35th Annual International Conference of the IEEE EMBS
Osaka, Japan, 3 - 7 July, 2013

In Vitro Bone Formation by Mesenchymal Stem Cells with 3D Collagen/β-TCP Composite Scaffold *

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Abstract**—** Recent years, various kinds of natural polymers and bioceramics has been used to develop porous scaffolds for bone tissue engineering. Among of them, collagen guarantees good biological conditions, and β-tricalcium phosphate (β-TCP) possesses good oseteoconductivity, cellular adhesion, accelerated differentiation and mechanical property. In this study, rat bone marrow mesenchymal stem cells (rMSC) were cultured in β-TCP/collagen composite scaffolds up to 28 days in order to assess the time-dependent behavior of the extracellular matrix formation and the mechanical performance of the scaffold-cell sysytem. The cell number and ALP activity were evaluated using a spectrophotometric plate reader. Gene expression of osteogenesis was analyzed using the real-time PCR reactions. Compression tests were also conducted periodically by using a conventional testing machine to evaluate the elastic modulus. The increasing behaviors of cell number and ALP activity in the composite scaffold were much better than in the collagen scaffold. The gene expression of osteocalcin and collagen type-I in collagen/β-TCP scaffold was higher than that of the collagen scaffold. The compressive modulus also increased up to 28 days. These results clearly showed that the distribution of micro β-TCP particles is very effective to increase the elastic modulus and promote cell growth.

I. Introduction

In bone tissue engineering, scaffolds influence the space of cells by functioning as an extracellular matrix (ECM), supplying the surface contact for cells and surrounding parts, mechanical stimulation for cells [1]. The ideal scaffolds are biodegradable with adjustable degradation rate that fits in the rate of tissue regeneration [2]. Moreover, the scaffolds should have high mechanical strength to protect the defect part from surrounding tissues and also highly porous structure to provide enough space for cell proliferation and ECM formation [3].

Porous structures of natural polymer such as collagen have widely been used as scaffolds in tissue engineering. Collagen guarantees excellent biological conditions, for example, it stimulates generation and differentiation of cells as extracellular matrix [4]. Collagen scaffolds usually have high porosity [5,6] and interconnected porous structures for cell proliferation [7]. On the other hand, bioactive ceramics such as β-tricalcium phosphate (β-TCP) and hydroxyapatite has also widely been used in bone tissue engineering because of excellent oseteoconductivity, cellular adhesion, accelerated differentiation and mechanical property. It is also noted that β-TCP has faster degradation rate than crystalline hydroxyapatite [8].

Recently, regenerated bone graft has been one of the primary concerns instead of autografts and allografts. A regenerated graft may be developed by culturing and differentiating mesenchymal stem cells (MSC) in a porous scaffold [9]. In this case, the biochemical and biomechanical culture conditions and the structures and compositions of the scaffold are important factors controlling the quality of the regenerated graft. It is therefore ideal that the bone graft has a mechanical compatibility with surrounding bone tissues such that mechanical stimulus is transferred into the bone graft to complete regeneration of perfect bone. However, detailed mechanisms of proliferation, differentiation and ECM formation of MSCs in a scaffold have not been well understood yet.

The primary aim of this study is to characterize the variational behavior of compressive mechanical property of collagen/β-TCP composite scaffold with MSCs during cell culturing period. The composite scaffold was fabricated by the freeze-drying method. Rat bone marrow mesenchymal stem cells (rMSC) were then cultured in the scaffold up to 28 days in order to characterize the ECM formation and the mechanical behaviors of the composite scaffold.

II. Experimental

A. Preparation of collagen/β-TCP scaffold

Type 1 collagen solution (Nippon Meat Packers Inc.) was used to fabricate pure collagen and collagen/β-TCP scaffolds by the freeze drying method. The collagen solution and β-TCP powder (weight ratio 90:10) were mixed by using a magnetic stirrer. The mixed solution was poured into silicon rubber molds, and then frozen at -80˚C in a freezer and freeze-dried using a vacuum pump. The freeze-dried scaffolds were cross linked by glutaraldehyde vapor at 37˚C for 4 hours. After cross-linking, the scaffolds were treated with 0.1M glycine water solution to block unreacted aldehyde, afterwards, they were washed by deionized water and lyophilized [10].The porous microstructures were observed by a field emission electron microscope (FE-SEM).

B. Cell culture

rMSC (DS Pharma Biomedical Co.) were cultured in cell growth medium consisting of alpha-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. 100,000 cells suspended in 10μl of α-MEM were seeded in each of the scaffolds and then they were incubated for 1 hour to make cells adhered. After 1 hour incubation, these scaffolds were transferred to a 12-well plate

*Resrach supported by JSPS KAKENHI (21360054, 23656090 and 23·1708).

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978-1-4577-0216-7/13/$26.00 ©2013 IEEE

containing 2 ml of differentiation medium per well. The differentiation medium was composed of cell growth medium and the supplement of osteoblast differentiation (KE-200, DS Pharma Biomedical Co.) including MEM, β-glycerophosphate, L-ascorbic acid and dexamethasone. The plate was then incubated at 37 ˚C in a humidified atmosphere of 5% CO_2. The α-MEM was changed twice per week.

C. Characterization methods

Compression tests of the scaffolds with proliferated cells were conducted periodically by using a conventional testing machine at a loading-rate of 1 mm/min to evaluate compressive mechanical properties. As the control group, scaffolds without cells were also tested in order to characterize the effects of β-TCP distribution.

The cell number and alkaline phosphatase (ALP) activity were also evaluated using a spectrophotometric plate reader with the wave lengths of 450 and 405 nm, respectively. Cell Counting Kit (DOJINDO) was used by following the instruction provided by the supplier. ALP was measured to evaluate the differentiation behavior quantitatively. Specimens for the assay of ALP were prepared by means of Fujita's protocol [11]. The LabassayTM ALP kit was used by following the instruction.

Gene expression of osteogenesis was analyzed using the real-time PCR reactions with primers of β-actin, collagen type I, osteopontin and osteocalcin. The scaffolds with rMSC were washed with PBS and frozen in liquid nitrogen. For each scaffold, three samples were prepared for the RT-PCR measurement. RT-PCR amplification was performed using the reagent (SYBR premix EX Taq Perfect real time kit, Takara Bio Inc.) with a RT-PCR system (the thermal cycler Dice RealTime System TP960, Takara Bio Inc.). β-actin was used as the house keeping control and results were quantified for collagen I, osteopontin, osteocalcin using the ΔΔCt relative quantification method[12].

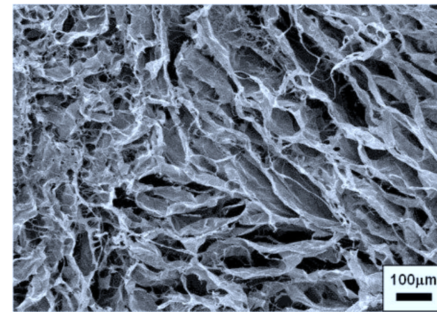
All data were presented as means ± SD and derived from three independent samples. Analysis of data was evaluated by t-test. Any difference was considered statistically significant when the p value was < 0.05.

The surfaces of the scaffold specimens were observed using FE-SEM in order to characterize the porous structures and the proliferation behaviors of the cells.

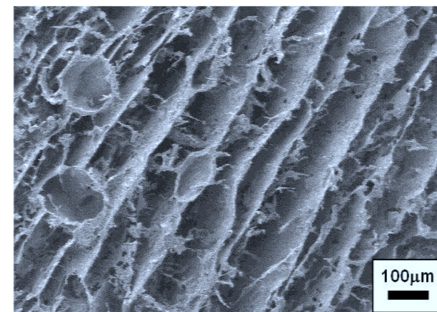
III. Results and Discussion

FE-SEM micrographs of the cross-sections of the scaffolds are shown in Fig.1. It is seen that continuous porous structure was well constructed. The ranges of pore diameter in the collagen scaffold and the collagen/β-TCP scaffold are from 50 to 200μm and from 50 to 150μm, respectively. It has been reported that the minimal pore size is from 100 to 150μm required for tissue ingrowth and cell adhesion [13].

The variation of the compressive moduli are shown in Fig.2. In the case of collagen scaffold with rMSC, the modulus tended to decrease up to 14 days and then recovered to the almost same value with the initial modulus on 28 days. The modulus of the collagen scaffold without showed the same tendency with the scaffold-cell system up to 14 days, and kept almost constant up to 28 days. In the collagen/β-TCP scaffold with rMSC, the modulus tended to increase up to 7 days, and then decreased from 7 to 21 days, afterwards, increase up to 28 days. The modulus of the composite scaffold without cells exhibited the same tendency with the scaffold-cell system. The modulus of the composite scaffold with rMSCs were higher than that of the scaffold without cells during the entire period.



(a) Collagen scaffold



(b) Collagen/β-TCP scaffold.

Fig.1. Micro structure of the scaffolds.

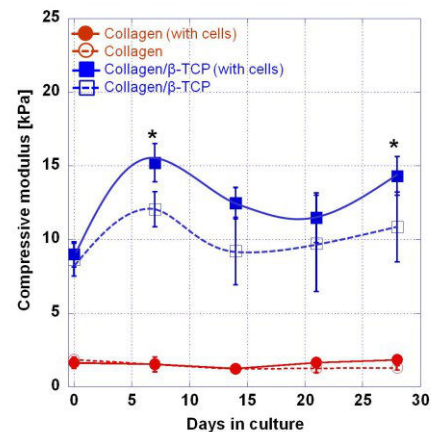


Fig.2 Variation of compressive modulus during cell culture. The result is presented as the means ± standard deviation for n=3.
* indicate p<0.05 vs. collagen/β-TCP scaffold on 0 day.

The variation of cell number is shown in Fig.3. In the case of the collagen scaffold, the cell number tended to increase up to 14 days and then, slightly decreased or almost kept constant until 28 days. On the other hand, in the composite scaffold, the cell number tended to increase gradually up to 28 days. The variation of ALP activity is shown in Fig.4. The ALP of the collagen scaffold reached 2.5 times larger than the initial

value on 14 days of culture. It is also seen that the ALP increased up to 14 days and afterwards, decreased until 28 days. Such increase of ALP activity up to 14 days is likely due to the differentiation of rMSCs to osteoblasts with increase of cell number. The decreasing behaviors of ALP activity and cell number from 14 to 28 days are thought to be strongly related to the differentiation of rMSCs and the mineralization by osteoblasts. In the composite scaffolds, the ALP activity dramatically increased from 14 to 21 days and became twice of the initial value for 21 days of culture. It is also found that the maximum value of ALP activity of the collagen/β-TCP scaffold was about twice as large as that of the pure collagen scaffold, indicating that the distributed β-TCP particles are very effective for differentiation of the rMSCs into osteoblasts.

The RT-PCR results for the osteogenic genes expression at defined time point were shown in Fig.5. In culture period of 7 and 14 days, the expressions of osteocalcin of the collagen scaffold were higher than those of the composite scaffold, on the other hand, the relationship was reversed from 21 to 28 days. Additionally, the osteocalcin level of the collagen scaffold reached the peak on 21 days whereas that of the composite scaffold increased gradually until 28 days. The expression of collagen I in the composite scaffold was similar to that of the collagen scaffold on 7 days. Afterwards, the composite scaffold showed higher expression than the collagen scaffold up to 28 days.

FE-SEM micrographs of the surface regions of the scaffolds with the proliferated cells after 14 days are shown in Fig.6. It is clearly understood that the surface was totally covered by the proliferated cells and ECM produced by the cells after 14 days. It was also observed that the morphology has been changed such that the tissue-like structure became thick as culture period increased. A magnified view of the surface of the collagen/β-TCP scaffold is shown in Fig.7. It was understood that the ECM consists of fibril structures of collagen and the mineralized nodules produced by the cells.

From these experimental results, it was found that the distribution of micro β-TCP particles in collagen matrix has the favorable effects on improvement of the compressive modulus of collagen scaffold, activation of rMSCs for proliferation and differentiation and formation of ECM such as collagen and mineralization. It is thus understood that such structural change, especially the formation of collagen fibril structures and mineralization, strongly affected the variational behavior of the composite scaffold shown in Fig.2.

IV. CONCLUSIONS

Effects of rMSC culture in two different types of collagen scaffolds were examined in this study. The macroscopic mechanical property such as the compressive modulus was correlated with the microscopic cell growth behavior within the porous structures of the scaffolds. The conclusions are summarized as follows:

(1) The compressive elastic moduli of the collagen/β-TCP scaffold tended to increase with increase of culture period and this is mainly due to the cell proliferation, differentiation and subsequent ECM formation and calcification. The modulus of the collagen scaffold tended to decrease up to 14 days due to

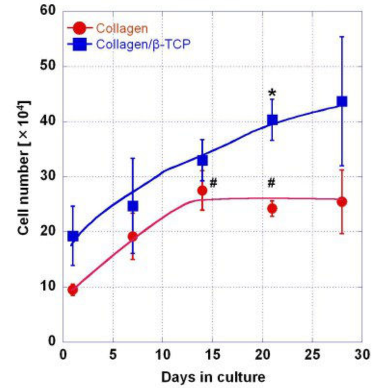


Fig.3 Variation of cell number during cell culture. The result is presented as the means ± standard deviation for n=3. *, # indicate $p<0.05$ vs. collagen/β-TCP scaffold for 1 day, $p<0.01$ vs. collagen scaffold for 1 day, respectively.

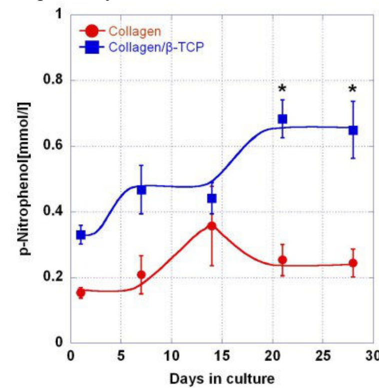
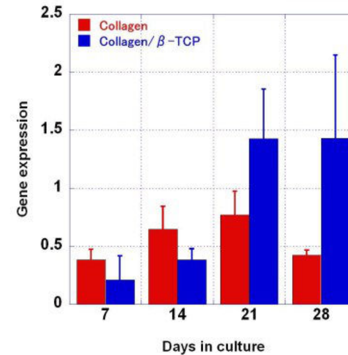
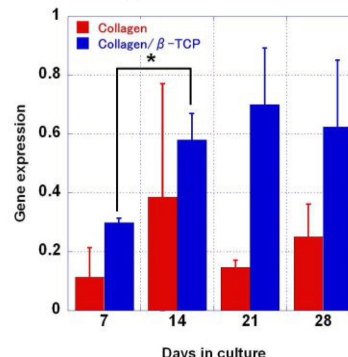


Fig.4 Variation of ALP activity during cell culture. The result is presented as the means ± standard deviation for n=3. * indicate $p<0.01$ vs. collagen/β-TCP scaffold for 1 day.



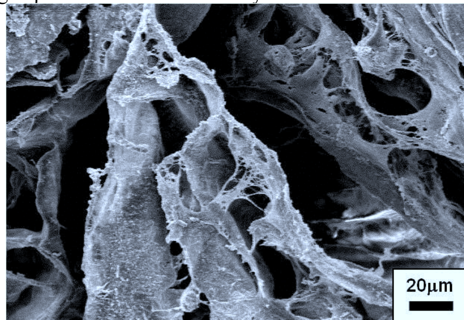
(a) Osteocalcin



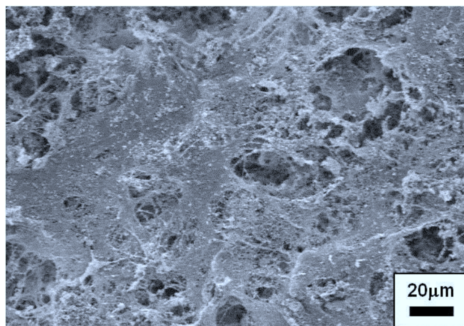
(b) Collagen type-I

Fig.5 Variation of gene expression during cell culture. The result is presented

as the means ± standard deviation for n=3. * indicate $p<0.05$ vs. collagen/β-TCP scaffold after 7 days in culture.



(a) Collagen scaffold,14 days



(b) Composite scaffold, 14 days.

Fig.6 Cell growth behavior in the scaffolds.

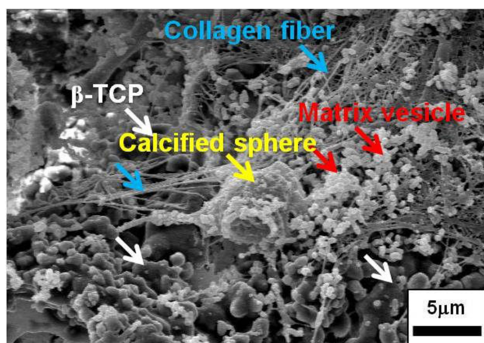


Fig.7 ECM formation in collagen/β-TCP scaffold.

the degradation of the scaffold and then increase up to 28 days because of the proliferation.

(2) The cell number and ALP activity in the collagen/β-TCP scaffold increased gradually. On the contrary, the cell number and ALP activity in the collagen scaffold increased up to 14 days, afterwards, decreased up to 28 days.

(3) The gene expressions of osteocalcin and collagen type-I in the collagen/β-TCP scaffold were much higher than those in the collagen scaffold, indicating the effectiveness of β-TCP distribution within the scaffold.

(2) FE-SEM studies showed that in the collagen/β-TCP scaffold, the rMSCs constructed thick ECM layer consisting of collagen fibril structures and the subsequent calcification, resulting in the increasing behavior of the compressive modulus.

ACKNOWLEDGMENT

This work was supported by JSPS KAKENHI (21360054, 23656090 and 23·1708) .

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