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SINTERED CARBONATE APATITES AS BONE SUBSTITUTES

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Abstract

Sintering of carbonate apatites was investigated and the usefulness of sintered specimens as bone substitutes was evaluated in vitro and in vivo. Osteoclasts appeared to be capable of resorbing sintered carbonate apatite, which was as soluble as deproteinated bone and much more soluble than sintered hydroxyapatite in weak acids. In skull defects of Wistar rats, sintered carbonate apatite particles resorbed to an appreciable extent, but the rate of resorption did not exceed that of new bone formation. At 4 weeks after implantation the defects were filled almost completely with new bone that compared favorably with the host bone. These findings suggest that sintered carbonate apatites would be useful as bioresorbable bone substitutes.

Key Words: Carbonate Apatite, sintering, bioresorbable, osteoclast, skull defect, bone substitute.

Introduction

Calcium phosphate biomaterials have been used safely and effectively during the past twenty years in a variety of restorative and preservative clinical applications [9, 27, 35]. Among calcium phosphate ceramic biomaterials, hydroxyapatite (HAp) has been shown to be somewhat more biocompatible and osteoconductive than β-tricalcium phosphate (β-TCP), primarily because of its structural similarity with the inorganic phase of bone. In tissues, however, HAp biomaterials do not undergo appreciable resorption when implanted [10, 23, 28, 35], whereas β-TCP biomaterials seem to resorb at a much faster rate [3, 21, 28]. Because of this property, β-TCP is thought to be better for bone repair, since new bone can be expected to form as it gradually resorbs [35]. β-TCP is a material foreign to living tissue and thus is likely to be phagocytosed by macrophages, evoking a local inflammatory response [30]. Thus, it would be desirable to develop more soluble sintered apatites which resorb in bone tissues at rates similar to bone itself during osteoclastic cell-mediated remodeling.

Carbonate-containing apatites [15, 32, 33] resemble bone apatites in their chemical properties more than pure HAp, the material generally used for sintering. Carbonated apatites, however, are non-stoichiometric and many investigators have been reluctant to use them for sintering, since non-stoichiometric apatites likely produce undesirable thermal by-products such as β-TCP, calcium oxide, or sometimes tetracalcium phosphate when heated over 1100°C for sintering [17, 18]. In the present study, it is shown that apatites that contain about 12 weight percent (wt%) carbonate can be made bioresorbable when sintered. The bioresorption was demonstrated in part in osteoclastic cell culture in vitro and in skull defects of Wistar rats in vivo.

Materials and Methods

Preparation of unsintered apatite

Carbonate apatite and HAp were prepared by mixing 2 liters of 1 mol/l calcium nitrate solution and 8...
liters of 0.15 mol/l disodium hydrogen phosphate solution containing, respectively, 6 and 0 mol disodium carbonate and stirring the resulting slurry for 3 days at 100°C and pH 9.0 ± 0.1 [17]. Each slurry was then centrifuged, the supernatant decanted, and the centrifugate washed 10 times with deionized, double-distilled water and then freeze-dried. All the precipitates were sieved to pass 390 mesh (38 μm) before use. With carbonate apatite some of the final centrifugate was oven dried at 100°C for one night and sieved to pass between 300 and 500 μm. These latter particles were used as sintering materials for animal experiment as described later.

**Thermal analysis**

Sieved samples were placed in a metal mold (7 mm in diameter and 10 mm in length), remolded at 15 MPa and further compacted isostatically at 600 MPa into cylindrical specimens for thermal analyses (DT-40 assembly, Shimazu, Kyoto, Japan). The thermal mechanical analysis (TMA) unit was used to record the thermal dimensional change of the specimens heated at a rate of 5°C/min in a stream of dry argon (20 ml/min) under a 5 g load.

**Preparation of sintered apatite**

The sintered HAp and carbonate apatite specimens were produced by heating the compacted samples (five samples for each) in a platinum crucible in an electric furnace (Nikkato Corp., Tokyo, Japan) for 2 hours at 1200°C and 750°C, respectively, with a temperature increase and subsequent decrease of 5°C/min.

Samples from some of the sintered specimens were powdered and sieved to pass 145 mesh (105 μm) for dissolution experiments. Twenty-four sections of approximately 1 mm thickness were cut from other sintered specimens with a diamond saw, some of whose surfaces were polished with 1 μm diamond paste (16 sections) or with #1000 water-proof sand paper (8 sections). The sections were subsequently cleaned ultrasonically and placed in 24-well plates for cell culture experiments.

For animal experiment, non-compacted samples of carbonate apatite particles with sizes between 300 and 500 μm were sintered under the same conditions as with the compacted samples of carbonate apatite.

**Bone samples**

Powder samples of compact bovine bone were prepared from the femora of 3-5 year-old cows obtained from a local slaughterhouse. After deproteination using hydrazine [14, 45], the powder samples were passed through a 105 μm sieve. Thin, devitalized bone slices [40] were also prepared from the same femora and placed in 24-well plates for cell culture experiments.

**X-ray diffraction**

The powder X-ray diffraction patterns of sintered samples were recorded with a Shimazu XD-3A diffractometer operated at a scanning speed of 2 degrees 2θ/min using CuKα (λ = 0.154 nm) radiation generated at 30 kV and 30 mA.

**Infrared spectroscopy**

Infrared spectra were obtained between 4000 to 400 cm⁻¹ using a Shimazu FTIR-4200 spectrophotometer (Shimazu, Kyoto, Japan). For carbonate determination, the total area of the peaks between 1630 and 1390 cm⁻¹ in the linear absorption mode was integrated and compared with that of standard carbonate apatite [17].

**Dissolution experiment**

Dissolution reactions were initiated by rapidly adding 100 mg powdered samples of the sintered and bone specimens to 50 ml of 10 mM/l acetic acid solutions adjusted to pH 5.00 contained in a double-walled glass reactor at 37°C. The solutions were stirred continuously at a rate of 500 rpm throughout the experiments. Reaction kinetics were followed by monitoring proton activity with a pH electrode and by analyzing solution calcium and phosphate. Results of three runs were averaged for each point. The solution composition thus obtained was further analyzed through the use of chemical potential plots as described previously [16].

**Osteoclastic cell culture**

Cell suspensions containing osteoclasts were obtained from the inside of the bony shafts of 1-day-old neonatal rabbits (Japan white) by scraping out the trabecular bone, suspending the bone particles in medium 199 (GIBCO, Life Technologies, Inc., NY) supplemented with 10% fetal bovine serum and antibiotics (100 μg/ml of penicillin G and 50 μ g/liter of gentamicin), and releasing the cells by pipetting. Cell suspensions (100 μl) containing 50-100 multinucleated osteoclasts per 100 μl were plated onto the cut sections of sintered apatites (12 sections; 4 sections each for three different sintered apatites, i.e., carbonate apatite, HAp with smooth surface, and HAp with rough surface) or the devitalized bone slices (4 slices) in the small wells of microculture plates. α-Modified minimum essential medium (α-MEM, GIBCO) supplemented with 10% fetal bovine serum and antibiotics was used as the plating medium. After incubation at 37°C in 5% CO₂/95% air for 90 minutes, the non-attached cells were gently washed off and the apatite and bone substrates transferred into the culture medium and then incubated at 37°C in 5% CO₂/95% air for periods of up to 48 hours. Osteoclasts were identified by staining for tartrate-resistant acid phosphatase (TRAP) after cyanuric chloride pre-treatment as described previously [19, 40]. Some cut sections of sintered apatites (total 12 sections) were incubated in the medium in the absence of cells under the same conditions as above.
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![Graph](image)

Figure 1. Comparison of linear shrinkage curves for two compacted specimens of carbonate apatite and hydroxyapatite. Specimens of hydroxyapatite (a) and carbonate apatite (b) contained, respectively, nearly zero and about 12 wt% carbonate. Each specimen was heated to 1200°C at a rate of 5°C/min.

Scanning electron microscopy (SEM)

After culturing, the substrates were fixed with 2% glutaraldehyde in 0.15 M/l sodium cacodylate buffer at room temperature for 2 hours. They were then dehydrated through graded alcohols, critical point dried from liquid CO₂, and sputtered coated with gold. Secondary electron images of osteoclasts and resorbed lacunae on the surface of the substrates as well as the surfaces of the substrates before and after incubation without cells were observed with a JEOL JSM-35C SEM (JEOL, Ltd., Tokyo, Japan).

Skull defects

Twelve Wistar rats weighing 300-350 g were anesthetized with a solution of pentobarbital sodium (Nembutal, Abbott Laboratories, North Chicago, Illinois) at 0.04 ml/100 g body weight, and the pericrania were stripped off the parietal skulls with a periodontal elevator. A 3 mm diameter defect (one defect for each rat) was made through each parietal bone with a trephine (Figs. 2a and 2b). This poorer resolution suggests that the sintered carbonate apatite was somewhat low in crystallinity than the sintered HAp. In the infrared spectra, the broad doublet-like bands in the range of 1600-1400 cm⁻¹ and the band at ~870 cm⁻¹, characteristic for carbonate ions [15, 33], were clearly observed in the compacted carbonate apatite (Fig. 2d). The estimated carbonate content, calculated from spectra recorded in the linear absorption mode, was 5.8 wt% for the sintered carbonate apatite, suggesting that approximately half the carbonate initially present still remained in the apatite lattice after sintering [17]. When viewed with SEM, the sintered carbonate apatite appeared to be composed of grains, ranging in size from 0.3-1 μm, whereas

Results

Physicochemical properties of sintered carbonate apatite

The carbonate apatite had a calcium content of 34.59 ± 0.08, a phosphate content of 13.22 ± 0.07, a sodium content of 4.18 ± 0.04, a carbonate content of 11.76 ± 0.69 wt% and a specific surface area of 81.6 m²/g before sintering [17], whereas the HAp used was nearly stoichiometric in composition, having a calcium phosphate molar ratio of 1.68 ± 0.02 with no carbonate, of which composition remained the same after sintering within experimental errors. As shown in Figure 1, it was found that the sintering temperature for an apatite specimen containing about 12 wt% carbonate was about 400°C lower than that needed for sintering a specimen containing no carbonate, i.e., HAp. The carbonate-containing specimen, however, expanded after rapidly shrinking when temperatures were further raised over 800°C, probably because of the evolution of carbon dioxide from the specimen [18]. Thus, the best sintering occurred when the carbonate-containing specimen was kept at temperatures between 600 and 800°C, preferably around 750°C where the shrinkage was greatest. Although calcium phosphate molar ratios and sodium contents were essentially the same as those before sintering, irrespective of the difference in sintering temperatures used, calcium and phosphate contents increased whereas carbonate contents decreased as the sintering temperature increased. For example, the carbonate apatite that was sintered at 750°C for 2 hours had a calcium content of 36.07 ± 0.21, a phosphate content of 13.78 ± 0.05 and a sodium content of 4.18 ± 0.04 that was essentially the same as that before sintering.
Figure 2 (at left). Comparison of the X-ray diffraction patterns (a and b), infrared spectra (c and d) and fractured surfaces (e and f) of sintered hydroxyapatite (a, c, and e) and sintered carbonate apatite (b, d, and f). The hydroxyapatite and carbonate apatite were sintered, respectively, at 1100 and 750°C for two hours. No phases other than apatite were evident in the X-ray diffraction patterns.

Figure 3 (above). Chemical potential plots of the solution composition in 10 mM acetic acid dissolution reactions for sintered hydroxyapatite (a), deproteinated bone (b), and sintered carbonate apatite (c). Figure 3a corresponds to the inset in Figures 3b and 3c. The numbers with small letters, m, h, and d, represent the reaction time in minute, hour, and day, respectively.
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Figure 4. Scanning electron micrographs of osteoclasts on the surfaces of a sintered hydroxyapatite with a smooth surface (a) and a rough surface (b), a sintered carbonate apatite (c) and a bone slice (d) after 2-day incubation. No resorption pits were evident on the surface of the sintered hydroxyapatite, irrespective of the difference in surface roughness, except for micropores that were present initially.

The sintered HAp had about ten times larger grains (Figs. 2e and 2f).

In 10 mM acetic acid solutions of initial pH of 5.00, the solution pH increased more rapidly with the sintered carbonate apatite and bone apatite than with the sintered HAp. For the sintered HAp, the solution calcium and phosphate continued to increase to the end of reaction, paralleled with the solution pH, whereas the solution calcium and phosphate for the sintered carbonate apatite and bone apatite decreased after the first 10 to 100 minutes. The decreases in solution calcium and phosphate concentrations indicate that other, less soluble calcium phosphates formed after rapid dissolution of the two specimens.

Figure 3 shows the chemical potential plots of the solution compositions of the three specimens, sintered HAp (Fig. 3a), sintered carbonate apatite (Fig. 3b) and bone apatite (Fig. 3c) in the 10 mM acetic acid solutions. The solution composition for the sintered HAp at 30 seconds was at a position far above the solubility line of HAp, i.e., extremely undersaturated with respect to HAp, indicating that more HAp could be dissolved in this region. The composition moved with time toward the HAp line and stopped at a position slightly below this line after 3.8 days. In contrast, the solution compositions of the sintered carbonate apatite and bone apatite even at 30 seconds were already at positions below the HAp line. The compositions initially moved toward the solubility lines of octacalcium phosphate (OCP) and \( \beta \)-TCP, then the bone apatite compositions moved parallel with or on the OCP and/or \( \beta \)-TCP line, while the solution compositions of the sintered carbonate apatite fell below the \( \beta \)-TCP line before moving back into the region above the OCP and \( \beta \)-TCP lines.

Osteoclastic response

Without cells each substrate surface after 2-day incubation was essentially the same as that before incubation and no pits except micropores present in sintered samples were evident. Osteoclasts appeared to attach to the surfaces of the three substrates: sintered HAp, sintered carbonate apatite and bone in, essentially the same
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manner after 1-day and 2-day incubations. With sintered HAp, however, no pits were observed after 2-day incubation, irrespective of the difference in surface roughness from the two polishing techniques used, i.e., the surface polished with 1 μm diamond pastes left no rifts (Fig. 4a) whereas the one polished with #1000 waterproof sand paper left remnants of the parallel rifts caused by the diamond saw (Fig. 4b). The number of osteoclasts, however, appeared less on the smooth surface, probably because some osteoclasts could have detached after washing treatment before incubation and/or during incubation [26]. On the other hand, with sintered carbonate apatite, many resorption pits, or lacunae were evident on the surface of this substrate. Some of the pits were clearly seen adjacent to, or beneath the filopodia-fringed osteoclasts (Fig. 4c). Particulates of sintered carbonate apatite crystals (approx. 5-10 μm in diameter) clearly show the crystals to be fused to each other (Fig. 5), and the pit morphology was distinctly different from that seen outside the pits. With bone, bundles of collagen fibrils appeared in the resorption pits (Fig. 4d).

Skull defects

Sintered carbonate apatite particles appeared to resorb in skull defects of Wistar rats, forming new bone without any significant invasion of inflammatory cells. Figure 6 shows photomicrographs of skull defects of Wistar rats, filled with sintered carbonate apatite particles, at 2 and 4 weeks after implantation. In the defect at 2 weeks, new bone formed at the edges of the defect (Fig. 6a). Some osteoblast-like cells were evident on the periphery of newly formed bone as indicated by an arrow head (Fig. 6b). Connective tissues embedding the new bone spanned the central part of the defect on the dura mater. Presence of a large void (M), which originated from the implant materials that were dissolved by demineralization in neutral ethylene tetraacetic acid, suggested that no significant implant material resorption, was evident at this time. At 4 weeks, however, the defect was almost completely filled with bone and the implant material had resorbed considerably (Fig. 6c). The new bone contained osteocytes and was as mineralized and dense as the host bone. Osteoclast-like cells were also evident in contact with the implanted materials as indicated by arrow heads (Fig. 6d). This evidence is consistent with that staining of sectioned specimens by tartrate-resistant acid phosphatase (TRAP) after cyanuric chloride pretreatment [19, 40] (data not shown) showed that there were many TRAP-positive cells around the resorbing material.

Discussion

Physicochemical properties

The present study clearly demonstrates that the presence of carbonate decreased the sintering temperature of apatite (Fig. 1) as was first shown by Ellies et al. [20] based on qualitative mechanical criteria. For example, an isostatically compacted specimen, containing initially about 12 wt% carbonate, sintered at approximately 400°C below that for relatively pure HAp [37, 39]. The mechanism by which carbonate lowers the sintering temperature is still unknown [17, 18]. However, the carbonate apatite used had finer particles with a greater specific surface area (81.6 m²/g) than the HAp (10.4 m²/g). Generally, initial sintering of ceramics occurs more easily with finer powders [29, 31, 34].

Although no phases other than apatite were detected in the sintered carbonate apatite (Fig. 2b), some of carbonate had decomposed during sintering, as suggested by the fact that the sintered material contained only about half the carbonate initially present. Probably because of the evolution of carbon dioxide [18], the density of the sintered carbonate apatite (2.8-2.9) was slightly lower.
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Figure 6. Photomicrographs of skull defects of Wistar rats, filled with sintered carbonate apatite particles, (a and b) 2 and (c and d) 4 weeks after implantation at lower (a and c) and higher (b and d) magnification. Implanted materials were dissolved after decalcification (m). New bone (b) is evident at the edge of the defect at 2 weeks (Fig. 6a). In the area (Fig. 6b) outlined in Figure 6a, some osteoblast-like cells appeared around the periphery of new bone, as indicated by arrows. In Figure 6d, arrows indicate osteoclast-like cells in contact with implanted materials. When implant materials were not used to fill the defects, no bone had formed by 4 weeks.

than that of the sintered HAp (≤ 3.1) and its micropores (≤ 3 μm) were about three times as large (Figs. 2e and 2f). Grain growth and grain size increase rapidly with sintering temperature [13], which could account in part for the smaller grains of the sintered carbonate apatite as shown in Figure 2f.

One of the most promising features of sintered carbonate apatite as a biomaterial is that it can be favorably compared with bone, especially with respect to its reactivity to acid media [32, 33]. In the acid media used in this study (10 mM acetic acid, pH 5.0), both the bone apatite and the sintered carbonate apatite rapidly dissolved to an appreciable extent (Fig. 3). Their solution compositions changed in an almost identical manner until toward the end of dissolution reaction. On the other hand, it took 3.8 days for the solution composition for sintered HAp to become comparable with that for sintered carbonate apatite at 30 seconds with respect to the degree of saturation, indicating the extreme difficulty in dissolving the former. Also of importance was the observation that sintered carbonate apatite could be favorably compared with β-TCP in solubility.

Osteoclastic cell response

Osteoclasts that have been identified under the microscope as TRAP positive giant cells and resorbed bone were capable of resorbing sintered carbonate apatite (Fig. 4). Particulates that were observed in resorption pits (Fig. 5) appeared to be similar in shape to those seen on the substrate surface which was acid-etched with 1 N lactic acid (data not shown) rather than those seen on the fractured surface shown in Figure 2f. The osteoclasts on the sintered carbonate apatite surface appeared essentially the same as those observed on the bone apatite and sintered HAp after 2-day incubation, suggesting that osteoclast-substrate interaction may be the same for all the substrates used, in agreement with the finding.
that osteoclasts display essentially the same life cycle even on a glass or plastic substrate [4]. After 2-day incubation, no pits, or lacunae, however, were evident on both smooth and rough substrates of HAp that was sintered at 1200°C for 2 hours (Figs. 4a and 4b). Evidence concerning osteoclast response to HAp substrates is still controversial. In some studies [8, 24, 26, 36], osteoclasts have been shown to resorb the substrate, but in other studies [8, 44], they did not. Many factors appear to govern osteoclast-related bioresorption of sintered HAp. However, if all other osteoclast activity were the same on HAp substrates, the resorption would be directly governed by the concentration of hydrogen ions that is secreted from the cell into the clear subosteoclastic zone that is sealed from the rest of the media by the cell boundary [2]. Resorption of the mineral in this sealed region, in which the acidity is brought down to slightly below pH 5.0 [42], may be regarded as acid promoted chemical dissolution. The finding from the dissolution experiments may lead us to speculate that lacunae development on sintered HAp is very unfavorable. This explanation, although speculative, is consistent in part with the evidence of Shimizu et al. [41] that no osteoclast-related bioresorption on sintered HAp was observed after 3-day incubation but that multiple resorption pits with well-defined margins were formed with acetic acid at pH 4.5 after one-day immersion, suggesting that buffer action of acid in the sealed region must be much weaker than that of acid they used, although acidity, pH in these both media is approximately the same.

Skull defects

As has been well demonstrated by many investigators [1, 7, 11, 12, 22, 25, 43], the skull defect is a useful model to evaluate osteoconductive properties of biomaterials, since it is difficult for new bone to form spontaneously in these defects in the absence of such materials. In fact, in the present study, no bone formed in the defects after 4 weeks when materials were not implanted. When carbonate apatite particles were implanted, however, new bone had formed in the defects at 2 weeks without any significant inflammatory cell involvement (Fig. 6a). At 4 weeks, the defect was almost completely filled with bone (Fig. 6b). This finding is favorably compared with that found with demineralized bone powders by Glowacki et al. [25], but contrast to the evidence that with sintered HAp particles skull defects of Wister rats were not completely filled with bone even at 42 days after implantation [12]. In no case did the rate of sintered carbonate apatite resorption exceed bone formation, thus, the material provided a scaffold for bone growth during the early stages of healing before eventually being resorbed completely. Staining of sectioned specimens by tartrate-resistant acid phosphatase (TRAP) after cyanuric chloride pretreatment [19, 40] (data not shown), showed that there were many TRAP-positive cells around the resorbing material, suggesting that the resorption of the sintered carbonate apatite particles is, in part, similar to osteoclastic bone resorption. Details concerning osteoclast-substrate interactions as well as bone ingrowth in the defect, however, remains to be studied by transmission electron microscopy [5, 6].

Finally, in regard to bioresorption sintering temperature, sintering time, porosity, surface roughness and grain size appear to be most important [8, 24]. In addition, carbonate contents in sintered apatites are also important in this regard [20]. The carbonate contents in sintered specimens could be controlled with ease by varying carbonate contents initially present in apatites [17, 18] and may lead to different rates of bioresorption.

Conclusion

Sintered carbonate apatites are effective materials as bioresorbable bone substitutes. Under appropriate conditions, the sintered mass remains a single apatitic phase, in which some lattice substituted carbonate remains. As a result, the sintered carbonate apatite more closely resembles bone apatite than sintered HAp especially with respect to its reactivity to acid media. Osteoclasts that resorbed bone were capable of resorbing the sintered carbonate apatite in vitro. In skull defects, new bone formed as the sintered specimen resorbed without any significant inflammatory cell reaction and the defect was filled with bone almost completely at 4 weeks after implantation.

Acknowledgements

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Discussion with Reviewers

J.D. de Bruijn: The author compared carbonate apatite with HAp in all experiments, except in the rat skull defect. If HAp had been included in this model, one would have been able to compare conventional HAp with carbonate apatite. Why was HAp not included in the rat skull defect?

Author: Your comment is correct, but we were not attempting here to elucidate difference of bioresorption between sintered carbonate apatite and sintered HAp implants. In the preliminary experiments, however, we implanted sintered HAp implants whose particle sizes (300-500 μm) were same as those of sintered carbonate apatite implants used. No resorption was evident after 4 weeks with HE stained specimens. Unfortunately, only one defect was used for this experiment and not included in the text. Also, in bone marrow spaces of femurs of 6-week Wistar rats, the same sintered HAp implants (300-500 μm) have been shown not to resorb at 4 weeks after implant [47]. Although this finding could not be extended to that of skull defect situations, it agrees well with the evidence that apatite biomaterials do not undergo appreciable resorption. Further studies, however, are underway at our laboratory to elucidate differences between carbonate apatite and HAp.

J.D. de Bruijn: Rats have a faster bone metabolism than humans. The author mentioned that in the rat skull defect model, implant resorption coincided with new bone formation. This equilibrium might be shifted toward faster implant resorption in the human situation due to a significant slower bone formation rate in man.

Author: As is well known, with regard to bioresorption, sintering temperature, sintering time, porosity, surface roughness and grain size appear to be the most important parameters. In addition, carbonate content in sintered apatites is also very important in this respect. The carbonate content of sintered specimens could be controlled with ease by varying the carbonate content initially present in the apatite structure and may lead to different rates of bioresorption. So when you need less resorbable sintered apatites you can use apatites with less carbonate.

J.D. de Bruijn: Although mentioned in the Materials and Methods section, nothing is said about the tartrate-resistant phosphatase (TRAP) positive cells in the osteoclast lineage. For example, were the resorbing cells TRAP positive, or more specifically, was the cell in Figure 4c TRAP positive?

Author: The localization of TRAP deposits in the cytoplasm of osteoclasts was verified prior to SEM observation. However, I cannot answer "yes" with absolute certainty to your specific question "was the cell in Figure 4c TRAP positive?". The giant cell which attached tightly to the surface has many cytoplasmic processes, a domed, round and flattened shape, characteristic for osteoclast lineage cells that were demonstrated with the TRAP staining in other specimens.

J.D. de Bruijn: The author mentioned in the Discussion that no bone is formed in the skull defects after 4
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weeks of implantation, in the absence of such implant materials. In Figure 6, however, it is clearly visible that no direct bone contact exists between the newly formed ingrowth bone and the material grains. A layer of soft-tissue with a width of approximately 60-120 μm (as based on the light micrographs) is present between the newly formed bone and the implant material. Therefore, since the material grains do not seem to give rise to osteoconduction or bone formation at their surface, in what way do the authors think the defect healing is induced by these materials without physically guiding bone formation at their surface.

Author: As is well known, with decalcified specimens nothing can be said about direct bone contact, though the possibility that the material might have had direct contact to bone cannot be excluded. Your suggestion that soft tissue with a width of approximately 60-130 μm (as based on the light micrographs) is present between the newly formed bone and the material is correct. However, this situation differed from specimen to specimen, in specimens prepared by sectioning the defect portion longitudinally. In specimens obtained from the central portion of the defect, no soft tissue appeared to be present between the newly formed bone and the material. One of the reasons I stated that the material used was osteoconductive is that without the material, new bone hardly formed in the defect at 4 weeks after implantation, but, in the defect filled with the material, new bone formed even at 2 weeks. However, I wonder whether the material could be resorbed by osteoclasts if the material gave rise to bone formation at its whole surface and was embedded in bone.

D.G. Nelson: Were there any changes in the a and c crystal unit cell dimensions between the sintered carbonate apatite and the sintered HAp?

Author: Yes, the a and c unit cell dimensions were different between the sintered carbonate apatite and the sintered HAp. Also the breadth of (300) and (002) reflections at one-half maximum intensity was different between them. It was greater for the sintered carbonate apatite, as demonstrated in our previous study [17].

D.G. Nelson: Is the increased rate of resorption for sintered carbonate apatite versus HAp only major reason for its better performance in stimulating new bone growth in the rat model? If so, could other implants with smaller particles size or less crystallinity (or other impurities) be used with similar results?

Author: The author feels that the increased rate of resorption for sintered carbonate apatite versus HAp is one reason for its better performance in stimulating new bone growth. As you pointed out, implants with smaller particle size would have potential to resorb faster. However, to have implants resorbed there must be cells in contact with implants. If you fill defects with smaller particles, there will no be significant, relatively large enough pores to allow for tissue ingrowth. This is why we used 300-500 μm particles. With these particles filled, you could expect to have pore sizes in the range 100-200 μm to allow for tissue ingrowth.

However, it is not evident to the author as to what you mean by "other implants with less crystallinity". When they are no carbonate apatite associated implants with less crystallinity, they hardly dissolve in biologic fluids since these fluids are generally supersaturated with respect to apatite with less crystallinity, such as, bone apatite. Resorption by cells becomes very important in this regard. But, liberated crystals appear to be phagocytosed by macrophages, which, I feel, is a signal reflecting an inflammatory response. So, the problem with these materials is how we could stick microcrystals together. If one uses a high temperature fusion process (e.g., sintering) to do so, it likely will produce undesirable byproducts such as β-TCP, CaO, or sometimes tetracalcium phosphate, as clearly stated in the Introduction. To overcome this problem, we have developed apatite-collagen complex [19] in which microcrystals of apatites with less crystallinity, which compared favorably to that of bone, were deposited on the collagen fibers. The adhesion of each crystal to collagen fiber was appreciably strong so that no apatite was visually observed to detach under deformation with fingers or a forceps and after ultrasonification for more than 10 minutes. These implants have been used as one of bone substitutes at our laboratory. In another way, Stupp and Ciegler [48] have used some organic polyelectrolytes to combine microcrystals of apatite with less crystallinity with organoapatites as they seemed to have also been shown to be effective as bioresorbable bone substitutes [46].

G. Daculsi: The decalcified method used to evaluate bone ingrowth in the skull defects give no information on the real interface of bone and carbonated apatite. Have you observed bone bonding or osseo-coalescence? Can you describe more precisely the interface and the cell populations observed on the sections (osteoclasts, macrophages, monocytes, lymphocytes, etc.).

Author: The author was much concerned about whether the implanted carbonate materials resorbed in skull defects and new bone formed as they resorbed. This could be evaluated with decalcified HE stained specimens. As you pointed out, in order to investigate the real interface of bone and carbonate apatite, some transmission electron microscopy of non-decalcified specimens is needed. Further studies are now being performed at our laboratory.
N. Nagai: It is stated that the starting carbonate apatite contains about 12 wt% of carbonate. How was this determined?

Author: Carbonate contents of our samples before and after sintering have been determined chemically by a microdiffusion technique and the IR method, and cross-determined with a carbon determinator with copper used as an oxidizer, as shown before [17].

N. Nagai: After sintering at 750°C, why the carbonate apatite contains as much as 5.8 wt% of carbonate, because the author himself showed the carbonate apatite shrank at between 600°C and 800°C, which should be attributed to the evolution of carbonate and because the many previous studies showed that after heating at over 400°C apatite losses the lattice carbonate.

Author: The statement that the many previous studies showed that after heating at over 400°C apatite loses the lattice carbonate is only partially correct, since it does not mean that all the lattice carbonate is lost after ignition at over 400°C. However, as shown previously [18], almost all the carbonate is lost after ignition at temperatures over 1000°C when powdered apatite are used. With apatites compacted at 200-600 MPa, however, it was not completely lost even after ignition at 1300°C. Our specimens did contain as much as 5.8 wt% carbonate even after sintering at 750°C. This is what we have been collaborating to develop bioresorbable sintered apatites in which some lattice-carbonate remains.

C.M. Müller-Mai: Please comment on the mechanical properties of the carbonate apatite.

Author: Mechanical properties of sintered carbonate apatites strongly depend on sintering temperature, sintering time, heating rate, and also contents of carbonate initially present as shown previously [17]. For example, the sintered sample that was obtained by heating 12 wt% carbonate-containing apatite at a rate of 2°C/min to 750°C and held at this temperature for 2 hours had Knoop hardness of about 500 and three-point bending strength of 95 ± 5 MPa. Fracture toughness, \( K_{IC} \), which was calculated by four-point bending mode using the Chevron notched beam, was 0.76 ± 0.09 MPam\(^{1/2}\). This fracture toughness compared favorably to that of HAp that was sintered at 1100°C for 2 hours (0.62 ± 0.07 MPam\(^{1/2}\)), but approximately 20% lower than that reported for sintered HAp (~ 1 MPam\(^{1/2}\)).

C.M. Müller-Mai: The author used implants with different particle sizes leading to different rates of resorption. There are several papers in the literature demonstrating different resorption rates of HAp implants with different particles sizes. HAp implants with particle sizes between 0.1 to 0.4 μm [24] showed superficial resorption. Shimizu et al. [41] did not observe resorption in vitro using polycrystalline implants with particle sizes about 1 μm and single crystal HAp at 3 days in vitro. Is there any relation between particle size and resorption rate especially considering that microcrystalline implants [24] did show comparable resorption rates as carbonate apatite?

Author: As stated in the text, smaller particles dissolve faster than larger particles, so one would expect that smaller particles resorb faster in tissues. However, to what extent calcium phosphate biomaterials dissolve is governed by their solubility products. I am not sure that your microcrystalline implants show comparable resorption rates as carbonate apatite used here. Nevertheless, it may be evident that the microcrystalline implants resorb faster than sintered HAp implants. We have compared dissolution behavior of the sintered carbonate apatites and microcrystalline apatites that had been prepared at 37°C for 1 week. We presume that our microcrystalline apatites could be compared to your microcrystalline apatites as far as X-ray diffraction patterns are concerned. We, however, have found that our microcrystalline apatites were less soluble than the sintered carbonate apatites in weak acid media, such as acetic and lactic acid (10-100 mM) solutions at pH 4.5-5.5 at 37°C.

C.M. Müller-Mai: Since there is a high dissolution rate of the carbonate apatite, one should look for significant amount of liberated implants particles in the surrounding tissue. Did the author observe particulate disintegration of the carbonate apatite to a higher extent than that of HAp?

Author: We have not yet performed any transmission electron microscopic observations with non-decalcified specimens at higher magnification. However, we feel that, as you pointed out, more liberated implant particles may be there in the surrounding tissues, since the carbonate apatite resorb to an appreciable extent, eventually resulting in complete resorption of fused portions. To answer your question, further studies are now underway at our laboratory.

Additional References

