Introduction

In order to reconstruct bone tissue effectively it was proposed that at least five factors must be taken into consideration. These are: 1) cells, 2) extracellular matrices (ECM), 3) vasculature, 4) regulators such as cytokines and finally 5) mechanodynamics.[1-6] Among these five factors, ECM is relatively more amenable for clinical use and can be artificially designed in order to be applied for oriented, directed and organized bone regeneration. Furthermore, it has recently been shown that the geometric properties
of artificial ECM are crucially important, along with their physical, chemical and biochemical properties. For example, if the geometry of the scaffold is different, the scaffold of the same composition induces the formation of different tissues: bone or cartilage, when combined with bone morphogenetic proteins (BMPs) and implanted into rat skin.[1-6]

It was shown that honeycomb-shaped hydroxyapatite (HC-HAP) with a tunnel diameter less than 100 μm induced endochondral ossification, while one with tunnels of 300-400 μm induced direct bone formation with haversian type geometry. [1, 2] From a series of experiments we deduced the importance of geometry of artificial ECM, above all that “vasculature-inducing geometry” is essential for successful development of bone by tissue engineering.[1] Geometry is defined in this case as the three-dimensional (3D) structures of artificial ECM in the order of micrometers, which direct growth of tissues and organs in vivo and in vitro.

To facilitate geometrical design, we have classified all the possible geometric forms of artificial ECM into ten categories among which tunnel type geometry seemed most effective for directing tissue and cell growth.[1] Previously, a tunnel structure obtained from natural resources, a biomaterial consisting of coral replicated hydroxyapatite, was confirmed to be effective in bone formation.[7] It was also shown that the size and shape of irregular tunnels or pores in hydroxyapatite are important for efficacy as BMP-carriers and the expression of bone or cartilage phenotype.[4-6] Recently, a straight and multiple tunnel structure, namely, honeycomb hydroxyapatite was created. [1, 2, 11] Interestingly, it turned out that if the tunnel diameters were large (about 350 μm), the scaffold directly generated a concentric, haversian-type bone formation. However, honeycomb hydroxyapatite scaffolds with small tunnel diameters (less than 100 mm) followed an endochondral ossification process, inducing first cartilage, gradually replacing it with bone.[1, 2, 11]

Concerning this interesting biphasic bone-cartilage phenomenon that was observed in hydroxyapatite ceramics, we wanted to know whether the same phenomenon occurs in β-TCP ceramics. We prepared honeycomb-shaped β-TCP ceramics of disk form with three-different tunnel sizes, 300, 75 and 50 μm, having tunnel numbers of 37, 568 and 907 per ceramic, respectively within a cross-section of 3 mm diameter. These honeycomb-shaped β-TCPs (HC-β-TCP) were implanted into rat skin, and time dependent changes of phenotype expression were analyzed histologically at 2 and 4 weeks after implantation.

Materials and Methods

1. Fabrication of HC- β-TCP

HC- β-TCP was prepared by the method which we have previously reported for the preparation of HC- hydroxyapatite.[1] Briefly, a paste of β-TCP powder (purchased from Sangi Co., Tokyo) was extruded through multi-nozzles, in the form of multiple rods, into a condensation space of tapered conical tubing. From the outer surface of the nozzles equipped disc, multiple guiding sticks were extruded perpendicularly toward the conical tubing. By these sticks, multiple tunnels were created as the β-TCP was pushed through the tapered tubing. The paste that contained tunnels was gradually reduced in diameter as it was pushed through the tapered tubing. The cylindrical products were sintered at 1,000–1,200°C and cut to the required lengths. The sizes of the cylindrical products, including tunnel diameters, were controlled. In this study, three different HC- cylinders with tunnel sizes, 300, 75 and 50 μm, were prepared, with tunnel numbers of 37, 568 and 907 per honeycomb, respectively within a cross-section of 3.1 mm diameter. As a control a solid cylinder without any tunnels was prepared at the same time. The average density of the solid β-TCP was 35-40%. They were cross sectioned with a thickness of 1 mm.

2. Implantations

A solution (5 μg/30 μL) of recombinant human BMP-2 (rhBMP-2) (a kind gift from Yamanouchi Co., Japan) was absorbed into the three sorts of HC-β-TCP as the scaffolds (carriers). The scaffold/rhBMP-2 composites were immediately lyophilized. Four-week-old male rats (Wistar, weighing 70-85 g) were anesthetized with sodium pentobarbital (3.6 mg/100 g body weight, Nembutal®, Dainippon Sumitomo
Pharma, Japan), and the samples were implanted subcutaneously into rats.

The animal experiments were approved by the Committee for Animal Experiments in Hokkaido University. Experimentation was carried out in accordance with guidelines proposed by the Institutional Animal Care and Use Committee. Different rats were used to examine implantation of four samples of the same carrier/rhBMP-2 composites. After weeks 2 and 4, the samples were removed for examination as described previously. [1-6, 8-10]

3. Alkaline phosphatase (ALP) activity
The retrieved samples were homogenized in 0.5 mL PBS (PBS homogenates). To a part of the PBS homogenate, an equal volume of 0.4% IGEPAL, 20 mM Tris-HCl, 2 mM MgCl2, (pH 7.5) was added and the mixture shaken vigorously for 30 minutes at 4 ºC. The supernatant after centrifugation was measured for ALP activity by the p-nitrophenol phosphate (Bessey-Lowry) method as described previously. [10]

4. Histological Observations
For histological observation, the implanted samples were carefully removed from the tissue as a single pellet, fixed in 10% neutral formaldehyde, and decalcified in Blank-Rychlo solution at 4°C for 5 hours. After washing with water, the pellets were dehydrated with alcohol and embedded in paraffin. 4-5 μm sections were cut and stained with hematoxylin and eosin. [8, 9]

Results
1. Observation by SEM of the products of HC-β-TCP
Figure 1 shows SEM images of the HC- TCP products with three different tunnel sizes: 300, 75 and 50 μm, with 37, 568 and 907 tunnels per 3.1 mm diameter cross-section. Pore sizes were uniformly distributed in each with minimum distortion. Numbers and sizes of tunnels were in good accordance with original designs.
2. Alkaline phosphatase activity
As shown in Fig. 2, ALP activity of the retrieved HC-β-TCP at 2 weeks after implantation indicated that HC-TCP 75/568 (the fraction indicates the tunnel size in μm/numbers of tunnels) expressed the highest ALP activity and the solid-TCP the lowest among the tested samples. The other two HC-TCPs 50/907 and 300/37 were considerably lower than HC-TCP 75/568.

3. Histological observations
Bone formation began at two weeks after implantation within the tunnels of the two scaffolds: HC-TCP 300/37 and 75/568 (data not shown). Figure 3A-F shows advanced bone formation in these HC-TCPs at 4 weeks. New bone formed in a concentric pattern, centering on a few capillaries. Particularly in the HC-TCP 300/37, concentric bone formation accompanying adipose tissue was eminent as shown in Fig. 3A and B. More active bone formation was seen in HC-TCP 75/568, as shown in Figs. 3C and D, in which tunnels were almost filled with bone tissue. There was also adipose tissue in some tunnels but the relative amount was less than that of HC-TCP 300/37.

Figures 3EF and GH show the histological observations of HC-β-TCP 50/907 retrieved at 4 and 2 weeks after implantation. Remarkably in this material chondrocytes and cartilage were detected within the tunnels at 2 weeks. However, no chondrocytes and cartilage were detected in HC-β-TCP 50/907 at 4 weeks and bone tissues were seen growing instead, as shown in Figs 3GH. In summary, all three of the geometrically different β-TCP scaffolds expressed specific patterns of tissue development within their tunnels. Only small amounts of bone formation were detected on the outer surface of disks.

Discussion
In this paper, we showed for the first time that HC-β-TCP induced bone tissue highly effectively, and opened up the possibility of a new application of β-TCP in bone regenerative medicine. Previously, we showed that HC-hydroxyapatite also induced bone and cartilage highly effectively, depending upon the diameter of the tunnels in HC-structure. Since ceramics composed of β-TCP are biodegradable in contrast to hydroxyapatite ceramics that are non-biodegradable, the clinical value of HC-β-TCP is self evident. Furthermore, we demonstrated that induction of bone by HC-β-TCP depends upon the diameters of tunnels in the honeycomb structure. Under the experimental conditions of this study, we clearly showed that HC-β-TCP with a diameter of 75 μm induced higher ALP activity than those with 50 or 300 μm.
Kuboki et al., HC-β-TCP as a bone substitute, Nano Biomedicine 2(2), 107-113, 2010

Figures 3
Histological profiles of HC-β-TCP retrieved at 2 weeks (G and H) and 4 weeks (A-F) after implantation
AB: HC-β-TCP 300/37, CD: 75/568, EF: 50/907, ACEG: at lower and BDFH: at higher magnifications
Bars indicate 500 μm in ACE and G, 100 μm in B, 50 μm in DF and H.
According to the recent studies scaffolds for bone reconstruction have to provide both vascular- and bone-inducing geometry. [1-3, 9, 11] One of the essential requirements for vascular and bone development is interconnecting pores within the solid matrix of these scaffolds. We concluded that 300-400 μm was the optimum size of irregular pores in the hydroxyapatite particles concerned 5). With regard to the optimal tunnel diameter of the HC-β-HAP, only one comparative study has been reported on two different tunnel diameters: 80mm and 350 mm, with the conclusion that the former induced enchondral ossification, while the latter direct-bone formation. [1-3, 9, 11]

In this study, Fig. 2 indicates that the highest ALP value was found in HC-β-TCP with a tunnel diameter of 75 μm compared with the other three scaffolds. Also from morphological observation (Figs. 4), it is estimated that HC-TCP with 75 μm tunnel produced the highest amount of bone at 4 weeks after implantation. Interestingly, an appreciable amount of cartilage formation was observed at 2 weeks in the HC-β-TCP with 50 μm tunnel diameter (50/907) HC, but not in the one with 75 μm (75/568), and as expected, the one with 300 μm (300/37) tunnels. These results showed considerable agreement with the previous conclusion on the tunnel size-dependent phenotype expressions by HC-β-HAP: enchondral ossification with 90-110 μm and direct bone formation with 350 μm, though there was a slight shift in the diameters which induced enchondral ossification. On the other hand, the previous conclusion that the optimal pore size is 300-400 mm in the irregular pores differed from the present one that the optimal tunnel size for bone formation was 75 μm.

This discrepancy makes an important argument that the size of a scaffold pore or tunnel is not the only determining factor influencing the efficacy of bone formation. Clearly, straight tunnels and irregular pores are geometrically different. Aside from the geometry of artificial ECM, different physical, chemical and biochemical properties of matrices have different effects on bone and cartilage induction (1-2). In this particular study, the slight discrepancy was explained by two aspects: (1) difference between non-biodegradable hydroxyapatite and biodegradable β-TCP, and (2) the difference between irregular pores and straight tunnels.

It is presumed that non-biodegradable hydroxyapatite will support firmly and constantly the developing cells on the inner surface of the tunnel or pore, while biodegrading β-TCP will not, which may promote enchondral development more rapidly than in the hydroxyapatite scaffold, resulting in the shift of optimal size for bone formation to smaller than that of hydroxyapatite. In regard to the feasibility of tissue ingrowth, it is reasonable to assume that straight tunnels in HC- are superior to irregular pore structures, such as in the products made by combusting polymer particles mixed in hydroxyapatite. Thus the optimal pore size of the scaffold for bone reconstruction must be carefully determined, taking into consideration the geometrical, physical and chemical properties of the scaffolds. In this particular case of HC-TCP in disk form, the tunnel structure of 75/568 showed a higher efficacy of bone formation than those of 50/907 and 300/37, estimated by ALP activity. Further comparative studies using honeycomb hydroxyapatite with identical geometry are being planned in this laboratory.

**Conclusion**

For the first time, HC- β-TCP scaffold was shown to be an effective tool for bone reconstruction. Three sorts of HC- β-TCP with different tunnel sizes of 50, 75, and 300 μm and tunnel number of 907, 56 and 37 respectively, were fabricated and tested for their efficacy as an artificial ECM, in a BMP-induced isotopic osteogenesis system. We found that HC- β-TCP with a tunnel size of 75 μm had a higher efficacy than the other two scaffolds with 50 and 300 μm tunnel diameter. Endochondral ossification was clearly observed in HC-β-TCP with tunnel size of 50 μm, 2 weeks after implantation.

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**References**


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