Evaluation of carbonate apatite as a bone substitute in rat extraction sockets from the perspective of mesenchymal stem cells

髙橋, 良輔

Dental Materials Journal 2023; : –

Evaluation of carbonate apatite as a bone substitute in rat extraction sockets from the perspective of mesenchymal stem cells

Ryosuke TAKAHASHI[1,2], Ikiru ATSUTA[2], Ikue NARIMATSU[1], Takayoshi YAMAZA[3], Xiaoxu ZHANG[1,2], Yuki EGASHIRA[1,2], Kiyoshi KOYANO[2] and Yasunori AYUKAWA[1]

[1] *Section of Implant and Rehabilitative Dentistry, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

[2] *Division of Advanced Dental Devices and Therapeutics, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

[3] *Department of Molecular Cell Biology and Oral Anatomy, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

Corresponding author, Ikiru ATSUTA; E-mail: atyuta@dent.kyushu-u.ac.jp

Carbonate apatite (CO_3Ap) is a major inorganic bone component and an effective bone substitute. To clarify the function of CO_3Ap, we compared differences among CO_3Ap, hydroxyapatite (HAp), and β-tricalcium phosphate (β-TCP) by focusing on mesenchymal stem cells (MSCs) that have a role in wound healing. For *in vivo* experiments, maxillary molars were removed and the bone substitute was inserted. MSC accumulation around extraction sockets was significantly promoted in CO_3Ap and β-TCP groups. For *in vitro* experiments, MSCs were cultured with bone substitutes. The differentiation potential and amount of calcium deposition were significantly lower in CO_3Ap and HAp groups than in the β-TCP group. Increases in insulin-like growth factor-I and vascular endothelial growth factor were found only in the CO_3Ap group. CO_3Ap-filled extraction sockets accumulated MSCs, and MSCs cultured in the presence of CO_3Ap produced large amounts of growth factors. These results suggest that CO_3Ap promotes healing of tooth extraction sockets.

Keywords: Carbonate apatite, Mesenchymal stem cells, Accumulation, Bone substitutes

INTRODUCTION

A bone substitute is used to fill a bone defect to provide options in terms of the implant depth and direction, and expand treatment possibilities. The rate of bone replacement is critical for the choice of bone substitute, because it is necessary to maintain space for bone formation[1-3]. Although many materials have been used to date[4], autologous bone is the first choice[5,6]. However, artificial bone is often used because of the limited amount of autologous bone that can be collected and the highly invasive nature of the procedure[7,8].

Artificial bone has been developed since the 1970s, and hydroxyapatite (HAp) and β-tricalcium phosphate (β-TCP) are used in the dental field. HAp is inorganic and similar to natural bone with high biocompatibility[9-11]. Because HAp bone substitutes are fabricated by sintering that eliminates carbonate groups[12], its resorption by osteoclasts is difficult, and HAp remains in the body for a long time. Conversely, β-TCP is highly soluble[13,14]. While early bone formation is expected in the area of resorption, soft tissue often invades before new bone formation. Ishikawa *et al*. developed carbonate apatite (CO_3Ap) as a bone substitute, which is the inorganic main component of bone[12,15]. CO_3Ap bone substitute has significantly higher solubility in a low acid environment than HAp and lower solubility than β-TCP[13,16-19].

However, it remains unclear how CO_3Ap differs from other materials in replacing bone. Therefore, in this study, we focused on elucidating the mechanism by which mesenchymal stem cells (MSCs) are involved in wound healing of the extraction socket. MSCs are present in almost all tissues of the body (*e.g*., bone marrow[20], fat[21], and gingiva[22]) and are easily isolated and cultured, facilitating their application in the field of regenerative medicine. In wound healing, endogenous MSCs play an important role by their multipotency to differentiate into bone cells, cartilage cells, adipocytes, cardiomyocytes, nerve cells, and vascular endothelial cells[23-27]. Additionally, MSCs stimulate tissue repair by controlling the activity of immune cells and eliciting anti-inflammatory and anti-immune effects[28].

In this study, on the basis of the hypothesis that CO_3Ap promotes the function of MSCs for hard and soft tissue repair compared with other bone substitute materials, we investigated the involvement and role of MSCs in the restoration of tooth extraction sockets with CO_3Ap bone substitutes. Furthermore, we compared localization of endogenous MSCs in three bone substitutes (CO_3Ap, HAp, and β-TCP) in animal experiments, and investigated the differentiation, proliferation, and growth factors of MSCs in culture experiments.

MATERIALS AND METHODS

Materials

Cytrans® (GC, Tokyo, Japan) was used as CO_3Ap. The granules were 300–600 μm in diameter (pore diameter: <0.5 μm; porosity: 26%[16])[19,29,30]. BONETITE (HOYA, Tokyo, Japan) was used as HAp. The granules were

Received Feb 17, 2022: Accepted Nov 1, 2022
doi:10.4012/dmj.2022-040 JOI JST.JSTAGE/dmj/2022-040

500–1,000 μm in diameter (pore diameter: 200 μm; porosity: 70%[31]). CERASORB M® (Curasa, Kleinostheim, Germany) was used as a representative β-TCP. This product is a porous granule with a particle size of 1,000–2,000 μm (pore diameter: 5–500 μm; porosity: ~65%[16]).

CO_3Ap, HAp, and β-TCP plates (diameter: 10.0 mm; thickness: 0.8 mm), which had a similar composition to the granules except for strength and shape, were kindly provided by GC.

Animals

Wistar rats received care following the guidelines established by Kyushu University (approval number: A29-222-0). The experimental model was established as reported previously[19]. Briefly, 6-week-old Wistar rats (30 males; 120–150 g) had maxillary first and second molars (80 teeth) extracted under systemic anesthesia. The extraction socket was enlarged to eliminate the alveolar septum with a dental round bur, and CO_3Ap, HAp, or β-TCP bone substitute was implanted. After each experimental period, the rats were deeply anesthetized and perfused intracardially with heparinized phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) (pH 7.4).

Tissue preparation

Tissues were prepared in accordance with the methods described in our previous studies[19,32]. Briefly, the oral mucosa surrounding the extraction socket was immersed in 4% PFA for 48 h at 4°C. The samples were snap frozen after decalcification with Kalkitox™ solution (Wako, Osaka, Japan) and cut into 10-μm-thick buccopalatal sections using a cryostat. The sections were stained with Ladewig's fibrin stain[32] and then observed under an optical microscope. Furthermore, the length of distance between the edge of the epithelial surface was calculated. All measurements were done three times and their averages were calculated.

Immunohistochemistry

The sections were stained immunohistochemically using mouse anti-rat CD90 (1:100, Sigma-Aldrich, St. Louis, MO, USA) and CD105 antibodies (1:100, Sigma-Aldrich) overnight at 4°C. Samples were then incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200, Jackson Immuno Research, West Grove, PA, USA) for 1 h at room temperature and mounted with 4′-6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) as described previously[33].

MSC isolation and culture

MSCs were isolated from bone marrow of rats with or without GFP expression as described previously[34]. Briefly, bone marrow cells were flushed out from femoral and tibial bone cavities. The cells were passed through a 40-μm cell strainer to obtain a single cell suspension. The single cell suspension was seeded in 100-mm culture dishes at 1×10^6 cells/dish. At 1 day after seeding, the cells were washed with PBS and cultured in growth medium consisting of alpha-minimum essential medium (Invitrogen, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (Equitech-Bio, Kerrville, TX, USA), 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen). After 1 week of culture, colony-forming unit-fibroblasts had formed colonies. Adherent MSCs in these colonies were detached with trypsin/EDTA (Invitrogen), reseeded as new cultures, and expanded for experiments. For *in vitro* assessment, MSCs were seeded in 35-mm dishes at 2×10^4 cells/dish and incubated with or without bone substitutes for 12 h at 37°C with 5% CO_2.

Scanning electron microscopy

At 3 and 7 days after MSCs were seeded on CO_3Ap, HAp, or β-TCP plates at 5×10^3 cells/mL, cell morphology was evaluated by scanning electron microscopy. Samples were fixed with 2.5% glutaraldehyde, dehydrated in graded ethanol solutions, and then freeze-dried. The samples were mounted on stubs, coated with an Au/Pd alloy, and evaluated microscopically[32]. The number of cells on materials was counted in a field of view.

Fluorescence staining

MSCs expressing GFP were seeded on CO_3Ap, HAp, or β-TCP in 35-mm dishes at 5×10^3 cells/mL and incubated for 12 h at 37°C with 5% CO_2. Then, the cells were fixed in 4% PFA for 5 min and incubated with TRITC-conjugated phalloidin (1:100; Sigma-Aldrich) and a mouse anti-rat GFP antibody (1:100, Sigma-Aldrich) overnight at 4°C. The cells were then incubated with a FITC-conjugated secondary antibody (1:200, Jackson Immuno Research) for 1 h at room temperature and mounted using VECTASHIELD® mounting medium containing DAPI (Vector Laboratories)[34,35].

Proliferation assay

MSCs were indirectly cultured in the bottom chamber of Transwells at 1×10^4 cells/mL for 3 days with or without bone substitutes in the upper chamber. A Transwell® with a 0.4-μm insert was used as a separator (upper chamber: blank, CO_3Ap, HAp, or β-TCP; bottom chamber: MSCs) to evaluate the indirect effects of MSCs with or without each bone substitute mediated through calcium ions and growth factors[36]. In this experiment, the number of cells on days 1 and 3 after culturing was counted, and the rate of increase was calculated.

Osteogenic differentiation assay

MSCs were indirectly cultured in the bottom chamber of Transwells at 1×10^4 cells/well in osteogenic culture medium containing 1.8 mM KH_2PO_4 and 10 nM dexamethasone (Sigma-Aldrich) with or without bone substitutes in the upper chamber. After 28 days of osteogenic induction, the cultures were stained with a 1% Alizarin Red S solution (Sigma-Aldrich)[33].

Adipogenic differentiation assay

MSCs were indirectly cultured in adipogenic culture

medium containing 0.5 mM isobutylmethylxanthine, 60 μM indomethacin, 0.5 μM hydrocortisone, and 10 μg/mL insulin (all from Sigma-Aldrich) with or without bone substitutes using Transwells. After 14 days of adipogenic induction, the cultures were stained with Oil Red O. Oil Red O-positive lipid droplets were observed under an inverted microscope (BZ-9000, Keyence, Osaka, Japan)[33].

Calcium measurement

CO_3Ap, HAp, or β-TCP was immersed in a 150-ppm calcium solution (0.04% $CaCl_2$ and 0.75% KCl), distilled water, or MSC medium (α-MEM with 20% FBS, 2 mM L-glutamine, 1×10^{-8} M dexamethasone, 55 μM 2-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin; 48 ppm calcium) at 37°C (n=8 each). After 7 days, each solution was evaluated using a calcium ion meter (LAQUAtwin Ca-11, Horiba, Kyoto, Japan) in accordance with the methods described in a previous study[37].

Enzyme-linked immunosorbent assays (ELISAs) of growth factors

MSCs were indirectly cultured with or without bone substitutes using Transwells with a 0.4-μm pore size, and culture supernatants were collected at 3 and 7 days. The samples were concentrated to a 5× concentration using an Apollo™ (Orbital Biosciences, Topsfield, MA, USA), and ELISAs were performed to measure endothelial growth factor (EGF), vascular endothelial growth factor (VEGF), and IGF-I (Invitrogen)[38].

Statistical analysis

Five samples in each group were used in experiments. An *a priori* Shapiro–Wilk test was performed to test for normality. One-way analysis of variance with Bonferroni's correction was performed. p<0.05 was considered statistically significant. Data are indicated as the mean±standard deviation.

RESULTS

Histology of early extraction socket healing

Figure 1 shows extraction socket healing on days 3 and 7 in models (HAp, β-TCP and CO_3Ap groups) with the bone substitute inserted into the socket compared with the extraction socket healing model (control group) without a bone substitute. There was almost no difference in the healing degree at 3 days. However, at 7 days, we observed promotion of epithelial tissue closure on the extraction socket in the CO_3Ap group compared with the other groups.

MSC accumulation around bone substitutes

Figure 2 shows MSC accumulation in experimental groups (control , HAp, β-TCP, and CO_3Ap). When endogenous MSC localization was observed by immunohistochemical staining of CD90 and CD105, the number of double immune-positive cells was significantly higher in β-TCP and CO_3Ap groups than the other groups (Figs. 2B, C). The MSCs were localized in soft tissues slightly distant from the extraction socket. However, in the HAp group, negligible MSC accumulation was observed, which was similar to the control group.

Effect of bone substitutes on MSC adhesion

MSCs were directly seeded on each bone substitute, and cell dynamics were compared after 3 and 7 days (Fig. 3A).

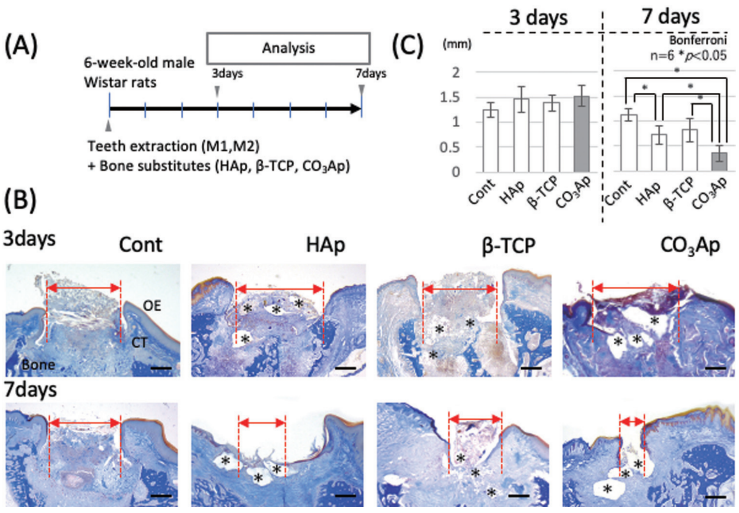

Fig. 1 Wound healing after tooth extraction.
(A) Experimental protocol of the animal experiments. (B) Ladewig's fibrin staining of the extraction socket with or without materials after 3 and 7 days. OE: oral epithelial tissue, CT: connective tissue. The black asterisk indicates the bone substitute. Bar=300 μm. (C) The distance between the epithelial edge of the extraction socket. The red double arrow indicates the distance measured (n=6, *p<0.05). HAp: hydroxyapatite, β-TCP: β-tricalcium phosphate, CO_3Ap: carbonate apatite

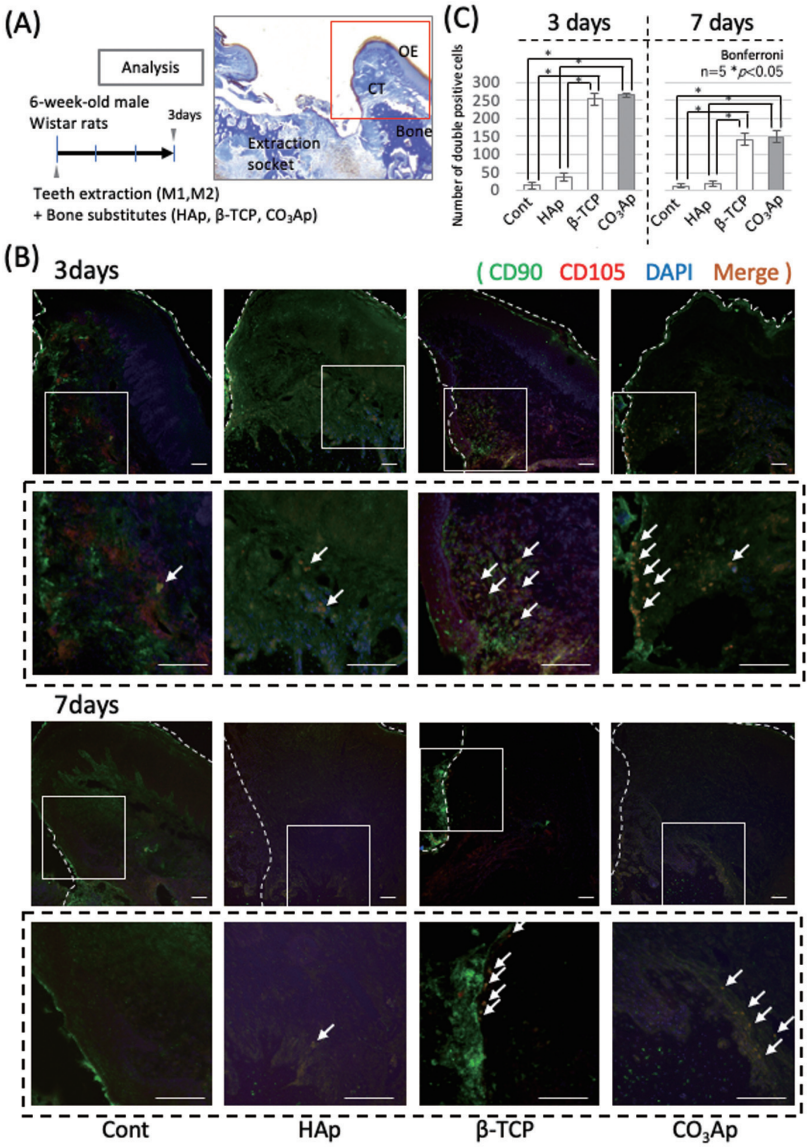

Fig. 2 MSC accumulation around extraction sockets with bone substitutes.
(A) Experimental protocol of the animal experiments. The red square indicates the following observation sites. This panel shows the control group. OE: oral epithelial tissue, CT: connective tissue. (B) Localization of endogenous MSCs observed by immunohistochemistry of CD90 and CD105 after 3 and 7 days. The white square indicates the following magnified view. White arrows indicate double positive cells. Bar=100 μm. (C) Number of accumulated cells around the extraction socket with or without bone substitutes (n=5, *p<0.05). HAp: hydroxyapatite, β-TCP: β-tricalcium phosphate, CO_3Ap: carbonate apatite

Morphology was observed at 3 and 7 days after seeding by fluorescence staining of actin and scanning electron microscopy (Figs. 3B, C). MSCs in the CO_3Ap group were more extended than those in the HAp group and the cells were in close contact with each other. Round cells were observed in the β-TCP group (Fig. 3C). The number of adherent cells was similar in CO_3Ap and HAp groups, and clearly higher than that in the β-TCP group (Fig. 3D). This difference did not change even after 7 days.

Effect of bone substitutes on MSC proliferation and differentiation potential

MSCs were cultured in a differentiation medium in accordance with each condition. Indirect effects of the bone substitute were evaluated (Fig. 4A). The control contained no bone substitute. Proliferation was similar among the CO_3Ap, HAp, and β-TCP groups, and significantly lower than in the control group (Fig. 4B). Although the bone substitute groups had lower values than the control, calcification was observed only in the

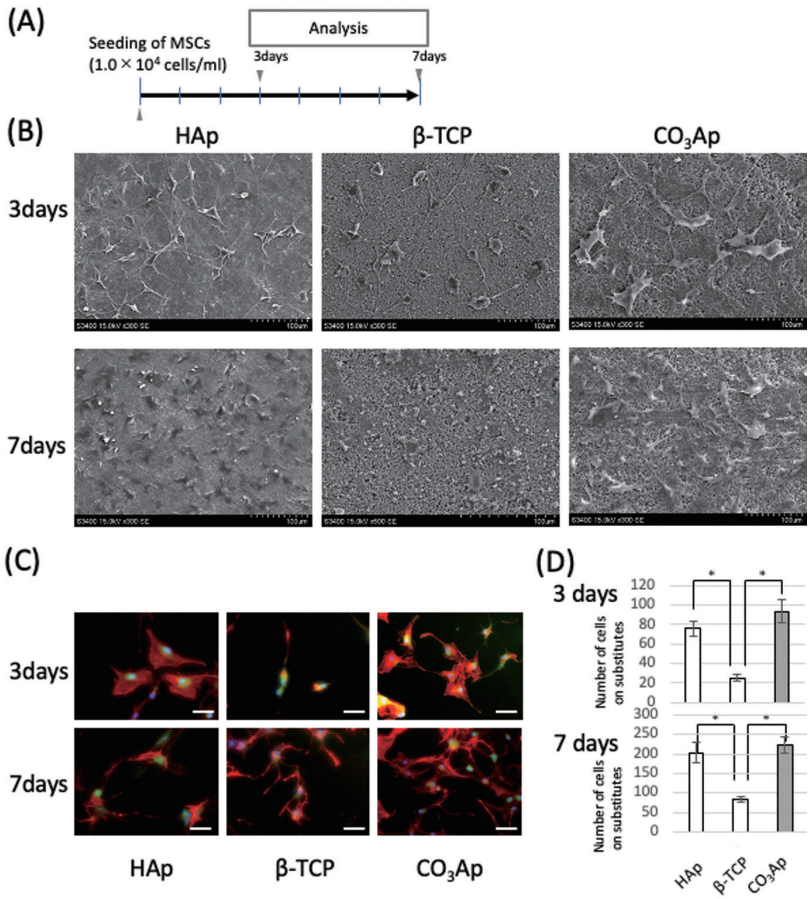

Fig. 3 Effect of bone substitutes on MSC adhesion.
(A) Experimental protocol of the *in vitro* experiments. (B) Scanning electron microscopy of MSCs on the materials. Expansion rate: ×300. (C) Immunofluorescence (actin and GFP) of MSCs on the materials. Bar=100 μm (D) Number of adhesive MSCs on the materials (n=4, *p<0.05). HAp: hydroxyapatite plate, β-TCP: β-tricalcium phosphate plate, CO_3Ap: carbonate apatite plate

β-TCP group (Fig. 4C). Conversely, there was no clear difference in the ability to differentiate into adipocytes among all groups (Fig. 4D).

Changes in the calcium concentration in culture medium

Bone substitutes immersed in high Ca ion concentrations were evaluated (Fig. 5A). The Ca ion level was decreased in CO_3Ap and HAp groups, although the Ca ion levels in β-TCP and control groups were similar. Conversely, in the absence of Ca ions, Ca ions were eluted from the materials in all groups, but the level was highest in the β-TCP group. When culture medium was used, only the CO_3Ap group showed a decrease compared with the control (Fig. 5B).

Effect of bone substitutes on MSC growth factor expression

The culture supernatants of MSCs cultured on bone substitutes for 3 or 7 days were collected and growth factors were measured by ELISAs (Fig. 6A). After 3 days, VEGF and IGF-I production in the CO_3Ap group was significantly higher than that in the other groups. After 7 days, VEGF tended to decrease in the CO_3Ap group, but the IGF-I level was similar to the HAp group and significantly higher than in control and β-TCP groups (Fig. 6B).

DISCUSSION

Healing of hard and soft tissues in extraction sockets filled with bone substitutes is greatly affected by the characteristics of the material, individual differences of patients, and the treatment site and size. In this study, we focused on the function of MSCs and considered the differences between three typical bone substitutes. MSCs are involved in many wound healing processes, including those in bone[38,39], and it has been predicted that their function would be influenced by bone substitutes. In previous studies, the conventional method to investigate bone substitutes was to insert them into the tibia or the top of the head and observe bone replacement over time.

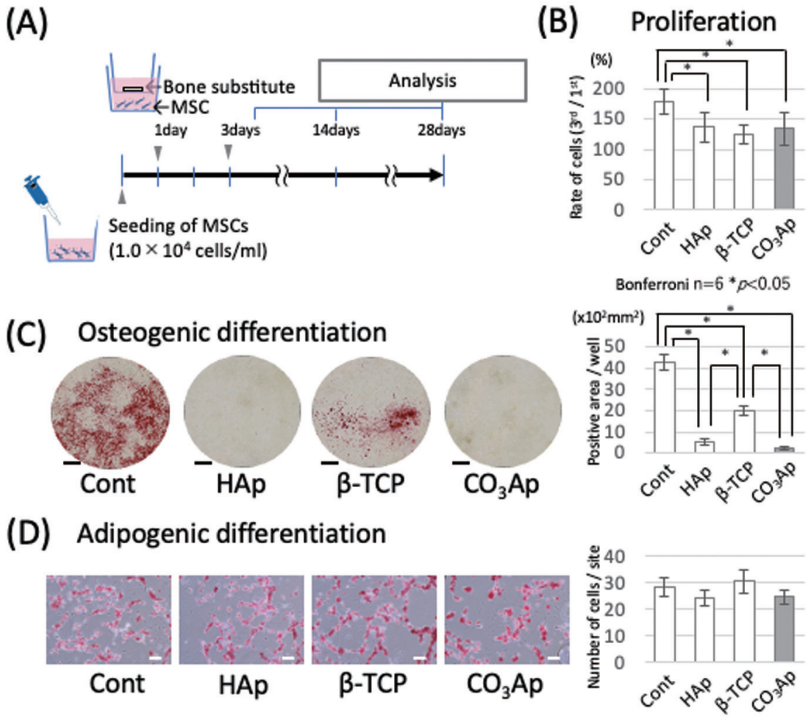

Fig. 4 Effect of bone substitutes on MSC proliferative and differentiation potential.
(A) Experimental protocol of the *in vitro* experiments. (B) Number of proliferated MSCs on the materials (n=6, *p<0.05). (C) Calcified deposits stained by Alizarin Red. (D) Oil Red O staining of adipocytes on the materials. Control: culture dish, HAp: hydroxyapatite, β-TCP: β-tricalcium phosphate, CO_3Ap: carbonate apatite

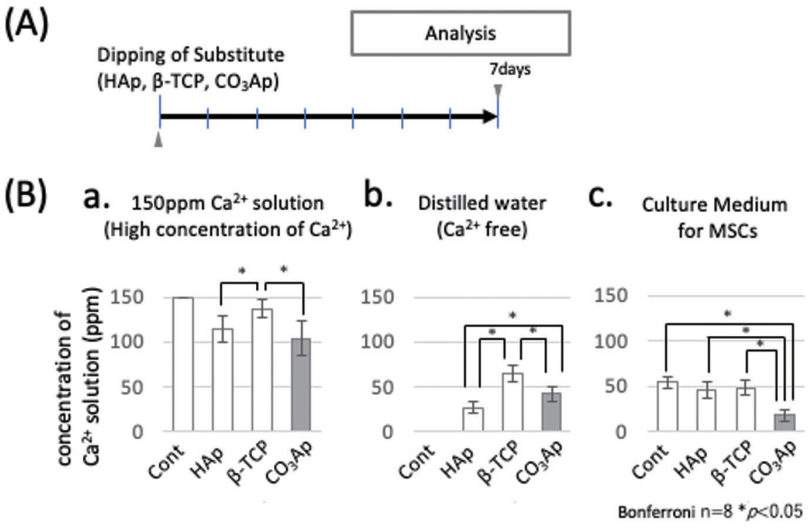

Fig. 5 Changes in the calcium concentration in the culture medium.
(A) Experimental protocol of the *in vitro* experiments. (B) Bone substitutes in (a) the presence of Ca ions, (b) the absence of Ca ions, and (c) culture medium. Control: culture dish, HAp: hydroxyapatite, β-TCP: β-tricalcium phosphate, CO_3Ap: carbonate apatite

However, cortical bones are thick in these areas and blood supply is poor[40]. Previous studies have reported that MSCs are abundant in the jawbone region[41]. Therefore, in this study, we established a model to insert a bone substitute into the extraction socket of a rat and evaluated the effects of the bone substitutes[19].

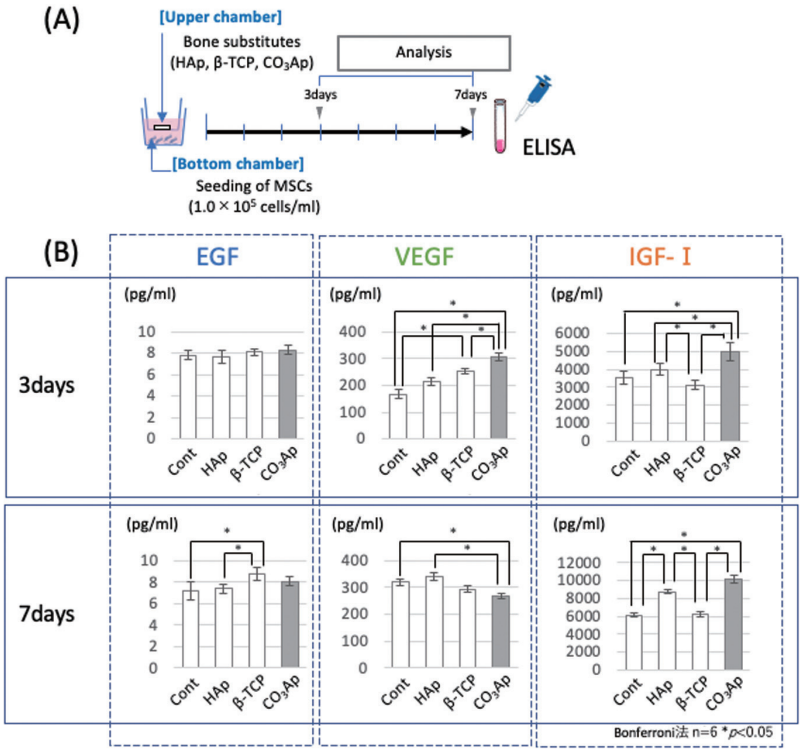

Fig. 6 Effect of bone substitutes on growth factor expression in MSCs.
(A) Experimental protocol of the *in vitro* experiments. (B) Measurement of growth factors (EGF, VEGF, and IGF-I) from MSCs cultured on each material by ELISAs (n=6, *p<0.05). Control: culture dish, HAp: hydroxyapatite, β-TCP: β-tricalcium phosphate, CO_3Ap: carbonate apatite

Our findings indicate that the use of this model may have important implications for the clinical use of bone substitutes.

There appeared to be no difference in the healing degree at 3 days as shown in Fig. 1B, but the CO_3Ap group exhibited promotion of epithelial tissue covering the extraction socket from the edges at 7 days. The immunohistology of MSC markers showed that endogenous MSCs in soft tissues surrounding CO_3Ap and β-TCP in the extraction socket had accumulated in large numbers. This tendency continued on day 7 (Fig. 2B), but disappeared at approximately day 14 (data not shown). In control and HAp groups, the number of accumulated MSCs was small, but the number of endogenous MSCs was initially small. Therefore, the presence of MSCs may trigger extraction socket healing[28].

When tissue is severely damaged by tooth extraction, which is similar to our model, the damaged cells attract lineage cells by releasing stromal cell-derived factor and high mobility group box 1 into blood[42,43]. MSCs also accumulate at the injured site through the action of these protein, and induce regeneration of the injured tissue by anti-inflammatory, fibrosis inhibitory, and tissue regeneration effects[44-46]. However, it was unclear which reaction affected MSCs among the materials. Therefore, we confirmed the characteristics of each material using *in vitro* experiments.

As shown in Fig. 3, rat bone marrow-derived MSCs were directly cultured on the materials to confirm MSC adhesion on the bone substitutes. MSCs are greatly influenced by the surrounding environment and change their properties accordingly[47,48]. Therefore, we evaluated what kind of the bone substitutes affected MSCs. In the β-TCP group, cells were round, and contact was lost between cells. Conversely, when MSCs had cell activity, they extended processes and their adhesion ability was promoted. In the CO_3Ap group, the cells had stretched and maintained contact with each other to the extent that they adhered to the culture dish.

However, as shown in Fig. 4, MSCs on HAp and CO_3Ap, which should have a strong adhesive ability, also had little ability to proliferate and differentiate. Conversely, β-TCP has a strong bone-forming ability, although not to the same extent as the control as shown by the Alizarin Red staining. In general, cells with strong adhesion have high proliferation and differentiation potentials[49]. However, *in vitro* experiments using MSCs, various culture media were used for proliferation, adhesion, or differentiation. Therefore, we were able to compare materials, but could not compare the correlation between MSC properties such as adhesiveness, differentiation, and proliferation. MSCs on β-TCP showed a strong bone formation ability. The number of adhesive cells was observed on day 7,

but Alizarin Red staining, which indicates the ability to form bone, revealed differences after 28 days of culture. Therefore, some material changes may have occurred over this long period.

The most probable factor is the elution of calcium ions. Previous studies have recorded the amount of calcium eluted from each material[13]. In this study, it was thought that the difference in culture conditions affected the elution of calcium ions from each material, and the calcium amount was confirmed in the solutions with different calcium concentrations in Fig. 5. These results suggest that CO_3Ap had adsorbed calcium in the calcium-rich cultures and eluted them in the absence of ions. The culture conditions in this study were related to the culture medium, and it was thought that calcium was absorbed from the culture solution (Fig. 5B). Therefore, under the culture conditions shown in Fig. 4, calcium ions may have been adsorbed and bone formation may have been suppressed.

Kondo *et al*. showed that systemically administered GFP-expressing rat bone marrow-derived MSCs had accumulated around tooth extraction and implant placement sockets, and promoted their healing[33]. However, the accumulated GFP-positive MSCs had disappeared by approximately 2 weeks, and it was believed that exogenous MSCs did not directly differentiate into the tissue to be repaired, but acted on the surrounding tissue. Therefore, in the animal experiments of this study, we focused on the growth factors released by MSCs as the reason why MSCs that accumulated around CO_3Ap promoted healing of the extraction socket. This was because MSCs release many growth factors depending on the surrounding environment[50], which were EGF, IGF-I, and VEGF as growth factors involved in wound healing in this study. In fact, IGF-1 is strongly involved in healing of the oral mucosa, and our previous study demonstrated that IGF-1 enhances epithelial formation around implants[33]. Additionally, VEGF promotes angiogenesis that is critical for bone formation[51,52], highlighting its importance in the study of bone substitutes.

CONCLUSION

CO_3Ap accelerates epithelial tissue closure when placed in an extraction socket. MSCs tend to accumulate around extraction sockets filled with CO_3Ap, and MSCs cultured in the presence of CO_3Ap have increased expression of growth factors such as VEGF and IGF-1. These results suggest that MSCs around CO_3Ap *in vivo* promote hard and soft tissue healing by releasing growth factors.

ACKNOWLEDGMENTS

We thank Helen Jeays, BDSc AE, and Mitchell Arico from Edanz (https://jp.edanz.com/ac) for editing drafts of this manuscript.

This study was supported by JSPS KAKENHI Grant Number JP 23592888 (to I. A) and JST SPRING, Grant Number JPMJSP2136.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

1) Yamada M, Egusa H. Current bone substitutes for implant dentistry. J Prosthodont Res 2018; 62: 152-161.
2) Zaki J, Yusuf N, El-Khadem A, Scholten R, Jenniskens K. Efficacy of bone-substitute materials use in immediate dental implant placement: A systematic review and meta-analysis. Clin Implant Dent Relat Res 2021; 23: 506-519.
3) Kolk A, Handschel J, Drescher W, Rothamel D, Kloss F, Blessmann M, *et al*. Current trends and future perspectives of bone substitute materials - from space holders to innovative biomaterials. J Craniomaxillofac Surg 2012; 40: 706-718.
4) Jordana F, Le Visage C, Weiss P. Bone substitutes. Med Sci (Paris) 2017; 33: 60-65.
5) Giannoudis PV, Dinopoulos H, Tsiridis E. Bone substitutes: An update. Injury 2005; 36 Suppl 3: S20-27.
6) Finnan RP, Prayson MJ, Goswami T, Miller D. Use of the Reamer-Irrigator-Aspirator for bone graft harvest: A mechanical comparison of three starting points in cadaveric femurs. J Orthop Trauma 2010; 24: 36-41.
7) Friedlaender GE. Bone grafts. The basic science rationale for clinical applications. J Bone Joint Surg Am 1987; 69: 786-790.
8) Zhao R, Yang R, Cooper PR, Khurshid Z, Shavandi A, Ratnayake J. Bone grafts and substitutes in dentistry: A review of current trends and developments. Molecules 2021; 26: 3007.
9) Kamadjaja MJK, Abraham JF, Laksono H. Biocompatibility of portunus pelagicus hydroxyapatite graft on human gingival fibroblast cell culture. Med Arch 2019; 73: 378-381.
10) Szcześ A, Hołysz L, Chibowski E. Synthesis of hydroxyapatite for biomedical applications. Adv Colloid Interface Sci 2017; 249: 321-330.
11) Drobeck HP, Rothstein SS, Gumaer KI, Sherer AD, Slighter RG. Histologic observation of soft tissue responses to implanted, multifaceted particles and discs of hydroxylapatite. J Oral Maxillofac Surg 1984; 42: 143-149.
12) Ishikawa K, Hayashi K. Carbonate apatite artificial bone. Sci Technol Adv Mater 2021; 22: 683-694.
13) Ishikawa K, Miyamoto Y, Tsuchiya A, Hayashi K, Tsuru K, Ohe G. Physical and histological comparison of hydroxyapatite, carbonate apatite, and β-tricalcium phosphate bone substitutes. Materials (Basel) 2018; 11: 1993.
14) Tripathi G, Ishikawa K. Fabrication and in vitro dissolution evaluation of low-crystalline β-TCP blocks through aqueous solution mediated phase conversion. Mater Sci Eng C Mater Biol Appl 2019; 101: 228-231.
15) Daitou F, Maruta M, Kawachi G, Tsuru K, Matsuya S, Terada Y, *et al*. Fabrication of carbonate apatite block based on internal dissolution-precipitation reaction of dicalcium phosphate and calcium carbonate. Dent Mater J 2010; 29: 303-308.
16) Mano T, Akita K, Fukuda N, Kamada K, Kurio N, Ishikawa K, *et al*. Histological comparison of three apatitic bone substitutes with different carbonate contents in alveolar bone defects in a beagle mandible with simultaneous implant installation. J Biomed Mater Res B Appl Biomater 2020; 108: 1450-1459.
17) Kudoh K, Fukuda N, Kasugai S, Tachikawa N, Koyano K, Matsushita Y, *et al*. Maxillary sinus floor augmentation using low-crystalline carbonate apatite granules with simultaneous implant installation: First-in-human clinical trial. J Oral Maxillofac Surg 2019; 77: 985.e1-.e11.
18) Nakagawa T, Kudoh K, Fukuda N, Kasugai S, Tachikawa

N, Koyano K, *et al*. Application of low-crystalline carbonate apatite granules in 2-stage sinus floor augmentation: a prospective clinical trial and histomorphometric evaluation. J Periodontal Implant Sci 2019; 49: 382-396.
19) Zhang X, Atsuta I, Narimatsu I, Ueda N, Takahashi R, Egashira Y, *et al*. Replacement process of carbonate apatite by alveolar bone in a rat extraction socket. Materials (Basel) 2021; 14: 4457.
20) Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luriá EA, *et al*. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974; 2: 83-92.
21) Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, *et al*. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 2004; 364: 149-155.
22) Najar M, Raicevic G, Boufker HI, Fayyad Kazan H, De Bruyn C, Meuleman N, *et al*. Mesenchymal stromal cells use PGE2 to modulate activation and proliferation of lymphocyte subsets: Combined comparison of adipose tissue, Wharton's Jelly and bone marrow sources. Cell Immunol 2010; 264: 171-179.
23) Motegi SI, Ishikawa O. Mesenchymal stem cells: The roles and functions in cutaneous wound healing and tumor growth. J Dermatol Sci 2017; 86: 83-89.
24) Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, *et al*. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.
25) Janeczek Portalska K, Leferink A, Groen N, Fernandes H, Moroni L, van Blitterswijk C, *et al*. Endothelial differentiation of mesenchymal stromal cells. PLoS One 2012; 7: e46842.
26) Chen SL, Fang WW, Ye F, Liu YH, Qian J, Shan SJ, *et al*. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 2004; 94: 92-95.
27) Liu Q, Cheng G, Wang Z, Zhan S, Xiong B, Zhao X. Bone marrow-derived mesenchymal stem cells differentiate into nerve-like cells in vitro after transfection with brain-derived neurotrophic factor gene. In Vitro Cell Dev Biol Anim 2015; 51: 319-327.
28) Fu X, Liu G, Halim A, Ju Y, Luo Q, Song AG. Mesenchymal stem cell migration and tissue repair. Cells 2019; 8: 784.
29) Lin X, Matsuya S, Nakagawa M, Terada Y, Ishikawa K. Effect of molding pressure on fabrication of low-crystalline calcite block. J Mater Sci Mater Med 2008; 19: 479-484.
30) Wakae H, Takeuchi A, Udoh K, Matsuya S, Munar ML, LeGeros RZ, *et al*. Fabrication of macroporous carbonate apatite foam by hydrothermal conversion of alpha-tricalcium phosphate in carbonate solutions. J Biomed Mater Res A 2008; 87: 957-963.
31) Yoshikawa H, Tamai N, Murase T, Myoui A. Interconnected porous hydroxyapatite ceramics for bone tissue engineering. J R Soc Interface 2009; 6 Suppl 3: S341-348.
32) Narimatsu I, Atsuta I, Ayukawa Y, Oshiro W, Yasunami N, Furuhashi A, *et al*. Epithelial and connective tissue sealing around titanium implants with various typical surface finishes. ACS Biomater Sci Eng 2019; 5: 4976-4984.
33) Kondo R, Atsuta I, Ayukawa Y, Yamaza T, Matsuura Y, Furuhashi A, *et al*. Therapeutic interaction of systemically-administered mesenchymal stem cells with peri-implant mucosa. PLoS One 2014; 9: e90681.
34) Matsuura Y, Atsuta I, Ayukawa Y, Yamaza T, Kondo R, Takahashi A, *et al*. Therapeutic interactions between mesenchymal stem cells for healing medication-related osteonecrosis of the jaw. Stem Cell Res Ther 2016; 7: 119.
35) Ueda N, Atsuta I, Ayukawa Y, Yamaza T, Furuhashi A, Narimatsu I, *et al*. Novel application method for mesenchymal stem cell therapy utilizing its attractant-responsive accumulation property. Appl Sci-Basel 2019; 9: 4908.
36) Kubosch EJ, Heidt E, Bernstein A, Böttiger K, Schmal H. The trans-well coculture of human synovial mesenchymal stem cells with chondrocytes leads to self-organization, chondrogenic differentiation, and secretion of TGFβ. Stem Cell Res Ther 2016; 7: 64.
37) Edanami N, Ibn Belal RS, Takenaka S, Yoshiba K, Yoshiba N, Ohkura N, *et al*. Apatite-forming ability of flowable vs. putty formulations of newly developed bioactive glass-containing endodontic cement. Applied Sciences 2021; 11: 8969.
38) Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 2008; 3: e1886.
39) Pajarinen J, Lin T, Gibon E, Kohno Y, Maruyama M, Nathan K, *et al*. Mesenchymal stem cell-macrophage crosstalk and bone healing. Biomaterials 2019; 196: 80-89.
40) Hopper RA, Zhang JR, Fourasier VL, Morova-Protzner I, Protzner KF, Pang CY, *et al*. Effect of isolation of periosteum and dura on the healing of rabbit calvarial inlay bone grafts. Plast Reconstr Surg 2001; 107: 454-462.
41) Matsubara T, Suardita K, Ishii M, Sugiyama M, Igarashi A, Oda R, *et al*. Alveolar bone marrow as a cell source for regenerative medicine: Differences between alveolar and iliac bone marrow stromal cells. J Bone Miner Res 2005; 20: 399-409.
42) Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. Annu Rev Immunol 2011; 29: 139-162.
43) Yin Y, Zhao X, Fang Y, Yu S, Zhao J, Song M, *et al*. SDF-1alpha involved in mobilization and recruitment of endothelial progenitor cells after arterial injury in mice. Cardiovasc Pathol 2010; 19: 218-227.
44) Kitaori T, Ito H, Schwarz EM, Tsutsumi R, Yoshitomi H, Oishi S, *et al*. Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. Arthritis Rheum 2009; 60: 813-823.
45) Kimura Y, Komaki M, Iwasaki K, Sata M, Izumi Y, Morita I. Recruitment of bone marrow-derived cells to periodontal tissue defects. Front Cell Dev Biol 2014; 2: 19.
46) Aoyagi H, Yamashiro K, Hirata-Yoshihara C, Ideguchi H, Yamasaki M, Kawamura M, *et al*. HMGB1-induced inflammatory response promotes bone healing in murine tooth extraction socket. J Cell Biochem 2018; 119: 5481-5490.
47) Wang T, Xu Z, Jiang W, Ma A. Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. Int J Cardiol 2006; 109: 74-81.
48) Loibl M, Binder A, Herrmann M, Duttenhoefer F, Richards RG, Nerlich M, *et al*. Direct cell-cell contact between mesenchymal stem cells and endothelial progenitor cells induces a pericyte-like phenotype in vitro. Biomed Res Int 2014; 2014: 395781.
49) Nagai H, Kobayashi-Fujioka M, Fujisawa K, Ohe G, Takamaru N, Hara K, *et al*. Effects of low crystalline carbonate apatite on proliferation and osteoblastic differentiation of human bone marrow cells. J Mater Sci Mater Med 2015; 26: 99.
50) Inukai T, Katagiri W, Yoshimi R, Osugi M, Kawai T, Hibi H, *et al*. Novel application of stem cell-derived factors for periodontal regeneration. Biochem Biophys Res Commun 2013; 430: 763-768.
51) Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 1999; 5: 623-628.
52) Zelzer E, Mamluk R, Ferrara N, Johnson RS, Schipani E, Olsen BR. VEGFA is necessary for chondrocyte survival during bone development. Development 2004; 131: 2161-2171.