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IMMUNOCYTOCHEMICAL AND LECTIN-GOLD CHARACTERIZATION OF THE INTERFACE BETWEEN ALVEOLAR BONE AND IMPLANTED HYDROXYAPATITE IN THE RAT

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Abstract

The tissue response to implanted hydroxyapatite (HAP) in rat alveolar bone was investigated using morphological and cytochemical approaches. High-resolution immunogold labeling was used to examine the distribution of two non-collagenous bone proteins, osteopontin and osteocalcin, and two plasma proteins, α2HS-glycoprotein (fetuin) and albumin. Glycoconjugates were characterized by lectin-gold cytochemistry using Ricinus communis I agglutinin and Wheat germ agglutinin. Transmission electron microscopy demonstrated the presence of an electron-dense, lamina limitans-like layer at the HAP particle-bone interface and organic material within the micropores of the particles. Mineral was observed in relation to both these sites. The HAP particle-bone interface was strongly immunoreactive for osteopontin; moderately to weakly reactive for osteocalcin, α2HS-glycoprotein and albumin, and rich in glycoconjugates containing galactose and/or N-acetyl-glucosamine/sialic acid. Organic material within the micropores of the HAP particles was also labeled with the various antibodies and lectins. These results indicate that the lamina limitans at the HAP particle-bone interface is enriched in osteopontin as well as in sugars present in this and other molecules. Similarly, certain bone and plasma proteins infiltrate the micropores of HAP and distribute differentially throughout the particles.

Key Words: Hydroxyapatite, biomaterial, bone, interface, ultrastructure, lectin cytochemistry, immunocytochemistry, osteopontin, osteocalcin, albumin, α2HS-glycoprotein.

Introduction

Hydroxyapatite (HAP), because of its biocompatibility, is commonly used as an implant material for bone augmentation and reconstruction, and in particular for dental applications (reviewed by Frame, 1987; Kenney and Lekovic, 1991; Matukas et al., 1991). Substantial information is available regarding the morphological events that occur following implantation of HAP into bone defects (Jarcho et al., 1977; de Lange, 1990). The sequence of events that take place in response to HAP include an inflammatory response that accompanies the surgical procedure, formation of granulation tissue, deposition of bone and its subsequent mineralization, and ultimately bone remodeling (Krizek, 1983; Ziats et al., 1988; Gross and Müller-Mai, 1990).

Integration of a biomaterial is in part due to its interaction with the host tissue, an event which occurs at the tissue-implant interface. Previous electron microscopic studies on HAP implanted into experimental bone defects have shown the presence of an electron-dense, granular layer at the HAP particle-bone interface (Denissen et al., 1980; Jarcho, 1981; Ganeles et al., 1985, 1990; van Blitterswijk et al., 1986; de Lange et al., 1990; van Blitterswijk et al., 1985, 1990; Ravaglioli et al., 1992). Although the origin and organic composition of this layer remain obscure, it has been speculated by these authors that it might play an important role in HAP-bone interactions. The purpose of this study was to examine, using a combination of morphological and cytochemical approaches, the tissue response to HAP implanted into an alveolar bone defect created in the rat maxilla. More specifically, we have focused on characterizing the extracellular matrix present at the HAP-bone interface.

Materials and Methods

Experimental model

Male Wistar rats (Charles River, St.-Constant, QC) weighing 200-250 g were used in this study. Three-walled periodontal osseous defects (approximately 1 mm x 1 mm x 1 mm) were created along the palatal root of
the maxillary first molar and sterile HAP particles (Kyo­
cera, Kyoto, Japan) were implanted into the defects as­
described previously (Kawaguchi et al., 1992). Precipi­
tated calcium-phosphate particles were sintered at 900°C
for 2 hours producing highly-crystalline HAP as deter­
minded by X-ray diffraction. Particles were a maximum
of 100 \( \mu m \) in diameter, had a 45% porosity, and the mi­
cropore diameter was approximately 100 nm as deter­
minded by transmission electron microscopy (TEM). The
animals were sacrificed at 2 and 3 weeks after implanta­
tion.

**Tissue preparation for morphological analyses**

Animals were fixed by perfusion through the heart
and into the aorta with 2.5% glutaraldehyde and 2% para­
formaldehyde buffered with 0.1 M sodium cacodylate
(pH 7.3). The maxillae were dissected and immersed in
the same fixative at 4°C for an additional 24 hours.
Some of the tissue specimens were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 2 months,
while others were prepared as non-decalcified samples.
After a brief washing with the same buffer containing
8% sucrose, tissues were post-fixed in 2% osmium te­
troxide for 2 hours, dehydrated through a graded series
of ethanol followed by pure propylene oxide, and em­
bedded in Epon 812. Thick sections (1-2 \( \mu m \) thick)
were cut with glass knives, stained with toluidine blue
and observed by light microscopy. Thin sections were
either left unstained or were stained with uranyl acetate
and lead citrate, and then examined with a JEOL JEM-
100S transmission electron microscope operated at an
accelerating voltage of 80 kV.

Some samples of the HAP particles were sputter­
coated with gold and examined in a JEOL JSM-6300F field emission scanning electron microscope (SEM)
operated at an accelerating voltage of 15 kV.

**Tissue preparation for immunocytochemistry and lectin cytochemistry**

Animals were fixed by perfusion with 1% glutaral­
dehyde in 0.08 M sodium cacodylate buffer (pH 7.3)
containing 0.05% calcium chloride. After tissue dissec­
tion, the samples were additionally fixed by immersion
in the same fixative for another 3 hours at 4°C. Follow­ing
decalcification for 1 week in 0.5 N HCl contain­ing
0.1% glutaraldehyde (McKee et al., 1991), the sam­
ple were washed and dehydrated through a graded series
of ethanol and embedded in LR White resin
(Marivac, Halifax, NS). Thin sections of the tissues
were cut with a diamond knife, recovered on Formvar­
and carbon-coated nickel grids, processed for immuno­
cytochemistry or lectin cytochemistry as described
below, and stained with uranyl acetate and lead citrate.
Sections were examined with a JEOL JEM-1200 EX-II
TEM operated at an accelerating voltage of 60 kV.

**Immunocytochemical procedures**

The protein A-gold method (reviewed by Bendayan,
1989) was used for immunocytochemical labeling of
bone and plasma proteins. Grid-mounted tissue sections
were first floated for 5 minutes on a drop of 0.01 M
phosphate-buffered saline (PBS) containing 1% oval­
bumin (Sigma Chemical Co., St. Louis, MO). They
were then transferred and incubated for 1 hour at room
temperature on a drop of goat anti-rat osteopontin anti­
serum diluted 1:20 in PBS (Mizuno et al., 1991), goat
anti-rat osteocalcin antiserum diluted 1:50 in PBS
(Hauschka et al., 1983), rabbit anti-rat \( \alpha_2 \)HS-glycopro­
tein (fetuin) antiserum diluted 1:30 in PBS (Mizuno et
al., 1991), or rabbit anti-rat serum albumin diluted 1:30
in PBS [Organon Teknika, Inc. (Cappel), Scarborough,
ON]. After antibody incubation, the thin sections were
washed in PBS and incubated with protein A-gold (8 or
14 nm in diameter) for 30 minutes at room temperature.
The grids were then washed with PBS and distilled
water. As controls, some sections were incubated with
protein A-gold alone, and non-immune serum or an un­
related antibody anti-amelogenin (Slavkin et al., 1982),

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**Figure 1.** Scanning electron micrographs of hydroxy­
apatite (HAP) particles prior to implantation. The mi­
croporous nature of these polymorphic particles can be
observed at higher magnifications (inset). Bars = 50
\( \mu m \) and 0.5 \( \mu m \) (inset).
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Figure 2. Light micrographs of surgical site 2 weeks after hydroxyapatite (HAP) implantation. A. HAP particles are clearly visible within the bony defect situated between the alveolar bone (AB) and the tooth. A portion of the HAP particles closest to the remaining alveolar bone are surrounded by newly-formed, woven bone (arrowheads). B. At higher magnification, the bone is associated with numerous osteoblasts (OB) and osteocytes (OC). HAP particles may be encased in bone (asterisks) or surrounded by other connective tissue elements and multinucleated giant cells (MNC). Bars = 0.5 mm (A); and 50 μm (B).

followed by protein A-gold.

Lectin-gold cytochemical procedures

Lectin-gold and glycoprotein-gold complexes were prepared using 8 nm gold particles (Roth, 1983; Nanci et al., 1989). *Ricinus communis* I agglutinin (RCA) was directly coupled to the colloidal gold, while *Wheat germ* agglutinin (WGA, *Triticum vulgaris*) binding was indirectly revealed using an ovomucoid-gold complex. RCA is specific for galactose (Gal) and WGA for N-acetylglucosamine/sialic acid (GlcNAc/NeuNAc) (reviewed by Benhamou, 1989). The grid-mounted tissue sections were floated on either RCA-gold (diluted 1:20 in PBS) for 1 hour or WGA (25 μg/ml PBS) for 1 hour followed by ovomucoid-gold (diluted 1:20 in PBS) for 30 minutes. As controls, grids were incubated with the lectins in the presence of 0.2 M of their corresponding competing saccharides. The lectins, ovomucoid and competing saccharides were purchased from Sigma.

Results

Morphological analyses

Observations of HAP particles by SEM prior to implantation showed that the particles varied greatly in size and shape (Fig. 1). At 2 weeks after implantation, new bone formation was observed around some of the HAP particles within the alveolar bone defect (Fig. 2A). Where bone had been deposited, numerous osteoblasts and osteocytes were present, whereas multinucleated cells were frequently observed in contact with HAP particles not yet surrounded by bone (Fig. 2B). In calcified sections viewed by TEM, HAP particles consisted of a polycrystalline mass (Fig. 3). At the outermost interface between the bone and the HAP particles, there was a mineralized layer differing in appearance from the
mineral in the adjacent bone or in the HAP particle. This mineralized layer appeared to extend into the micropores of the HAP particles (Fig. 3). Demineralized sections of similar regions showed that the interfacial layer consisted of an organic, lamina limitans-like layer (Figs. 4-8; see a discussion of terminology regarding laminae limitantes and cement lines in McKee and Nanci, 1993), which in some places was continuous with cement lines in bone and additional organic material within the micropores. Indeed, the lamina limitans at the surface of the particles, and the content of the superficial micropores, showed a similar electron density and texture, and an apparent absence of collagen fibrils (Fig. 5B). Putative preosteoblasts (Fig. 4A) or bone (Figs. 5-8) were occasionally found in contact with this structure. Although not always present, the lamina limitans showed considerable variation in its thickness and continuity (Fig. 5A). Micropores near the HAP particle surface generally showed a denser accumulation of organic material than those of the innermost regions of the particles (Fig. 7A).

Immunocytochemical labeling
Sections incubated with osteopontin antibody showed numerous gold particles over the electron-dense, lamina limitans-like layer at the surface of the HAP particles, and over the organic matrix within the micropores (Figs. 4 and 5). Bone was also characteristically labeled with this antibody over 'patches' of granular or reticular material routinely found in mineralized bone ("grey patches"; discussed in McKee et al., 1990, 1991). Osteoid showed little or no labeling. In addition, there was a gradient of osteopontin labeling over the organic material within the micropores (Figs. 7A and 7B), with the number of gold particles varying in proportion to the density of organic matrix. This labeling was greatest at the periphery of the particles. Similar to the reaction observed after incubation with anti-osteopontin, although more diffuse and less intense, immunolabeling for osteocalcin was present over bone matrix and the organic content of the micropores (Fig. 6A). Somewhat less labeling was seen over the lamina limitans at the HAP particle-bone interface. With this antibody, the labeling...
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4A

Bone

HAP

OB

N

pOB

5A

Bone

HAP

OB

N

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Figure 8 (above). Lectin-gold cytochemistry showing binding sites for *Ricinus communis* I agglutinin (A, RCA) and *Wheat germ* agglutinin (B, WGA) to reveal the sugar residues galactose and N-acetyl-glucosamine/sialic acid, respectively. Using these lectins, patches (asterisks) in mineralized bone, and organic material at the hydroxyapatite (HAP) particle-bone interface (arrows) and in the micropores (arrowheads), all label intensely. Insets illustrate the interfacial region in controls where sections were incubated with lectin and the corresponding competing saccharide. CP: cell process. Bars = 1 μm (A and B); and 0.5 μm (insets).

of the intrapore material did not seem to correlate with the density of organic material contained therein.

For plasma proteins, the labeling pattern obtained after incubation with anti-α₂HS-glycoprotein was very similar in distribution and intensity to that obtained with anti-osteocalcin (compare Figs. 6A and 6B). Following incubation of tissue sections with anti-albumin, the *lamina limitans* and organic material within the micropores near the surface showed weak labeling, while the content of the micropores in the more central portion of

Figure 6 (facing page, top). Similar interfacial region as in Figure 5 except after incubation with anti-osteocalcin (A, OC), anti-α₂HS-glycoprotein (fetuin; B, Fet) and anti-albumin (C, Alb). For osteocalcin and α₂HS-glycoprotein, moderate labeling is observed over mineralized bone, the electron-dense, *lamina limitans*-like layer at the hydroxyapatite (HAP) particle-bone interface (arrows) and the content of the micropores (arrowheads). In both cases, the labeling appears more diffuse than for osteopontin (compare to Figure 5). For albumin, some gold particles are present over mineralized bone and the intrapore material, but practically no labeling is observed at the HAP particle-bone interface. CP: cell process. Bars = 1 μm.

Figure 7 (facing page, bottom). Electron micrographs of the porous region of the hydroxyapatite (HAP) particles. Intrapore organic material is generally denser at the periphery of each particle (bottom of each Figure), and diminishes as a gradient more centrally (top of each Figure). Osteopontin (OPN) labeling (A, B) is greatest towards the periphery of the particles, whereas albumin (Alb) labeling (C) is strongest more centrally. Bars = 1 μm (A); 0.5 μm (B and C).
the HAP particle was more intensely labeled (Fig. 7C), as was the surrounding bone matrix (Fig. 6C).

In all control incubations, only a few randomly-distributed gold particles were found over the tissue sections (Fig. 5B, inset).

**Lectin-gold labeling**

RCA labeling showed an intense reaction over the lamina limitans-like layer, 'patches' of mineralized bone matrix and over the organic matrix within the micropores (Fig. 8A). WGA showed a similar distribution of gold particles, however, there also seemed to be an additional, more diffuse labeling over some regions of the bone matrix (Fig. 8B).

Control incubations, in the presence of the corresponding competing saccharide, resulted in a significant reduction in labeling (Figs. 8A, inset; 8B, inset).

**Discussion**

The present results show that during bone formation around implanted HAP particles, an electron-dense, lamina limitans-like layer forms at the interface between the bone and the HAP particles. This finding is consistent with previous reports dealing with the HAP-bone interface in decalcified sections. Denissen et al. (1980) and Jarcho (1981) reported the presence of an amorphous "bonding zone" at the interface between bone and HAP. This layer stained darkly with alcin blue, thus suggesting a proteoglycan content similar to that found in "natural bone cementing substance" (Jarcho et al., 1978). Additional ultrastructural observations have been made on this interfacial layer by Ganeles et al. (1986). Since this electron-dense layer showed features similar to cement lines in bone, it has been speculated that it is similar in nature to this normal bone counterpart (Hench and Paschall, 1974; van Blitterswijk et al., 1985, 1990; de Lange et al., 1990; Davies et al., 1991; Ravaglioli et al., 1992). Continuity between cement lines and laminae limitantes with the interfacial layer at the HAP particle surface was observed (data not shown). In agreement with previous reports (Frank et al., 1991; Neo et al., 1992, 1993), we have observed the presence of a distinct mineralized zone separating the HAP particle from the bone matrix proper on sections of calcified material. Indeed, this interfacial layer, which is more readily apparent in demineralized preparations (Daculsi et al., 1990a), also extends within the micropores of the HAP particles. In decalcified specimens, both these regions show the presence of organic material which labels specifically for various bone non-collagenous and plasma proteins. As for the bone-titanium interface in vivo (Albrektsson et al., 1983), implanted HAP rods are surrounded by a "cement line-like" layer which mineralizes (Orr et al., 1992). This layer, however, was not consistently present at all sites around a particle and varied in thickness. Similar data have been reported in other experimental animal models (Neo et al., 1992) and in clinical applications (van Blitterswijk et al., 1990). The observed regional variation in the presence and thickness of this interfacial layer may reflect substantial heterogeneity in both local cellular activity and extracellular matrix events (McKee et al., 1992, 1993). Cultured osteoblasts and bone marrow cells on HAP surfaces also express an electron-dense layer at the HAP-mineralized tissue interface (Brook et al., 1991; Sautier et al., 1991, 1992; de Bruijn et al., 1992).

The initial host response after HAP implantation is generally marked by an inflammatory reaction (Krizek, 1983). During this early post-implantation phase, inflammatory cells and proteinaceous materials accumulate in the vicinity of the implant and some of these proteins adsorb onto the implant surface (Klein et al., 1980; Gross and Müller-Mai, 1990; Nishiyama et al., 1990). Products released by the cells, including those adsorbed onto the HAP surface, may change the properties of the surface and, thereby, may influence subsequent events (Thomsen and Ericson, 1991). Whether this initial layer of adsorbed material persists following the various osteogenic activities that occur around the implanted material is unclear. In this regard, prior to bone formation around the HAP particles, multinucleated cells appear to partially excavate both newly-formed bone and the HAP particles themselves (Ogilvie et al., 1987; Kawaguchi et al., 1992; Takeshita et al., 1992; Balsé et al., 1993). Although the tissue response may vary depending on the exact nature of the ceramic implant material used, ultimately, the turnover of matrix components and mineral that occurs during normal bone remodeling around an implant is clearly another means by which an implant-bone interface might be modified. Indeed, interfacial adsorbed molecules may directly or indirectly mediate these resorptive events.

Osteoblasts secrete various non-collagenous proteins including osteopontin, a highly-phosphorylated, sulfated glycoprotein found in bone and a number of other tissues (reviewed by Butler, 1989; Sodek et al., 1992), and osteocalcin, a low-molecular weight, gamma-carboxyglutamic acid-containing protein (reviewed by Hauschka, 1986; Cole and Hanley, 1991). Although the precise roles of these non-collagenous bone proteins are still unknown, they have been shown to have a high affinity for calcium ions and mineral (reviewed by Boskey, 1992; Young et al., 1992). At the electron microscopic level, immunocytochemical studies have characterized the distribution of these two proteins, and more recent reports in chicken and rat have shown that osteopontin is concentrated at cement (reversal, resting) lines, the laminae
Limitantes at cell-lined bone surfaces and the bone/calcified cartilage interface (reviewed by McKee and Nanci, 1993). Based on the structural and functional properties of osteopontin, it is probable that it plays a multifunctional role, including regulation of mineralization and mediation of cell dynamics during osteogenic processes (reviewed by Butler, 1989). However, where bone interfaces with biomaterials, such as HAP, little information is available concerning the nature of the interfacial organic material. Here, we present immunocytochemical evidence showing that osteopontin, osteocalcin and \( \alpha_2 \) HS-glycoprotein comprise, at least in part, the organic layer at the HAP particle-bone interface. Osteopontin, in particular, appears to be a major component of this interfacial zone. The ultrastructure and composition of the lamina limitans-like layer seen at the HAP particle-bone interface, a structure similar to the planar arrangements of protein present as cement lines and laminae limitantes in normal bone, suggest that this 'coating' of protein (containing osteopontin as a major constituent) may serve similar functions at implant surfaces with regard to adhesion, cell signalling and/or the regulation of mineralization.

The osteopontin-containing lamina limitans at the surface of the HAP particles was sometimes present without adjacent osteoid and without the immediate presence of clearly identifiable cells of the osteoblastic lineage. In these cases, it seems likely that osteogenic cells, or other cells, in the vicinity of implanted HAP release osteopontin that subsequently diffuses to exposed surfaces of mineral and functions at these more distant sites. Polyanionic proteins, such as many of the non-collagenous bone proteins, when immobilized on a stable support in vitro, induce and support mineral deposition (Linde and Lussi, 1989). Therefore, it is conceivable that some of the non-collagenous bone proteins, and in particular osteopontin, by being immobilized onto the surface of the particle itself, or onto the initial mineral deposited onto the implanted HAP, may play a role in the early mineralization events that occur at this site.

Some of the plasma proteins synthesized in the liver, such as \( \alpha_2 \) HS-glycoprotein and albumin, are also found in bone (Ashton et al., 1974; Trifitt and Owen, 1977; Smith et al., 1985). \( \alpha_2 \) HS-glycoprotein, in particular, is enriched in bone matrix and has a strong affinity for hydroxyapatite (Ashton et al., 1976; Mbuyi et al., 1982). Furthermore, it has been suggested that \( \alpha_2 \) HS-glycoprotein co-localizes with other bone matrix proteins, such as osteopontin and osteocalcin, and these molecules may thus interact at sites of tissue mineralization (Ritter et al., 1992; McKee et al., 1993). In the present study, the co-localization of these proteins in the lamina limitans-like layer at the HAP particle-bone interface may like-wise reflect these potential mutual inter-

actions. Alternatively, it may simply reflect an affinity of one or more of these proteins for the solid, mineral phase of the HAP particle. However, more careful analysis of early events (i.e., less than 2 weeks) is necessary to elucidate details of the initial sequence of appearance of proteins at the interface and within the micropores. The present lectin-gold cytochemical results demonstrate the presence of Gal and GlcNAc in the organic matrix at the HAP-bone interface. Osteopontin, a glycoprotein having approximately 17% carbohydrate composition by weight, contains, among other post-translational modifications, significant amounts of Gal sugar residues (Prince et al., 1987). Thus, accumulation of osteopontin in the extracellular matrix of bone and at the HAP-bone interface clearly contributes to the labeling patterns observed here with RCA. Furthermore, the intense labeling obtained with WGA also indicates the presence of other glycoconjugates at the sites of labeling. Considering the less specific, but nonetheless selective, nature of lectin cytochemistry relative to immunocytochemistry, it is reasonable to expect that additional, as yet unidentified classes of glycoconjugates exist at the interfacial regions of implanted HAP. Further studies are required to more fully characterize the organic material present at these sites. Indeed, the selective binding of lectins may be useful for the biochemical extraction and purification of this material.

With regard to the material found within the micropores of the HAP particles, a previous ultrastructural study on HAP implantation into human intrabony defects showed the presence of a "honeycomb-like meshwork" of organic material which extends from the electron-dense, granular surface layer throughout the particle (Ganeles et al., 1986). They speculated that this meshwork represents adsorption of organic material onto the individual crystallites composing the particle, a process occurring during infiltration of tissue fluids after implantation. With regard to the mineralization of the micropores, Ogilvie et al. (1987) reported that the overall mineralization pattern within implanted HAP particles occurs either from the surface outwards into the surrounding tissue, or from the 'inside out', in which calcification of the particle is initiated centrally and spreads peripherally. Whichever the case, the formation of small, needle-like microcrystals within the micropores has been demonstrated using a variety of different calcium-phosphate ceramics, regardless of the osseous or non-osseous site of implantation (Daculsi et al., 1990b). These investigators suggested that partial dissolution of the implanted HAP releases calcium and phosphate ions, thereby increasing local mineral ion concentrations to levels capable of precipitating carbonato-apatite. Immediately following implantation, HAP particles are exposed to a myriad of molecules present in plasma and
known to circulate within the bloodstream (reviewed by Weiner, 1986). In the present study, we have shown the extent to which proteins diffuse throughout the micropores (as discussed by Daculsi et al., 1990b) may occur in the absence, or presence, of a proteinaceous milieu. However, it is generally accepted that the normal physiological deposition of mineral in most cases is guided by an organic matrix (reviewed by Weiner, 1986). In the present study, we have shown the co-existence of mineral and organic matrix within the micropores, and have further identified some of the organic constituents contained therein (e.g., osteopontin, osteocalcin, \( \alpha_2 \)HS-glycoprotein, and albumin). The presence and interactions of the identified plasma and non-collagenous bone proteins found at these sites most likely influence this mineralization process.

It could be expected that the initial infiltration of organic material into HAP implants may depend in part on micropore and molecule size. The micropore size of the HAP particles used in the current experiments was approximately 100 nm, a dimension clearly large enough to accommodate the proteins examined in this study. Excluding differences in mineral affinity, it is reasonable to assume that smaller proteins present in the tissue fluid at the time of HAP implantation, or shortly thereafter, may penetrate further into the particles. However, the relatively low molecular weight \( (\sim 6 \text{kDa}) \) of osteocalcin may only partially explain the deep penetration and uniform distribution of this protein within the HAP particles since a larger molecule such as albumin is also found in large amounts in the center of the HAP particles. With the arrival of osteoblasts and following the secretion of non-collagenous bone matrix proteins by these cells, protein-protein and protein-mineral interactions undoubtedly influence the diffusion and accumulation of proteins within the micropores of the HAP particles. Based on similarities in the density, texture and composition of the organic material within the more peripheral micropores and the lamina limitans-like layer surrounding the HAP particle, it is probable that the bulk of this material is similar, only adapting spatially to the surrounding physical constraints afforded by the three-dimensional architecture of the individual HAP particles themselves. Ultimately, it would appear that the accumulation of organic material at bone-implant interfaces is a more general phenomenon similar to events taking place at normally-occurring bone interfaces (reviewed by McKee and Nanci, 1993).

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Discussion with Reviewers

C.A. van Blitterswijk: In earlier studies a lamina limitans-like layer was also observed for the HAP/soft tissue interface at some distance from osseous tissue. Do the authors suspect that this layer would have a similar proteinaceous content as the same structure at the bone/ HAP interface. If the answer to this question is no, and in view of the fact that this fibrous tissue interface is also calcified, then would it not seem unlikely that such bone proteins are related with the mineralization process at the HAP surface.

Authors: Since it is known that plasma proteins can adsorb onto hydroxyapatite [Ellingsen JE (1991) A study on the mechanism of protein adsorption to TiO_2. Biomaterials 12: 593-596], and despite the observation that the soft-tissue-HAP interface appears to be calcified, the lamina limitans-like layer at this interface may indeed show a different composition from that found in a bony environment. However, it cannot be excluded that non-collagenous bone proteins circulating in blood (e.g., osteocalcin) or proteins produced locally by mesenchymal cells may interact with the HAP resulting in their accumulation at the surface of the material.

C.A. van Blitterswijk: It is known that tricalcium phosphate may show a focal deposit at its interface with bone in contrast to the more continuous precipitations with HAP. Do you think that this reflects a different protein coating or is it related to variations in calcium phosphate precipitations (HAP versus tri-calcium phosphate).

Authors: Regarding the appearance of organic and inorganic material at an implant interface, conceptually, there are several possibilities that can be envisaged. Firstly, an initial coating of protein may influence subsequent mineralization events. Secondly, initial mineral may spontaneously precipitate at the surface of the implant and subsequently interact with protein. A third possibility would be that protein and mineral appear simultaneously at the surface of the implant. It could be expected that different materials may influence these processes.


Authors: At the time intervals examined (2 and 3 weeks), we have not observed such globular accretions, although examination of the interface at earlier intervals should provide some insight on the sequence of early events occurring in our system as proposed by Davies et al. (cited in the question) for the various implant materials.

J.-M. Sautier: Several authors assume that there might be a relationship between the bone bonding properties of bioactive material and the composition of the lamina limitans-like structure adjacent to HAP. Do you think that the incorporation of organic components at the interface play a role in the bone-bonding process?

Authors: Whereas non-collagenous bone proteins are known to bind tightly to mineral via certain structural motifs (e.g., polyacidic amino acid domains), and although proteins are known to adsorb onto the surface of biomaterials, the contribution to the "bone-bonding" and biocompatibility process of proteins at the tissue-implant interface remains to be determined. Since the implanted particles sit amidst extensive osteogenic activity, it is likely that some bone will be formed and its individual proteins will be deposited on the particles. Indeed, osteoblasts may view the HAP surface as an interface between an older and a younger matrix, and thereby secrete the equivalent of a cement line onto the particles [for further discussion, see: Nanci A, McCarthy GF, Zalzal S, Clokie CML, Warshawsky H, McKee MD (1994) Tissue response to titanium implants in the rat tibia: Ultrastructural, immunocytochemical, and lectinocytochemical characterization of the bone-titanium interface. Cells and Materials, vol. 4, accepted for publication]. However, when considering these processes related to different implants, it is reasonable to expect that the surface characteristics of the implant itself play a role in the interactions between the implant and bone constituents.

J.-M. Sautier: Intriguing is the fact that immunoreactivity for osteopontin was not localized inside the cell. Do you think that this absence of labeling may be due to the fact that osteopontin is expressed at an early stage of bone cell differentiation and deposited into the extracellular matrix prior to mineralization or to the fact that, in part, osteopontin is taken up from biological fluids?

Authors: Since non-collagenous proteins represent a minor portion of the secretory output of osteogenic cells and since during histological processing of tissues not all
antigenicity will be preserved, it is to be expected that only cells with a significant, and perhaps maximal, synthetic activity will show unequivocal intracellular labeling for proteins such as osteopontin. In this regard, at the post-implantation time intervals examined, the lamina limitans was well-established, and therefore, cells may no longer intensely produce its constituents, thus reducing the immunocytochemical probability of visualizing a reaction in cells. Indeed, even in normal untreated bone, not all osteoblasts will show intracellular immunoreactivity for osteopontin, an observation which suggests that these cells do not function synchronously, in accordance with current concepts of bone modeling and remodeling (reviewed in McKee and Nanci, 1993). Furthermore, we have recently obtained initial data (unpublished) suggesting that treatment of rats with drugs which interfere with the intracellular processing or transport of secretory products (e.g., vinblastine sulfate) may result in an increase in the frequency of osteoblasts showing a distinct intracellular immunoreactivity for osteopontin, an observation consistent with the notion that the absence of immunolabeling in osteoblasts at the bone-HAP interface may in part reflect cellular levels of protein below the detection limit.

J.E. Davies: One small, but troublesome, point arises when comparing the scales of the various transmission electron micrographs. If one measures the implant grain size distribution in Figure 3, simple measurement yields a range of 0.03 to 0.8 \( \mu \)m (approximately measured using a ruler and the scale bar provided). If one carries out the same measurement in, for example, Figure 4 (a, b and c) then the voids (decalcified grains) yield a range of 0.13 to 0.4 \( \mu \)m. This discrepancy applies to both the other transmission electron micrographs and similar measurements of the pore size. Please comment.

Authors: The apparent HAP particle, grain and micropore dimensions vary for calcified specimens visualized by SEM or TEM when compared to histological preparations of decalcified tissues, in part because of the tissue shrinkage known to occur during histological processing. Plane of section most probably also contributes to the range of measurements observed. Furthermore, smaller particles were not excluded from the HAP preparations used in these studies.

G.L. de Lange: What is the opinion of the authors with regard to: (1) Dynamic events which take place at the bone-implant interface? (2) Are there differences between 2 or more weeks after implantation? and (3) What gives the HAP the high bone biocompatibility? Is this caused by non-specific adsorption of osteopontin/osteocalcin and other glycoproteins, or is this due to a more specific extracellular matrix production by osteoblastic cells?

Authors: The issues raised by these questions have been addressed in the answers provided above for the questions of Drs. van Blitterswijk, Gross, and Sautier.

G.L. de Lange: Do the authors have more information on the connective tissue-HAP interface? What difference do they find comparing HAP particles embedded in bone and in periodontal ligament? Do periodontal ligament fibroblasts have a different reaction on HAP compared with other (gingival) fibroblast?

Authors: We have not examined aspects of the soft connective tissue-HAP reaction in the present study and therefore, regretfully, cannot offer answers to these questions.