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Review

Creation of highly functional CO_3Ap-collagen scaffold biomaterials

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Physicochemical properties of apatites are affected dramatically by the substitution of trace elements. Especially, biological apatites constituting bone and teeth contain several wt% of CO_3^{2-} ions, which are related to the crystallinity and solubility. Recently, scaffold biomaterials are being developed with a shape-maintaining property in addition to large pores and high porosity, into which cells can easily invade. To develop a new biodegradable scaffold biomaterial, bone-like carbonate apatites (CO_3Ap) were synthesized and CO_3Ap–collagen scaffolds were created. This scaffold biomaterial is useful for regions with bone regeneration ability. When these sponge-frame complexes with rh-BMP2 were implanted beneath the periosteum cranii of rats, sufficient new bone was created at the surface of the periosteum cranii after 4 weeks' implantation. Furthermore, when a CO_3Ap-collagen sponge containing SVVYGLR peptide was implanted as a graft into a tissue defect created in rat tibia, the migration of numerous vascular endothelial cells, as well as prominent angiogenesis inside the graft, could be detected after 1 week. Thus, the modification of higher functions such as cytokine and angiogenesis factors is effective for low regeneration regions using tissue engineering biomaterials.

Keywords: Scaffold biomaterials, CO_3Ap-collagen, High function

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INTRODUCTION

Inorganic materials of human hard tissues such as bone and teeth are composed with hydroxyapatite. However, biological apatites contain many trace elements and have different crystallographic properties. Tooth enamel is well-crystallized, contrary to poorly-crystallized dentine and bone (Fig. 1). It is well known that a number of elements can substitute into the apatite crystal structure[1]. The behaviors of many trace elements have been reported. It is said that most elements on the periodic table can be substituted into apatite crystals. Both cationic and anionic ions can substitute into the Ca^{2+} position and PO_4^{3-} or OH^- position (Fig. 2). Furthermore, some of these substituted ions contribute to metabolism in the human body and cell adhesion.

CO_3^{2-} ION SUBSTITUTION

It is well known that an increase in the carbonate content in enamel is correlated with increased caries susceptibility[2]. In addition, wide variations in the reported values for the carbonate content in enamel may reflect actual changes in the carbonate distribution during and after mineralization[3]. These variations are closely linked to caries susceptibility.

Biological apatites contain a several wt% CO_3^{2-} ions. For many years, it was speculated that bone mineral might be composed of calcium phosphate and calcium carbonate $CaCO_3$. However, LeGeros clarified that the inorganic composition of hard tissues such as bone and teeth are composed of carbonate apatites[4]. In general, CO_3^{2-} ions can substitute into both the PO_4^{3-} position and OH^- position. It is said that the apatite synthesized in an aqueous system contains CO_3^{2-} ions in the PO_4^{3-} position. When ions substitute into positions, the a-axis dimension decreases. The crystallinity of CO_3apatites decreases with increasing CO_3^{2-} content and solubility increases[5], *i.e.* the caries susceptibility and bone metabolism increase with increasing CO_3^{2-} content.

EFFECT OF CO_3^{2-} IONS ON BONE FORMATION

To examine the interrelation of CO_3^{2-} contents in apatite crystals and biocompatibility, hydroxyapatite and CO_3apatites with different carbonate contents were synthesized[6], mixed with atelocollagen, and made into sponge scaffolds[7]. The scaffolds were implanted into the bone sockets of the femurs of male New Zealand white rabbits. Histological observation indicated that the CO_3Ap-collagen scaffolds with higher carbonate contents were gradually deformed throughout the implantation period, and CO_3Ap-collagen scaffolds with similar crystallinity and chemical composition to human bone showed the highest bone area ratio of all of the scaffolds during the experimental period (Fig. 3). After 24 weeks, CO_3Ap-collagen scaffolds with higher carbonate contents showed uniform surrounding bone and could not be distinguished, although hydroxyapatite- and CO_3Ap-collagen scaffolds with lower carbonate contents still partially remained and were slightly opaque on X-ray radiography. This suggested that a CO_3Ap-collagen scaffold with carbonate content similar to that of human bone has optimal bone formation ability.

FUNCTIONALLY GRADED Mg-CONTAINING CO_3Ap-COLLAGEN COMPOSITE

Interface affinity (wettability) is affected by morphology, surface mobility, surface composition, electrical charge *etc.*[8]. Since hydroxyapatite is an ionic

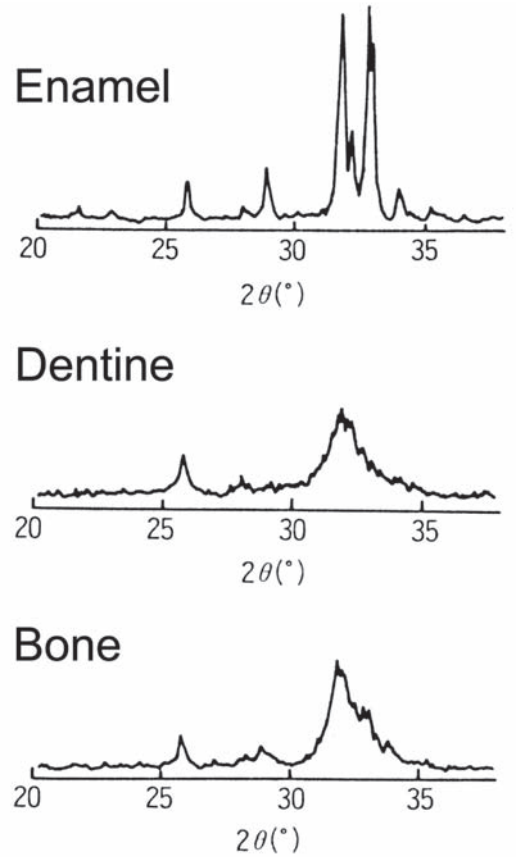

Fig. 1 X-ray diffraction patterns of human enamel, dentine and bone.

crystal, the interface affinity is strongly related to the surface composition and electrical charge. Adhesion molecules such as those of the integrin family were examined in terms of cell structure and function. Divalent ions affect cell adhesion in relation to the integrin molecule, an adhesion molecule at the cell surface. Especially, it has been reported that Zn^{2+} and Mg^{2+} ions promote cell adhesion[9]. Integrins are crucially important receptor proteins because they are the main way that cells both bind to and respond to the extracellular matrix. They are composed of two noncovalently associated transmembrane glycoprotein subunits called α and β, both of which contribute to the

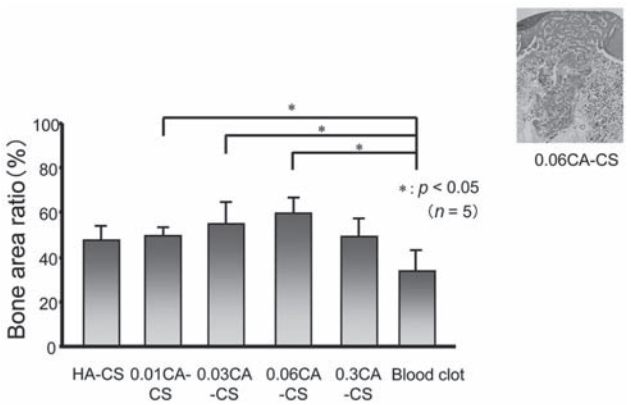

Fig. 3 Bone formation ability of CO_3Ap-collagen sponge scaffolds with different carbonate contents implanted into a rabbit femur after 3 weeks.

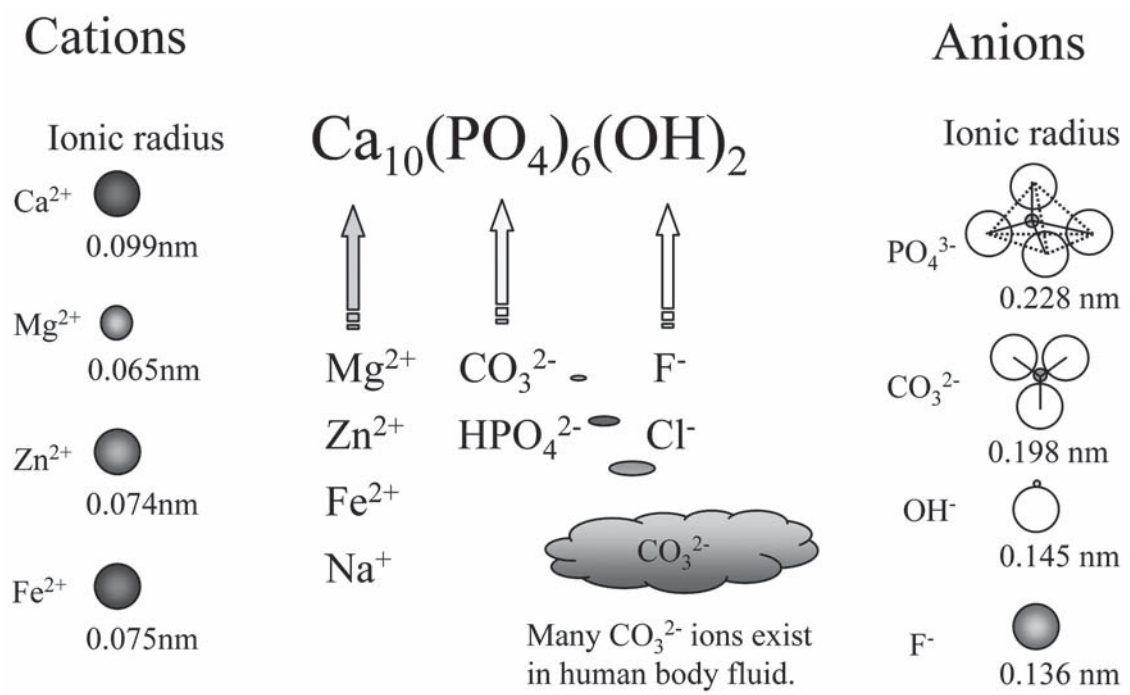

Fig. 2 Substitution of trace elements with different ionic radii into hydroxyapatite crystal.

binding of the matrix protein (Fig. 4). The binding of integrins to their ligands depends on extracellular divalent cation, reflecting the presence of three or four divalent-cation-binding domains in the large extracellular part of the α chain[10].

Mg^{2+} ions also play a role in cell adhesion. Thus, magnesium seems to be an important factor in controlling *in vivo* bone metabolism since it plays a part in both bone formation and resorption[11]. Mg^{2+} ions may contribute to the bone metabolism of osteoclast and osteoblast action with the integrins at their cell surfaces.

Recently, scaffold biomaterials have been studied in the tissue engineering field. In a continuation of those studies, functionally graded CO_3apatite containing Mg, producing a negative gradient of magnesium concentration from the surface toward the core, was synthesized[12]. The degree of cell adhesion to a composite that was made by mixing the $FGMgCO_3$apatite and collagen to facilitate bonding and processing was investigated. Furthermore, the biocompatibility and effect of magnesium on bone formation by implanting the same $FGMgCO_3$Ap-collagen composite into the rabbit femur was examined[13].

ESCA analysis clearly showed a negative gradient distribution of Mg1s intensity (atomic concentration) of magnesium from the crystal surface toward the inner core (Fig. 5). The WST cell growth assay of mouse MC3T3-E1 osteoblast cells incubated on the surface of the composites showed similar optical densities between the control CO_3Ap-collagen composite and $FGMgCO_3$Ap-collagen. This suggests that cell growth was good and that there was no significant inhibition in relation to the components of each sample. In a cell adhesion assay, after the nonadhering cells were rinsed off, the optical density of $FGMgCO_3$Ap-collagen composite was higher than that of the CO_3Ap-collagen composite. Furthermore, a cell adhesion assay with a radioisotope showed that the adhesion fraction of the $FGMgCO_3$Ap-collagen composite was higher than that of the CO_3Ap-collagen composite and much higher than that of the Ti plate as a control (Fig. 6). After 4 weeks of incubation, many more osteoblasts adhered to the

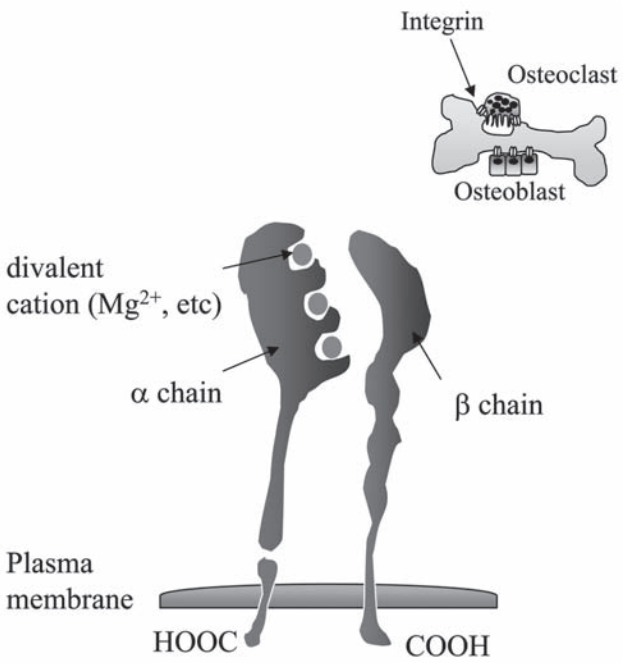

Fig. 4 Integrin as an adhesion molecule related to divalent cation such as magnesium ions.

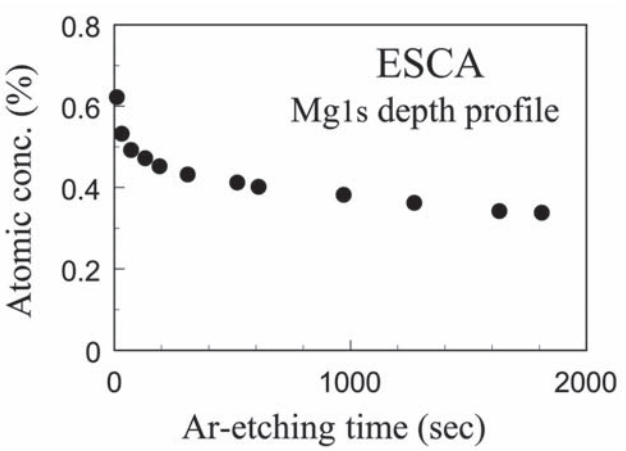

Fig. 5 Magnesium concentration depth profile in functionally graded Mg-containing CO_3Ap ($FGMgCO_3$Ap).

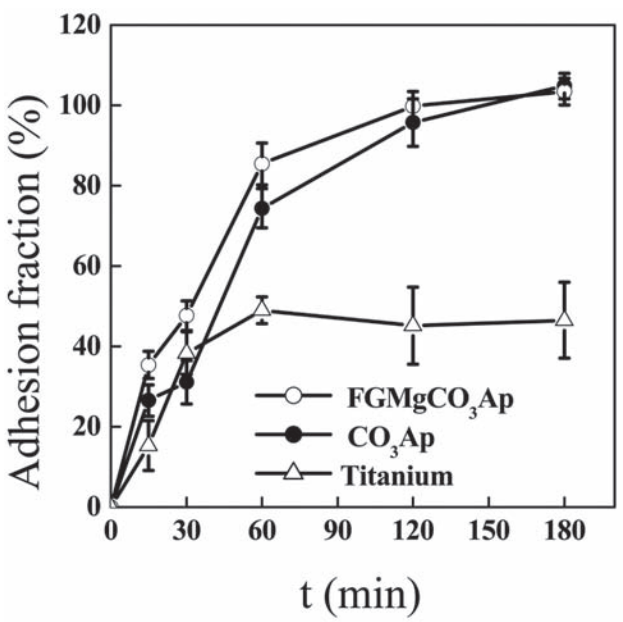

Fig. 6 Initial osteoblast adhesion on an $FGMgCO_3$Ap-collagen composite.

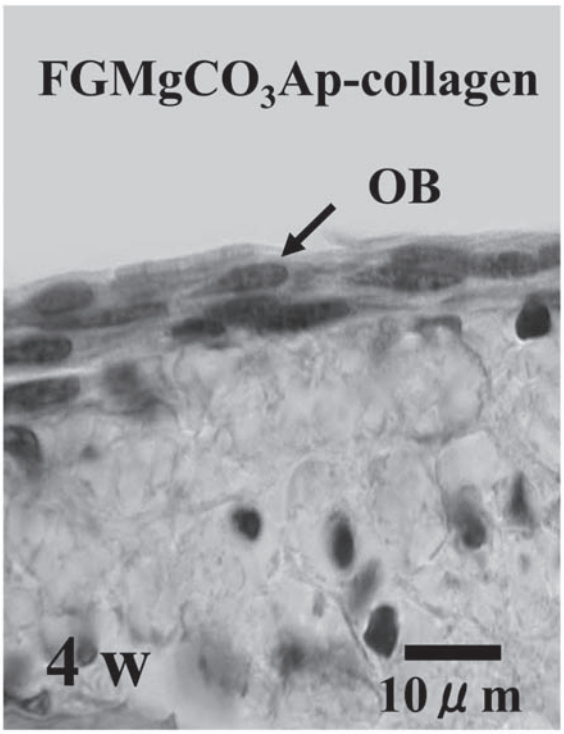

Fig. 7 Hematoxylin-eosin staining of an $FGMgCO_3Ap$-collagen composite 4 weeks after the osteoblast adhesion experiment.

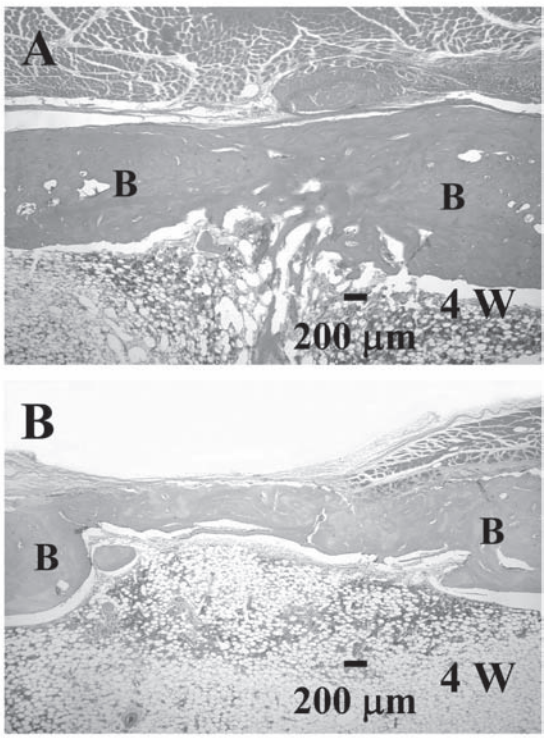

Fig. 8 Hematoxylin-eosin staining of bone formation of an $FGMgCO_3Ap$-collagen composite implanted into a rabbit femur (A) and control (B) after 4 weeks. B: bone.

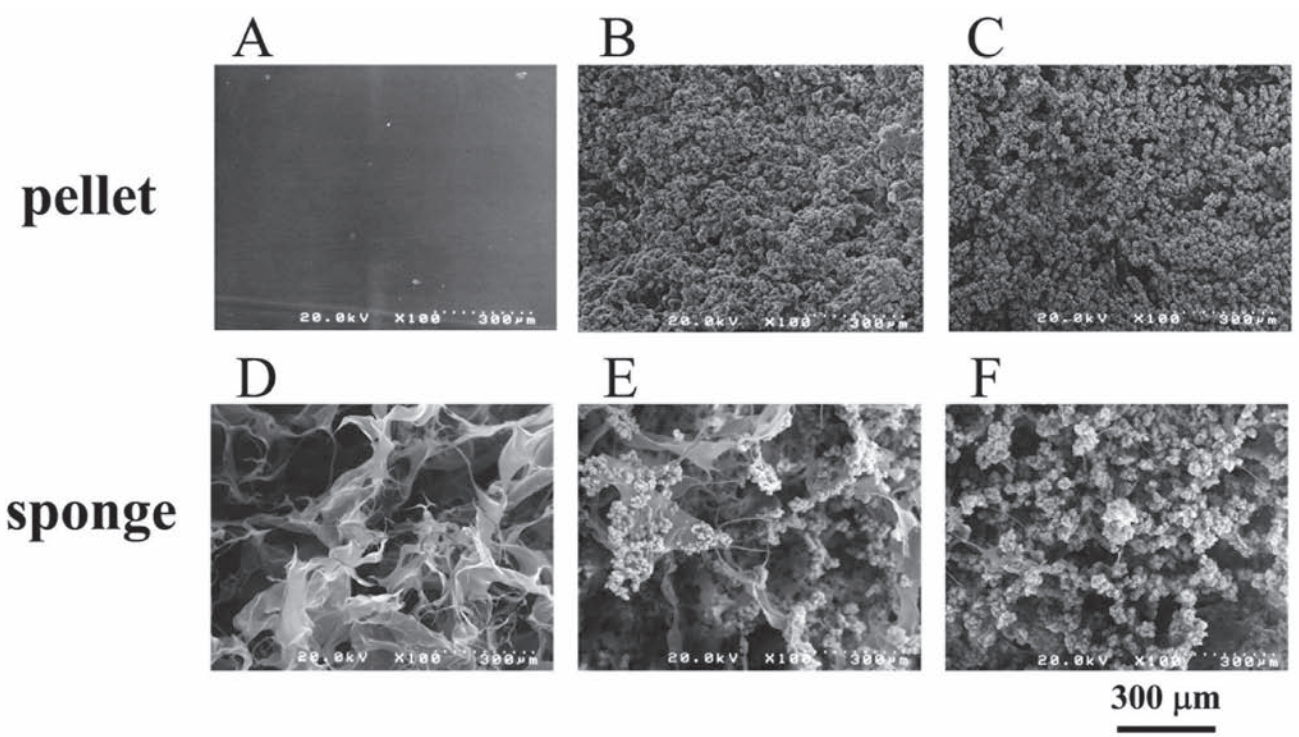

Fig. 9 SEM photos of CO_3Ap-collagen pellets (A, B, C) and sponges (D, E, F) with different CO_3Ap contents. Pure collagens (A, D), 70 wt% (B, E) and 90 wt% (C, F) CO_3Ap-collagen pellets and sponges, respectively.

FGMgCO_3Ap-collagen composite than to the CO_3Ap-collagen composite and the layer they formed was thicker (Fig. 7).

After 1 week of implantation into rabbit femurs, both the FGMgCO_3Ap-collagen composite and the CO_3Ap-collagen composite showed no clear difference from the control in histological results. However, hematoxylin-eosin stains of magnified new bone revealed the existence of many osteoblasts (OB) 2 weeks after implantation. Four weeks after implantation, both the FGMgCO_3Ap-collagen composite (Fig. 8) and the CO_3Ap-collagen composite showed clear bone formation, although the control hole with no implantation also appeared to have been repaired with a thinner layer of new bone. The bone density of the FGMgCO_3Ap-collagen composite was higher than that of the CO_3Ap-collagen composite.

Also, modification of gradational Mg^{2+} ions on the apatite crystals was successfully achieved. The apatite crystals showed no special function on the surface, but did promote cell adhesion. Furthermore, as a scaffold material, the FGMgCO_3Ap-collagen composite was suggested to contribute to bone formation. Since this composite is easy to process into any form, it is extremely useful in the reconstruction of bone defects. Thus, trace elements in apatite crystals are closely related to our biological metabolism and physiology. Bone apatites contribute to storage of trace elements in addition to the skeletal structure.

SPONGE-FRAME COMPLEX

A recent focus has been how cells can invade scaffold materials and a 3D cell culture can be established. Ohgushi *et al.*[14] reported that a porous hydroxyapatite with a several hundred μm pore size is open to osteoblast invasion. We also successfully created a 70 wt% CO_3Ap-collagen sponge with 50 – 300 μm pore sizes[15] (Fig. 9). This sponge has a chemical composition and crystallinity similar to bone. X-ray high-resolution microtomography revealed a clear image of the 3D structure of the sponges. The porosity of 70 wt% CO_3Ap-collagen sponges was 72.6±2.4% and appeared to be the most favorable biomaterial from the viewpoint of natural bone properties. Osteoblasts could invade through the sponge bottom; however, the sponge material appeared to shrink during culture or animal experiments. First, we considered ensuring enough space in which new bone could easily form, utilizing the concept of guided bone regeneration (GBR)[16], and we newly created a sponge reinforced with a porous HAp-frame[17]. Our concept is that sponge as cancellous bone and the frame as trabecular bone are hybridized similar to natural bone. Bone formation was carried out on frame-reinforced CO_3Ap-collagen sponge scaffolds, after which they were implanted beneath the periostum cranii of rats. Unfortunately, bone formation was not sufficient.

CO_3Ap-COLLAGEN SCAFFOLD WITH CYTOKINES

Cytokines such as BMP2[18] and BMP7 have been successfully used to create and promote new bone growth using fixing biomaterials[19]. We investigated the acceleration of bone formation with rh-BMP2 using frame-reinforced CO_3Ap-collagen sponge scaffolds. To develop a new biodegradable scaffold biomaterial reinforced with a frame, synthesized CO_3Ap was mixed with neutralized collagen gel, and the CO_3Ap-collagen

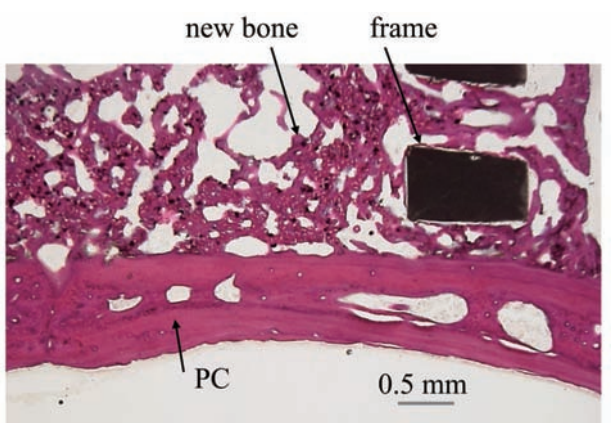

Fig. 10 Hematoxylin-eosin staining of reinforced CO_3Ap-collagen sponge scaffold with rh-BMP2 implanted beneath the periosteum cranii of a rat after 4 weeks. (rh-BMP2: Peprotech Co.Ltd).

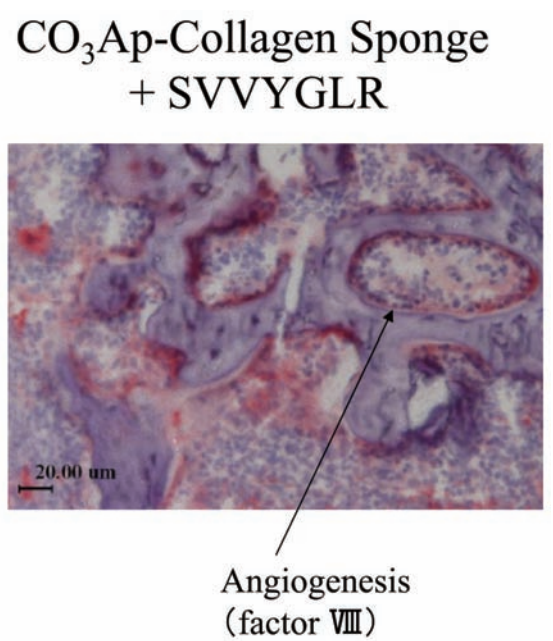

Fig. 11 Immunostaining of factor VIII in CO_3Ap-collagen sponge with an SVVYGLR peptide implanted into the bone marrow of the rat tibia after 1 week. S: Serine, V: Valine, Y: Tyrosine, G: Glycine, L: Leucine, R: Arginine.

mixtures were lyophilized into sponges in a porous HAp-frame ring. X-ray diffraction and FT-IR analyses together with chemical analysis indicated that synthesized CO_3Ap had crystallinity and a chemical composition similar to bone. SEM observation showed that the CO_3Ap-collagen sponge had a suitable pore size for cell invasion. In proliferation and differentiation experiments with osteoblasts, ALP and OPN activity were clearly detected. When these sponge-frame complexes with rh-BMP2 were implanted beneath the periosteum cranii of rats, sufficient new bone was created at the surface of the periosteum cranii 4 weeks after implantation (Fig. 10)[20]. These reinforced CO_3Ap-collagen sponges with rh-BMP2 are expected to be used as hard tissue scaffold biomaterials for therapeutic purposes to aid rapid cure.

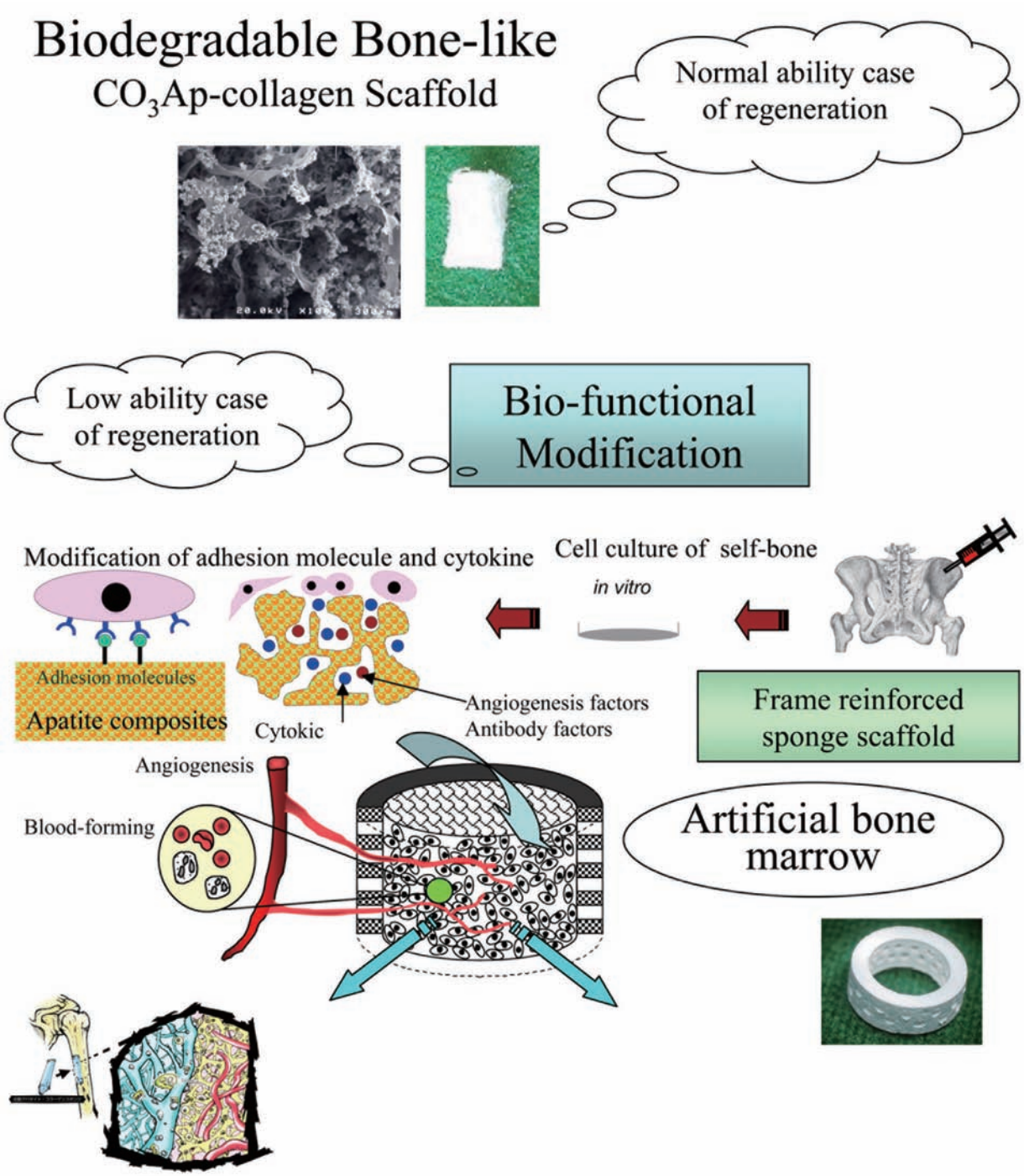

Fig. 12 Concept of biodegradable bone-like CO_3Ap-collagen scaffolds with highly functional ability.

CO_3Ap- COLLAGEN SCAFFOLD WITH ANGIOGENESIS FACTOR

To apply tissue engineering to Japan's aging society, recently the development of hard-tissue biomaterials from a different viewpoint using the regeneration of myeloid tissues has been investigated. Regeneration of the vessels that supply oxygen and nutrients to cells is essential to allow defective tissues to regenerate and biomaterials to engraft and express sufficient function. Blood vessels form a crucial lifeline for the maintenance and growth of bone, in addition to providing hybrid functions to hard-tissue scaffold materials.

The process of angiogenesis starts from digestion of the basement membranes of blood vessels by endothelial cells[21]. Cells subsequently migrate, proliferate and form tube-like structures. Numerous researchers have reported that these cellular responses are carefully regulated by signals from various growth factors and cytokines, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and interleukin 8[22-26].

Osteopontin, one of the extracellular matrix proteins, is a phosphoric acid protein containing a large quantity of sialic acid. This protein is widely distributed in areas such as bone tissue, kidney, brain and skin. Osteopontin participates in bone metabolism and mediates inflammatory responses and angiogenesis[27].

Recently, the novel binding sequence Ser-Val-Val-Tyr-Gly-Leu-Arg (SVVYGLR) has been identified as an amino acid sequence in OPN involved in angiogenesis[28,29]. This motif might be important in pathological conditions, as SVVYGLR is adjacent to the RGD sequence in osteopontin and is exposed by thrombin cleavage. Synthesizing a sequence of SVVYGLR as a neovascularization growth factor has been done artificially[30]. Based on the finding that OPN is a protein that is widely distributed in osseous tissue, the involvement of SVVYGLR in osseous tissue and its effects on bone marrow mesenchymal stem cells have been investigated. Cellular proliferation tests were conducted using bone marrow mesenchymal stem cells and an animal study using grafts made of a CO_3Ap-collagen sponge as a scaffold biomaterial containing the SVVYGLR motif, to assess the effects of SVVYGLR on bone formation.

To modify the angiogenesis property of CO_3Ap-collagen sponges, osteopontin-derived peptide SVVYGLR was synthesized with high purity. When CO_3Ap-collagen sponges with the synthetic motif SVVYGLR peptide were implanted beneath the back skin of the rat, new blood tubes were dramatically induced in 1 week (Fig. 11)[31], while no blood tubes were observed with the control sponge without SVVYGLR. SMA staining also indicated smooth muscular actin of blood tubes was stained red for the sponge with SVVYGLR. These results suggest that CO_3Ap-collagen sponges combined with SVVYGLR form a useful high-quality scaffold biomaterial contributing to the angiogenesis necessary for bone formation. They will be useful as scaffolds for bone construction and regeneration during artificial tooth implantation and setting dentures on weak alveolar bone.

CONCLUSION

CO_3Ap-collagen sponges are useful by themselves for therapeutic applications in normal cases of regeneration without any modifications. Furthermore, they can be utilized as a rapid cure biomaterial, by emphasizing the adhesion motif and cytokines such as growth factor BMP or angiogenesis factor SVVYGLR in poor regeneration cases (Fig. 12). Further investigation into artificial bone marrow with a blood cell creation function is in progress.

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