Osteoblastic Cell Attachment to Hydroxyapatite-Coated Implant Surfaces In Vitro

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Hydroxyapatite (HA) used as a coating for implants can exhibit varying levels of interaction with the biologic environment. The crystallinity of the HA-based coating has been shown to control the rate of dissolution and appears to play a role in the initial cellular interaction with the implant surfaces. An osteoblastic cell attachment assay was employed to examine the cell attachment to untreated and pretreated (pH 5.2, 24 hours) titanium and HA coatings of low (50%), medium (75%), and high (90%) crystallinity. A slightly higher percentage of cell attachment (%CA) was found on untreated and pretreated HA surfaces as compared to the titanium surface. No significant difference could be found in the %CA between the 3 levels of crystallinity. However, higher levels of %CA were observed on pretreated HA surfaces than on untreated HA surfaces (*t* test, $P < .05$). Elevated calcium and phosphate levels in culture medium did not have any effect on cell attachment. Scanning electron microscopic examinations revealed surface degradation of the HA coating following pretreatment in the simulated inflammatory media (pH 5.2, 24 hours). The results suggest that the altered surface topography may influence the initial cell attachment to HA surfaces.
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Key words: cell adhesion, crystallinity, dissolution, hydroxyapatite, osseointegration, osteoblast

Calcium phosphate biomaterials, primarily hydroxyapatites (HA), have been used as coatings on metal implants for many years in the field of oral implants with favorable results.[1] Hydroxyapatite is generally accepted as an osteoconductive biomaterial that is associated with accelerated bone healing and close adaptation of bony tissue. Animal studies have shown faster bony healing around implants[2] and increased amount of bone-to-implant contact when implants are coated with HA.[3–7] Some investigators believe there is a direct physicochemical bonding of HA to bone and describe the phenomenon as "biointegration,"[8] but the exact mechanism underlying this phenomenon is still unclear.

Hydroxyapatite coatings demonstrate dissolution and interaction with biologic fluid when exposed to physiologic environments. Several factors, such as the chemical composition, porosity, grain size, and crystallinity of the material, have been shown to influence the rate of degradation of HA coatings.[9–11] The degree of dissolution is accelerated by acidic conditions and is inversely related to the level of coating crystallinity.[12–15] It is believed that the release of calcium and phosphate ions into the vicinity of the implant following degradation and the subsequent reprecipitation of supersaturated ions onto the coating surface may play a role in the acceleration of bony adaptation to these surfaces.[16–20]

The primary goal of this study was to examine the initial cellular interaction with HA coatings of varying levels of crystallinity. Using osteoblast-like rat cells as an in vitro model, a well-established

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cell attachment assay was employed to evaluate the attachment behavior on HA-coated surfaces. To investigate the biologic influence of the change in local ion environment following dissolution of the coatings, a similar assay was performed under elevated concentrations of calcium and phosphate.

Materials and Methods

Implant Surface and Coating Preparation. Titanium discs were prepared from commercially pure titanium (Johnson Matthey Alfa Aesar, Ward Hill, MA) by cutting rod stock 12.7 mm in diameter into 5-mm-thick discs. The discs were polished with Carbimet silicon carbide paper discs (Buehler, Lake Bluff, IL) to a 600-grit finish and then sandblasted using 50-μm aluminum oxide particles. The specimens were ultrasonically cleaned in 75% ethanol for 10 minutes, followed by rinsing twice in organic-free, ion-free Milli-Q water (Milli-Q Plus Water Systems, Millipore Corporation, Bedford, MA) and air-drying in a vacuum desiccator. These were then submitted to an industrial coating company (Steri-Oss, Yorba Linda, CA) for application of HA coatings of various levels of crystallinity using a proprietary plasma-spraying technique. Three levels of crystallinity were obtained and characterized by x-ray diffraction. These were reported to have approximately 50% (low), 75% (medium), and 90% (high) crystallinity.[15]

Uncoated titanium discs were prepared by polishing and sandblasting as previously described. These were then solvent-cleaned in methylethyl ketone for 5 minutes and washed in Milli-Q water for 15 minutes. Further acid passivation was performed in 30% nitric acid for 30 minutes, followed by a final, 20-minute rinse in Milli-Q water.

Half of the prepared discs were subjected to pretreatment in CMRL 1066 media (Gibco, Gaithersburg, MD) with 10% fetal bovine serum (Intergen, Purchase, NY) at a constant pH of 5.2. This was maintained for 24 hours to simulate initial contact with host body fluid during the early stage of the inflammatory response. This was achieved by dispensing 1 mol/L hydrochloric acid from a 655 Multi-Dosimat (Brinkman Instruments, Westbury, NY) controlled by a 614 Impulsomat (Brinkman Instruments) and monitored by an E 632 Digital-pH-Meter (Brinkman Instruments). The specimens were then washed with Milli-Q water and air-dried. Before conducting the cell attachment assay, all surfaces were sterilized with ultraviolet light (Model UVGL-58, UVP, San Gabriel, CA) for 20 minutes (254 nm, 200 $\mu W/cm^2$).

Osteoblastic Cell Culture. Rat calvarial osteoblast-like cells were used in this work. According to the method of Ecarot-Charrier et al,[21] cells were harvested from calvarial parietal plates of 3-day-old Sprague-Dawley rats. Isolated calvaria were washed in phosphate-buffered saline with glucose solution to remove all debris. All attached periosteum and endosteum were scraped off, followed by removal of sutures. Parietal plates were then chopped into small pieces and transferred to CMRL 1066 medium with 5% fetal bovine serum and antibiotics (50 μg/mL penicillin, 50 μg/mL streptomycin, and 1.25 μg/mL fungizone). The bone chips were grown at 37°C in 5% CO_2 for 14 to 16 days, with changing of media every other day. Cellular outgrowth was then isolated by a combination of sterile 0.01% trypsin (Difco, Detroit, MI) and 0.1% collagenase (Boehringer-Mannheim, Indianapolis, IN) in Ca^{+2} and Mg^{+2} free saline glucose solution. After transferring to the medium containing serum, the enzyme was inactivated and the cells were pelleted. Packed cells were then resuspended in medium and the cell number was determined by a hemocytometer. The final cell concentration was adjusted to 100,000 cells/mL by adjusting the volume of suspending medium.

Cell Attachment Assays. Tygon tubing (Fisher Scientific, Pittsburgh, PA) was coated with Prosil-28 (PCR, Gainesville, FL) and fitted around the prepared discs to limit cell adhesion to the test surfaces. The cell number in the media suspension was determined with a Coulter counter (Coulter Electronics, Hialeah, FL) before performing the assay. One-half milliliter of cell suspension (50,000 cells/5.0 mL) was incubated on the test surface of interest for 60 minutes. At the end of the incubation period, cell suspensions were carefully pipetted and the surfaces were rinsed twice with Hematall Isotonic Diluent (Fisher Scientific) to remove nonadherent cells on the surface. The unattached cells were then counted by a Coulter counter and the percentage of cells attached was calculated by the methods previously described.[22,23] The sandblasted titanium surface served as the control, while tissue culture polystyrene (PS) plate was used as the reference surface. The percentages of cell attachment on test surfaces were then normalized by the percentage of cell attachment on PS surfaces according to the following equation. This is necessary to minimize the variation in percentage of cell attachment (%CA) on PS surfaces between each test run.

$$\text{CA on test surface} = \frac{\text{attached cell count on test surface / average cell count in media}}{\text{attached cell count on PS / average cell count in media}} \times 100\%$$

Since the increased local concentration of calcium and phosphate ions following coating dissolution is considered to be a potential mechanism for the enhanced osteoblastic activity, a series of cell attachment assays were performed on PS surfaces for 15 min, 30 min, 60 min, and 120 min in regular CMRL 1066 medium (having inherently 1.8 mmol/L Ca^{+2} and 1 mmol/L Pi) and in a medium supplemented to a final concentration of 3.6 mmol/L Ca^{+2}, 1 mmol/L Pi, and 5 mmol/L ß-glycerophosphate (ß-GP).

According to previous experiments[22,24] and the results from the first assay on the PS surface, cell attachment on titanium and tissue culture PS reached a plateau phase after approximately 60 minutes. Thus, a second series of cell attachment assays were conducted on pretreated and untreated titanium and HA surfaces for 60 minutes to evaluate the attachment phenomenon on HA coatings of varying levels of crystallinity.

All assays were performed on 2 runs of 3 samples each, totaling 6 samples for each experimental group. The influence of attachment time and media supplementation on cell attachment was examined by 2-way analysis of variance (ANOVA). Student's *t* test was used to find differences between untreated and pretreated surfaces. Analysis of variance with Bonferroni multiple range test was employed to detect differences among various test surfaces.

Scanning Electron Microscopic Examination of Cellular Morphology. Representative specimens of the test surfaces were selected for morphologic study of the cell-substrate interface under scanning electron microscopy. Immediately following cell attachment assays, specimens were fixed in 3% formaldehyde-glutaraldehyde in sodium cacodylate buffer (Tousimis, Rockville, MD), dehydrated in graded acetone (30%, 50%, 70%, 95%, 100%), processed through critical-point drying (CPD030, Blazers Union, Liechtenstein), and sputter-coated with gold-palladium (SCD040, Blazers Union). The processed samples were then evaluated using an Amray 1820 scanning electron microscope (Bedford, MA) with beam voltages of 10 to 20 kV.

Results

Cell Attachment Assays. Gross inspection of the coated discs after pretreatment in simulated inflammatory media showed that there was considerable dissolution of the low-crystallinity surfaces in comparison with medium- and high-crystallinity surfaces. Exposure of titanium substrates was readily evident on the low-crystallinity disc (Fig 1). The first triplicate of samples revealed that the cell counts on low-crystallinity surfaces were even higher than the average counts before the assay. This led to the assumption that particles dissolved off the coating had also been counted. To correct this situation, another triplicate of samples were subjected to the same pretreatment at pH 5.2 for 24 hours. With no cells in culture media, dislodged HA particles were counted after 1 hour of incubation and were used to correct the original counts. Table 1 shows the data of cell counts on 3 samples. Particle counts dissolved off the low-crystallinity surface were about 30 to 50 times higher than those on medium- and high-crystallinity surfaces.

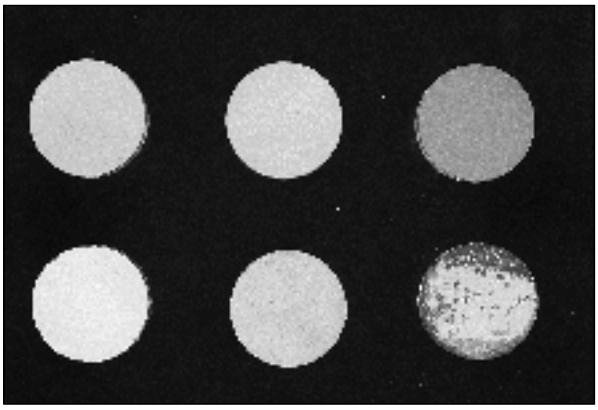


Fig 1 Gross appearance of HA coatings after pretreatment for 24 hours at pH 5.2. The low-crystallinity surface sustained considerable degradation and exposure of titanium substrate. *Top row* = untreated samples; *bottom row* = pretreated samples; *left* = high-crystallinity surfaces; *center* = medium-crystallinity surfaces; *right* = low-crystallinity surfaces.

Figure 2 reveals the results of assays performed on PS surfaces with regular media and supplemented media. Cell attachment increased with time and reached a plateau at about 60 minutes. Two-way ANOVA showed that cell attachment on tissue culture PS was significantly affected by attachment time ($P < .05$) but was not affected by media supplementation ($P > .05$).

Cell attachment on untreated and pretreated titanium and HA surfaces is illustrated by bar charts grouped by implant surfaces (Fig 3) and pretreatment schemes (Fig 4). It was found that mean %CA on PS surfaces varied from 50% to 60% between test runs. To minimize the influence of this variation, all %CA values were normalized by %CA on PS surfaces. A significantly higher %CA was found on pretreated medium- and high-crystallinity surfaces (*t* test, $P < .05$) than the untreated counterparts (Fig 3). On either untreated

Table 1 Cell Counts on Triplicate of HA-Coated Samples

Implant surface	Sample	Unattached cell count	Attached cell count	Particle count	Corrected unattached	Corrected attached	%CA	Normalized %CA†
PS	1	1253	1644	—	—	—	56.7	—
PS	2	1291	1606	—	—	—	55.4	—
PS	3	1376	1521	—	—	—	52.5	—
HA-low	1	3453*	—	2240	1213	1684	58.1	105.9
HA-low	2	4217*	—	2240	1977	920	31.8	57.8
HA-low	3	3879*	—	2240	1639	1258	43.4	79.1
HA-med	1	1449	1448	81	1368	1529	52.8	96.1
HA-med	2	1442	1445	81	1361	1536	53.0	96.6
HA-med	3	1293	1604	81	1212	1685	58.2	105.9
HA-high	1	1198	1669	44	1154	1743	60.2	109.6
HA-high	2	1139	1758	44	1095	1802	62.2	113.3
HA-high	3	1179	1718	44	1135	1762	60.8	110.8

*Low-crystallinity samples showed higher counts than average (average = 2897). Cell counts were corrected by subtracting particle counts from original counts.
†Final %CA was normalized by average %CA on tissue culture polystyrene.
%CA = percentage of cell attachment; PS = tissue culture polystyrene; HA-low = hydroxyapatite with low crystallinity; HA-med = hydroxyapatite with medium crystallinity; HA-high = hydroxyapatite with high crystallinity.

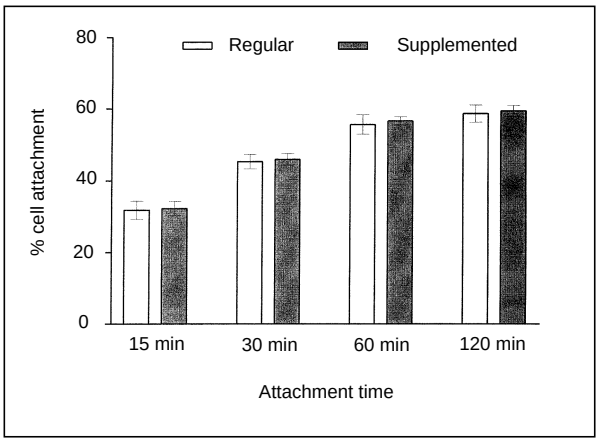


Fig 2 Graph showing cell attachment on PS in CMRL 1066 media and CMRL 1066 media with 3.6 mmol/L Ca^{+2} and 5 mmol/L ß-GP. Percentages of cell attachment increased with time but were not affected by calcium and phosphate supplementation (2-way ANOVA, $P > .05$).

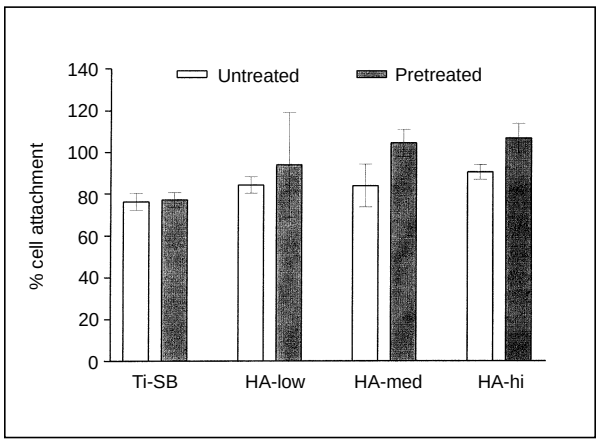


Fig 3 Graph indicating cell attachment after 1 hour to untreated and pretreated titanium and HA surfaces with regard to surface type. All %CA were normalized by %CA on PS surfaces. Sample size for each experimental group was 6. Pretreated medium- and high-crystallinity HA surfaces were shown to have significantly higher %CA than untreated surfaces (*t* test, $P < .05$). Ti-SB = sandblasted titanium; HA-low = low-crystallinity hydroxyapatite; HA-med = medium-crystallinity hydroxyapatite; HA-hi = high-crystallinity hydroxyapatite.

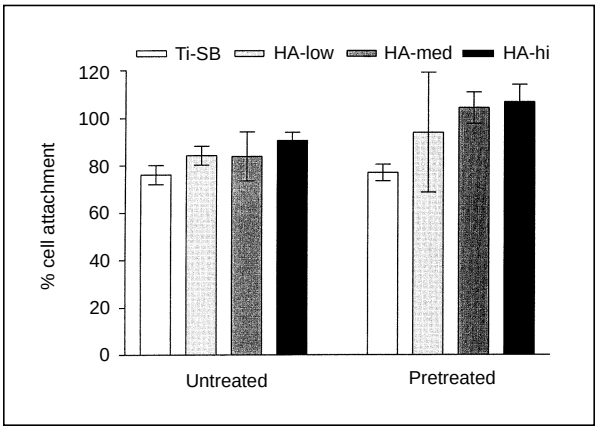


Fig 4 Graph indicating cell attachment after 1 hour to untreated and pretreated titanium and HA surfaces with regard to pretreatment methods. All %CA were normalized by %CA on PS surfaces. Sample size for each experimental group was 6. Analysis of variance revealed significantly higher %CA on untreated and pretreated high-crystallinity HA surfaces than the corresponding titanium surfaces ($P < .05$). Ti-SB = sandblasted titanium; HA-low = low-crystallinity hydroxyapatite; HA-med = medium-crystallinity hydroxyapatite; HA-hi = high-crystallinity hydroxyapatite.

or pretreated surfaces, there was no significant difference for %CA among the 3 HA surfaces of varying crystallinity (ANOVA, $P > .05$). However, the high-crystallinity HA surface was shown to have higher %CA than a sandblasted titanium surface (ANOVA, $P < .05$), with or without pretreatment (Fig 4).

Scanning Electron Microscopic Evaluations. Surface morphologies of titanium and HA coatings before and after pretreatment are shown in Fig 5. Titanium surfaces remained the same after pretreatment. In contrast, topographic changes on HA coatings were evident after pretreatment in the simulated inflammatory environment. These changes suggest partial dissolution of the coating and formation of spherical precipitates as a result of interaction between the surface and the medium. The observed changes were more pronounced on low- and medium-crystallinity surfaces than on high-crystallinity surfaces. Figure 6 illustrates the cellular morphologies after 60 minutes of incubation. Cells appeared to be globular in shape, with filopodial extensions reaching out to surrounding areas, indicating an early stage of attachment. No obvious differences were noted among the attachment morphology on titanium and the 3 HA surfaces, either treated or pretreated.

Discussion

Cellular attachment to the implant surface is an important step in the process of tissue-implant interaction. The aim of this project was to investigate the effect of increased calcium and phosphate ions subsequent to coating degradation on osteoblastic cell attachment and the influence of the crystallinity of HA coating on cell attachment behavior.

The first series of cell attachment assays performed in regular and supplemented (3.6 mmol/L Ca^{+2}, 1 mmol/Pi, and 5 mmol/L ß-GP) medium was intended to investigate the influence of calcium and phosphate released from the HA coatings. The general trend of percentage of cell attachment increasing over time and then reaching a plateau is consistent with a previous study.[24] Results from this part of the study indicated that elevated calcium and phosphate concentrations did not alter osteoblastic cell adherence (Fig 2). Therefore, the release of calcium and phosphate ions into the media as a result of dissolution did not appear to affect cell adherence to implant surfaces.

The attachment result obtained from each test run was normalized by the %CA on tissue culture polystyrene surfaces. This procedure was necessary to minimize the variation between each test run (about 17%), thus yielding more consistent results across all test runs. The use of a primary bone culture, which presented more variable behavior among batches of cultures compared to a transformed cell line, seems to have contributed to this observation.

Several reports[25,26] revealed the active nature of HA coatings when exposed to the biologic fluid. According to Gross et al,[26] degradation of HA coatings in Ringer's solution by cracking and dissolution was followed by precipitation of crystalline apatite that modified the coating topography. Dissolution occurred probably in the amorphous phase, causing liberation of the crystalline segments. As a result of these changes, Anselme et al[25] found relatively low levels of cell attachment and growth on HA coatings. These active and ongoing physicochemical transformations rendered the surface so unstable that it prevented the cellular attachment and growth in vitro. Therefore, it is necessary to pre-expose the coatings to simulated physiologic solution to allow the completion of the dissolution-reprecipitation process leading to a stable HA layer. In the present study, it was decided to pretreat samples in culture medium under pH 5.2 for 24 hours so as to simulate the initial host inflammatory response. Varying degrees of dissolution were observed on HA surfaces. Further, the exfoliation of particles from low-crystallinity surfaces confounds the interpretation of the cell attachment assay. Although the results were corrected by additional control tests, this perplexing factor manifested as a great variability among %CA on low-crystallinity HA surfaces (Fig 3).

Protein adsorption onto an implant surface can take place immediately following contact with serum.[27,28] The protein layer formed by pretreatment in a serum-containing medium may have some influence over cell adherence onto implant surfaces. Okamoto et al[29] recently investigated the adhesion of human osteoblasts onto HA and titanium under various precoating conditions for up to 180 minutes. A significantly higher number of cells were found to adhere to HA than to titanium under serum-precoated or non-precoated conditions. These findings suggest that HA by itself has a higher affinity for osteoblasts in vitro than does titanium. Additional results showed that integrin-related peptides remarkably inhibited the tight adhesion and spreading of osteoblasts onto HA, while no evident inhibition was found on titanium. These authors speculated that HA has a strong adsorptive property for protein, so that serum pro-

Fig 5 Scanning electron microscopic photomicrographs of *(a*) untreated titanium, *(b)* untreated low-crystallinity HA, *(c)* pretreated low-crystallinity HA, *(d)* untreated high-crystallinity HA, and *(e)* pretreated high-crystallinity HA surfaces. Pretreatment was performed by immersion in CMRL 1066 medium at pH 5.2 for 24 hours (original magnification ×1000; bar = 10 μm).

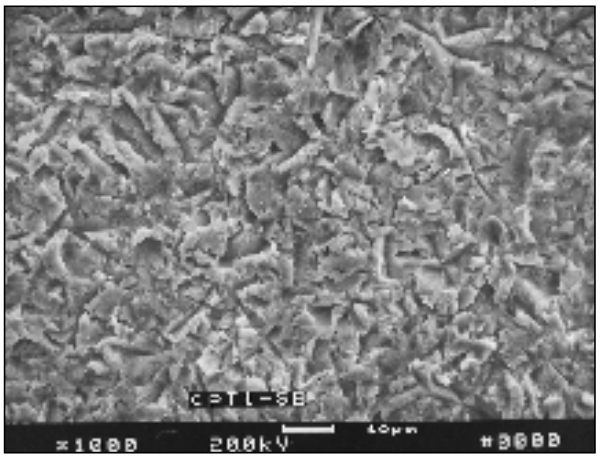


Fig 5a

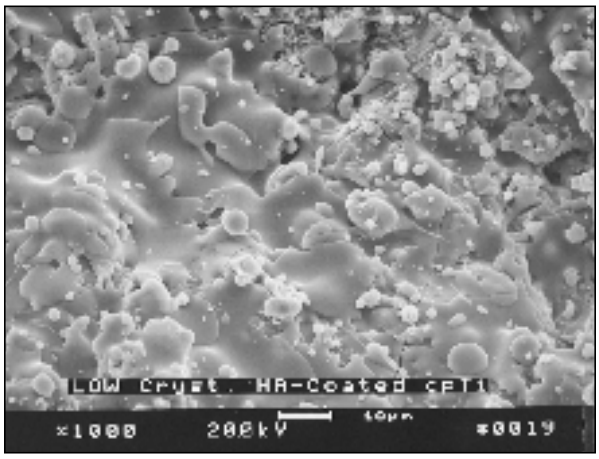


Fig 5b

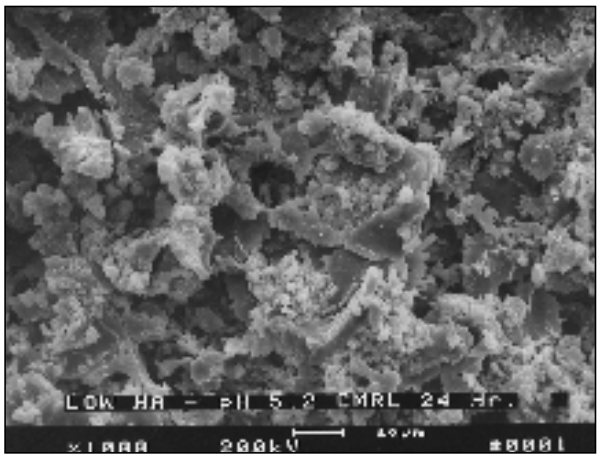


Fig 5c

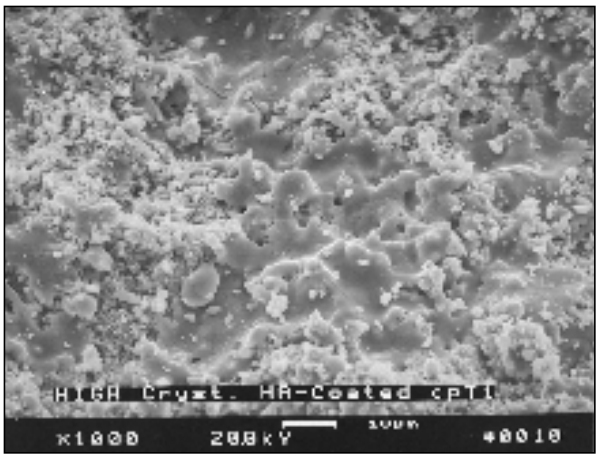
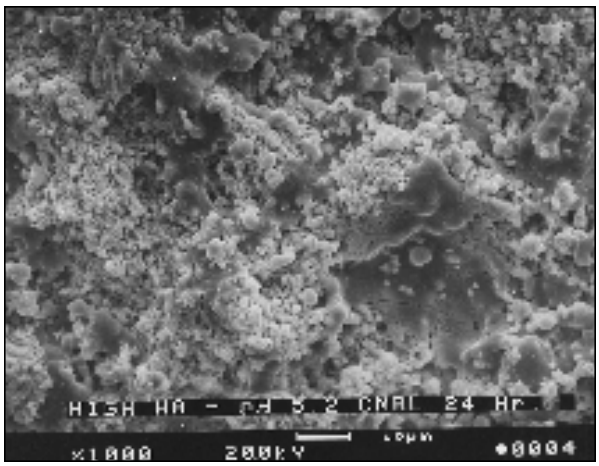


Fig 5d



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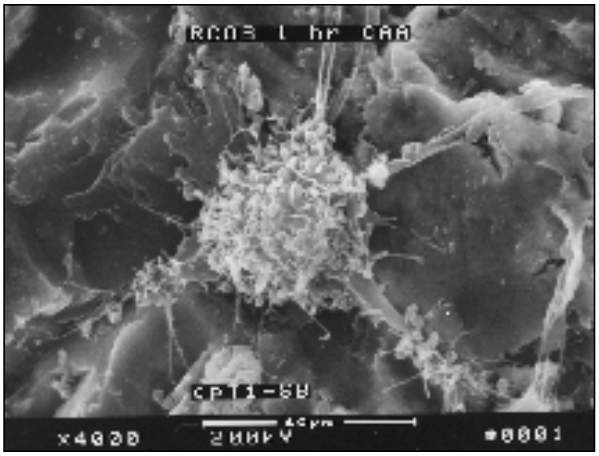

Fig 6a

Fig 6b

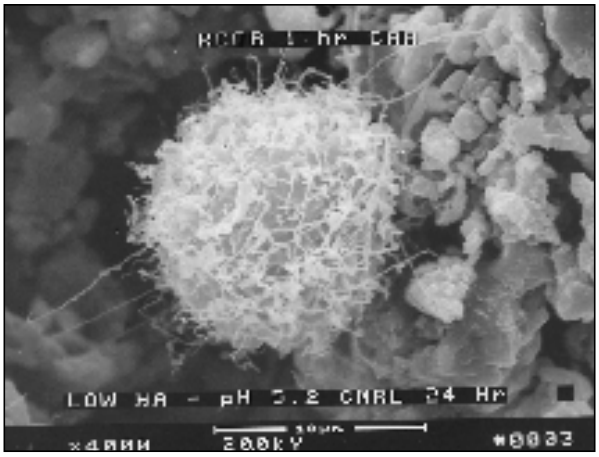

Fig 6c

Fig 6 Cellular attachment morphology on *(a)* untreated titanium surface, *(b)* untreated low-crystallinity HA surface, and *(c)* pretreated low-crystallinity HA surface. Photographs were taken after 1 hour of incubation (original magnification ×4000; bar = 10 μm).

teins are adsorbed to HA and then mediate the specific adhesion of osteoblasts. Results from this study bear a resemblance to the experiment already mentioned showing higher %CA on high-crystallinity HA surfaces than on titanium surfaces (Fig 4). This further supports the notion that serum protein affinity for HA is higher than for titanium. Although the authors did not investigate this phenomenon on HA surfaces, it would be an interesting area worth exploring.

Attachment assays performed on the HA surfaces were unable to distinguish any difference between the 3 levels of crystallinity (Fig 4). Two factors may have accounted for this observation. The dissolution of the HA coating and the subsequent dislodgment of crystalline particles may have masked the difference by contributing to a greater variability in the test group. It is also possible that the affinity of serum protein components for HA surfaces was so high that no apparent difference could be found among the 3 HA surfaces.

Scanning electron microscopic observation of the implant surfaces demonstrated that HA is a bioactive material undergoing constant interaction with the physiologic milieu. Gross et al[26] investigated the in vitro changes of HA coatings and showed degradation of both crystalline and amorphous coatings by cracking and dissolution, as well as formation of precipitated crystalline apatite secondary to supersaturation. Our observations are in general agreement with their findings. Low-crystallinity coatings, having relatively higher amorphous phase content, were particularly prone to dissolution in the simulated inflammatory condition.

Previous investigations in this laboratory demonstrated that higher levels of cellular attachment were found on rough, sandblasted titanium surfaces than on smooth, polished surfaces.[30] This may indicate a potential role of surface topography on cellular attachment in vitro. As mentioned earlier, topographic changes were evident after pre-

treatment in simulated inflammatory conditions, leading to an even more irregular surface microtexture. The present study did not characterize the surface roughness or surface area of the implants. It would be of interest to know and compare the surface roughness as well as the cell attachment on unit surface area.

Ongoing studies indicate that, in addition to precoating with serum proteins, other factors may also contribute to cellular attachment on HA surfaces. Nishizawa et al[31] measured the zeta potentials of hydroxyapatite/tricalcium phosphate sinters and revealed negative potentials of –2 mV to –6 mV. They were able to modify the surface potential through the use of silane coupling agents. Further analysis of initial cell anchorage ratio and adhesive strength on the ceramics led to the conclusion that the cell adhesiveness depended on the zeta potential of the material and that a negative potential was effective in increasing the cellular adhesiveness on these ceramics. Another study conducted by the same group[32] showed that the zeta potentials of TCP and HA ceramics shifted toward a more negative potential after immersion in culture medium. In view of the higher %CA on pretreated HA surfaces observed in this study, the change in surface potential may also play an important role in cell adherence.

Osteoblastic morphology after 1 hour of attachment observed under SEM revealed a globular shape with protruding cellular extensions typical of the early stage of cellular attachment and showed no discernible difference on all test surfaces. Okamoto et al[29] made some interesting observations of the morphology of osteoblasts adhering to HA and titanium after incubation for 12 hours. Osteoblasts closely adhered to and spread on both serum-precoated HA and titanium discs, whereas cells adhered loosely to non-precoated discs without spreading. They also showed that integrin-related peptides inhibited cell spreading on HA discs, while titanium discs were unaffected by the peptides. In the present study, observation beyond 1 hour of incubation, which would allow further cellular spreading on the surface, may reveal subtle differences between the test materials.

The attachment assay used in this study determined the amount of cells attached to the material surface indirectly. It is a relatively simple and easy procedure. However, the assay presented some variables and limitations. First, the number of attached cells was determined indirectly by counting the cells remaining in the media. The particles dislodged from the HA coatings further complicated the situation. Some investigators[29] measured the radioactivity of radiolabeled cells directly from the surface, giving a more accurate count of cell adherence. Furthermore, this approach was unable to measure the strength of adhesion to the test material. In 1996, Nugiel et al[33] developed a micropipette aspiration technique to measure the adhesive force of individual cells on various materials. The adhesion strength was measured by applying negative pressure to the micropipette and then removing the pipette from the surface to detach the cells. This technique requires delicate instrumentation and skillful manipulation. Another method of determining adhesive strength was developed by Suzuki et al,[32] who counted the number of released cells after trypsination in static conditions. Combined quantitative and qualitative analysis of cellular attachment to implant surfaces should provide a more detailed description of attachment behavior.

Conclusions

Hydroxyapatite coatings on titanium were altered by interaction with culture medium. Dissolution results in topographic changes of the surface. Cellular attachment to HA surfaces was slightly higher than titanium surfaces, thus confirming the bone-enhancing properties of HA. The level of HA crystallinity seems to have no influence over initial cell adherence in either the untreated or pretreated conditions. However, the higher osteoblastic attachment on pretreated HA surfaces observed in this study implies that the active interaction of HA with living tissue plays an important role in the initial cellular response to this material.

Acknowledgment

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