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Transplantation of cultured bone cells using combinations of scaffolds and culture techniques

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Abstract

The transplantation of cultured bone cells is expected to become a candidate for bone regeneration therapy. For the clinical application of this therapy, there remain several problems to be overcome, for example, the improvements of scaffolds and culture techniques. In this review article, two kinds of porous ceramics, a novel sintered porous hydroxyapatite and a porous beta-tricalcium phosphate (TCP), as well as a collagen-phosphosphoryn sponge are introduced as new scaffolds for bone regeneration. The former two ceramic scaffolds proved to be applicable for bone regeneration therapy. The collagen-phosphophoryn sponge proved to have bone formation ability in vivo. Moreover, for the application of this therapy to the regeneration of large bone defects, we improved the culture method by applying a low-pressure system and a perfusion system. Both culture systems accelerated the formation of bone in vivo in this transplantation model. Combinations of the scaffolds and culture techniques might be considered when designing therapeutic strategies.

Keywords: Bone; Regeneration; Hydroxyapatite; Beta-TCP; Low-pressure; Perfusion culture

1. Introduction

The transplantation of cultured bone cells is expected to become a candidate for bone regeneration therapy. First developed by Caplan and Bruder [1–3], this technique will be applicable to patients who have lost large segments of bone due to bone tumors, etc. The procedure is outlined in Fig. 1 as follows; mesenchymal stem cells (MSCs) are isolated from bone marrow and expanded in number in culture. When sufficient numbers of cells are available, they are loaded into a porous ceramic scaffold and surgically inserted into the excision defect. Yoshikawa and Ohgushi [4–6] improved the culture method for osteoblastic cells by introducing the method of Maniatopoulos [7].

The transplantation of cultured bone cells is clinically important, however, several problems remain to be solved before any clinical applications. First of all, appropriate materials are necessary for cell scaffolding. Our study focuses on the improvement of scaffolds and

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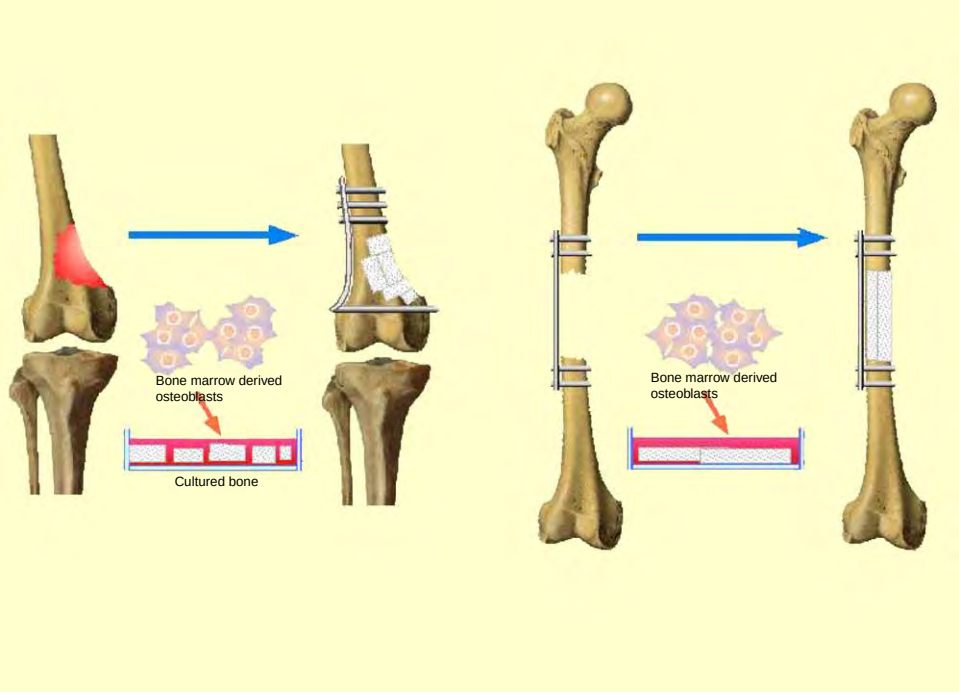

Fig. 1. Concept of the transplantation of cultured bone cells.

the in vitro culture method. Several kinds of porous ceramic scaffolds have been examined for this transplantation. For clinical usage, the ideal scaffolds would be a porous ceramic material not only with good biocompatibility and osteointegrative properties but also with high mechanical strength and high biodegradability. Some ceramics have the former two properties, however no porous scaffolds satisfy both of the latter two properties. A typical ceramic material with good mechanical properties is hydroxyapatite (HA). It is true that bulk HA has high mechanical strength, but past synthetic methods had not succeeded in producing strong porous HA materials available for clinical usage. The compressive strength of macroporous calcium phosphate ceramics is very weak; ranging from 0.5 to 10 MPa, depending mainly on the porosity percentage, pore size, chemical composition, grain size, and synthesis procedure [8]. Another problem is the interconnection of porous HA, which is incomplete, making vasculature invasion difficult. High compressive strength and good vasculature are important features of scaffolds for bone regeneration. To overcome these problems, two of the authors (M.K. and J.T.) developed a novel synthetic porous HA, which is stronger with more interconnections. Using the technique of the transplantation of cultured bone cells, we examined this novel porous HA as a scaffold for bone tissue regeneration and concluded that a composite of the novel HA and cultured bone marrow cells had good osteogenic ability in vivo [9,10].

It is true that the application of porous HA materials for bone tissue engineering has the advantage of stability and maintenance of compressive strength. However, the use of a rigid non-resorbable HA for skeletal reconstruction is associated with potential long-term interference with mechanical stress and strain in load-bearing areas. Furthermore, HA cannot be replaced by new bone. A fracture might occur either in the HA itself or at the interface with host bone. Therefore, a biodegradable material that can be replaced by bone tissue is important. The most popular ceramic with high biodegradability and biocompatibility is beta-tricalcium phosphate (TCP) and porous beta-TCP has attracted much attention among biomaterials scientists [11–14]. However, some reports on biodegradable porous beta-TCP alone implanted at extraskeletal sites suggest that degradation occurred quickly with no bone formation [13,15]. Owing to the rapid degradation and weak mechanical properties of beta-TCP, much research has focused on mixed calcium phosphates (a mixture of beta-TCP and HA, or beta-TCP and polymer). Moreover, the purity of beta-TCP also influences bone formation and biocompatibility. To try to overcome these difficulties, a highly pure porous beta-TCP was manufactured as an implantable material, Osferion (Olympus CO Ltd.). This biodegradable beta-TCP was combined with bone marrow-derived osteoprogenitor cells (BMO) and cultured in osteogenic medium in vitro, then implanted into subcutaneous sites in rat. We examined the ability for bone formation, absorbability,

and biochemical and mechanical properties of the beta-TCP/BMO composite and evaluated the beta-TCP porous material as a scaffold for bone tissue engineering [16,17].

For the clinical application of the transplantation of cultured bone cells for patients who have lost large segments of bone, a large scaffold is necessary. However, as the size of the scaffold increases, the subculture of cells into the central area of porous materials and perfusion of culture medium in the central area in vitro become more difficult. Osteoblasts need a supply of oxygen and nutrients from adjacent blood vessels in vivo, however, the osteoblasts inside the porous ceramics obtain no supply from blood vessels during the culture in vitro. To solve these problems, we designed and examined two kinds of culture techniques, a low pressure system and a perfusion culture system [18–20].

Moreover, we are designing and developing a new type of scaffold for bone tissue engineering, a collagen-phosphophoryn sponge. Phosphophoryn is the most abundant of the non-collagenous proteins of dentin. It is deposited directly at the advancing mineralization front of dentin. Previously, one of the authors (T.S.) has shown that type I collagen alone will not nucleate mineral formation from metastable calcium phosphate solutions while phosphophoryn covalently cross-linked to the collagen has good ability to form HA [21,22]. Taking advantage of this property, we synthesized a collagen-phosphophoryn sponge and tested whether it could work as a scaffold for bone tissue engineering by examining in vivo bone formation in Fischer rats orthotopically transplanted with the sponge and a bone marrow osteoblast composite (bone defect model).

All these topics will be introduced in this review article.

2. Materials and methods

2.1. Preparation of sintered porous HA and porous beta-TCP materials

A sintered porous HA was prepared by the following procedure. The HA slurry was foamed by adding polyoxyethylenelaurylether (PEI) and mixing. The pores were fixed by crosslinking PEI with diepoxy compounds and the HA porous body was sintered at 1200°C for 3 h. The HA sintered porous body had a high porosity (77%), and was completely interconnected. The average pore diameter was 500 μm and the interconnecting path was 200 μm in diameter. The compressive strength and three-point bending strength was 17 and 7 MPa, respectively. Used porous beta-TCP (Osferion) was kindly donated by Olympus Optics, CO Ltd. The compressive strength was 2.2 MPa. The average pore was 200–400 μm in diameter. Almost all pores were interconnected via a 100–200 μm path.

2.2. Preparation of collagen-phosphophoryn composite sponge

Phosphophoryn was prepared from molar teeth extracted from 8–9 month-old calf jaws. The powdered dentin was decalcified with 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.4, including protease inhibitors (100 mM 6-aminohexanoic acid, 5 mM benzamidine-HCl, and 1 mM phenylmethylsulfonyl fluoride). The extract was dialyzed against distilled water and precipitated with 1 M calcium chloride. Then, phosphophoryn was purified by a DEAE-cellulose column chromatography. Type I collagen was extracted from 2-yr-old calf skin, and was fibrilized at neutral pH at 37°C to produce reconstituted collagen fibrils. Phosphophoryn was cross-linked to the reconstituted collagen fibrils with divinylsulfone (Sigma Chem. Co., St. Louis, MO). To remove any phosphophoryn that was not covalently bound, the complex was washed with 0.05 M Tris buffer (pH 7.4) containing 0.5 M NaCl. Then, the complex was lyophilized.

2.3. Procedure of transplantation of cultured bone cells

We used osteoblastic primary cells using the modified culture method of Maniatopoulos [7]. Fig. 2 shows the procedure for the transplantation of cultured bone cells. Briefly, osteoblastic primary cells were obtained from the femur of 7-week-old male Fischer rats. The bone marrow cells were cultured in a standard medium of Eagle-minimum containing 15% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When culture dishes became near confluent after 10 days, they were treated with trypsin and subcultured onto porous ceramic blocks at a concentration of 10^6 cells/ml and incubated for 2 h at 37°C. After that, the cells/porous ceramic composites were cultured in an osteogenic medium consisting of the standard medium supplemented with 50 μg/ml of vitamin C phosphate, 10 mM β-glycerophosphate, and 10^{-8} M dexamethasone for 2 weeks. Then the cells/porous ceramic composites were implanted at subcutaneous sites in the rats.

2.4. Low pressure system

The low pressure system consisted of an Ulvac G-5 and rotary vacuum pump, Iuchi vacuum controller VC-100 and Iuchi Polycarbonate vacuum dessicator, connected to each other by rubber and silicon tubes as shown in Fig. 3(A). We examined six groups with different pressures, 760 (normal atmospheric pressure), 500, 250, 100, 50 and 10 mmHg. After treatment with the low-pressure system, the culture dish was incubated for 2 h at 37°C at normal atmospheric pressure. After that, the same procedure was used.

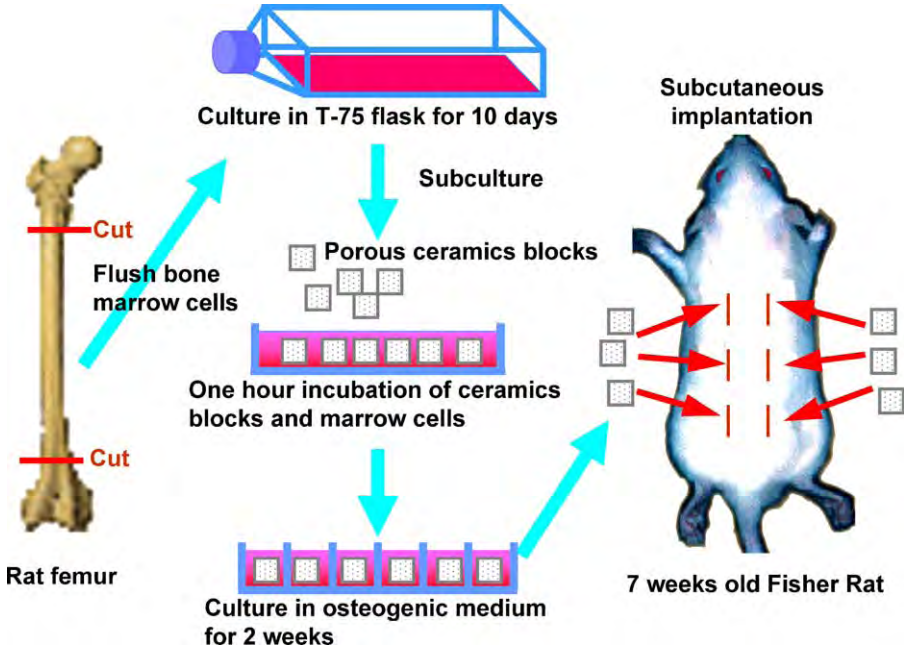

Fig. 2. Animal model system for the transplantation of cultured bone cells.

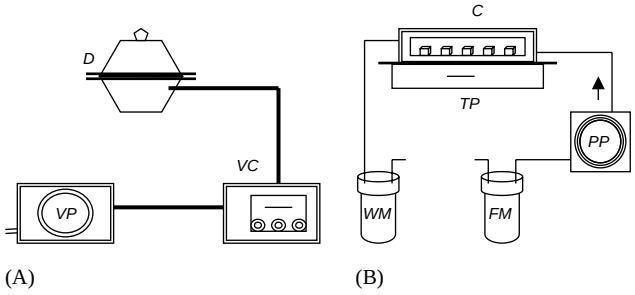

Fig. 3. Schematic diagram of the low-pressure system (A) and perfusion culture system (B). In (A), D stands for polycarbonate vacuum dessicator, and V_c for vacuum controller; in (B), C stands for perfusion container, TP for thermoplate, PP for peristaltic pump, FM for fresh medium, and WM for waste medium.

2.5. Perfusion culture system

A perfusion culture system, as shown in Fig. 3(B), was used in our study. After the subculture of BMO with porous ceramics, the BMO/ceramic composites were transferred into the perfusion culture container (Minucells and Minutissue, Bad Abbach, Germany). The medium flow was adjusted to a rate of 2 ml/h. The perfusion system was maintained at 37°C by a thermo plate.

3. Results and discussion

3.1. Transplantation of cultured bone cells using HA and beta-TCP porous ceramic scaffolds

We examined the transplantation of cultured bone cells using two kinds of ceramic scaffolds; a novel porous HA [9,10] and porous beta-TCP [16,17]. Figs. 4(A) and (B) show SEM photographs of the former and the latter material, respectively. The porosity of each was 77% and 75%.

Activity of alkaline phosphatase was detected in HA/BMO composite grafts at 1–8 weeks post-implantation. The alkaline phosphatase activity increased gradually and peaked at 3 weeks. A high level of activity was maintained for 8 weeks. Bone osteocalcin could be detected at 1 week post-implantation and showed a steady increase with time until 8 weeks. These biochemical data supported the high osteogenic activity of HA/BMO composites. At two weeks post-implantation, the decalcified sections stained with HE showed bone formation in some pores of HA. At 4 weeks after implantation, mature bone areas and the number of active osteoblasts facing the bone increased in pores. Fig. 5 shows the HE staining of the composite at 8 weeks post-implantation. The quantity of bone increased. These results suggested that the sintered porous HA, which has a cancellous bone-like microstructure, proved to be a good scaffold for osteogenic differentiation of bone marrow-derived osteoblasts and provided a bone forming biomaterial in vivo.

Biodegradable beta-TCP may also be a good candidate for a scaffold in bone tissue engineering. We examined beta-TCP porous materials using the same method applied for the sintered porous HA materials. Alkaline phosphatase activity and osteocalcin content had a similar time course to the case of porous HA. In the light microscopic analysis, the decalcified sections stained with HE showed immature bone had formed in some pores of the implant at 2 weeks post-implantation.

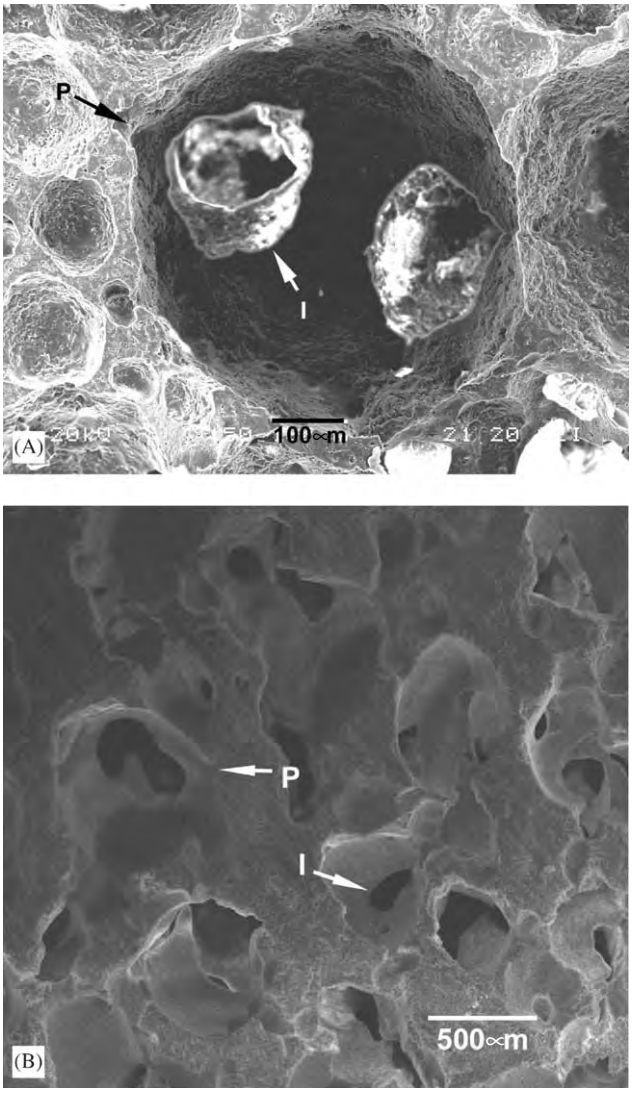

Fig. 4. SEM photomicrograph of microstructures of the novel porous hydroxyapatite (A) and beta-TCP(B). P indicates pore and I indicates interconnecting path.

At 4 weeks, areas of mature bone existed in the pores. At 8 weeks, the amount of mature bone had increased in the pores. Fig. 6 shows the HE staining of the BMO/beta-TCP composite at 16 weeks post-implantation. Blood vessel formation was observed, supporting bone formation. These results also suggest the beta-TCP porous materials are good candidate for scaffolds in bone tissue engineering. The most serious problem for clinical application is the mechanical weakness of beta-TCP porous materials with 2 MPa of compressive strength. Many orthopaedic surgents have doubts over clinical applications due to the rapid degradation of beta-TCP in vivo. In some reports, it is suggested that bone formation cannot follow the degradation of beta-TCP. However, this difficulty proved to be overcome by tissue engineering treatment of the beta-TCP materials. Fig. 7 shows the time course of the compressibility of the beta-TCP/BMO composite after implantation. The compressive strength of beta-TCP/BMO before implantation was weak at 2.25 MPa, however, it had improved to 4.92 MPa at 12 weeks post-implantation. Moreover, it reached 11.2 MPa at 24 weeks post-implantation. These results suggested that tissue engineering treatment using porous beta-TCP increased the mechanical strength of the composites in vivo.

3.2. *Application of the low-pressure system and perfusion culture system*

The advantage of the transplantation of cultured bone cells is the ability to regenerate bone in large segments lost. For that, it is necessary to achieve a homogeneous bone formation in large porous ceramics in vivo. However it is more difficult to insert cells into the central area of porous ceramics by normal subculture procedures and for them to differentiate into mature osteoblasts because of the poor flow of culture medium

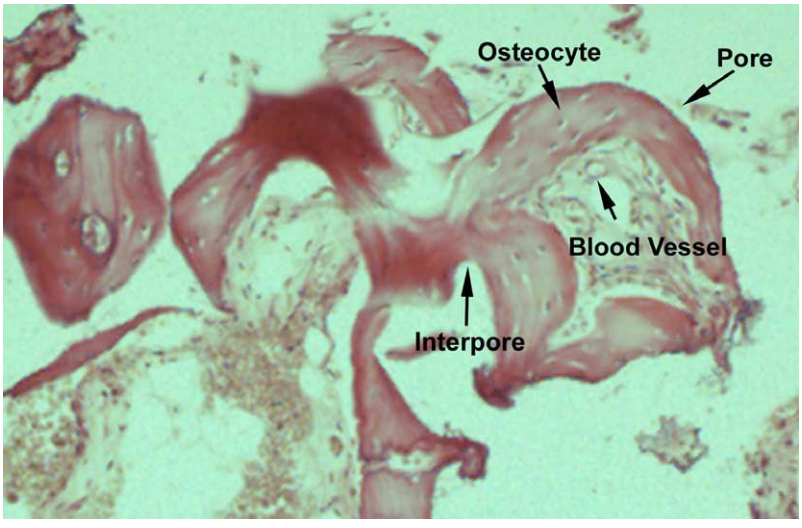

Fig. 5. HE staining of HA/BMO composite at 8 weeks post-implantation.

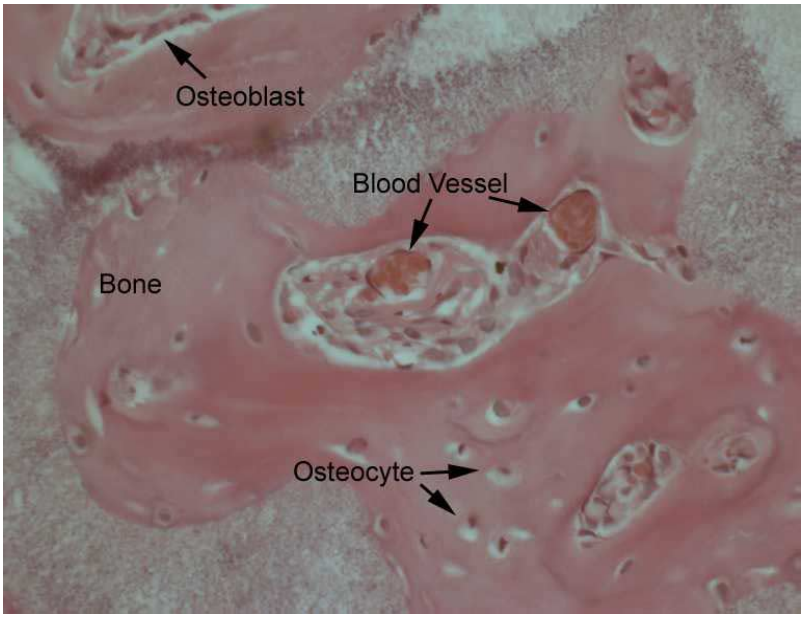

Fig. 6. HE staining of beta-TCP /BMO composite at 16 weeks post-implantation.

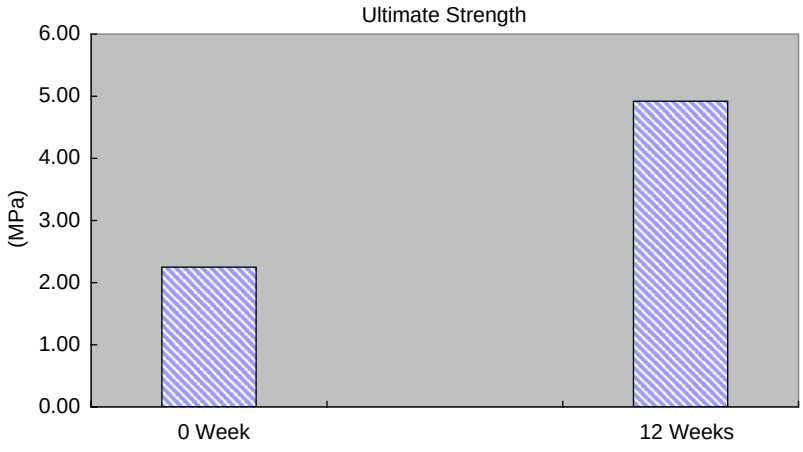

Fig. 7. Compressive strength of beta-TCP/BMO composite at 0 and 12 weeks.

there. To overcome these difficulties, the culture technique was modified by introducing a low-pressure system and a perfusion culture system. Fig. 8(A) shows the pressure dependence of the alkaline phosphatase activity of the BMO/porous ceramic composites at 4 and 8 weeks post-implantation. As expected, the lower the pressure applied to cells and porous ceramics at subculture, the stronger the alkaline phosphatase activity became. However, the level of the activity reached a maximum of around 100 mmHg and decreased below 50 mmHg. This means that there exists an appropriate pressure for the application of this low-pressure system. It is concluded that the bone formation was significantly improved by using a low-pressure system with appropriate pressure. Fig. 8(B) indicates the effect of the perfusion culture system applied to cultured bone transplantation. The time course of alkaline phosphatase activity of the BMO/porous ceramics is shown with and without the perfusion of medium. The alkaline phosphatase activity improved two-fold compared to normal culture and the perfusion of medium significantly improved bone formation in vivo.

3.3. Transplantation of cultured bone cells using a collagen-phosphophoryn sponge

In the light microscopic study, the decalcified sections stained with HE showed that immature bone had formed in the same parts adjacent to collagen fibers in the composite of collagen-phosphophoryn and marrow cells at 1 week post-implantation. No bone formation was observed in the control composite at 1 week. At 2 weeks, the area of mature bone had expanded in the experimental composite. Bone formation was induced

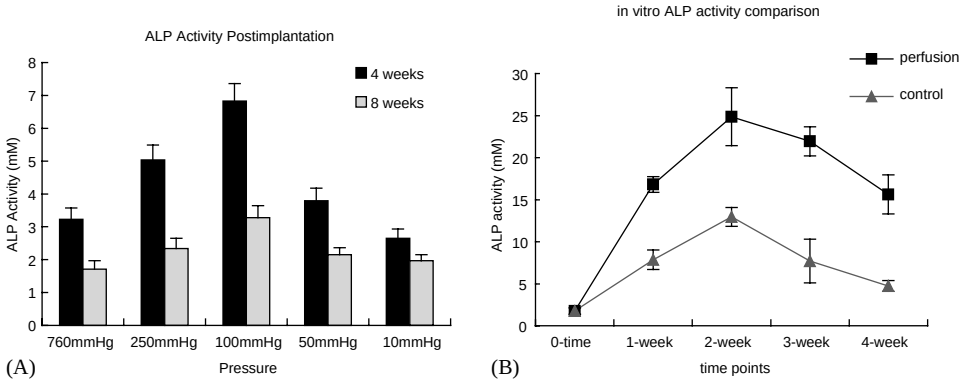

Fig. 8. (A) Temporal changes in ALP activity of subcultured composite grafts treated with different pressures at 4 and 8 weeks. Significant differences compared with values for the 760-mm Hg group. (B) Temporal changes in ALP of in vitro cultured composites. The difference is significant between the perfusion group and the control.

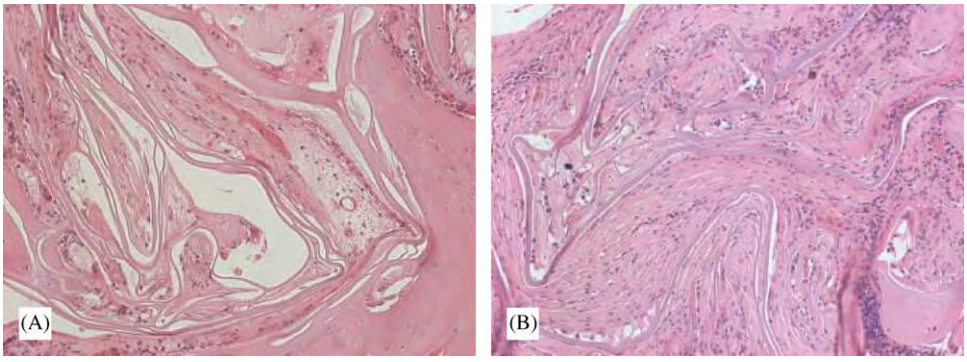

Fig. 9. Typical results with the phosphophoryn-collagen sponge as a scaffold. Control group (Bone marrow cells/collagen sponge) (A) and experimental group (bone marrow cells/phosphophoryn-collagen sponge) (B) 2 weeks after implantation. Hematoxylin and eosin stain; original magnification × 100.

slightly in the control composite at 2 weeks. Even at 8 weeks, the quantity of bone formed in the composite of collagen-phosphophoryn and marrow cells was greater than that in the control as shown in Fig. 9. The collagen-phosphophoryn sponge was not degradated at 8 weeks in either composite.

These results clearly showed that more bone formation was observed in the collagen-phosphophoryn sponge and osteoblast composites than the control composites from 1 week to 8 weeks. In a previous study, we demonstrated that the collagen-phosphophoryn sponge has good ability for apatite induction in vitro. The present study showed that tissue engineering treatment on incubating the composite of collagen-phosphophoryn and BMO in osteogenic medium results in good osteogenic activity. The collagen-phosphophoryn sponge is a good candidate as a scaffold for bone tissue engineering.

3.4. Discussion

Reconstructing large bone defects is one of the major topics in the orthopedic field. Many approaches have been made using autografts, allografts, or artificial materials alone or with bone grafts [23]. The major problem with autografts is an insufficient supply and surgical morbidity with donor site pain and loss of function [24]. Moreover, patients have to suffer surgery twice. Allografts are also associated with infection and inflammation [25]. For overcoming these problems, tissue engineering strategies have recently been developed. The basic concept is the imitation of the natural process of bone repair by using cell sources differentiating into osteoblasts and scaffolding matrices which support cellular attachment, differentiation and proliferation [1–3]. Bone marrow contains MSCs [26], known as multipotent stem cells capable of differentiating into osteoblasts, chondrocytes, myoblasts, adipocytes, etc. Preclinically, the osteogenic potential of MSCs in porous ceramic scaffolds has been proved [27–30]. For cell-based strategies, unfractionated fresh bone marrow, purified culture-expanded MSCs, differentiated osteoblasts or chondrocytes, or genetically modified cells expressing bone morphogenic protein (BMP) are implanted. This type of bone tissue engineering is not only exciting and useful for clinical application of MSCs, but

also improving our basic understanding of stem cells. Notably, the effect of aging on MSCs is quite important in clinical applications of MSCs for tissue engineering.

When designing therapeutic strategies, orthopedic surgeons should take the position and size of the bone defect into consideration for any cell-based approach. For that, the mechanical and structural properties of materials are very important when choosing appropriate scaffolds.

Porous HA functions effectively in conducting osteogenesis. A number of HAs have been tested and approved for clinical application since the 1980s. The nature of porous synthesized HAs facilitates bone ingrowth. HA are not osteoinductive, but they can support bone formation from marrow cells, even at extraosseous sites. However, there have been no materials that can satisfactorily meet clinical demands. Furthermore, there are still some problems with the application, for example, the mechanical properties of HA. The compressive strength of macroporous calcium phosphate ceramics is very weak; ranging from 0.5 to 10 MPa, depending mainly on the porocity percentage, pore size, chemical composition, grain size, and synthetic procedure [31]. Another problem is the interconnection of porous HAs, which is incomplete, making vasculature invasion difficult. Blood supply guarantees further growth of bone tissue in porous HA. High compressive strength of the bone graft material and good vasculature are important features for bone repair in orthopedics. In this regard, the results of the sintered porous HA combined with BMO and implanted into the rat body, well satisfied the above demands as a scaffold for bone tissue engineering. Furthermore, the chemical composition of a major factor in osteoinduction, HA, which was secreted by osteoblasts, has good biocompatibility without immunological and toxic reactions [32]. Moreover, it has good osteoinduction within the bone microenvironment maintaining normal MSC development [5]. Therefore, osteoblasts and mineralized bone matrix can be directly deposited upon the inorganic surface of pores within the HA block [33]. This property is important for bone-graft substitutes to avoid fibrous tissue interventing at the interface between bone tissue and the graft, causing loosening, which can destroy the architecture around and in the bone-graft substitute. The sintered porous HA, which has a cancellous bone-like microstructure, proved to be a good scaffold for osteogenic differentiation of BMOs and provided a bone-forming biomaterial in vivo.

It is true the application of HA has the advantage of stability and maintenance of compressive strength as a scaffold for bone tissue engineering. However, the use of a rigid non-resorbable HA for skeletal reconstruction is associated with potential long-term interference with mechanical stress and strain in load-bearing areas. Furthermore, HA cannot be replaced by new bone. Therefore, a fracture might occur either in the HA itself or at the interface with host bone. The clinical application of HA has thus been restricted due to its poor absorbability and brittleness. Degradable materials that can be replaced by bone tissue are also important for bone repair. Bioactive glass has been reported to be completely resorbed at skeletal sites within 16 weeks, but is a solid [34]. Porous beta-TCP has been attracting much attention due to its biodegradability and biocompatibility [11–14]. However, while some reports on biodegradable porous beta-TCP alone implanted at extraskeletal sites suggest that degradation occurred quickly with no bone formation [13,15], at least one study on porous TCP implanted at skeletal sites found that the bone which formed during the initial stages was resorbed later on, so that bone repair after 1 year was not significantly enhanced [35]. Moreover, some solid beta-TCP material have been reported to be completely resorbable at skeletal sites, although they were not porous but granular [36,37]. Owing to the rapid degradation and weak mechanical properties of TCP, much research has focused on mixed calcium (a mixture of TCP and HA, or TCP and polymer) [38–45]. The purity of beta-TCP, however, also influences bone formation and biocompatibility [46]. These difficulties were overcome by using a highly pure porous beta-TCP as an implantable material; Osferion. Our results on porous beta-TCP as a scaffold for bone tissue engineering suggest that the TCP/BMO composites cultured in osteogenic medium were capable of forming bone in vitro and in vivo. Furthermore, we tested the mechanical properties of the TCP/BMO composites. We found that the compressive strength was much greater than that of the dry beta-TCP and increased over time. This means that bone formed in beta-TCP faster than the material biodegraded and the biomechanical properties of beta-TCP were enhanced. At 6 months post-implantation, the compressive strength was similar to that of cancellous bone. In conclusion, through tissue engineering, a biodegradable and highly pure porous beta-TCP was combined with BMO incubated in osteogenic medium to produce a composite with good osteogenic activity, enhanced biomechanical properties, and low biodegradability.

Even if the porous structure of the ceramic is ideal, as the size of the scaffold material increases, the subculture of the cells into the central area of the porous scaffold and perfusion of culture medium there in vitro become more difficult. To overcome these difficulties, two culture methods were introduced in this paper; the low-pressure system and the perfusion culture system. Both methods improved the activities of cells in the central area of the porous materials.

Which kind of scaffolds should be used, and is the low-pressure or perfusion culture necessary or not? The answer might depend on the kind of therapy, age of

patients, kind of bone defects, etc. The choice is up to the surgeon. For example, if the bone defect is small, one need not use the perfusion culture. However, if the defect is large, and fixation is difficult, the porous HA with low-pressure and/or perfusion culture might be appropriate. If the mechanical load on the implantation is small by fixation, the biodegradable beta-TCP might be appropriate. A more detailed study is necessary for supplying information to surgeons who will apply these techniques and is now in progress.

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