion behavior of cells was also studied to improve the surface condition of the HA scaffold.

II. Materials and Methods

A. Materials

Micro-HA powder (Sangi Co. Ltd.) and polyurethane (PU) foam template (HR-40, 37-43cells/25mm, Bridgestone Co.) were used to fabricate HA scaffolds. Polyvinyl alcohol (PVA), α-minimum essential medium eagle with L-glutamine and phenol red (MEMα), ethanol and 10percent formalin solution were purchased from Wako Pure Chemical Industries, Ltd. Phosphate buffered saline (PBS), trypsin and fetal bovine serum (FBS) from Gibco, penicillin-streptomycin (Pen-Strep) from Sigma Life Science, cell counting kit from Dojindo Molecular Technology, INC., and Arg-Gly-Asp (RGD) from Bachem AG were also prepared for the study.

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B. Scaffold fabrication

Porous HA scaffolds were fabricated by the template method as summarized in Fig.1. HA slurry was prepared by mixing 1 g of the micro-HA powder with 1 ml of PVA solution of 5wt% using a centrifuge medium. Cubic PU sponges of 1x1x1cm^3 were immersed into the slurry. The PU sponges absorbing HA/PVA were then fully compressed to remove excess slurry and to disperse the slurry uniformly. The templates were dried in an oven at 60^{o}C for 24hours. The dried templates were heated up to 400^{o}C in an electric oven and kept for 6hours to remove PU and PVA completely, then sintered at 1300^{o}C for 4hours to solidify the HA porous structures. The heating rate was chosen to be 10^{o}C/min.

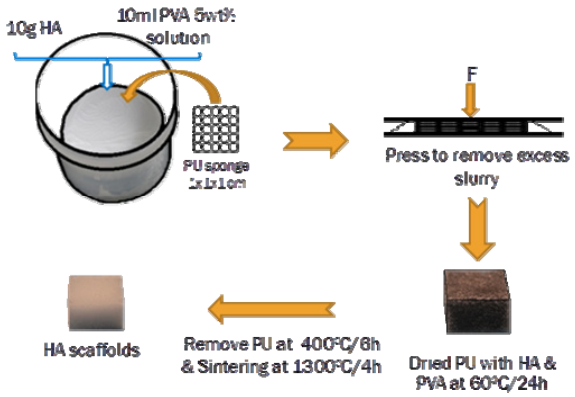


Fig.1 Fabrication procedure of HA scaffold

C. Cell culture and maintenance

hMSCs were cultivated in MEMα supplemented with 10 percent of FBS and 1 percent of Pen-Strep. Cells were maintained under the standard cell culture condition at 37^{o}C in an atmosphere of 5percent CO_2 and 70percent humidity. The medium was changed twice per week. When cells reached sub-confluence, they were harvested with trypsin and sub-cultured. All the experiments were performed under the identical condition. Cells from passage 4 were used for seeding on the porous HA scaffolds.

In seeding process, HA scaffolds placed in 24-well plate were exposed to UV for 1hour prior to cell seeding. 1.5×10^5cells per scaffold were seeded and placed under cell culture environment for 2, 3 and 4 weeks.

For cell adhesion assay, porous HA scaffolds were incorporated with 0.1percent RGD solution and kept overnight at 37^{o}C. Then the RGD coated HA scaffolds were moved to a fresh 24-well plate and washed with MEMα and subsequently seeded with 1.5×10^5 cells for 6, 12, 18 and 24hours. HA scaffolds without RGD were also maintained at the identical condition throughout the experiment as a control.

D. Cell counting protocol

Proliferation of cells in a HA scaffold was determined using the cell counting kit from Dojindo. The medium was removed and the scaffold with cells was washed twice with PBS then 1000 µl of PBS with 100µl of cell counting kit was added, followed by incubation at 37^{o}C for 2hours. Subsequently, 110 µl of the solution from each sample was placed in the well of a 96-well plate and optical densities were evaluated by a plate reader (Perkin Elmer Arvo X2) at 37^{o}C with wavelength of 450nm.

E. SEM images of cellular morphology

The morphology of the scaffold was observed by a field-emission scanning electron microscope (FE-SEM, S-4100, Hitachi, Ltd.). Scaffolds with hMSCs were washed with PSB, fixed with 10percent formalin solution and subjected to graded alcohol dehydration. Then the scaffolds were sputter-coated with Pt-Pb and observed by FE-SEM.

F. Compression mechanical test

Compression tests were performed to determine the mechanical properties using Shimadzu Compact Tabletop Testing Machine EZTest equipped with 500N load cell and a crosshead speed of 1mm/min. Load-displacement relations were recorded and converted to stress-strain relations. Compressive elastic modulus and strength were evaluated from the stress-strain relations.

III. Results and Discussion

SEM microimage of HA scaffold is shown in Fig.2. Three-dimensional and fully interconnected porous structure was obtained with pore size of about 100 to 500 µm. The pore size is known to be one of the most important requirements for tissue formation [2-5]. On the surface of the strut, HA crystals are seen to be firmly connected each other. No micro-pore was observed at the HA inter-crytals. Munar et al. [18] investigated the physical and compositional properties of porous HA foam under the effects of sintering temperature above 1300 ^{o}C, and reported that micro-pores were observed at the inter-crytal regions.

Fig. 3 shows the hMSC proliferation result in HA scaffolds for 2, 3 and 4 weeks after seeding. The absorbance of OD corresponds to the increase of cell number. The absorbance showed sharp increase at 2weeks, indicating that the hMSC was greatly proliferated. Whereas after 2weeks seeding, the absorbance slowly increased, indicating limited cell proliferation [11]. This is because the most of stem cells might undergo differentiation process to form a mature cell

type. Only the cells that remain undifferentiated stage undergo proliferation through cell cycle which is responsible for the little enhancement of the absorbance at 3 and 4 weeks.

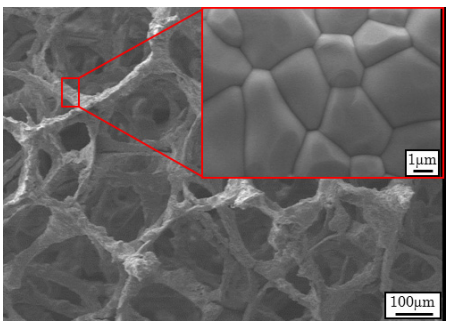


Fig. 2 FE-SEM microsmages of HA scaffold

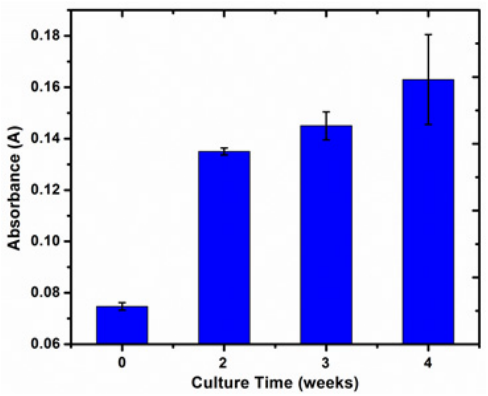
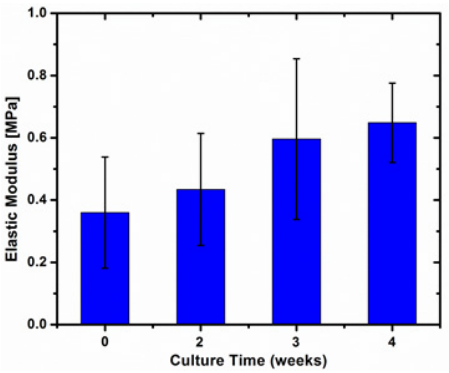


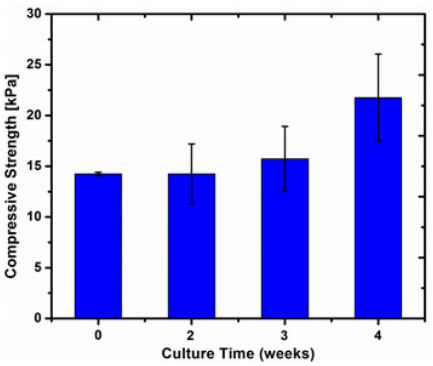
Fig. 3 Proliferation of hMSC on HA scaffolds for 2, 3, and 4weeks

Variations of compressive mechanical properties such as elastic modulus and strength during cell culture are shown in Fig.4. These properties tend to increase with increasing culture time. Comparing Figs. 3 and 4, it is obviously recognized that the variation of the mechanical properties well correspond to that of proliferation of hMSCs. During the proliferation, cells spread and develop cytoplasmic extension and cell-cell junctions, resulting in strengthening the scaffold structures.

Fig. 5 shows SEM images of cells attached to HA crystals where cells noted as 'C'. The cells spreaded over the HA surfaces and developed firm attachment to the surfaces that proves biocompatibility of the HA scaffolds. It is known that cellular spreading occurs through cyto-skeletal stretching over the material surface [19]. Surface roughness and rigidity possess significant effects on cellular spreading [5,20]. Previous studies reported better cellular spreading and proliferation on rough and rigid surface than soft and smooth surface [21]. Moreover, filapodial extensions from cellular plasma membrane that contains meshwork of actin-containing microfilaments permit the movement of cells along a substratum [15,19]. In addition, such filamental extensions form inter-cellular bridge, illustrated as 'J'. Mammalian cells share several biomolecular signals through the inter-cellular bridge that have significant influence on cell fate decision including cellular proliferation, differentiation or apoptosis [19].



(a) Elastic modulus



(b) Compressive strength

Fig.4 Variation of compressive properties during cell culture

Fig. 6 shows the hMSC attachment results to the pure HA and the RGD-coated HA scaffolds for 6, 12, 18 and 24hours culture. 'Control' indicates the absorbance of hMSCs seeded on the specimen. The RGD-coated HA scaffold shows rapid adhesion, and after 6hours almost all the seeded cells were attached to the surface of the scaffold. However, on the HA scaffold, it took about 24hours until the cells reach adequate attachment. This is because the surface of the RGD-coated HA scaffolds is rougher than that of the HA scaffolds [5, 20, 21], which provide strong accessibility for cellular receptors to attach, spread and proliferate.

In conclusions, the compressive mechanical properties of the continuous porous HA scaffold with hMSCs tend to

increase with increasing culture time. Proliferated cells are considered to be strengthen the HA strut structures. It was also found that introduction of RGD improves the cell attachment to the HA surface.

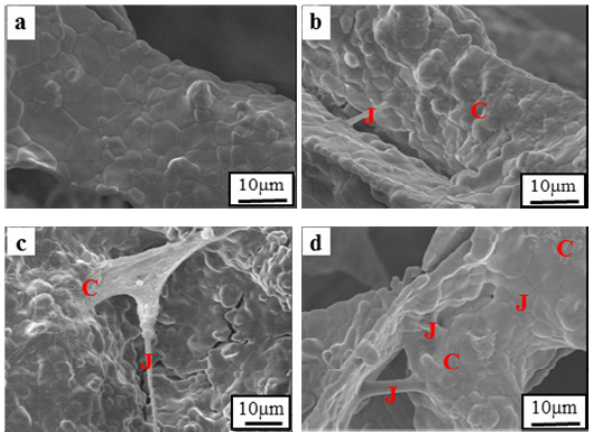


Fig. 5 SEM microimages of cells attached on the scaffold surfaces (a) control, (b) 2weeks, (c) 3 weeks and (d) 4weeks. C: hMSC and J: cell-cell junctions.

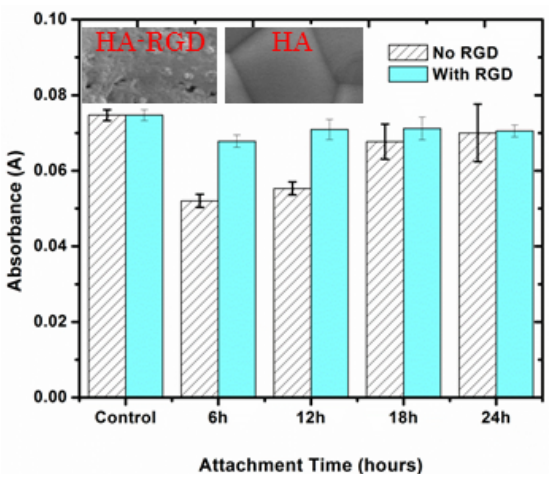


Fig. 6 Cell adhesion assay of HA scaffolds with and without RGD-coating up to 24hours. Images on the top indicate RGD-coated HA and HA surface.

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