The purpose of this study was to investigate the effect of sintering different calcium phosphate (CaP) surfaces on protein adsorption and osteoblast cell response. As-received and sintered hydroxyapatite (HA) and brushite were used in this study. X-ray diffraction revealed a poorly crystallized HA structure for the unsintered HA and a highly crystallized HA for the sintered HA surfaces. A brushite-type structure was indicated on the unsintered brushite surfaces, whereas sintered brushite surfaces contained mixtures of different CaP phases. Using 1 mg/mL albumin solution, protein was suggested to selectively adsorb on the CaP surfaces. A statistically higher albumin adsorption was observed on unsintered HA (9.5 μg/mL) and unsintered brushite (50.1 μg/mL) surfaces compared to sintered HA (3.2 μg/mL) and sintered brushite (3.4 μg/mL) surfaces. In the in vitro study using osteoblast cells, no statistical responses were observed between cells cultured on sintered HA and sintered brushite after 8 days of incubation. However, statistical differences in osteocalcin and protein production were observed between the unsintered HA and unsintered brushite. In addition, statistical differences in protein production, alkaline phosphatase activity, and osteocalcin production were observed between sintered CaP and unsintered CaP surfaces. From the protein adsorption and cell responses observed in this study, it was concluded that CaP surfaces need to be fully characterized prior to implantation.

INTRODUCTION

The study of tooth mineral has been linked to the investigation of calcium phosphate (CaP) minerals, namely hydroxyapatite (HA). Like other bioactive materials, such as bioactive glasses, HA has been shown to bond directly with bone, resulting in the formation of a uniquely strong bone-implant interface.1-4 In spite of the desirable properties of HA, it is not strong enough for load-bearing areas.5-7 Thus, in an attempt to improve osseointegration of implants in the bone and surrounding tissue, HA and other calcium phosphate ceramic coatings are being used.8 Extensive in vivo research has indi-
icated that plasma-sprayed HA implants are biocompatible, with reports of early skeletal attachment. However, the nature of HA properties on tissue responses has not been fully investigated. A significantly higher osteogenesis level was observed in the presence of HA compared to other biomaterials. However, tissues respond differently to biomaterials of different crystallinity. Major differences in the adhesive response of epithelial cells to different crystallographic structures were reported, even though these structures were chemically identical.

In other studies, an adverse effect of amorphous HA coatings on the establishment of an interface with bone was observed, whereas in yet other studies, amorphous HA coatings have been thought advantageous for a more stable interface with the biological environment. It must also be noted that in many of these animal and clinical studies, the physical and chemical characteristics of plasma-sprayed HA were either unknown, poorly known, or left unstated.

Although CaP coatings on titanium implants are used to improve initial osseointegration, the data are still inconclusive as to what the nature of the CaP coatings should be to obtain optimum bone growth. Thus, the objective of this study was to investigate the effect of sintering different CaP surfaces on protein adsorption and osteoblast cells in vitro.

**Materials and Methods**

**Calcium phosphate (CaP)**

Brushite and HA were the two CaP materials used in this study. Brushite powders were obtained from Aldrich Chemical Company, Inc (Milwaukee, Wisc). HA disks were prepared by CeraMed Corporation (Lakewood, Colo) by a wet reaction between calcium nitrate and ammonium phosphate. The HA precipitate was collected and washed several times with water. The HA paste was then dried in a spray dryer to obtain dry HA powders. The dry powders were then pressed into as-received HA disks (12 mm diameter by 2.5 mm thick) using a hand press at a pressure of 20,000 psi. Sintered HA and brushite were produced by sintering the as-received HA and brushite disks (atmospheric pressure) at 1100°C over 7 hours. The CaP disks were sterilized using dry heat sterilization prior to the study.

**X-ray diffraction**

X-ray diffraction analyses were carried out on a Syntag Model PAD V (Cupertino, Calif) diffractometer at 45 kV and 40 mA using Cu K radiation. The diffractometer was equipped with a solid-state detector, and the diffraction pattern was collected as a continuous scan at 1.5 degrees/minute.

**Protein adsorption**

The protein adsorption study was performed using 1.5 mL of albumin solution (1 mg/mL of albumin/Dulbecco's Modified Eagle's Medium [DMEM] solution) in a 12-well plate containing sterile CaP samples. The plate was then placed in a sterile humidified incubator at 37°C for 15 minutes. The samples were then washed twice in distilled water to remove the nonadherent proteins and were placed in a fresh 12-well plate. Two milliliters of sodium dodecyl sulfate (SDS) solution (1%) was added to the plate containing the
FIGURE 3. X-ray diffraction of the as-received brushite samples, showing data collected from 20 to 60 degrees 2 theta.

samples and was incubated at 50°C for 15 minutes. The plates were then placed on a plate shaker for 10 minutes, and the solution was saved. Four more SDS washes were performed, saving the solution between each wash. Protein concentrations were analyzed using the microbicinchoninic acid (BCA) protein assay. The protein concentration between each wash was cumulated, and differences in protein adsorption between the different CaP surfaces were statistically compared using the analysis of variance (ANOVA) test.

Cell culture

Bone cell activity studies were carried out using ATCC CRL 1486 human embryonic palatal mesenchyme cell line (HEPM), a precursor of osteoblastlike cells. The cells were seeded onto the HA disks in 24-well culture plates at a density of 10,000 cells/cm²/mL in DMEM containing 7% fetal bovine serum, 1% antibiotic-antimycotic solution, 50 µg/mL ascorbic acid, and 4 mM glycerophosphate. The study was conducted in an incubator at 37°C with a humidified atmosphere of 95% air and 5% CO₂. The culture medium was changed every 2 days with complete DMEM media. At 0-, 4-, and 8-day postconfluency, triplicate samples from each treatment were assayed for protein synthesis, alkaline phosphatase activity, and osteocalcin production.

Cell surface and matrix-associated protein synthesis

Total cell surface and matrix-associated protein synthesis was measured using the Pierce BCA protein assay (Pierce, Rockford, Ill). On the day of the assay, media were removed from the cell culture and the cell layers lysed with 1 mL of Triton X-100 (0.2%). An aliquot of the triton lysate (50 µL) was added to 200 µL of BCA working reagent, and the samples were incubated for 30 minutes at 37°C. The concentration of cell surface and matrix-associated protein synthesized was determined from the absorbance read at 570 nm by a microplate reader. Alkaline phosphatase (ALP) activity was determined from the absorbance using a standard curve prepared from p-nitrophenol stock standard (Sigma). The ALP specific activity was statistically compared using ANOVA.

1,25 (OH)&& vitamin D3 stimulated osteocalcin production

On the day of the assay, the medium was removed from the cultures and frozen at −20°C until assayed. The samples was then lyophilized using a speed-vac (Savant, Holbrook, NY). The sample residues were reconstituted at one-fourth the original starting volume using glass distilled water. The 1,25 (OH)&& vitamin D3 stimulated osteocalcin production was then measured using a commercially available human osteocalcin radioimmunoassay kit (Biomedical Technologies, Stroughton, Mass). Differences in 1,25 (OH)&& vitamin D3 stimulated osteocalcin production were statistically compared using the ANOVA test at a value of 0.05.

RESULTS AND DISCUSSION

Depending on the properties of biomaterials, different rates of cellular responses have been observed in vitro.19–22 These differences have been attributed to varying surface chemistries and crystallinities. X-ray diffraction analyses for all HA samples indicated an HA-type structure, with as-received HA samples having broad HA peaks (Fig 1), whereas the sintered HA samples (Fig 2) displayed more distinct, sharper x-ray diffraction peaks, indi-
cating higher crystallinity. It has been reported in previous studies that the crystallite size of sintered HA samples exceeded 2500 Å and was different from the crystallite size of calcined HA (275 Å) and as-received HA (200 Å) samples.23 Crystallite sizes of about 5000 Å have been reported for HA powders.24,25 Similarly, x-ray diffraction analyses of as-received brushite indicated a brushite-type structure, whereas sintering of brushite produces mixed CaP phases with higher crystallinity (Figs 3, 4). These differences in crystallinity/crystallite size have been associated with varying degrees of dissolution rates, with smaller, more imperfect crystals being subject to greater dissolution.26-28 The increase in crystallite size during sintering concurs with observations made by other investigators.29 Studies using high resolution transmission electron microscopy have shown that a higher annealing temperature not only increases grain size but also improves crystal perfection by minimizing the number or preventing the formation of crystal void defects.30 As the grains become larger, the gaps between grains become smaller, and the material undergoes densification.

Depending on the surfaces, albumin was suggested to selectively adsorb on the CaP surfaces, with a statistically higher albumin adsorption on as-received HA (9.5 µg/mL) and as-received brushite (50.1 µg/mL) surfaces compared to sintered HA (3.2 µg/mL) and sintered brushite (3.4 µg/mL) surfaces. It has been reported that the presence of surface calcium plays an important role in the adsorption of proteins to implant surfaces through a mechanism involving calcium bridging.31 Calcium bridges were also observed to bind to glycosaminoglycans (GAGs), an important component of proteoglycans.32 As reported in other studies, the method of action of protein/GAG adsorption to the implant surfaces was proposed to be related to the surface affinity for a variety of cations, which then lend themselves to bind electrostatically to a variety of substances, including proteins.31 With changes in surface properties such as crystallinity, a change in ionic interaction was suggested, which thus affected the amount of calcium ion ligands readily available to electrostatically bind to proteins.31

In in vitro cell culture studies, protein synthesis is an important marker for evaluating cell function. Matrix proteins in bone have been reported to play a crucial role in the calcification and architectural construction of these hard tissues.33 In this study, no statis-
tical difference in the cellular protein production was observed on sintered HA and brushite surfaces (Fig 5). However, a statistically higher protein production was observed when cells were seeded on as-received CaP surfaces compared to sintered CaP surfaces, suggesting the possible influence of heat treatments of CaP surfaces on protein synthesis. As-received brushite surfaces were also observed to induce a higher protein production compared to the as-received HA surfaces. As reported in previous studies, the surface chemistries of brushite and HA are different. Using x-ray photoelectron spectroscopy, an oxygen component at 533.1 eV was observed for brushite and was not reported on HA surfaces. The presence of an oxygen component at 533.1 eV was attributed to the presence of H$_2$O. Thus, statistical differences in protein production by cells cultured on as-received HA and as-received brushite surfaces also suggested the influence of surface chemistry on protein production.

Two other biochemical markers, the ALP specific activity and osteocalcin level, are used as markers for determining osteoblast phenotype and are considered to be important factors in determining bone mineralization. As shown in Fig 6, cells grown on the sintered HA and brushite surfaces were observed to exhibit a significantly higher ALP specific activity during the 8 days postconfluence when compared to the as-received HA and brushite surfaces, indicating a higher cellular differentiation and mineralized matrix production on sintered CaP surfaces. However, as shown in Fig 7, the 1,25(OH)$_2$ vitamin D$_3$ stimulated osteocalcin production was observed to be significantly higher for sintered HA and brushite surfaces compared to as-received HA and brushite surfaces, suggesting the mineralization phase of the cells grown on sintered CaP surfaces. No statistical difference in ALP specific activity and 1,25(OH)$_2$ vitamin D$_3$ simulated osteocalcin production was observed when cells were cultured on as-received HA and as-received brushite surfaces. Similarly, no statistical difference in ALP specific activity and 1,25(OH)$_2$ vitamin D$_3$ simulated osteocalcin production was observed when cells were cultured on as-received HA and as-received brushite surfaces. The low ALP specific activity and high osteocalcin production on sintered CaP surfaces suggested that the cells cultured on sintered CaP surfaces differentiate and mineralize at a faster rate than cells cultured on as-received CaP surfaces. This difference in the rate of differentiation and mineralization could be also attributed to the selective
protein adsorption as observed in this study. Further studies are required to confirm this hypothesis.

Conclusions

Overall, this study has shown the importance of characterizing HA surfaces. As indicated by x-ray diffraction, differences in the structure of the sintered and as-received CaP surfaces were observed. Statistical differences in protein adsorption and osteoblast cell responses were observed between the sintered and as-received CaP surfaces, indicating the need to fully characterize the CaP surfaces prior to implantation. The osteoblast-like phenotype displayed by the cells cultured on as-received and sintered HA and brushite surfaces suggested that the structural and chemical composition of CaP surfaces play an important role in governing the expression of osteoblast characteristics.

Acknowledgments

This study was supported by the Whitaker Foundation, NIH/NIDR DE 07160, and CeraMed Corporation.

References


