Protein Adsorption and Osteoblast Precursor Cell Attachment to Hydroxyapatite of Different Crystallinities

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Purpose: *The effect of hydroxyapatite (HA) crystallinity on protein adsorption and osteoblast precursor cell attachment to HA was investigated.* ***Materials and Methods:*** *Different weight ratios of 100% crystalline HA and 100% amorphous calcium phosphate powders were mixed and pressed into disks (0.5 g) of different crystallinities—either 0% (HA0), 30% (HA30), 50% (HA50), 70% (HA70), or 100% (HA100).* ***Results:*** *X-ray diffraction indicated differences in HA crystallinities. In addition, dissolution of the HA was dependent on its crystallinity, with an increase in phosphorus dissolution as the degree of crystallinity was decreased. No significant difference in albumin adsorption and initial osteoblast precursor cell attachment was observed in the range of HA0 to HA70 surfaces. However, a significantly lower albumin adsorption and initial osteoblast precursor cell attachment were observed on HA100.* ***Discussion:*** *It was suggested that changes in ionic interactions as a result of a change in crystallinity affect the amount of calcium ion ligands readily available to electrostatically bind to proteins.* ***Conclusion:*** *It was thus concluded from this study that HA crystallinity affects the amount of albumin adsorbed and initial osteoblast attachment.* Int J Oral Maxillofac Implants 2005;20:187–192

Key words: *crystallinity, hydroxyapatite, osteoblast, protein*

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] Although HA has desirable properties, 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 CaP ceramic coatings are being used.[8,9]

Extensive in vivo research has indicated that plasma-sprayed HA implants are biocompatible; there have been reports of early skeletal attachment.[10,11] However, the effects of HA properties on tissue responses has not been fully investigated. A significantly higher level of osteogenesis has been observed in the presence of HA compared to other biomaterials.[12] However, tissues respond differently to biomaterials of different crystallinities. Major differences in the adhesive response of epithelial cells and osteoblast precursor cells to different crystallographic structures with identical chemical formulas have been reported.[13–15] In other studies, amorphous CaP coatings have been observed to have an adverse effect on the establishment of an interface with bone,[10,14] whereas in yet other studies, amorphous CaP coatings have been thought advantageous for a

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more stable interface with the biologic environment.[16–20] 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, explained only briefly, or left unstated.[21]

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.[22] Thus, the objective of this study was to investigate the effect of different crystallinities of HA on protein adsorption and osteoblast cells in vitro.

MATERIALS AND METHODS

Materials

One hundred percent crystalline commercial HA powder with a particle size of 50 to 75 μm and amorphous commercial CaP powder with particles only nanometers wide (Hitemco Medical Applications, Old Bethpage, NY) were mixed according to the following weight ratios: 70% crystalline HA to 30% amorphous CaP (HA70), 50% crystalline HA to 50% amorphous CaP (HA50), and 30% crystalline HA to 70% amorphous CaP (HA30). To ensure the uniformity of the mixture, the mixture powders were fully ground; 0.5 g of the mixed ground powders was then cold dry pressed into disks (13 mm × 13 mm × 4 mm). One hundred percent crystalline (HA100) and amorphous commercial CaP powder (HA0) were used as controls in this study.

X-ray Diffraction

A D8 advanced x-ray diffractometer (Bruker AXS, Madison, WI) was used to characterize the structure of different disks. The x-ray diffractometer was equipped with a single Gobel mirror to yield a diffracted parallel beam while removing the K_β radiation. Using a grazing incidence attachment, a 0.35-degree soller slit, and a lithium fluoride (LiF) (100) flat crystal monochromator to improve resolution and peak-to-background ratios, triplicate samples per group were analyzed using Cu K_α radiation at 40 kV and 30 mA and were scanned from 25 to 35 degrees 2θ at a scan rate of 0.1 degree per minute.

Dissolution Study

The HA disks were immersed in a 1.0 mol/L TRIS buffer (Fisher Chemical, Fair Lawn, NJ) containing 80 μmol/L sodium chloride (NaCl) (Fisher Chemical). The pH of solutions was balanced at 7.4 prior to the dissolution study. The experiment was performed in a sterile and humidified incubator (95% air, 5% carbon dioxide [CO_2] atmosphere) at 37°C. It was imperative that the pH of 7.4 was achieved at 37°C for each solution after all chemical components were added to the media. A TRIS buffer was used to maintain a constant pH of 7.4 around each sample during the incubation period. The specimens were incubated in buffer solution at 37°C for 14 days. The buffer medium was changed daily to ensure that all parameters were equal with respect to the composition of the media between each of the test specimens, especially with respect to quantities of calcium and phosphorus. As the buffer medium was collected each day, the volume withdrawn and pH was recorded. Each dissolution sample was then saved for subsequent analysis of phosphorus release.

Measurement of Inorganic Phosphorus

The amount of phosphorus ions released in solution each day was measured colorimetrically by utilizing the reaction of ammonium molybdate and ascorbic acid with the inorganic phosphate to obtain a molybdenum blue complex. The reaction was done in a 96-well microtiter plate. Each sample was diluted tenfold to make a 100-μL solution. Solution A was made by combining 2 parts double-distilled water, 1 part 5.0-N sulfuric acid (Baker Analyzed, Phillipsburg, NJ) 1 part 0.01 mol/L ammonium molybdate tetrahydrate (Sigma Chemical, St Louis, MO) in water, and 1 part 10% ascorbic acid (Sigma Chemical). Solution A was made fresh for each assay. To the 100-μL sample dilution, 100 μL of solution A was added. The complex was allowed to form for 1 hour at room temperature and was subsequently read at 750 nm on a Dynatech MR5000 microplate reader (Dynatech Laboratories, Chantilly, VA). Each phosphorus concentration was calibrated using a known phosphorus concentration standard curve. Statistical analysis was performed using analysis of variance (ANOVA), and differences were considered significant if $P < .05$.

Protein Adsorption Study

Bovine serum albumin, fraction V (PERBIO; Pierce Chemical, Rockford, IL) was used as the model protein in this study. Purity of the proteins was confirmed by the manufacturer. Three hundred microliters of each protein solution (1 mg/mL protein/saline solution) was pipetted on HA surfaces in a 6-well plate (Fig 1) and incubated in a sterile humidified incubator at 37°C for 3 hours. Nonadherent proteins were removed and washed twice using saline solution. The removed solution was then saved, and the total volume collected was recorded. Aliquots of 100 μL of the initial and removed solutions were mixed with 150 μL of micro bicinchoninic acid (Pierce Chemical) working reagent in a 96-well plate,

incubated at 37°C for 120 minutes, and read using an Opsys MR microplate reader (Dynex Technologies, Franklin, MA) at 595 nm. Each protein concentration was calibrated using a standard curve. The degree of adsorption was determined by subtracting the residual protein from the initial added protein. Measurements were performed in 5 samples for each time point. Mean adsorption protein concentration between the different surfaces was statistically analyzed using ANOVA, and differences were considered significant if $P < .05$.

Cell Attachment Study

The cell attachment study was conducted using CRL 1486 human embryonic palatal mesenchymal cells (American Type Culture Collection, Manassas, VA), an osteoblast precursor cell line. The cells were incubated in Dulbecco's modified Eagle's medium (DMEM), which contains 7% fetal bovine serum, penicillin (5,000 units/mL^{-1}), streptomycin (5,000 μg/mL^{-1}), and Fungizone (250 μg/mL; Bristol-Myers Squibb, New York, NY), in a 5% CO_2 humidified incubator at 37°C, and the medium was changed twice a week. Osteoblasts from preconfluent cultures were harvested with 0.25% trypsin and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) (GibcoBRL; Life Technologies, Grand Island, NY) and were centrifuged to produce a cell suspension with serum-free DMEM. The cells were then seeded onto the HA surfaces in 6-well culture plates at a density of 10,000 cells per sample and incubated in a 5% CO_2 humidified incubator at 37°C for 180 minutes. Cell concentration was analyzed using the Vybrant cell adhesion assay (Molecular Probes, Eugene, OR). The nonadherent cells were removed and washed twice using serum-free DMEM. The removed solution was then saved, and the total volume of removed solution was recorded. Calcein AM (from the Vybrant adhesion assay kit) was added to the initial and removed solution at a concentration of 5 μmol/L. Calcein AM is nonfluorescent but, once loaded into cells, is cleaved by endogenous esterases to produce highly fluorescent calcein. Aliquots of the initial and removed solutions (100 μL) containing calcein AM were mixed with 100 μL of phosphate-buffered saline (PBS) in a 96-well plate, incubated at 37°C for 120 minutes, and fluorometrically analyzed using a SPECTRAmax GEMINI XS microplate reader (Molecular Devices, Sunnyvale, CA) at an absorbance maximum of 494 nm and an emission maximum of 517 nm. Readings were then correlated to a standard curve of known cell concentration. The degree of cell attachment was determined by subtracting the residual cell from the initial cell concentration seeded. Mean attachment cell concentration between the different HA surfaces was statistically analyzed using ANOVA, and differences were considered significant if $P < .05$.

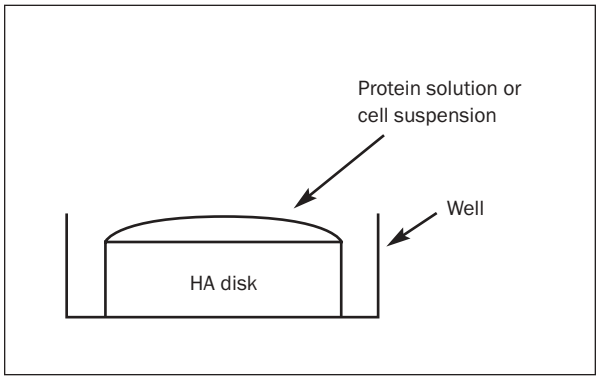

Fig 1 Schematic drawing showing how protein suspension and cell suspension were placed on HA disk surfaces.

RESULTS

X-ray Diffraction

The x-ray diffraction (XRD) patterns for HA samples are shown in Fig 2. No reflection peaks were observed for HA0 samples, whereas reflection peaks were observed for HA100, HA70, HA50, and HA30 samples. These peaks correspond to standard synthetic HA (JCPDS 09-0432).

In addition, a change in peak intensities was observed with HA surfaces of different crystallinities.

Dissolution Study

Phosphorus ions released for all the HA samples are shown in Fig 3. HA100 was observed to release a significantly lower concentration of phosphorus ions ($P < .001$). No significant difference in phosphorus ion release among HA70, HA50, HA30, and HA0 groups was observed ($P > .05$).

Protein Adsorption

The adsorption concentration of albumin after a 3-hour incubation period was 216 ± 1 on HA0, 210 ± 3 on HA30, 208 ± 2 on HA50, 199 ± 2 on HA70, and 179 ± 5 on HA100. No significant difference in albumin adsorption was observed among HA0, HA30, HA50, and HA70 surfaces ($P > .05$). However, HA100 surfaces were observed to have significantly lower albumin adsorption compared to the other HA crystallinities ($P < .02$).

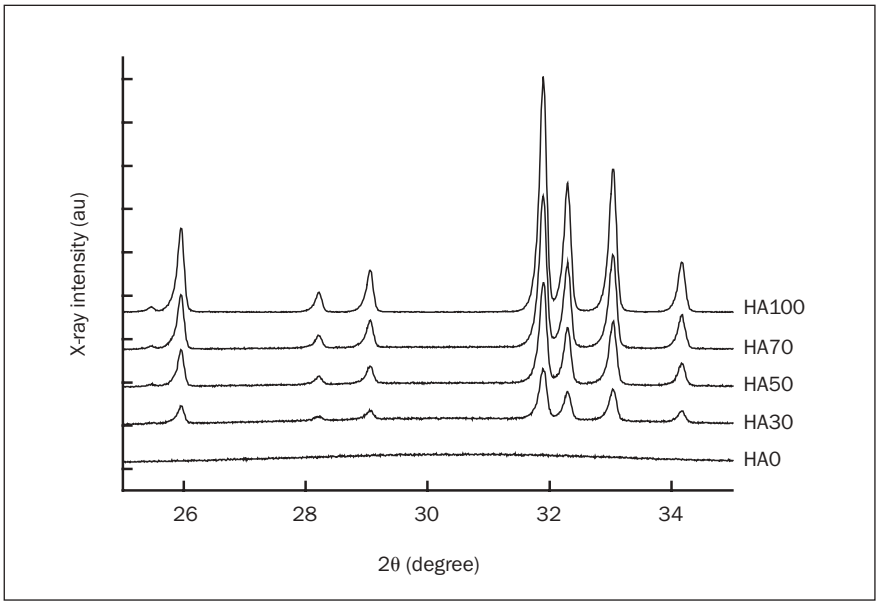

Fig 2 X-ray diffraction of HA disks of different crystallinity (n = 3). au = arbitrary unit.

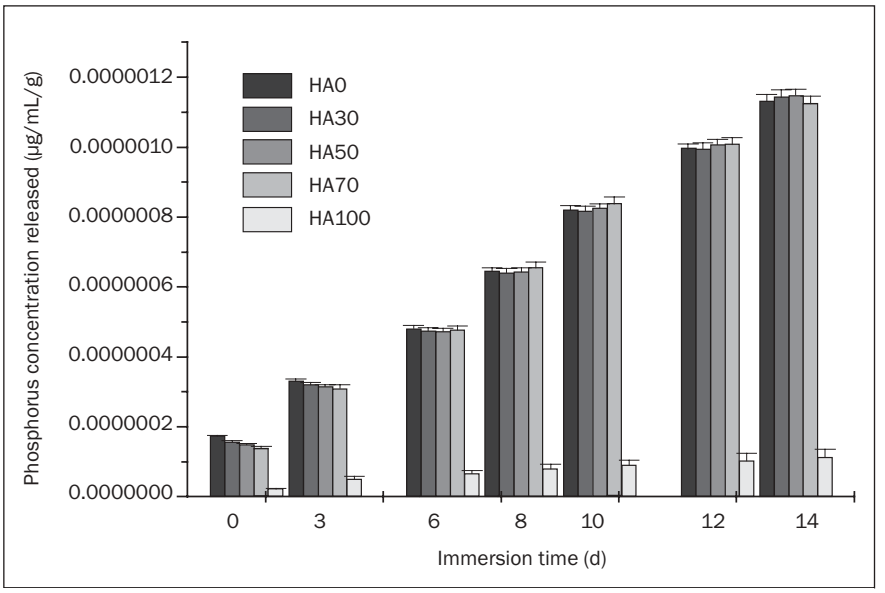

Fig 3 Phosphorus release in a 1.0 mol/L TRIS buffer for HA disks of different crystallinities.

Cell Attachment

Osteoblast precursor cell attachment on HA surfaces after 3 hours incubation was 98% ± 1% for HA0, 98% ± 1% for HA30, 97% ± 1% for HA50, 95% ± 1% for HA70, and 67% ± 10% for HA100. Osteoblast precursor cell attachment on HA100 surfaces was observed to be significantly lower compared to other HA crystallinities ($P < .05$). No significant differences in initial osteoblast precursor cell attachment were observed among the HA0, HA30, HA50, and HA70 surfaces.

DISCUSSION

Depending on the properties of biomaterials, different rates of cellular responses have been observed in vitro.[14,20,23–26] These differences have been attributed to varying surface chemistries and crystallinities. With the exception of HA0 samples, which exhibited no reflection peaks using x-ray diffraction, reflection peaks were observed on all HA samples. These peaks correspond to a standard synthetic HA, which sug-

gests an HA-type structure for the HA100, HA70, HA50, and HA30 samples. In addition, several studies have indicated a change in measured x-ray intensities as a result of a change in the degree of crystallinities.[27–29] These changes in peak intensities confirmed the HA with different crystallinities used in this study.

In addition to the change in crystallinities, the 14-day dissolution study indicated a significantly lower amount of phosphorus ions released from the HA100 disks as compared to the other groups. However, no significant difference in phosphorus ion release was observed between the HA70, HA50, HA30, and HA0 samples, suggesting that the amount released chiefly came from the amorphous component in the dissolution study. Other investigators have reported that these differences in crystallinity have been associated with varying degrees of dissolution rates, with smaller, more imperfect crystals being subject to greater dissolution.[30–33]

In the protein adsorption and cell attachment study, significantly lower albumin adsorption and cell attachment were observed on HA100 surfaces as compared to HA0 or other HA surfaces with varying degrees of crystallinity. 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.[34–37] Moreover, it has been suggested that changes in ionic interactions as a result of a change in crystallinity affect the amount of calcium ion ligands readily available to electrostatically bind to proteins.[34–37] Depending on the degrees of crystallinity, albumin solution or cell suspension were suggested to selectively adsorb on the HA surfaces. The lack of difference between the HA70, HA50, HA30, and HA0 may be the result of the sensitivity of the assay used to measure protein. In another study, cell attachment has also been observed to alter with the HA crystallinity.[38] As such, this study demonstrated the importance of determining the effects of the physical and chemical characteristics of HA surfaces on cell activity.

SUMMARY AND CONCLUSION

Dissolution of HA was dependent on its crystallinity, with dissolution increasing with decreasing degrees of crystallinity. Albumin adsorption and cell attachment were seen to selectively adsorb on the HA surface, and the degree of adsorption was dependent on HA crystallinity. However, no significant differences in albumin adsorption and cell attachment were observed for HA0, HA30, and HA50 and HA70 surfaces. It was thus concluded from this study that HA of 100% crystallinity resulted in reduced initial osteoblast attachment and albumin adsorption.

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