

Bone Formation on Synthetic Precursors of Hydroxyapatite

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SUZUKI, O., NAKAMURA, M., MIYASAKA, Y., KAGAYAMA, M. and SAKURAI, M. *Bone Formation on Synthetic Precursors of Hydroxyapatite.* Tohoku J. Exp. Med., 1991, 164 (1), 37-50 — The aim of this study was to investigate the reaction of skeletal tissue to various synthetic calcium phosphate (Ca-P) compounds in vivo. Five synthetic Ca-P compounds were implanted into the subperiosteal area of the calvaria of 7-week-old BALB/c mice for one to 15 weeks. Synthetic compounds were dicalcium phosphate (DCP), octacalcium phosphate (OCP), amorphous calcium phosphate (ACP), Ca-deficient hydroxyapatite and hydroxyapatite (HA). Implanted DCP, OCP and ACP were found to be converted to apatitic phase by x-ray microdiffraction analysis using undecalcified specimens. Structure of bone was found out on all of Ca-P compounds eventually at late stage under the light microscope, but the rate of bone formation calculated from a number of experiments varied on respective synthetic Ca-P compound. It was high as 80% for DCP, OCP and ACP, but was low as 5.6% for Ca-deficient HA, and no reaction was found for HA at the stage of 3 weeks. Fine filaments and granular materials in the newly formed bone matrix were detected at 7 days around the remnants of OCP particles which already converted to apatitic phase by ultrastructural study of decalcified specimens. These structures were very similar to the components of bone nodules seen in intramembranous osteogenesis. It is postulated that the precursors of HA have an important role in intramembranous osteogenesis. ——— calcium phosphate (Ca-P) compounds; precursors; hydroxyapatite; bone formation; bone matrix

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Abbreviations: Ca-P, calcium phosphate; DCP, dicalcium phosphate; OCP, octacalcium phosphate; ACP, amorphous calcium phosphate; Ca-deficient HA, calcium deficient hydroxyapatite; HA, hydroxyapatite; SSA, specific surface area; HE, hematoxylin and eosin; JCPDS, Joint Committee on Powder Diffraction Standards; β -TCP, β -tricalcium phosphate; CPP, calcium pyrophosphate; DCPD, dicalcium phosphate dihydrate; PSPC/MDG, microdiffraction goniometer equipped with a position sensitive proportional counter.

It is generally accepted that hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$; HA) in hard tissue such as bone and tooth enamel is calcium deficient carbonate apatite (Brown 1966; Simpson 1972; Young 1974; Nelson and Featherstone 1982). In supersaturated calcifying solutions, calcium deficient HA ($\text{Ca}_{10-x}\text{H}_x(\text{PO}_4)_6(\text{OH})_{2-x}$, $0 \leq x \leq 2$; Ca-deficient HA) (Winand 1965) has been shown to precipitate via the formation of one or more precursors such as amorphous calcium phosphate ($\text{Ca}_3(\text{PO}_4)_2 \cdot n\text{H}_2\text{O}$; ACP) (Eanes and Meyer 1977; Meyer and Eanes 1978a, b; Tung and Brown 1983). For that reason, it is believed that precursor phases such as ACP, dicalcium phosphate dihydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$; DCPD) and octacalcium phosphate ($\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$; OCP) may participate in biological mineralization (Nancollas et al. 1989). In fact, ACP has been detected within matrix vesicles isolated from epiphyseal calcified cartilage (Wuthier and Gore 1977; Ali et al. 1978), and also OCP has been detected as one of the components of dental calculus (Schroeder and Bambauer 1966).

Within the bone forming tissue, both HA and/or the precursors of HA might interact with various tissue components such as inorganic ions and organic macromolecules. HA has been shown to interact with collagens (Glimcher et al. 1957), and noncollagenous proteins such as osteocalcin (Hauschka et al. 1975), bone phosphoproteins (Glimcher et al. 1979), sialoproteins (Fisher et al. 1987) and osteonectin (Termine et al. 1981). OCP, one of the precursor of HA, has been reported to have high affinity to F^- (Brown et al. 1962), succinate (Monma and Goto 1983) and DNA (Spencer and Grynblas 1978). Termine and Conn (1976) have presented that phosphorylated metabolites and macromolecules are incorporated into ACP, and inhibit amorphous-crystalline conversion in vitro. Although the precursors of HA were suggested to be associated with the nucleating focus in organic matrix components (Eanes et al. 1973), functional role of the precursor phases of HA in vivo still remains to be not clear.

This study is the preliminary report dealing with the process of bone formation on synthetic precursors and on synthetic HA implanted into the tissue close to bone. The result suggested that the synthetic precursors converted to apatitic phase in vivo and the bone formation on the synthetic precursors appeared obviously earlier than that on HA or Ca-deficient HA.

MATERIALS AND METHODS

Synthesis of Ca-P compounds

Reagent grade chemicals were used without further purification. DCP, Ca-deficient HA and HA were prepared using a reactor which was made from pyrex glass and was composed of three entrances to flow the reactant and an exit to flow the synthesized deposit (Japan Fine Ceramics Co. Ltd., Sendai). For the preparation of DCP, 1,360 ml of 1 M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ solution, 1,360 ml of 1 M $(\text{NH}_4)_2\text{HPO}_4$ solution and 680 ml of H_2O were introduced into the reactor at 45°C , taking a time of 20 min. The precipitate was introduced to a beaker and allowed to mature with stirring at 600 r.p.m. for 30 min at the reaction temperature using a teflon-coated agitator composed of aluminum rod and blade.

The matured precipitate was filtered and washed with distilled water, then dried at 120°C. For the other Ca-P compounds except for ACP, the procedure after precipitation was carried out in the same manner as that of DCP. The modified method of Eanes (1970) for synthesis of Ca-deficient HA was used as follows: 1,500 ml of 1.5 M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ solution, 1,500 ml of 1 M $(\text{NH}_4)_2\text{HPO}_4$ solution and 750 ml of 1.1 M NH_4OH solution were introduced in 30 min into the reactor at 45°C. The precipitate was allowed to mature at 45°C for 30 min before filtration. The method of Moreno et al. (1968) was modified for synthesis of HA as follows: 2,500 ml of 1.7 M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ solution, 2,500 ml of a mixture of 1 M $(\text{NH}_4)_2\text{HPO}_4$ and 0.2 M NH_4OH solution and 1,250 ml of 0.9 M NH_4OH solution were introduced, taking a time of 30 min into the reactor at 45°C. The precipitate was allowed to mature at 45°C for 30 min and at 80°C for one hr before filtration. OCP was prepared using a method by LeGeros (1985). 250 ml of 0.04 M $\text{Ca}(\text{CH}_3\text{CO}_2)_2 \cdot \text{H}_2\text{O}$ solution was slowly added as taking one hr to 250 ml of 0.04 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ solution which was stirring at 400 r.p.m. at 67.5°C. The method of Eanes et al. (1965) was modified for synthesis of ACP as follows: 4,800 ml of a mixture of 1.5 M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 0.1 M NH_4OH solution was quickly mixed with a mixture of 4,800 ml of 1 M $(\text{NH}_4)_2\text{HPO}_4$ and 6.3 M NH_4OH solution which was stirring at 300 r.p.m. at 25°C. The precipitate was immediately filtered and washed with acetone, then dried at 120°C.

Characterization of precipitates

Powder x-ray diffraction patterns of precipitates were obtained by step scanning with Cu K α x-rays on a Rigaku Electrical Co. Ltd. (Tokyo), RAD-2B diffractometer at 40 KV, 30 mA. The Ca/P molar ratios of precipitates were determined by a method using a tool of Inductively Coupled Plasma (UB-I Mark IIS Kyoto Koken Co. Ltd., Kyoto). Specific surface areas (SSA) of precipitates were measured by BET method (Brunauer et al. 1938) using nitrogen as the adsorbate. To clarify the microstructural characteristics, the precipitates were mounted on aluminum blocks, coated with carbon, and observed by a JEOL T-300 scanning electron microscope. Furthermore, in order to know the degree of the thermal degradation, precipitates were sintered at 1,100°C for one hr in air and analyzed by powder x-ray diffraction at 40 KV, 30 mA.

Implantation procedure

In the process of drying after precipitation procedures, the precipitates except for DCP were found to be aggregated. The grade of aggregation of OCP was lower than that of ACP, Ca-deficient HA, and HA. The dried precipitates were softly ground into the granules of adequate size by the use of the pestle and the mortar. The ground granules except for DCP were sieved, and the granules between 16 and 32 mesh were used for implantation. The sieved granules and powdery DCP were sterilized by heating at 120°C for over night and implanted into the subperiosteal area of the calvaria of 7-week-old BALB/c mice under anesthesia by diethyl ether. Concretely, after the capilli of parietal region was removed using a depilatory agent, skin and periosteum of facies parietal is were aseptically incised through sagittal direction in about 4 mm length. Furthermore, the periosteum of parietal region was flayed with a raspatorium. Three spoonfuls of granule of Ca-P compounds and powdery DCP were placed adjacent to exposed region of parietal bone of each mouse with stainless steel microspatula and then the cranial skins of mice were sutured leaving the periosteum as had been flayed. Finally, the sutured regions of the cranial skins were sterilized by Nobecutane® spray (Yoshitomi Co. Ltd., Tokyo). Three spoonfuls of granule of each compound was identical to about 30 pieces of granule except for DCP. And the weight of three spoonfuls of Ca-P compounds were approximately 7.1 mg for DCP, 2.9 mg for OCP, 4.6 mg for ACP, 8.0 mg for Ca-deficient HA and 6.3 mg for HA. Four mice with each of Ca-P compounds were used for each experimental period.

Preparation of histological sections

Mice were sacrificed at every 2 weeks, from one to 15 weeks, after implantation, and the both the calvaria and implanted Ca-P compounds were dissected in one piece and fixed with neutral buffered 10% formalin. After dehydration with graded series of ethanol specimens were embedded in methylmethacrylate at 36°C. The 100 μm sections were cut at intervals of 0.5 mm with a band saw along the frontal direction to measure x-ray diffraction. Further, 100 μm sections were ground to 20 μm thick with sandpaper to stain with hematoxylin and eosin (HE). Several cross sections having various dimensions of a compound were visible on a section.

X-ray diffraction of implanted synthetic Ca-P compounds

Using 100 μm sections, x-ray microdiffraction patterns of implanted Ca-P compounds were measured with Cu K α X-rays on a Rigaku Electrical Co. Ltd., microdiffraction goniometer equipped with a position sensitive proportional counter (PSPC/MDG) using 30 $\mu\text{m}\phi$ collimator at 50 KV, 100 mA.

Light microscopic investigation

The ground sections were stained with HE and examined by light microscope to determine the rate of bone formation on Ca-P compounds. The rate of bone formation was expressed by percentage for total number of specimens in which new bone formed adjacent on Ca-P compounds to the number of all specimens examined. The number of numerator was expressed as one even if new bones were separately observed on several compounds on a specimen. New bone was defined as the bone matrix including osteocyte.

Transmission electron microscopy

At one week after OCP implantation, the calvaria containing an implant with cranial skins were dissected and fixed with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3-7.4. After decalcification with 5% EDTA in 0.1 M cacodylate buffer, specimens were post-fixed with 1% OsO₄, dehydrated with graded series of ethanol, passed through propylene oxide and embedded in Epon 812. Ultra-thin sections mounted on copper grids were stained with uranyl acetate and lead citrate and examined with a Hitachi H-700 transmission electron microscope at 100 KV.

RESULTS

Characterization of precipitates

The standard patterns agreed in line intensity and position with the Joint Committee on Powder Diffraction Standards (JCPDS) for DCP, OCP and HA. ACP was non-crystalline which showed no discrete diffraction pattern. X-ray diffraction pattern of Ca-deficient HA showed that of apatitic structure. The Ca/P molar ratio (± 0.02 SD) and SSA of precipitates were summarized in Table 1. Ca-P compounds obtained had approximately stoichiometric Ca/P molar ratio except for Ca-deficient HA. The higher Ca/P molar ratios of the compounds were, the greater their SSAs were. Scanning electron microscopic observation of the dried precipitates showed the aggregated morphology composed of plate-like crystals for DCP, small acicular and frequently mixed with aggregates of short platy crystals for OCP, and spherule-like crystals for ACP, Ca-deficient HA and HA. Furthermore, ACP and Ca-deficient HA were found to crystallize into

TABLE 1. *Specific surface area, theoretical and analytical Ca/P molar ratio of synthetic calcium phosphate compounds*

Calcium phosphates	Specific surface area (m ² /g)	Theoretical Ca/P	Analytical Ca/P
DCP ^a	0.7	1.00	1.01 ± 0.02 ^b
OCP	25	1.33	1.32 ± 0.02
ACP	36	1.50	1.47 ± 0.02
Ca-deficient HA	52	1.33-1.67	1.49 ± 0.02
HA	92	1.67	1.68 ± 0.02

^aAbbreviations: DCP, dicalcium phosphate; OCP, octacalcium phosphate; ACP, amorphous calcium phosphate; Ca-deficient HA, Calcium-deficient hydroxyapatite; HA, hydroxyapatite.

^bMean ± s.d.

β -tricalcium phosphate (β -TCP) by sintering at 1,100°C. This is consistent with the result by Eanes (1970) who showed that ACP and Ca-deficient HA having nearly 1.5 for Ca/P molar ratio crystallized to β -TCP by heat treatment. By the same heat treatment, HA increased crystallinity without thermal degradation and DCP crystallized to calcium pyrophosphate (CPP), and OCP was led to be decomposed into two crystalline phases. One was β -TCP and the other was CPP.

Phase conversion in vivo

X-ray microdiffraction patterns of implanted Ca-P compounds revealed conversion of the precursor to apatitic phase (Table 2). OCP and ACP were completely converted to apatitic phase at one week after implantation. Phase conversion of DCP to apatitic phase was slower than OCP and ACP. DCP

TABLE 2. *Crystalline phases of calcium phosphate compounds which were implanted in subperiosteal area of the calvaria of BALB/c mice*

Period (weeks)	X-ray diffraction results				
	DCP ^a	OCP	ACP	Ca-deficient HA	HA
Unimplanted	DCP	OCP	Non-crystalline	Apatite	Apatite
1	DCP	Apatite	Apatite	Apatite	Apatite
3	Apatite + DCP	Apatite	Apatite	Apatite	Apatite
5	Apatite + DCP	Apatite	Apatite	Apatite	Apatite
7	Apatite + trace DCP	Apatite	Apatite	Apatite	Apatite
9	Apatite + trace DCP	Apatite	Apatite	Apatite	Apatite
11	Apatite + trace DCP	Apatite	Apatite	Apatite	Apatite
13	Apatite + trace DCP	Apatite	Apatite	Apatite	Apatite
15	Apatite + trace DCP	Apatite	Apatite	Apatite	Apatite

^aAbbreviations are the same as in Table 1.

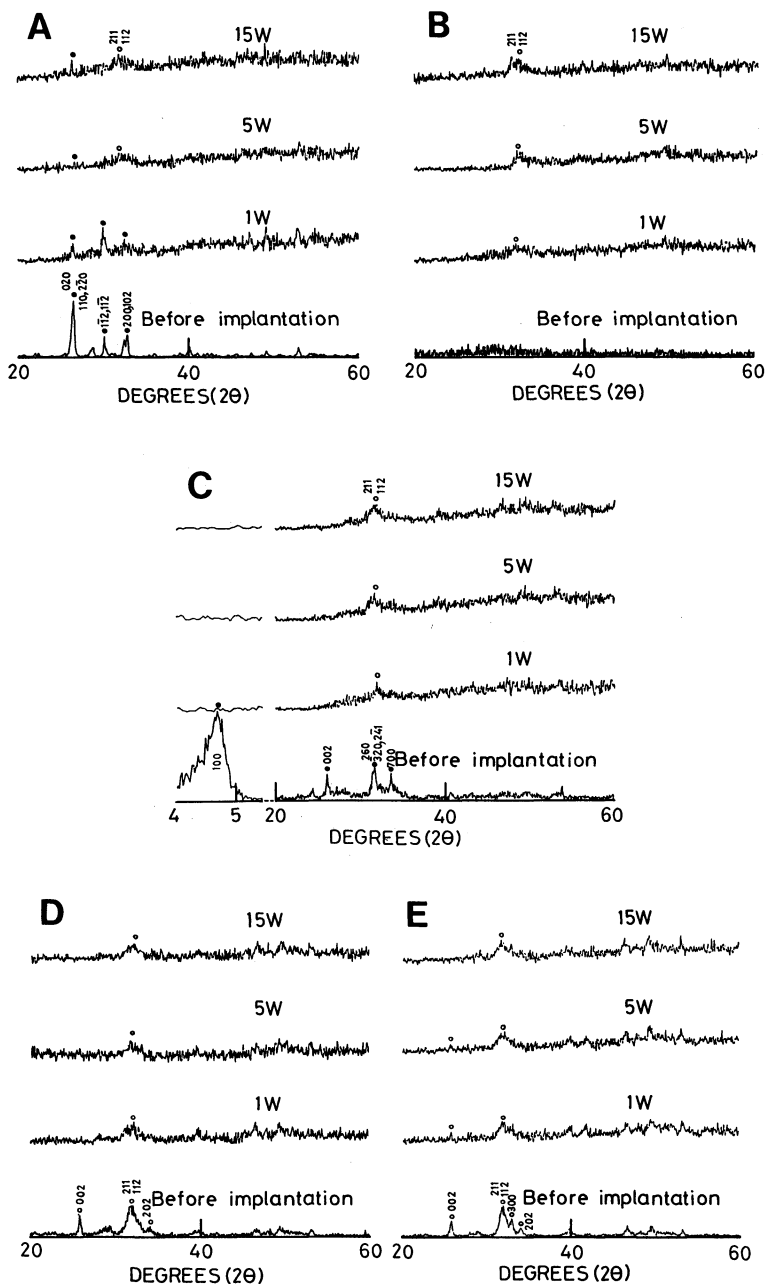


Fig. 1. X-ray diffraction patterns of DCP (A), ACP (B), OCP (C), Ca-deficient HA (D) and HA (E) which were implanted in subperiosteal area of the calvaria of BALB/c mice. Closed circles and open circles indicate diffraction peaks from lattice planes of crystal in each precursor phase of hydroxyapatite and apatitic phase respectively. W, weeks.

converted to apatitic phase partially remaining DCP crystalline at 3 weeks after implantation. To be concrete, in DCP implantation, except for the peak corresponding to the (020) reflection [$2\theta \doteq 26.4$ degrees] overlapping with (110, $2\bar{2}0$) reflection [$2\theta \doteq 26.6$ degrees], ($1\bar{1}2$, $1\bar{1}\bar{2}$) reflection [$2\theta \doteq 30.2$ degrees] and (200, 102) [$2\theta \doteq 32.9$ degrees] reflection disappeared and the reflection specific to apatitic phase ((211) [$2\theta \doteq 31.8$ degrees] overlapping with (112) reflection [$2\theta \doteq 32.2$ degrees]) appeared by implantation (Fig. 1A). In ACP implantation, the

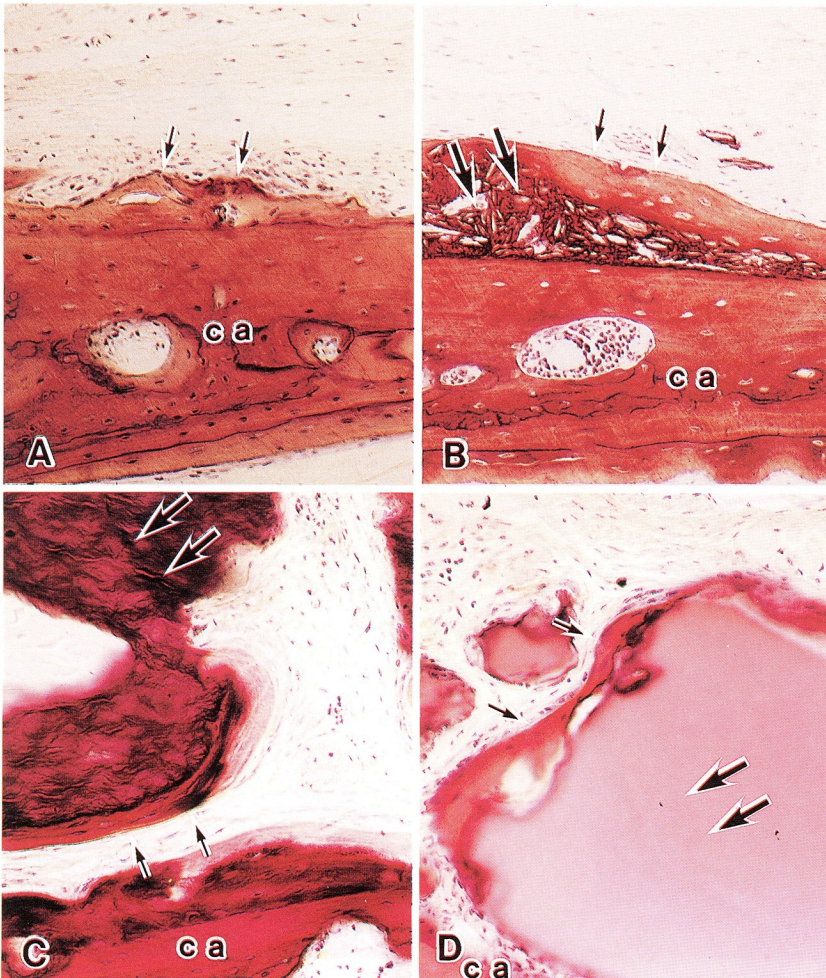


Fig. 2. Photographs of undecalcified histological sections after 5 weeks of implantation. (A) a control. (B) DCP implantation, (C) OCP implantation, (D) ACP implantation. Note the additional bone formation (small arrows) on the calvarium of a control mouse, also the bone formation (small arrows) around DCP, OCP and ACP. The areas labeled "ca" were identical to the calvaria. Large arrows indicate implanted calcium phosphate compounds. Hematoxylin and Eosin stain, $\times 144$.

pattern which showed no discrete diffraction changed to apatitic pattern (Fig. 1B). In OCP implantation, the peak corresponding to the (100) [$2\theta \cong 4.7$ degrees] and the (700) [$2\theta \cong 33.5$ degrees] reflection disappeared and the patterns specific to apatitic phase appeared at one week after implantation (Fig. 1C). In contrast, x-ray pattern of Ca-deficient HA and HA remained unchanged even at 15 weeks after implantation, suggesting their original crystalline structure (Fig. 1D, 1E).

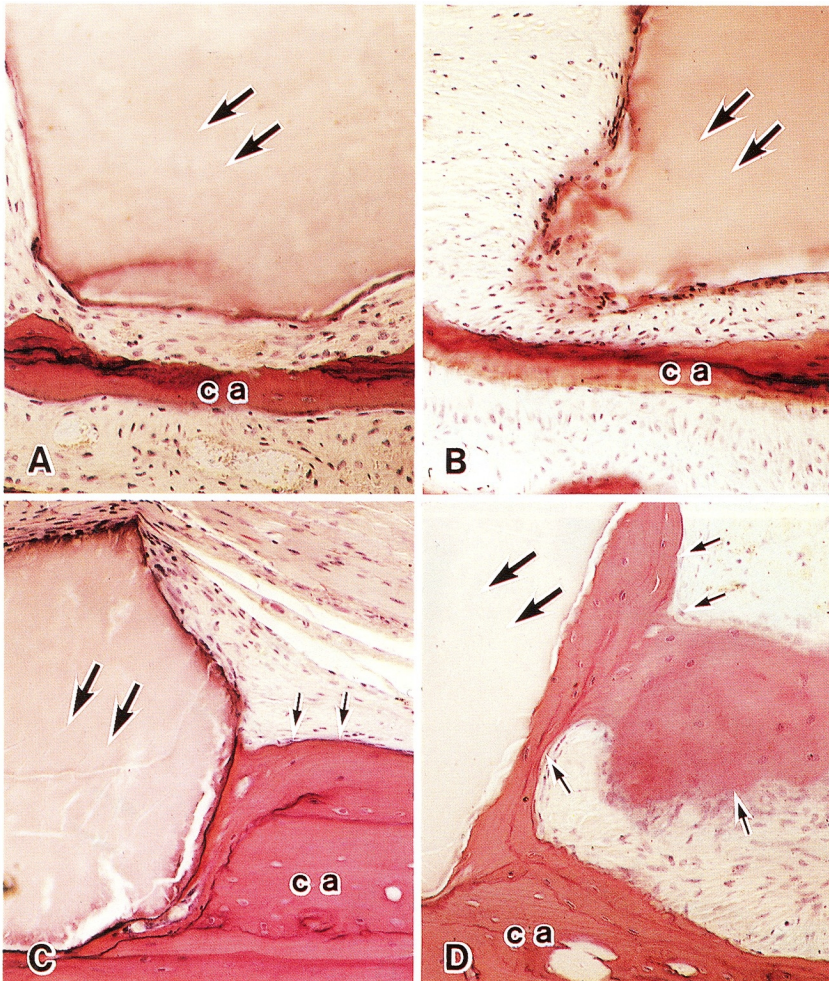


Fig. 3. Photographs of undecalcified histological sections after implantation. (A) Ca-deficient HA implantation at 5 weeks, (B) HA implantation at 5 weeks, (C) Ca-deficient HA implantation at 11 weeks, (D) HA implantation at 15 weeks. Note the bone formation (small arrows) around Ca-deficient HA at 11 weeks and HA at 15 weeks, but no bone formation around Ca-deficient HA at 5 weeks and HA at 5 weeks. The areas labeled "ca" were identical to calvaria. Large arrows indicate implanted calcium phosphate compounds. Hematoxylin and Eosin stain, $\times 144$.

Light microscopic findings

Undecalcified histological section after 5 weeks of sham operation (control experiment) showed that additional bone was formed on the calvaria of mice at the site where the periosteum had been flayed (Fig. 2A). Bone formation was apparently observed around DCP, OCP and ACP at 5 weeks (Fig. 2B-2D). In case of Ca-deficient HA and HA implantation, only little bone formation was observed at 5 weeks but it became obvious on Ca-deficient HA at 11 weeks as well as HA at 15 weeks (Fig. 3A-3D). The rate of bone formation on Ca-P compounds was calculated and summarized in Table 3. It was more than 80% for DCP, OCP and ACP while 5.6% for Ca-deficient HA and 0% for HA at 3 weeks after implantation. Although the rate of bone formation was 0% for HA at 3 weeks, it increased in accordance with prolonged implantation, namely 10.5% at 5 weeks and more than 80% at 15 weeks. Similarly, the rate of bone formation was more than 80% for Ca-deficient HA at 15 weeks. Comparing with crystalline phases of implanted Ca-P compounds in Table 2, this result indicates that bone formation on DCP, OCP and ACP which converted to apatitic phase occurred earlier than that on Ca-deficient HA and HA which are of apatitic structure.

Ultrastructural observations

Osteoblasts aligned on the newly formed bone matrix have well developed rough endoplasmic reticulum and Golgi complexes indicating the active synthesis of the bone matrix. Decalcified sections revealed that the remnants of implanted

TABLE 3. *The rate of bone formation on synthetic calcium phosphate compounds which were implanted in subperiosteal area of the calvaria of BALB/c mice*

Period (weeks)	The rate of bone formation ^a				
	DCP	OCP	ACP	Ca-deficient HA	HA
1	0/10(0.0%)	1/16(6.3%)	0/ 7(0.0%)	0/ 7(0.0%)	0/13(0.0%)
3	8/10(80.0%)	12/13(92.3%)	5/ 6(83.3%)	2/36(5.6%)	0/ 8(0.0%)
5	9/10(87.5%)	10/12(83.3%)	9/10(90.0%)	5/28(17.9%)	2/19(10.5%)
7	7/ 8(85.7%)	6/ 6(100.0%)	9/10(90.0%)	6/17(35.3%)	5/12(41.7%)
9	6/ 6(100.0%)	6/ 6(100.0%)	5/ 6(83.3%)	5/ 6(83.3%)	6/ 6(100.0%)
11	6/ 6(100.0%)	6/ 6(100.0%)	4/ 6(66.7%)	6/ 9(66.7%)	4/ 5(80.0%)
13	6/ 6(100.0%)	6/ 6(100.0%)	8/ 8(100.0%)	4/ 7(54.1%)	6/ 6(100.0%)
15	7/ 7(100.0%)	6/ 6(100.0%)	8/ 9(88.9%)	4/ 5(80.0%)	5/ 6(83.3%)

^aThe rate of bone formation was expressed by percentage for total number of specimens in which new bone formed adjacent on calcium phosphate compounds to the number of all specimens examined. The number of specimen more than one at least was examined from a mouse. In the periods around when new bone was formed adjacent to each Ca-P compound, plenty of specimens was examined than the periods around after bone was formed. Abbreviations are the same as in Table 1.



Fig. 4. Ultrastructure of bone matrix after one week implantation of OCP. Arrows indicate the remnants of implanted OCP particles. Many fine filaments and granular materials are localized around OCP particles (arrow heads), $\times 31,200$.

OCP particles which already converted to apatitic phase were observed as a denser structure than other components in the bone matrix. Although the main components of bone matrix were thick collagen fibers, many fine filaments and small granular materials were also observed between collagen fibers and especially accumulated around the remnants of implanted OCP particles (Fig. 4).

DISCUSSION

X-ray diffraction patterns of synthetic precursors (DCP, OCP, ACP) changed to that of apatitic phase for the period of one to 3 weeks, while that of synthetic Ca-deficient HA and HA did not change even 15 weeks after implantation. Many studies have been reported about the conversion of nonapatitic solid phases of Ca-P to HA in cell free condition. For example, DCP was shown to convert to carbonate-containing apatite in hot carbonate solution (LeGeros et al. 1971). Under physiological condition spontaneously precipitated ACP was shown to convert to an OCP-like crystalline phase which was subsequently hydrolyzed to apatitic phase (Eanes and Meyer 1977 ; Meyer and Eanes 1978a, b). The presence of magnesium (Boskey and Posner 1974) or adenosine triphosphate (Blumenthal et al. 1977) in solution was shown to delay the process of conversion of ACP to

HA. Therefore, although the effect of these elements could not be evaluated, the conversion of the synthetic precursors to apatitic phase in this study may be caused by the change from metastable Ca-P compounds to the most thermodynamically stable HA under physiologic conditions (Brown et al. 1981).

The most interesting finding in the present study is that the bone formation on the synthetic precursors occurred earlier than that on Ca-deficient HA and HA. Biological significance of this result is not clear yet. However, various evidences have suggested that interaction between Ca-P compounds and macromolecules might be involved in bone development. The interaction has been suggested between HA crystals and collagens (Glimcher et al. 1957) or noncollagenous proteins in bone and teeth such as osteocalcin (Hauschka et al. 1975), osteonectin (Termine et al. 1981), bone phosphoproteins including osteopontin (Glimcher et al. 1979) and sialoproteins and proteoglycans (Fisher et al. 1987). Eanes et al. (1973) have suggested that ACP could be the nucleating locus for bone nodules which were described as the developmental units of bone (Bernard and Pease 1969), because of general similarity in appearance between bone nodules and clusters of synthetic HA formed by conversion of ACP. Nelson and Barry (1989) have postulated that the involvement of biological macromolecules with crystal nucleation and growth processes are most probably by steric interactions, adsorption, and crystal growth inhibition or possibly by reducing the energy barrier for nucleation of the OCP seed. Furthermore, Termine and Conn (1976) have shown in vitro an evidence that phosphorylated metabolites and macromolecules were incorporated into ACP and they inhibited amorphous-crystalline transformation. However, these facts were induced from in vitro experiments and there have been no reports on the biological role of precursors of HA in vivo.

The rapid bone formation on implanted synthetic precursors observed in the present study may suggest different interactions between the macromolecules and the synthetic precursors or HA. Ca/P ratio of crystallines formed within matrix vesicles has been shown to be lower than that of crystallines extended from matrix vesicles to extracellular matrix and calcospherulites using freeze substitution methods in the young rats (Ozawa 1986). There was a tendency that extent of specific surface area (SSA) of Ca-P compounds was enlarged in accordance with the increase of Ca/P molar ratio. Lower value of Ca/P molar ratio or SSA seems to be related to the earlier occurrence of bone formation. However, Ca/P molar ratio of the Ca-deficient HA can be variable between 1.33 and 1.67 (Winand 1965) and the Ca/P molar ratio of the present materials was 1.49. So that it is not clear whether lower value of Ca/P molar ratio is directly related to the earlier occurrence of bone formation.

Under physiological conditions, the solubility of DCP, OCP and HA is decreased in order as can be seen in the turn of solubility isotherms for several calcium phosphates studied by Brown et al. (1981). This also gives the sequence of possible transformations to HA which is the most thermodynamically stable

Ca-P phase under physiological condition. ACP precipitates have been shown to form OCP which subsequently hydrolyzed to apatitic phase at pH 7.4 (Eanes and Meyer 1977), although ACP also was shown to directly convert to apatitic phase under restricted condition (Tung and Brown 1983). In the present study, therefore, implanted DCP or ACP should be expected to have converted to apatitic phase via OCP. From these consideration and the evidence presented by the studies in vivo, OCP is supposed to be the direct precursor to apatitic phase, and possible interaction between OCP and macromolecules may involve in the bone formation.

As Bernard and Pease described (1969), the primary bone matrix is filled with many spherical bone nodules with inorganic phase of Ca-P in intramembranous osteogenesis. The organic components within bone nodules are consisted of fine filaments, small granular materials and no collagen fibers. In the process of bone development, these bone nodules disappear and are replaced by collagen-rich matrix. Ultrastructural observations on 7-day-implantation of OCP onto the calvaria indicated that the matrix components around the remnants of implanted OCP particles which were shown to already have been converted to apatitic phase were fine filaments and small granular materials. These components appear to be almost identical to the components of bone nodules which were indicated by Bernard and Pease (1969). These findings lead to the hypothesis that these extracellular matrix components in the primary intramembranous osteogenesis may interact with precursors of HA as suggested by Eanes et al. (1973). It can not be excluded the possibility that the matrix components interacting with implanted precursors may involve growth factors such as bone morphogenetic protein (Urist 1976), transforming growth factor β and so on (Canalis et al. 1988).

We expect that further investigation will elucidate molecular nature of the matrix components accumulated around the implanted precursors, which lead to further understanding of the mechanism of ossification.

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