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Formation of osteon-like structures in unidirectional porous hydroxyapatite substitute

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Abstract: Unidirectional porous hydroxyapatite (UDPHAp) bone substitute comprises a microstructure of cross-sectionally oval pores with diameters ranging from 30 to 300 μm. Bone remodeling within the UDPHAp is expected upon implantation into bone; however, the mechanism and factors influencing this bone growth remain unclear. The objectives of the present study were to assess the vasculature and microstructure of newly formed bone and to determine how bone formation is affected by load transfer and UDPHAp pore size. Formation of osteon-like structures, defined by the presence of lacunae, canaliculi and a central lumen containing capillaries, was observed within the implanted UDPHAp material in all animals after six weeks. The number of osteocytes and osteon-like structures in areas adjacent to the cortex of recipient bone was significantly higher than in areas next to the medullary cavity throughout the recovery period. Notably, osteon-like structures tended to form in smaller diameter pores. Continuous bone remodeling might be promoted by the rapid formation of unidirectional capillaries and the osteocyte lacunae-canalicular system. Load transfer and smaller pore size could positively affect cortical bone regeneration. © 2018 The Authors Journal of Biomedical Materials Research Part B: Applied Biomaterials Published by Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater. 106B:2665–2672, 2018.

Key Words: bone remodeling, osteon-like structure, unidirectional porous hydroxyapatite bone substitute

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INTRODUCTION

Cortical bone is formed of concentric ring-like structures, called osteons, which are arranged parallel to the long axis in long bones, forming the microstructure of bone. Osteons contain a lumen, called the Haversian canal, which surrounds the blood vessels and nerve cells within bone. The Haversian canal facilitates communication of blood vessels with mature bone cells (osteocytes) through canaliculi. Osteocytes reside in lacunae within the bone matrix and are linked dendritically by gap junctions,[1] forming a cellular network known as the osteocyte lacunar-canalicular system. This system contributes to dynamic load sensing[2] and the transport of nutrients and oxygen[1] and is, therefore, critical for bone remodeling and homeostasis.[3] Therefore, the osteocyte lacunar-canalicular network needs to be re-established after successful bone healing.

We have developed a bone substitute material that is composed of unidirectional porous hydroxyapatite (UDPHAp). UDPHAp has a microstructure consisting of unidirectional, cross-sectional oval pores with diameters of 30–300 μm.[4] The osteoconduction and resorption of this material have been previously reported earlier.[5,6] In a previous study, we implanted UDPHAp blocks into a rectangular cortical bone defect in rabbit tibia and reported statistically greater new bone formation after 12 weeks in cortical areas than in the medullary cavity.[7] This difference of bone growth between the cortical area and medullary cavity was also observed throughout the 2-year period of recovery.[8] Based on these findings, we speculated that the unique structure of UDPHAp promotes cortical bone formation under the mechanical stress of weight bearing. However, the mechanism by which bone remodeling occurs within UDPHAp remains unclear. As the porosity and pore size of bone substitutes are critical factors for bone formation, it is likely that vasculature and microstructure play important roles in this process.[9]

The objectives of the present study were to assess the vasculature and microstructure of newly formed bone within UDPHAp and to determine how these parameters are affected by load transfer and pore size. To this end, we implanted UDPHAp blocks implanted into a cortical bone defect in an animal model and newly formed bone and vasculature were evaluated microscopically and macroscopically using a vascular cast.

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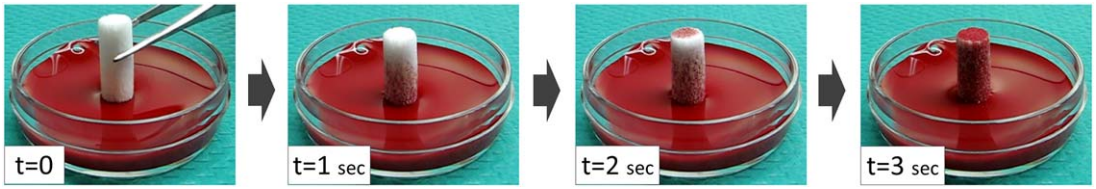

FIGURE 1. Rapid perfusion of heparinized rabbit blood in the material.

MATERIALS AND METHODS

Implant material

UDPHAp blocks were obtained from Kuraray Co., Ltd. (Okayama, Japan). The materials were prepared from a slurry of hydroxyapatite [$Ca_{12}(PO_4)_6(OH)_2$], using a freeze-casting technique, with a compressive strength of 14 MPa for the load parallel to the direction of pores and 2 MPa for the perpendicular load.[4] Pore diameter varied between 30 and 300 μm, with an overall porosity of 75%.[4] The high interconnectivity of the UDPHAp material was confirmed by the rapid and complete perfusion of the material with blood through capillary action, as represented in Figure 1. A UDPHAp block with a diameter of 6 mm and height of 15 mm was used. A final volume of 0.3 mL of blood was perfused into the material for 3 s. The shape and size of the UDPHAp block did not change visibly after perfusion (Figure 1).

Cortical bone defect model

Twenty-eight adult Japanese white rabbits, 28- to 32-weeks-old were used. All animals were housed in individual cages and acclimated in the laboratory for 7 days before the experiments. The study protocol was approved by the Tsukuba University Committee for Animal Experimentation.

General anesthesia was induced by intramuscular injection of a mixture of ketamine (50 mg/kg body weight) and xylazine (14 mg/kg body weight). A 2.5-cm longitudinal skin incision was performed in the right leg. The fascia was dissected to expose the tibia and a 5 × 8 mm area of the periosteum was then resected. A cortical bone defect with a 4 × 7-mm rectangular shape was generated using a high-speed bur. A dry trapezoidal column-shaped UDPHAp block was then implanted into the cavity. The dimensions of the block were as follows: upper bottom, 3 × 7 mm; lower bottom, 5 × 7 mm; height, 5 mm. The direction of the unidirectional pores was parallel to the long axis of the tibia [Figure 2(A)]. The UDPHAp block was fixed in the bone defect without any implant, reaching into the marrow cavity [Figure 2(B)]. The resected periosteum was not placed back. Animals were allowed to weight-bear on their legs immediately after surgery.

The animals were sacrificed at 2 ($n = 4$), 6 ($n = 6$), 9 ($n = 6$), 12 ($n = 6$), and 29 ($n = 6$) weeks after surgery. A calcein fluorochrome label (8 mg/kg body weight; Wako, Osaka, Japan) was injected subcutaneously at 2 and 9 days prior to euthanasia.

Preparation of a vascular cast

A vascular cast was prepared just before euthanasia. General anesthesia was induced by intramuscular injection of a mixture of ketamine (50 mg/kg body weight) and xylazine (14 mg/kg body weight). The femoral artery was cannulated with a 24-gauge venula needle and perfused with heparinized saline. The femoral vascular bundle was clamped proximally and the femoral vein was prepared as an outlet by cutting or cannulation with a venula needle. Perfusion was continued until the effluent from the femoral vein was clear and did not contain blood. A contrast medium (MV-120 [blue] Microfil; Flow Tech, Inc.) was then injected into the femoral artery. Microfil is a liquid silicone rubber formulation that cures in approximately 60 min after the addition of stannous octoate and ethyl silicate, and can be injected into vascularized structures to form a solid cast.[10]

The perfusion pressure during Microfil injection was also monitored and was maintained between 90 and 140 mmHg. After injection of 20–30 mL of Microfil, and confirmation that the effluent consisted of 100% Microfil solution, the femoral vascular bundle was clamped distal to the injection and outlet sites. After allowing the Microfil to cure (approximately 90 min), the right tibia was harvested after euthanasia, which was performed by administering an overdose of pentobarbital, through the auricular vein, during saline perfusion.

Histological analysis

The tibiae were fixed in a neutral buffered 10% formalin solution and were then cut longitudinally at the center of the UDPHAp insert into halves of approximately equal size.

One half of the tibia was used for preparing undecalcified sections, as previously described.[7] Briefly, the bone specimen was embedded in methyl methacrylate, sectioned near the cut surface using a diamond saw, and ground to a 30-μm thickness. The obtained sagittal section was subjected to Villanueva Goldner staining. In addition, an axial section was cut 3 mm from the proximal end of the UDPHAp which was not subjected to staining.

The other half of the tibia was used for preparing decalcified sections. The bone samples were decalcified using K-CX solution (Meiji Seika Pharma, Inc.), with hydrochloric acid as the main component. After decalcification, the bone samples were embedded in paraffin. The central region of the UDPHAp block near the surface was sectioned into two 5-μm slices using a microtome (sagittal section). One section was used for hematoxylin and eosin staining and the other for immunostaining with cathepsin K. An axial section was also prepared at a location 1 mm from the proximal end of the UDPHAp implant and subjected to Bodian's staining. Bodian's staining is a type of silver impregnation method first reported by Bodian in 1936.[11] Kusuzaki et al.[12] reported that this is a suitable

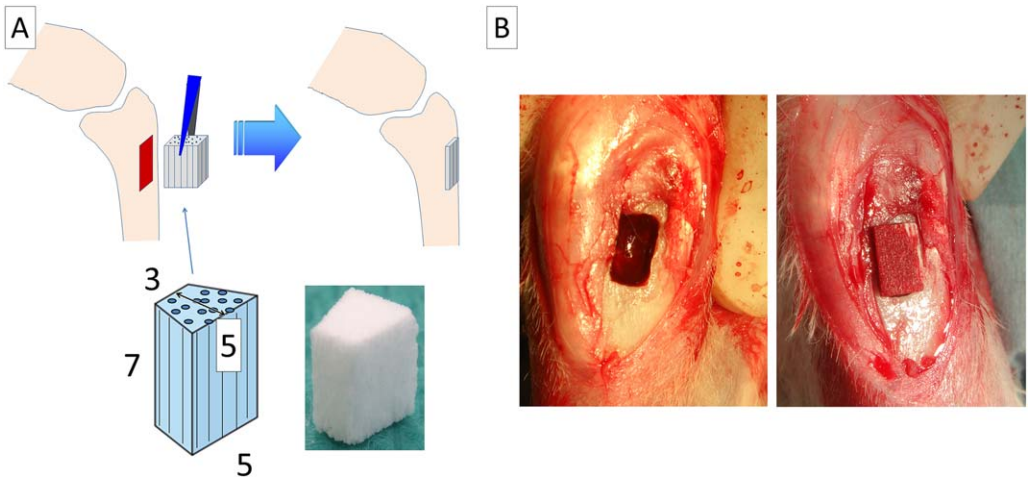

FIGURE 2. Schematic representation of rabbit bone defect model generation and UDPHAp bone material implantation (A). Photographic images of bone defect (B; left) and after filling implant (B; right).

method for histological identification of the details of bone canaliculi structure during the bone repair process.

Histomorphometric analysis

Bone formation was analyzed histomorphometrically at 2, 6, 9, 12, and 29 weeks after surgery. The implanted UDPHAp material was divided into cortical and medullary areas based on the positional relationship to the recipient bed bone. The region of UDPHAp that was continuous with the cortical bone of the recipient bed bone was defined as the cortical area, with the region adjacent to the medullary cavity defined as the medullary area (Figure 3). To examine the relationship between load transfer and newly formed bone, bone formation was compared between the cortical and medullary areas. For analysis, five fields in the unstained axial sections were randomly selected in both the cortical and medullary areas and imaged at a 400× magnification. The number of bone lacunae (osteocytes) in each field was counted. The area of the material (pore area) was measured using image analysis soft Image J, and the number of lacunae per mm^2 pore area was calculated. Newly formed bone with a central lumen and surrounding lacunae was defined as the osteon-like structure. The number of pores fully occupied by the osteon-like structures was counted. The number of pores with osteon-like structures was compared in one field at 100× magnification of each cortical and medullary region from unstained axial sections.

Pore sizes were measured in the axial sections. For the analysis, pores were approximated to be elliptical in shape, and the major axes were measured using Image J software to determine pore size. The relationship between bone formation and pore size was also analyzed. For the analysis, the number of osteon-like structures in one field within the cortical area of the implanted UDPHAp material from unstained axial sections at 100× magnification was counted. The relationship between pore size and number of osteon-like structures was then analyzed and compared over the 29-week recovery period.

Statistical analysis

Analysis of variance was used to compare the cortical and medullary areas. A p values of <0.05 was considered to be statistically significant. Moreover, receiver operating characteristic (ROC) analysis was used. The ROC curve is the plot

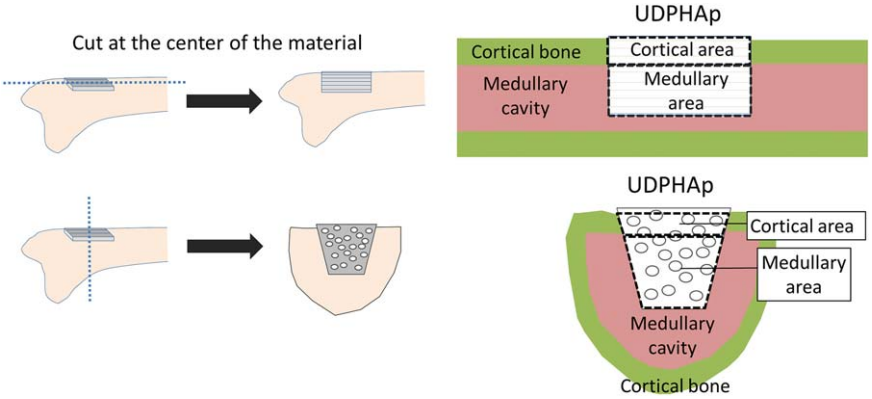

FIGURE 3. Schematic representation of the sagittal and axial sections.

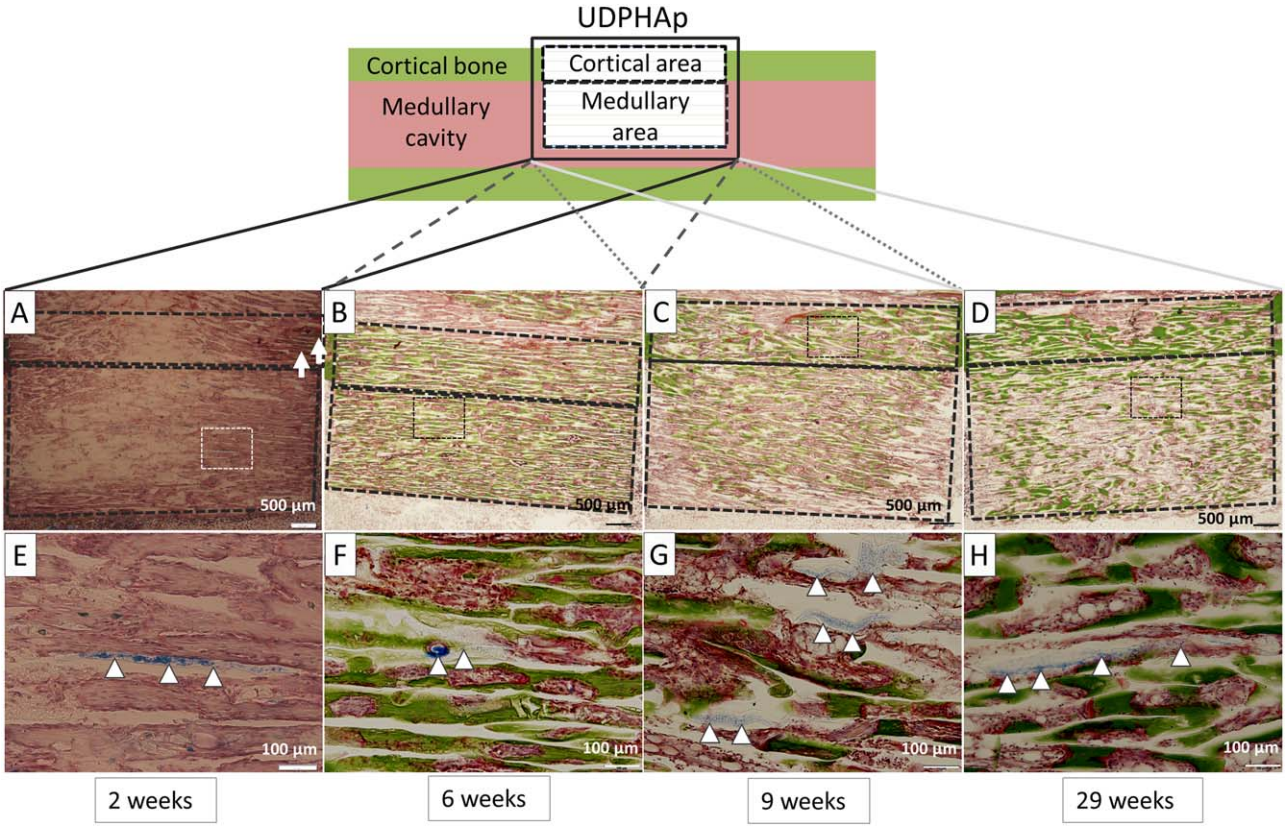

FIGURE 4. Schematic representation of the sagittal sections and undecalcified sagittal sections subjected to Villanueva Goldner stain. Sagittal sections at low (12.5×) and high (100×) magnification are displayed in the upper and lower images, respectively. Newly formed bone was observed as a green area at the edge of the material at 2 weeks (white arrow in A). Thereafter, new bone formation (green area) was evident in all the areas of UDPHAp bone substitute at 6 weeks or later (B–D). Newly formed capillaries with the UDPHAp material were observed as blue area of Microfil (white arrowheads in E–H).

of sensitivity versus 1-specificity and is used for evaluating diagnostic ability and determining the optimal cut-off values. We used this method to analyze the pore size as a prognostic parameter for the formation of osteon-like structures in UDPHAp.

RESULTS

Rabbit tibial bone defect model

The UDPHAp blocks were stably inserted into the tibial bone defect of all animals. No breakage of the material was visibly observed at the time of implantation and specimen collection. All animals maintained good physical condition after implantation. Weight loss or infections at the implantation site were not observed at the time of specimen collection.

Histological findings

In the undecalcified sagittal sections subjected to Villanueva Goldner stain, bone formation in the pore of the material was observed. Bone formation at the edge in the cortical area of the implanted UDPHAp block was detected at 2 weeks [Figure 4(A)], and bone formation was observed throughout the material at 6, 9, 12, and 29 weeks [Figure 4(B–D)]. The bone formation was visibly concentrated more in the cortical area than in the medullary area at 9 and 29 weeks [Figure 4(C,D)]. At high magnification (100×), Microfil was observed as blue particles in lumen structures at 2, 6, 9, and 29 weeks [Figure 4(E–H)]. The lumen structures containing Microfil were observed adjacent to pores and reached the center of the UDPHAp implants by 2 weeks.

In the undecalcified axial sections with no staining, random bone tissue was observed along pore walls at 2 weeks. The bone tissue formed regular lamellar structure, which was first detected at 6 weeks. In several pores within the UDPHAp implants, the newly formed bone was arranged concentrically around the lumen structures containing capillaries [Figure 5(A)]. Lacunae were also observed in the newly formed bone and were connected through canaliculi with other lacunae and the central lumen. These structures resembled osteons that are found in cortical bone [Figure 5(B)] and were therefore termed “osteon-like” structures. In several pores, the newly formed bone had a random non-osteon-like structure with a capillary [Figure 5(C)].

On dark-field images, an extensive fluorochrome label targeting bone formation was observed along the pore walls at 2 weeks. At 29 weeks, the label was clearly observed around the lumen structures that had formed within the UDPHAp implants [Figure 5(D)]. The label was more clearly visible in the cortical [Figure 5(E)] than in the medullary area [Figure 5(F)].

In decalcified sagittal sections stained with hematoxylin and eosin, coagulated blood was observed inside the implanted

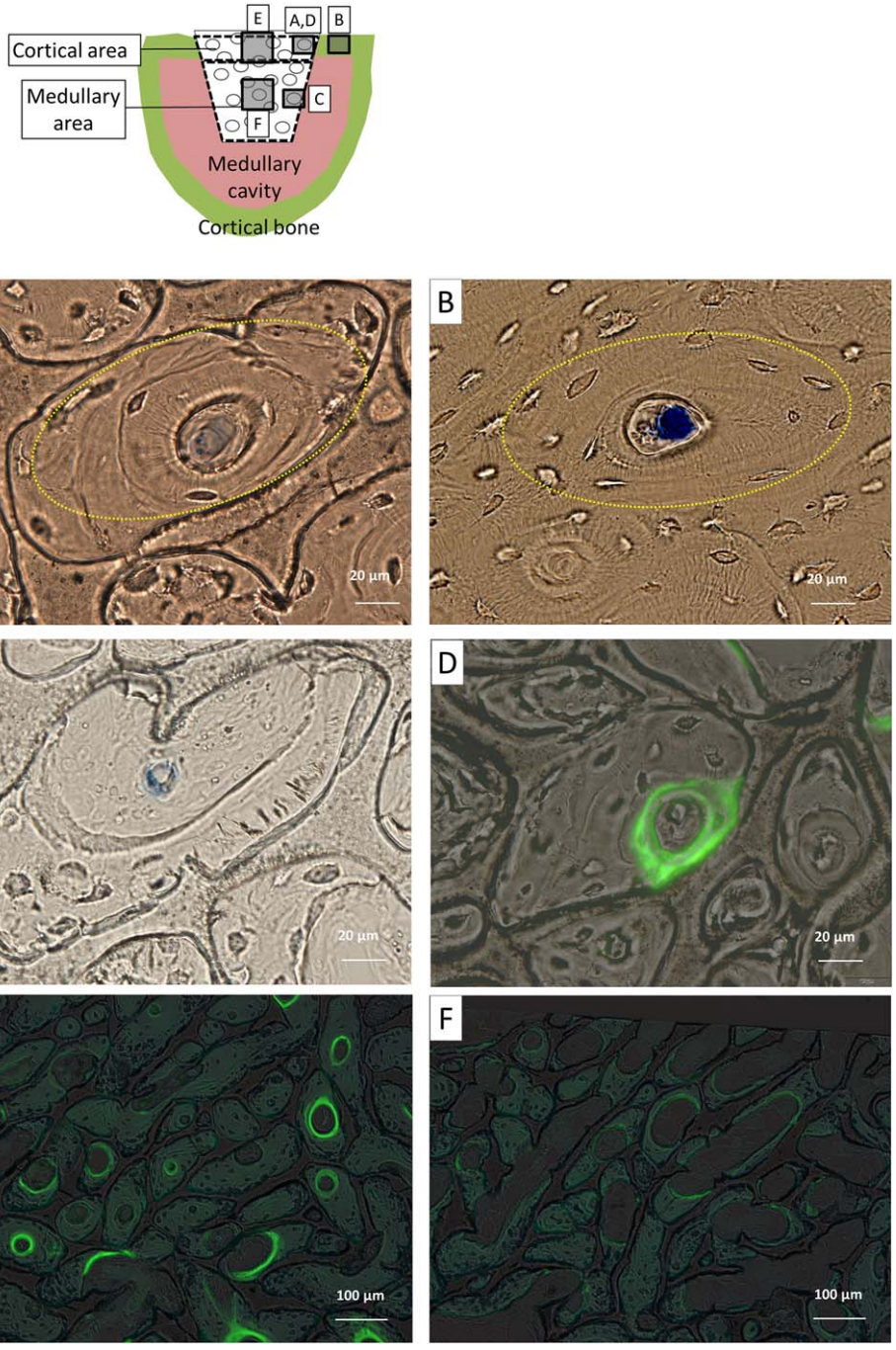

FIGURE 5. Unstained axial section of UDPHAp bone substitute and cortical bone and combined dark- and light-field images of UDPHAp at 29 weeks with 400× (A–D) and 100× magnification (E,F). Osteon-like structures were detected (A, yellow dot circle). Osteons were also present in recipient cortical bone (B, yellow dot circle). Microfil particles (blue) were detected in the central lumen of both the osteon-like and the osteon structures. A non-osteon-like structure was also observed in another pore (C). Double calcein labeling was observed around the central lumen in the osteon-like structures at 29 weeks (D). Labeling was more prominent in the cortical area (E) than in the medullary area (F).

UDPHAp material at 2 weeks. At 9 weeks, newly formed bone, fibrous tissue and lipid droplets were found within the material [Figure 6(A)]. No osteochondral lesions were evident during the 29-week recovery period. Many large multinuclear cells were cathepsin K–positive and were present within the newly formed bone [Figure 6(B)].

In the Bodian-stained decalcified axial section, lacunae and canaliculi of osteon-like structures inside the implanted UDPHAp material appeared black. Cell bodies were observed within the lacunae. Osteons in the recipient cortical bone exhibited similar characteristics to the osteon-like structures [Figure 6(C,D)].

Histomorphometric analysis

The mean number of lacunae per pore area in the cortical and medullary areas of UDPHAp was $813 \pm 244/mm^2$ and

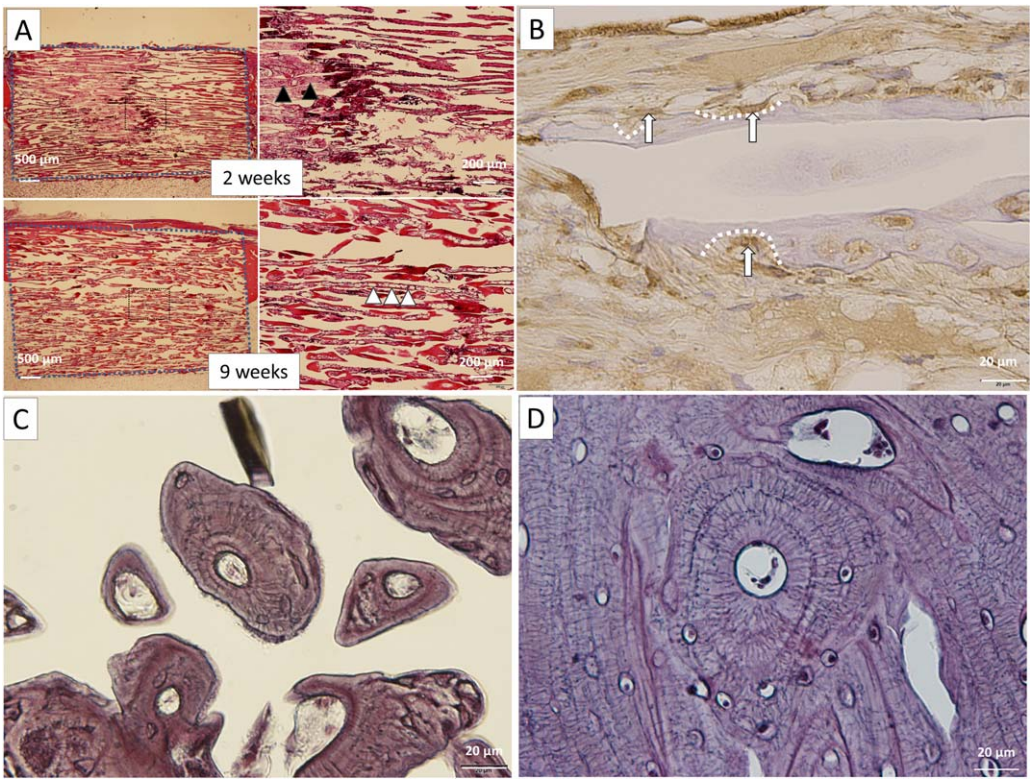

FIGURE 6. Decalcified specimen with hematoxylin and eosin staining (A), immunostaining for cathepsin K (B), and Bodian's staining (C,D). Fibrin was observed in the UDPHAp bone material at 2 weeks (A, black arrowhead). Lipid droplets (A, white arrowhead) were first detected at 9 weeks. Large multinuclear cells were positive for immunostaining for cathepsin K within the newly formed bone (B,white arrow). Howship's lacunae formed near the large multinuclear cells (B, white dot line). Osteon-like structures within the UDPHAp material (C) and osteons in the recipient bone (D) contained a central lumen, lacunae and canaliculi, which were similarly stained black.

$397 \pm 103/mm^2$, respectively, at 6 weeks, $794 \pm 192/mm^2$ and $301 \pm 138/mm^2$ at 9 weeks, $792 \pm 178/mm^2$ and $304 \pm 140/mm^2$ at 12 weeks, and $916 \pm 105/mm^2$ and $341 \pm 170/mm^2$ at 29 weeks. Significantly more lacunae were observed in the cortical region than in the medullary area at all time points of examination (Figure 7).

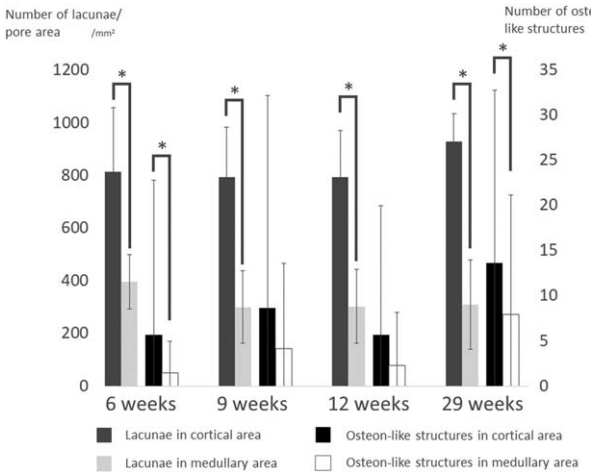

FIGURE 7. Number of lacunae per pore area and number of osteon-like structures in the cortical and medullary areas of UDPHAp bone implants between 6 and 29 weeks after implantation. The differences in the number of lacunae between the cortical and medullary areas were significant at all examined time periods. The number of osteon-like structures was different at 6 and 29 weeks. $*p < 0.05$.

The mean number of osteon-like structures in the cortical and medullary areas was 6 ± 17 and 2 ± 4, respectively, at 6 weeks, 9 ± 24 and 4 ± 9 at 9 weeks, 6 ± 14 and 2 ± 6 at 12 weeks, and 14 ± 19 and 8 ± 13 at 29 weeks. The difference in the number of osteon-like structures between the cortical and medullary areas was significant at 6 and 29 weeks (Figure 7).

Osteon-like structures were observed in 202 of 654 pores within the UDPHAp specimens that were examined throughout the 29-week recovery period. A histogram of osteon-like structure formation relative to pore size is shown in Figure 8. Osteon-like structures tended to form in smaller pores (<119 μm). ROC analysis showed that the optimal cutoff value for pore size was 105.1 μm. The area under the curve in the ROC analysis was 0.61. On examination 150 of the 397 pores (37.8%) with a diameter of <105.1 μm contained osteon-like structures, whereas 52 of the 257 pores (20.2%) with a diameter of >105.1 μm contained osteon-like structures.

DISCUSSION

In the present study, we evaluated the microstructure and vasculature of newly formed bone within UDPHAp bone substitutes implanted into a cortical bone defect animal

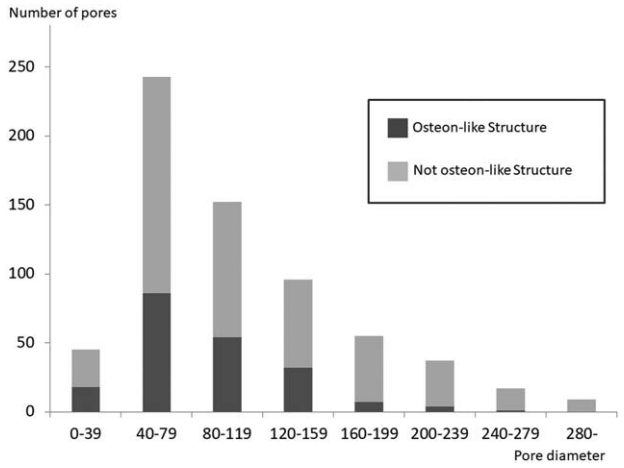

FIGURE 8. Histogram representing pore size distribution for pores containing osteon-like structures. Osteon-like structures tended to form in smaller pores.

model. During the 29-week recovery period, osteon-like structures consisting of osteocytes, canaliculi and capillary-containing central lumen were formed within the UDPHAp implants. Notably, the formation of osteocytes and osteon-like structures was more prevalent in the cortical areas of the implanted UDPHAp blocks than in the medullary areas. These findings suggest that load transfer significantly affected the formation of osteocytes and osteon-like structures. Moreover, the osteon-like structures tended to form in smaller pores within the UDPHAp implants, indicating that pore size is a contributing factor to osteon-like structure formation and bone tissue regeneration.

Artificial bone substitutes used in bone repair must efficiently promote capillary formation. Nakasa et al.[13] reported capillary formation in a sponge-like structure of interconnected porous hydroxyapatite. Histological data also showed several newly formed capillaries in various directions in the implanted material. In the present study, the formation of unidirectional capillaries was rapidly observed within the implanted UDPHAp material. We speculate that the newly formed capillaries rapidly reached the interior areas of the implanted material guided by the unidirectional pore structure within UDPHAp.

Within the implanted UDPHAp material, newly formed bone showed osteon-like structures. The formation of osteon-like structures would be expected to contribute to the mechanical strength of the repaired defect site and promote bone union between the recipient and newly formed bone. The finding is consistent with a previous study by Ryu et al.,[14] who reported that osteon-like structures were present in one-dimensional porous hydroxyapatite (HA), but were not detected in sponge-type HA, which displayed irregular woven bone formation. The strength of the present study is the confirmation, through the examination of unstained undecalcified section, that capillaries formed inside the central lumen of osteon-like structures within the UDPHAp. Moreover, the lacunae and canaliculi were stained black in Bodian-stained decalcified sections, indicating that these structures were similar those found in physiological bone. Taken together, our findings indicate that the unidirectional formation of bone tissue and capillaries promotes the formation of osteon structures.

Iwasashi et al.[7,8] analyzed bone formation within UDPHAp blocks implanted into the same animal bone defect model used in the present study and found that bone formation was greater in the cortical regions of the UDPHAp compared to medullary areas. Consistent with this previous finding, more lacunae and osteon-like structures were found in the cortical areas in the present study. Nguyen and Jacobs[2] reported that the osteocyte lacunar-canalicular system perceives mechanical loading by deflections in cilia and plays an important role in bone remodeling depending on the surrounding environment. The present results suggest that the differences in bone formation within the UDPHAp implants in the cortical and medullary areas were influenced by the number of osteocytes, whose formation is responsive to environmental stimuli through the lacunar-canalicular system. In addition, the positive calcein fluorochrome labeling and cathepsin K staining of the newly formed bone inside the UDPHAp blocks indicate that the bone remodeling was responsive to the surrounding environment.

The osteon-like structures did not form in all pores, suggesting that bone regeneration is influenced by factors, such as load transfer and pore size. The ideal pore size of artificial bone substitutes is a topic of debate. Earlier, Hulbert et al.[15] reported that a minimum pore size of 100 μm was required for osteogenesis. However, Karageorgiou and Kaplan[9] recommended that implant material should contain pore sizes >300 μm. Small pore size may induce osteochondral formation before osteogenesis as a result of hypoxic conditions.[16] In contrast, Chang et al.[17] reported active bone formation in 50-μm pores arranged in parallel. In this study, more osteon-like structures appeared to be present in pores with a diameter of <105 μm. Notably, capillary formation inside the UDPHAp material was also observed, whereas osteochondral formation was not detected. Taken together, our results suggest that early capillary formation through unidirectional pores induces osteogenesis, even in relatively smaller-diameter pores, and might avoid the onset of hypoxia. Moreover, the ideal pore size of artificial implant material with unidirectional pores might be smaller than previously concluded based on the increased formation of osteon-like structures in smaller-diameter pores (40–120 μm). However, further studies are needed to determine the ideal pore size of artificial bone substitutes. In addition, the diameter of osteons in rabbits is approximately 100 μm, which is markedly smaller than human osteons, which have a diameter of 200 μm. Thus, differences in bone structure might affect the relationship between osteon-like structure formation and pore size. The ideal pore size may need to be determined for individual species.

Two limitations of this study warrant mention. First, because we did not perform mechanical testing, the relationship between bone and osteon-like structure formation and mechanical strength is not clear. Second, since we used a single cortical bone defect model, in which the defect was

not of critical size, bone formation in larger UDPHAp implants that would be needed to repair larger bone defects encountered in the clinical setting and the size limit for UDPHAp implants to allow the formation of osteon-like structures are not clear.

Osteon-like structures were formed within the UDPHAp blocks implanted in a cortical large-bone defect rabbit model. Continuous bone remodeling was observed within the UDPHAp material and may be promoted by the rapid formation of unidirectional capillaries and osteocyte lacunae-canalicular system. These findings demonstrate that UDPHAp is a promising material for promoting cortical bone regeneration, although further studies in human subjects are needed.

CONCLUSIONS

Osteon-like structures were formed within the UDPHAp blocks implanted in a cortical large-bone defect rabbit model. Load transfer positively affected the formation of osteocytes and osteon-like structures. Pore size might be one of a key factor influencing bone formation, with more osteon-like structures forming in smaller pores.

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