EFFECTS OF DISSOLVED CALCIUM AND PHOSPHOROUS ON OSTEOBLAST RESPONSES

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The dissolution behavior of hydroxyapatite (HA) and its effect on the initial cellular response is of both fundamental and clinical importance. In this study, plasma-sprayed HA coatings were characterized by X-ray diffraction and Fourier transform infrared spectroscopy (FTIR). Calcium (Ca) and inorganic phosphorous (Pi) ions released from plasma-sprayed HA coatings within 3 weeks were measured by flame atomic absorption and colorimetrically molybdenum blue complex, respectively. To investigate the effect of dissolution of HA coatings on osteoblast response, additional Ca and Pi were added into the cell culture media to simulate the dissolution concentrations. Human embryonic palatal mesenchyme cells, an osteoblast precursor cell line, were used to evaluate the biological responses to enhanced Ca and Pi media over 2 weeks. Osteoblast differentiation and mineralization were measured by alkaline phosphatase-specific assay and 1,25 (OH)₂ vitamin D₃ stimulated osteocalcin production. The coatings exhibited an HA-type structure. FTIR indicated the possible presence of carbonates on the coatings. A dissolution study indicated a continual increase in Ca and Pi over time. In the cell culture study, enhanced osteoblast differentiation occurred in the presence of additional Ca concentration in the cell culture media. However, additional Pi concentration in the cell culture media was suggested to slow down osteoblast differentiation and mineralization.

INTRODUCTION

Dental and orthopedic implant surfaces have been altered with hydroxyapatite (HA) and calcium phosphate (CaP) coatings, with the assumption that osteointegration of the implants can be improved. As such, properties of HA and CaP ceramics and coatings have been extensively studied. In particular, the crystallinity of HA and other CaP ceramics are of great interest because of their dissolution properties. It is known that amorphous or smaller imperfect crystals have a higher dissolution rate compared with crystalline compounds. It has also been reported that the incorporation of sodium and carbonate ions in the HA and CaP structure greatly increases the dissolution rate.
Despite the slow dissolution rate of highly crystalline HA ceramics, it was also reported that the in vivo cellular response could be compromised by its high crystallinity, indicating that some amorphous or more soluble phases in the coatings would be more desirable and result in a more stable interface with the biological environment. However, the science of the bone-implant interface is still not fully understood. As reported in our previous studies, the ultimate interfacial strength and bone-implant contact length were higher for more amorphous CaP-coated implants than compared with more crystalline CaP-coated implants. As such, in this study, it was hypothesized that osteoblast differentiation and onset of mineralization would be affected by the dissolved calcium (Ca) and inorganic phosphorous (Pi) concentration in the tissue culture media. Thus, the objective of this study was to evaluate the properties of plasma-sprayed HA coatings and to measure the effect of additional Ca and Pi concentration on osteoblast response.

**Materials and Methods**

**Hydroxyapatite**

Sterile plasma-sprayed HA-coated disks (10.1 mm diameter × 2.15 mm thick) were obtained from Friatec AG (Mannheim, Germany).

**X-ray diffraction**

X-ray diffraction (XRD) was performed to evaluate the structure of the HA coatings before the experiment. A Siemens D500 diffractometer (New York, NY) using Cu Kα radiation with energies of 40 keV and 30 mA was used. The incident X rays passed through 3° and 1° slits before impinging upon the CaP coatings. Diffracted X rays passed through 1°, 0.6°, and 0.05° slits at the X-ray counter. Three HA samples were analyzed and the data were collected from 20° to 55° at 20° per minute scan rate. Crystalline coatings were identified by matching the peaks with standard synthetic HA. The lattice parameters and crystallite size (1 SD) were calculated based on the 410 reflection for the a-lattice spacing and 004 reflection for the c-lattice spacing. The crystallite size was calculated by the Scherrer equation.

**Fourier transform infrared spectroscopy**

The molecular composition and structure of HA coatings were evaluated with a Magna-IR Spectrometer 550 ( Nicolet Instrument Corp, Madison, Wis) interfaced with a SpectraTech microscope (Stamford, Conn). Triplicate samples were collected at a resolution of 4 cm⁻¹ and a scan number of 100. The Ti surface was used as a reference for background subtraction.

**Dissolution study**

Hydroxyapatite-coated disks were immersed in a 2.5-mL 1.0-M Tris buffer containing 80 μM NaCl with pH of the solution balanced at 7.4 before the study. The study was performed in triplicates in a sterile and humidified atmosphere of 95% O2 air and 5% CO2 at 37°C for 21 days. The buffer medium was changed daily. As the buffer medium was collected each day, the volume withdrawn and pH were recorded. Each withdrawn buffer medium was saved for subsequent analysis of Ca and Pi ions released.

**Measurement of Pi**

Released Pi ions were measured colorimetrically by using the reaction of ammonium molybdate and ascorbic acid with the Pi to obtain a molybdenum blue complex. The reaction was done in a 96-well microtiter plate. Each sample was diluted 10-fold to make a 100-μL solution. A working solution was made by combining 2 parts double-deionized water, 1 part 5.0 N H2SO4 (Baker analyzed), 1 part 0.01 M ammonium molybdate tetrahydrate (Sigma Chemical Co, St Louis, Mo) in water, and 1 part 10% ascorbic acid (Sigma). The working solution was made fresh for each assay. A 100-μL working solution was added to 100-μL sample. After 1 hour at room temperature, the complex was read at 750 nm on a Dynatech MR5000 microplate reader (Dynex, Middlesex, UK). The 1.0-M Tris buffer containing 80 μM NaCl (pH 7.4) was used as a baseline reference. At an 0.05, statistical analyses for Pi release was carried out by analysis of variance (ANOVA).

**Measurement of inorganic Ca ions**

Released Ca ions were measured with a flame atomic absorption spectrophotometer. The blank was prepared by adding 90.0 mL double-distilled deionized water to 10.0 mL 10× 1% LaCl3 20% HNO3. Samples were prepared by combining 2.0 mL of sample from dissolution media to 0.2 mL 10× 1% LaCl3 20% HNO3. Samples were diluted with 0.1% LaCl3 2.0% HNO3. The samples were measured at 422.7 nm and energy of 49 keV by using a Perkin Elmer 3030 atomic absorption spectrophotometer (Wellesley, MA) with a Perkin Elmer intenstron calcium lamp with a slit of 0.7° and a current of 10 A. The 1.0-M Tris buffer containing 80 μM NaCl (pH 7.4) was used as a baseline reference.
At an $\alpha$ level of 0.05, statistical analyses for Ca release was carried out by ANOVA.

**Preparation of cell culture medium**

Four groups of Dulbecco modified eagles media (DMEM) containing different Ca and Pi concentrations were prepared. The low and high Ca and Pi correspond to the dissolution measured at day 3 and day 21, respectively (Table). In this study, the low and high Ca media were prepared by adding 0.1 and 0.2 $\mu$g/mL of CaCl$_2$ into DMEM media, respectively. The low and high Pi media were prepared by adding 2.3 and 6.1 g/mL of NaH$_2$PO$_4$ into the DMEM, respectively. In addition, a DMEM without additional Ca or Pi was used as control.

**Cell culture study**

Titanium (Ti) disks of 13 mm in diameter and 2 mm thick were ground to 600 grits, ultrasonically cleaned with acetone and ethanol, and passivated with 40% (volume) HNO$_3$ at room temperature. The disks were then sterilized with ultraviolet light for 48 hours before placing them in 24-well tissue culture plates. American type culture collection 1486 human embryonic palatal mesenchyme cells, an osteoblast precursor cell line, were then seeded on the Ti surfaces at a concentration of 20 000 cells/mL. Five groups of media were used for cell culture, and the media was changed twice a week. Triplicate samples were analyzed for cellular differentiation by measuring the alkaline phosphatase (ALP)-specific activity over 9 days post-confluency. Differences in cellular responses to dissolved Ca and Pi were statistically compared by the ANOVA test.

**ALP-specific assay**

On the day of the assay, medium was removed from the cell cultures and the cell layers were lysed with 1 mL Triton X-100 (0.2%). An aliquot of the triton lysate (50 $\mu$L) was added to 50 $\mu$L of working reagent containing equal parts (1:1:1) of 1.5-M 2-amino-2-methyl-1-propanol (Sigma), 20 mM p-nitrophenyl phosphate (Sigma), and 1 mM magnesium chloride. The samples were then incubated for 1 hour at 37$^\circ$C. After incubation, the reaction was stopped with 100 $\mu$L of 1 N NaOH, and the absorbance was read at 410 nm with a microplate reader. Alkaline phosphatase activity was determined from the absorbance by a standard curve prepared from p-nitrophenol stock standard (Sigma). The ALP-specific activity was statistically compared by ANOVA.

**1,25 (OH$_2$) vitamin D$_3$ stimulated osteocalcin production**

1,25(OH$_2$) vitamin D$_3$ stimulated osteocalcin production was measured with a commercially available midtact human osteocalcin EIA kit (Biomedical Technologies Inc, Staughton, Mass). On the day of the assay, the medium was removed from the cultures and stored at 4°C until assayed, when the samples were thawed to room temperature. The samples (25 $\mu$L) or human osteocalcin standard (25 $\mu$L) were added to the microtiter plate that came with the kit. This was followed by the addition of the osteocalcin antiserum (100 $\mu$L). The microtiter plate was then swirled gently for 1 minute and covered, followed by incubation at 37°C for 2½ hours. The solution was then aspirated and the plate was washed 3 times with 0.3 mL of phosphate buffer solution. After washing, 100 $\mu$L of streptavidin-horseradish peroxidase reagent was added to all wells, swirled, and incubated at room temperature for 30 minutes. The media was again aspirated and the plate was washed 3 times with 0.3 mL of phosphate buffer solution. A mixture of 100 $\mu$L of 3,3',5,5' tetramethylbenzidine and hydrogen peroxide solution (1:1) was then added to all wells and incubated in the dark at room temperature for 15 minutes. This was followed by the addition of H$_2$SO$_4$ (100 $\mu$L) to stop the reaction. Absorbance was then immediately read at 450 nm. Osteocalcin concentrations were determined by a standard curve prepared from the osteocalcin kit. Differences in 1,25(OH$_2$) vitamin D$_3$ stimulated osteocalcin production were statistically compared by the ANOVA test at an $\alpha$ value of 0.05.

**RESULTS**

**X-ray diffraction**

As shown in Figure 1, HA coatings were observed to have HA-type structure, with the peaks matching Joint Committee on Powder Diffraction Standards (JCPDS) 9-0432. The sharp and distinct peaks indicated a more crystalline coating. However, a slight shift in the XRD peaks was observed as compared with the peak positions reported in the

<table>
<thead>
<tr>
<th>Day</th>
<th>Released Ca (µg/mL)</th>
<th>Released Pi (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.14 ± 0.01</td>
<td>2.55 ± 0.09</td>
</tr>
<tr>
<td>7</td>
<td>0.17 ± 0.01</td>
<td>3.70 ± 0.13</td>
</tr>
<tr>
<td>14</td>
<td>0.22 ± 0.01</td>
<td>5.48 ± 0.11</td>
</tr>
<tr>
<td>21</td>
<td>0.27 ± 0.02</td>
<td>6.82 ± 0.03</td>
</tr>
</tbody>
</table>

**Table**

Release of calcium (Ca) and inorganic phosphorous (Pi) ions from hydroxyapatite surfaces after 21 days in solution

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JCPDS index. The a- and c-lattice spacings for the HA coatings were 9.39 ± 0.002 Å and 6.88 ± 0.0004 Å, respectively. Crystallite size was 2779 ± 3 Å in the c direction and 461 ± 11 Å in the a direction.

Fourier transform infrared spectroscopy

A representative Fourier transform infrared spectroscopy (FTIR) spectrum of HA coatings is shown in Figure 2. Broad absorption bands in the range 865 to 1039 cm\(^{-1}\) and 1108 to 1414 cm\(^{-1}\) were observed for the coatings, indicating presence of PO\(_4\). The broad band in the region of 1400 cm\(^{-1}\) also indicates possible traces of CO\(_3\). A strong OH band at 3568 cm\(^{-1}\) was also observed for the coatings.

Ca release

During the 21-day period, no significant change from the initial pH of 7.4 was observed. As shown in the Table, a continual increase in Ca release was observed for HA coatings immersed in Tris buffer (Table). Released Ca was observed to increase from 0.14 ± 0.01 µg/mL at day 3 to 0.27 ± 0.02 µg/mL at day 21.

Pi release

Similarly, a continual increase in Pi was also observed for HA coatings immersed in Tris buffer (Table). Released Pi was observed to increase from 2.55 ± 0.09 µg/mL at day 3 to 6.82 ± 0.03 µg/mL at day 21.

ALP-specific activity

From the dissolution study, 0.2 µg CaCl\(_2\)/mL (high Ca), 0.1 µg CaCl\(_2\)/mL (low Ca), 6.1 µg NaH\(_2\)PO\(_4\)/mL (high Pi), or 2.3 µg NaH\(_2\)PO\(_4\)/mL (low Pi) were added to DMEM. As shown in Figure 3, osteoblast precursor cells were observed to induce statistically higher ALP-specific activity when cultured with media containing additional Ca compared with the cells cultured with media containing additional Pi and control media. The ALP-specific activity for cells cultured on high and low Pi media was observed to remain significantly low compared with cells cultured with the control media.

1,25(OH\(_2\)) vitamin D\(_3\) stimulated osteocalcin production

Similar to the ALP-specific activity, the 1,25(OH\(_2\)) vitamin D\(_3\) stimulated osteocalcin production for cells cultured in high and low Pi media was observed to remain significantly low compared with cells cultured with the control media (Figure 4). No significant difference of 1,25(OH\(_2\)) vitamin D\(_3\) stimulated osteocalcin produced by the cells in control media and media containing additional Ca was observed.

DISCUSSION

In many dissolution studies reported in the literature, acidic, basic, and buffered physiological salt solutions have been used.\(^7,13,14\) In addition, the success of an implant was recently suggested to depend on its ability to resorb or degrade, thereby allowing cellular penetration.\(^15,16\) All CaP coatings will degrade or dissolve to some degree, regardless of the degree of crystallinity.\(^17\) Because amorphous phases are expected to dissolve more rapidly than crystalline phases,\(^18\) it is possible that the amorphous CaP phases could control the initial biological response.

In this study, the dissolution properties of HA were investigated with a commercially avail-
able plasma-sprayed HA coating. Because it is known that surface properties of implants play critical roles in bone-implant interactions, the HA coatings were characterized before the cell culture study.

By using XRD analyses, the HA coatings were observed to exhibit a crystalline HA-type structure. No significant difference in the c-lattice spacings was observed between the coatings and the spacings reported in the JCPDS index; however, the a-lattice spacings observed in the coatings was 9.36 ± 0.002 Å compared with the 9.41 Å reported in the JCPDS index. The slight contraction in the a-lattice spacing has been attributed to many factors, such as the presence of carbonates. The crystallite size of the coatings in the c and a directions was 2779 ± 3 Å and 461 ± 11 Å, respectively, suggesting a hexagonal structure. A crystallite size of about 5000 Å has been reported for HA powders. These differences in crystallite size and lattice spacings have been associated with the alteration of structural properties during the plasma-spraying process. In addition, a slight shift in the XRD peak positions of the coatings, as compared with the JCPDS index, suggested a strain associated with interfacial interactions between the Ti substrate and the coatings and also the presence of other contaminants.

FTIR analyses indicated broad absorption bands in the range 865 to 1039 cm⁻¹ and 1108 to 1414 cm⁻¹ for the coatings, suggesting the presence of PO₄. The presence of a band in the region of 1400 cm⁻¹ also indicated the presence of CO₃ in the coatings. A strong absorption band at about 3568 cm⁻¹ was observed in the HA coatings, indicating bounded crystalline OH. This strong OH absorption band indicated that the hydroxyl group was not lost during the plasma-spraying process.

During the 21-day immersion study, a daily change in buffer was used to minimize or eliminate any changes in pH. As expected, the pH of the media remained stable at 7.4 ± 0.1. In a dissolution study, HA was reported to continue to dissolve as long as it is subjected to an undersaturated environment, regardless of the crystalline phase. This continuous release of Ca and Pi was observed in this study. As with this study and in previous studies, Pi released as a result of dissolution of HA into an undersaturated solution consequently yielded a continual increase in Pi in the solutions. However, as observed in this study, the rate of Pi dissolution from HA coatings was different from the dissolution rate of Ca. Difference in rate of Ca and Pi released may be due to the binding of Ca and Pi in the form of a phosphate and the equilibrium possibility of different ion species in solution. It is worth nothing that the Pi release is enhanced in the presence of protein.

In in vitro cell culture studies, 2 other biochemical markers, the ALP-specific activity and osteo-
calcin level, are used as markers for determining osteoblast phenotype and are considered to be important factors in determining bone mineralization.\textsuperscript{25-27} Cells grown on media containing high and low Ca\textsuperscript{2+} were observed to exhibit a significantly higher ALP-specific activity over the course of the study, indicating significantly greater cellular differentiation. It has been suggested that the low ALP-specific activity and 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} simulated osteocalcin production on media containing high and low Pi could be attributed to many factors, including apoptosis of osteoblast cells in culture. In other studies, Pi-treated cells have been reported to display profound loss of mitochondrial membrane potential, suggesting that Pi activated the death program through the induction of a mitochondrial potential, suggesting that Pi activated levels of Pi are at a distance from the site of active bone resorption. In addition, a recent in vivo study reported that the poorly crystalline HA and ß-TCP ceramics inhibited bone regeneration when compared with crystalline HA in a healing tibial wound.\textsuperscript{30} The inhibition of bone regeneration is probably attributed to the local elevated Pi concentration released from poorly crystalline HA and resorbable ß-TCP in vivo.

These observations indicate that the HEMP cells displayed a more differentiated osteoblast-like phenotype on biomaterials surfaces that can release Ca, suggesting that surfaces capable of releasing more Ca may be more advantageous for bone-biomaterial interface reactions. In addition, this study shows the importance of characterizing HA surfaces and the governing effect of Ca and Pi released on the expression of osteoblast characteristics in vitro.

**Conclusions**

Osteoblast cells were observed to respond differently to the different concentration of Ca and Pi in the media. In this study, enhanced osteoblast differentiation occurred in the presence of additional Ca concentration in the cell culture media. However, additional Pi concentration in the cell culture media was suggested to slow down osteoblast differentiation and mineralization.

**Acknowledgment**

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**References**


