In vitro and in vivo evaluation of carbonate apatite-collagen scaffolds with some cytokines for bone tissue engineering

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INTRODUCTION

Tissue engineering is required as an emerging technology to reconstruct damaged bone because of tumors, trauma or pathologic disease. The bone formation using bone graft material is a widely accepted technique in the surgical field. In order to repair damaged bone, autogenous bone has been the ideal choice to fill the defect site. However, autografts sometimes have significant limitations coming from a donor site morbidity, a limited donor bone supply, and an inadequate resorption rate during the healing process. These limitations have prompted

### Abstract

**Background:** Collagen is regarded as one of the most useful biomaterials. We tried to combine collagen and carbonate apatite (CA) with some cytokines in order to enhance bone formation ability. In this study, we found that CA-collagen sponge (CA-CS) was a possible candidate of newly graft material for bone formation. **Materials and Methods:** CA-CS was fabricated by the following procedure. One wt% of pig hide collagen solution (Nippon Meat Packers. Inc., Tokyo, Japan) was neutralized with 0.1 N NaOH, and then mixed immediately 243 mg apatite powder with 0.06 M carbonate contents. After centrifugation at 1500 rpm for 10 min, excess water was removed, and the mixture was packed into Teflon molds (5.0 mm × 2.0 mm). Each 10 µg of basic fibroblast growth factor (bFGF) and recombinant human bone morphogenetic protein-2 (rh-BMP2) were involved in these sponges. Then these scaffolds frozen at −80°C for 2 h and dried in a freeze dry machine for 24 h. CA-CS without cytokines were also prepared as a control. Mouse osteoblast-like cell (MC3T3-E1) proliferations in these scaffolds were investigated by 3-day in vitro cell culture using MTT assay examination. Ten New Zealand rabbits (weight: 3–3.5 kg) were used in this in vivo study. After 3 weeks of placement, the scaffolds, rabbits were sacrificed, and bone formation in the sockets was evaluated histologically and histomorphometrically. **Results and Conclusion:** By histological observation and measurement of bone area ratio, CA-CS with cytokines showed higher bone formation ability (bFGF/CA-CS: 50.7 ± 7.3%, rh-BMP2/CA-CS: 54.2 ± 5.0%) than other groups. From the limited results of this study, it is suggested that CA collagen scaffolds with some cytokines may become an attractive scaffold for bone regeneration.

**Key Words:** Bone tissue engineering, carbonate apatite, collagen

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increasing interest in alternative bone grafts. Allografts and xenograft are alternative and attractive sources but have several problems such as the risk of disease transmission, inflammation, immunogenicity, loss of biologic and mechanical properties, and religious concerns.[3] Consequently, significant efforts are being made to develop ideal bone graft material to induce the formation of new bone to bridge the defect.

The use of artificial bone graft material to replace bone defect may have many useful benefits, such as the elimination of the risk of disease transmission, fewer surgical procedures, reducing the risk of infection or immunogenicity, and the abundant availability of synthetic scaffold materials.[4] The characteristics of ideal bone graft material would be biocompatible, maintain volume over time, suitable mechanical property, controllable biodegradability, and be replaced entirely by new bone ingrowth.[5]

Many natural and synthetic materials have been developed and characterized, but satisfactory bone-formative materials have not been introduced. Current attempts are focused on bioceramics such as calcium phosphate which is found in the body, demonstrated excellent cellular and tissue responses in vitro and in vivo, and shows promises of biocompatibility, osteoconductivity, and biodegradability.[6,7] The types of calcium phosphate materials which have developed and investigated as bone graft material are hydroxyapatite (HA, Ca$_{10}$(PO$_4$)$_6$(OH)$_2$), β-tricalcium phosphate (β-TCP, Ca$_3$(PO$_4$)$_2$), biphasic calcium phosphate, and multiphasic bio-glasses.[7,8] However, some problems arise such as difficulties to control absorption time period of HA and it has been reported that HA produced from coral has the inability to control the pore size and chemical composition, thereby resulting in unpredictable outcomes.[9-11] In addition, resorption time of β-TCP is faster 10–20 times than HA, the macroscopic mechanical properties of β-TCP are inadequate for load bearing surface due to the inherent brittleness.[12] Therefore, to overcome these problems, we focused on carbonate apatite (CA) as a biodegradable material. The main reason is because CA resembles bone apatite more closely than any other calcium phosphate. The main inorganic content of bone is CA which contains about 7% carbonate by weight.[13] CA easily dissolves under acidic condition, and thermodynamically under neutral and basic condition. It was reported by the histological observation that CA was gradually replaced by regenerated bone. Considering these properties, CA is expected to become an ideal bone replacement material, which possesses both osseoconductivity and bioresorbability.[14]

To obtain the biomimetic bone graft material, combination of CA with Collagen has been developed. CA and collagen were combined to create new materials CA-collagen sponges (CA-CSs) with unique structural and mechanical properties appropriate for the bone graft material. It is reported that 0.06 M CA-CS has space maintenance ability and show bone formation ability higher than other concentration in bone defect of rabbit’s femur.[15-17]

However, osteoinductive proteins play an important role to enhance bone formation ability. There have been a variety of growth factors were identified and isolated for in vitro and in vivo including bone morphogenetic protein (BMP), fibroblast growth factor (FGF), platelet derived growth factor, and transforming growth factor (TGF). In this study, we focused on BMP and FGF because it has been reported that BMP and basic FGF (bFGF) can induce bone formation and have been used clinically within scaffolding biomaterials carriers. The term “BMP” was introduced to describe the substance(s) in the demineralized bone matrix responsible for the phenomenon. At least 15 BMPs are currently recognized (BMPs 1–15).[18,19] Recently, bone morphogenetic protein-2 (BMP2) is the leading osteoinductive growth factor used clinically in bone-related regenerative medicine. The osteoinductive properties of BMPs have been evaluated extensively both in vitro and in vivo.[18,20] On the other hand, bFGF is known to be a potent mitogen of bone cells in vitro produced by osteoblasts and stored in the bone matrix. It has been demonstrated that a topical application of exogenous bFGF also enhances the healing process of bone fractures.[18] Furthermore, bFGF can accelerate ideal periodontal regeneration without ankylosis or epithelial downgrowth.[19,20]

Based on these recent findings concerning the relationship of CA-CS with BMP2 and bFGF, the combined application of these two factors could be expected to enhance synergistically bone formation. Therefore, we examined the effect of CA-CS with recombinant human BMP2 (rh-BMP2) and bFGF on cell proliferation using MC3T3-E1 in vitro and initial bone formation in vivo.

**MATERIALS AND METHODS**

**Preparation of sponges**

CA-CS was fabricated using lyophilization techniques. To fabricate CA-CS, 10 ml of 1.0 wt% of pig hide collagen solution (Nippon Meat Packers, Inc., Tokyo, Japan) was neutralized with 0.8 ml of 0.1 N NaOH. Adding 243 mg of CA of 70 wt% dry weight and then mixed immediately. After centrifugation at 1500 rpm for 10 min, excess water was removed, and the mixture was packed into 2 types of Teflon molds (5 mm in diameter × 2 mm in height and 3 mm in diameter × 5 mm in height). The molds were frozen at −80°C for 2 h and dried in a freeze dry machine (EYELA Co. Ltd.,
Tokyo, Japan) for 24 h. CA-CS were subjected to UV radiation for 2 h. As a control, we fabricated CS, by not adding 243 mg of CA of 70 wt% dry weight after neutralizing with 0.8 ml of 0.1 N NaOH and then next procedure was the same as described above.

Preparation of recombinant human bone morphogenetic protein-2 and basic fibroblast growth factor solutions
rh-BMP2 and bFGF (PeproTech, Inc., London, UK) were dissolved in phosphate buffered saline and combined with CA-CS in each 20 µg/mL. Then stored at -80°C. Before cell seeding by using MC3T3-E1, CA-CS + rh-BMP2 and CA-CS + bFGF dried in a freeze dry machine (EYELA Co. Ltd., Tokyo, Japan) for 24 h.

Microstructure observation
To ensure that the shape of the sponge was kept after addition of cytokine, we observed the microscopic structure and porosity of CS, CA-CS, CA-CS + rh-BMP2 and CA-CS + bFGF by scanning electron microscopy (SEM) (3D Microscope VE-8800, Keyence, Japan).

Cell viability assay
Mouse osteoblast cells line MC3T3-E1 (RIKEN BioResource Center, Tsukuba, Japan) were cultivated on cell-culture plate in Dulbecco's Modified Eagle's Medium (DMEM) medium (Sigma, USA) supplemented with 2% penicillin/streptomycin, 10% fetal bovine serum and passage every 3–4 days. MC3T3-E1 cells between 2 × 10^4 cells in DMEM medium were statistically seeded onto CS, CA-CS, CA-CS + rh-BMP2, and CA-CS + bFGF. Cells were left in DMEM medium for 3 h, and then 980 µl of the culture medium was added to each well of 24-well culture plates. Cells were cultivated in 5% CO_2 at 37°C in 95% relative atmospheric humidity for 1, 3, 5, and 7 days. The culture medium was changed every 3 days.

Cell viability in the porous structure was examined by MTT assay (Cell Counting Kit-8, Dojindo Molecular Technologies, Inc., Rockville, Maryland, USA). Following incubation with 550 µl/well for 2 h in the incubator (in 5% CO_2 at 37°C). The solution was then transferred to 96 wells and the absorbance of the solution in each well was measured by using a microplate reader (BiotrakTM II, Amersham/Bioscience, Australia) at wavelength 450 nm.

Measurement of alkaline phosphatase activity
Cells were seeded on 24-well tissue culture treated plates at a density of 2 × 10^4 cells/well. At each time points, cells were washed 3 times with saline solution. The cell suspension was then transferred to a centrifuge tube, treated by ultrasonication and centrifuged (7200 g for 10 min, 4°C). The alkaline phosphatase (ALP) activities of the samples were determined by a colorimetric assay using an ALP reagent containing p-nitrophenyl phosphate (p-NPP) as substrate (BioAssay, Hayward, CA, USA). The absorbance of p-nitrophenol formed by the hydrolysis of p-NPP, catalyzed by ALP, was measured using microplate reader at 405 nm wavelength.

Animal experiments
In vivo study of this experiment was approved by Research Facilities Committee for Laboratory Animal Science, Hiroshima University School of Medicine. Sixteen New Zealand rabbits (weight: 3–3.5 kg) were used. Four bone sockets (3 mm in diameter, 5 mm in height) were prepared in right femur of a rabbit [Figure 1]. Four types of scaffolds (CS, CA-CS, CA-CS + rh-BMP2 and CA-CS + bFGF) were randomly implanted into the bone socket, and one socket was kept with blood clot as a control. 2 and 3 weeks after implantation, rabbits were sacrificed. Tissue block, including the sample were cut and observed on X-ray radiography. Then fixed in 10% neutral formalin for 1 week and embedded with paraffin through dehydration with ethanol. Specimen were cut into 5 µm thickness and stained with hematoxylin and eosin.

Images of new bone formation were examined by light microscopy and counting number with an image analyzing software (Image J, National Institutes of Health, Bethesda, USA). The percentage ratio of the newly formed bone area against the total compact bone area was analyzed statistically by ANOVA.

RESULTS

Microstructure observation
Microstructures of the scaffolds were examined by SEM Figure 2 showed that all of the scaffolds had three-dimensional porous structures together with good interconnections between...
the pores. This structural characteristic was preserved after cytokine incorporation.

**Cell viability assay**
The significant increase of cell numbers was seen in both of bFGF/CA-CS and rh-BMP2/CA-CS than in CA-CS at 3 days ($P < 0.05$) [Figure 3]. There was no significant difference between bFGF/CA-CS and rh-BMP2/CA-CS.

**X-ray observation**
X-ray examination was done after 2 weeks and 3 weeks implantation at femur of rabbits. X-ray observation of CA-CS + rh-BMP2, CA-CS + bFGF, CA-CS, CS, and open defect are shown in Figure 4. There are some high-density developments in the bone defect area. It meant that the new bone had formed at week 3 [Figure 4b].

**Histological observation**
The results of histological observation of CS, CA-CS, CA-CS + rh-BMP2, CA-CS + bFGF, and open defect after 2 and 3 weeks implantation at femur of rabbits with hematoxylin and eosin staining are shown in Figures 5 and 6. It could be observed that more new bone formation had developed. This is supported by the results of the calculation from the percentage ratio of the newly formed bone area at 3 weeks implantation [Figure 7].

**DISCUSSION**
In this study, we demonstrated a novel method to fabricate CA-CS based on lyophilization technique. CA was combined with collagen to improve the biological stability and strength of CA-CS to reach the demand of an application in the bone graft material. It has been known that the function of scaffolds not only as a delivery vehicle for growth factors and living cells but also support and regulate bone formation.[21]

To allow ingrowth of cells and migration of vascular tissue, porous structure is needed.[22] From the SEM images of this study revealed that CA-CS had much porous and three-dimensional structure and also attachment of CA powders on CA-CS were observed. We created CA-CS with pore size approximately 50–300 µm. These pore sizes had sufficient space for cells to invade. Highly porous and interconnected pore structures were required to ensure that the biological environment is conductive to cell attachment, proliferation, tissue growth and adequate nutrient flow.

To improve bone formation ability, growth factors are needed. A variety of growth factors participates in the regulation of cell proliferation, differentiation, and bone metabolism. Osteoblasts differentiation and matrix mineralization are regulated by the actions of systemic and local signaling factors. Currently, many cytokines have been evaluated and investigated including rh-BMP2 and bFGF and it was identified that rh-BMP2 and bFGF can accelerate bone formation.[16]

BMP2 belong to the TGF superfamily, has a powerful inducer of osteoblast differentiation in vitro and bone formation in vivo. BMP2 are required for bone regeneration. On the other hand, FGF was originally identified from extracts of the pituitary gland and brain and has been demonstrated to stimulate the proliferation of fibroblasts. It has been established that bFGF is a potent mitogen not only for fibroblasts, but also for other mesoderm-derived cells including osteoblasts and vascular endothelial cells.[21–23]

Most in vitro studies have reported that bFGF can influence the differentiation of bone marrow stromal cells (BMSCs), and enhance chondrogenic and osteogenic differentiation of BMSCs.
BMP2 and bFGF are growth factors that act as prototypical mitogen and morphogen, respectively. The combination of growth factors has been shown to result in synergistic effects that might further stimulate the complex cellular events and interactions that lead to new bone formation.

Cell viability of MC3T3-E1 in CA-CS was measured by MTT assay. The significant increase of cell numbers were seen in both of bFGF/CA-CS and rh-BMP2/CA-CS than in CA-CS at 3 days ($P < 0.05$). There was no significant difference between bFGF/CA-CS and rh-BMP2/CA-CS. All sponges supported the growth of cells on three-dimensional structures in a similar manner. Moreover, rh-BMP2/CA-CS had a tendency to promote the growth of cells to a greater extent than other.

The histological observations show that the combination of CA-CS with rh-BMP2 and bFGF promotes bone formation. There were trabecular bone included many osteocytes and regularly lined with many osteoblasts, indicating the bone-forming activity was observed. There was no evidence of inflammation or foreign body reaction in the host tissue adjacent to the new bone. After 3 weeks implantation, the percentages of newly formed bone at femur of rabbits were 23.7% in bone defect, 25.8% in CS, 38.1% in CA-CS, 50.7% in CA-CS + bFGF and 54.2% in CA-CS + rh-BMP2, respectively, indicating that new bone formation ratio of CA-CS with rh-BMP2 was significant higher than those of other group. This demonstrated that there were significant differences in the amount of new bone formed in response to the different cells.

CONCLUSION

We have shown that the combination of CA-CS with rh-BMP2
and bFGF may induce cell proliferation and the results of the in vivo study showed that CA-CS + rh-BMP2 and CA-CS + bFGF had the ability to enhance bone formation. It may be suggested that combined application of CA-CS with rh-BMP2 and bFGF can provide as a novel bone graft material for bone formation in clinical use.

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Conflict of interest
There are no conflict of interest.

REFERENCES

1. Kenar H, Köse GT, Hasirci V. Tissue engineering of bone on micropatterned biodegradable polyester films. Biomaterials 2006;27:885-95.
2. Lee JY, Kim KH, Shin SY, Rhyu IC, Lee YM, Park YJ, et al. Enhanced bone formation by transforming growth factor-β1-releasing collagen/chitosan microgranules. J Biomed Mater Res A. 2006;76:530-9.
3. Meyer U, Joos U, Wiesmann HP. Biological and biophysical principles in extracorporeal bone tissue engineering. Part III. Int J Oral Maxillofac Surg 2004;33:635-41.
4. Hong KS, Kim EC, Bang SH, Chung CH, Lee YI, Hyun JK, et al. Bone regeneration by bioactive hybrid membrane containing FGF2 within rat calvarium. J Biomed Mater Res A 2010;94:1187-94.
5. Keiichi K, Mitsuobu K, Masafumi S, Yutaka D, Toshiaki S. Induction of new bone by basic FGF-loaded porous carbonate apatite implants in femur defects in rats. Clin Oral Implants Res 2009;20:560-5.
6. Suh H, Song MJ, Ohata M, Kang YB, Tatsuaki S. Ex vivo mechanical evaluation of carbonate apatite-collagen-grafted porous poly-L-lactic acid membrane in rabbit calvarial bone. Tissue Eng 2003;9:635-43.
7. Oh S, Oh N, Appleford M, Ong JL. Bioceramics for tissue engineering applications – A review. Am J Biochem Biotechnol 2006;2:49-56.
8. Paul W, Sharma CP. Ceramic drug delivery: A perspective. J Biomater Appl 2003;17:253-64.
9. Gao Y, Cao WL, Wang XY, Gong YD, Tian JM, Zhao NM, et al. Characterization and osteoblast-like cell compatibility of porous scaffolds: Bovine hydroxyapatite and novel hydroxyapatite artificial bone. J Mater Sci Mater Med 2006;17:815-23.
10. Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials 2006;27:3413-31.
11. Mangano C, Bartolucci EG, Mazzocco C. A new porous hydroxyapatite for promotion of bone regeneration in maxillary sinus augmentation: Clinical and histologic study in humans. Int J Oral Maxillofac Implants 2003;18:23-30.
12. Kwon SH, Jun YK, Hong SH, Kim HE. Synthesis and dissolution behavior of beta-TCP and HA/beta-TCP composite powders. J Eur Ceram Soc 2003;23:1039-45.
13. LeGeros RZ. Calcium Phosphates in Oral Biology. Basel: Karger; 1991.
14. Suh H, Park JC, Han DW, Lee DH, Han CD. A bone replaceable artificial bone substitute: Cytotoxicity, cell adhesion, proliferation, and alkaline phosphatase activity. Artif Organs 2001;25:14-21.
15. Matsuura A, Kubo T, Doi K, Hayashi K, Morita Y, Yokota R, et al. Bone formation ability of carbonate apatite-collagen scaffolds with different carbonate contents. Dent Mater J 2009;28:234-42.
16. Hirata I, Nomura Y, Ito M, Shimaizu A, Okazaki M. Acceleration of bone formation with BMP2 in frame-reinforced carbonate apatite-collagen sponge scaffolds. J Artif Organs 2007;10:212-7.
17. Itoh M, Shimaizu A, Hirata I, Yoshida Y, Shintani H, Okazaki M. Characterization of CO3Ap-collagen sponges using X-ray high-resolution micromotography. Biomaterials 2004;25:2577-83.
18. Lan J, Wang Z, Wang Y, Wang J, Cheng X. The effect of combination of recombinant human bone morphogenetic protein-2 and basic fibroblast growth factor or insulin-like growth factor-I on dental implant osseointegration by confocal laser scanning microscopy. J Periodontol 2006;77:357-63.
19. Yamagiwa H, Endo N, Tokunaga K, Hayami T, Hatano H, Takahashi HE. In vivo bone-forming capacity of human bone marrow-derived stromal cells
is stimulated by recombinant human bone morphogenetic protein-2. J Bone Miner Metab 2001;19:20-6.
20. Zara JN, Siu RK, Zhang X, Shen J, Ngo R, Lee M, et al. High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. Tissue Eng Part A 2011;17:1389-99.
21. Mathew PN, Binulala SV, Selvarurugan N, Tamurac H, Jayakumara R. Novel biodegradable chitosan-gelatin/nano-bioactive glass ceramic composite scaffolds for alveolar bone tissue engineering. Chem Eng J 2010;158:353-61.
22. Wang L, Huang Y, Pan K, Jiang X, Liu C. Osteogenic responses to different concentrations/ratios of BMP-2 and bFGF in bone formation. Ann Biomed Eng 2010;38:77-87.
23. Wen B, Karl M, Pendlry D, Shafer D, Freilich M, Kuhn L. An evaluation of BMP-2 delivery from scaffolds with miniaturized dental implants in a novel rat mandible model. J Biomed Mater Res B Appl Biomater 2011;97:315-26.
24. Alam S, Ueki K, Marukawa K, Ohara T, Hase T, Takazakura D, et al. Expression of bone morphogenetic protein 2 and fibroblast growth factor 2 during bone regeneration using different implant materials as an onlay bone graft in rabbit mandibles. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;103:16-26.
25. Presta M, Dell’Era P, Mitola S, Moroni E, Ronca R, Rusnati M. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. Cytokine Growth Factor Rev 2005;16:159-78.