Mineralized Matrix Production by Osteoblasts on Solid Titanium In Vitro

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MINERALIZED MATRIX PRODUCTION BY OSTEOBLASTS ON SOLID TITANIUM IN VITRO

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Abstract

Rat bone marrow cells were cultured on solid commercially pure titanium discs. Extracellular matrix (ECM) formed by the cells and the ECM/metal interface developed were examined by both scanning and transmission electron microscopy. The ECM most intimately associated with the substratum comprised afibrillar calcium phosphate globular accretions produced by the colonizing osteoblasts. The presence of calcium and phosphorus was confirmed by energy dispersive X-ray analysis. This initial layer acted as a site of anchorage for collagen fibres, produced by the osteoblasts. However, flaps of tissue elaborated during the culture period and manually reflected created a tissue division immediately above the afibrillar layer which indicated that the latter was adherent to the underlying metal oxide surface. The collagen matrix, consisting of networks of fibres, became mineralized with time in culture and also enveloped osteocytes which possessed radiating cell processes to form a bone nodule. This *in vitro* study suggests that while a calcified matrix layer, produced by osteoblasts, may adhere to titanium surfaces, subsequently formed bone is separated from this layer by a zone rich in both proteoglycans (as demonstrated by ruthenium red staining) and collagen fibres.

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Introduction

The clinical success of endosseous implants is dependent not only on surgical technique but also on the biological response to the implant surface in the bony healing compartment. Bioactive ceramics, which may generate biological bonding to their surfaces by collagen interdigitation, are used as bulk implants or coatings on metals. However, the most favourable response to metal surfaces is generally believed to be so-called "osseointegration" which was first described in relation to commercially pure titanium implants (Branemark et al. 1977). Such osseointegrated implants are considered to generate direct bone/implant contact at light microscopic levels while, ultrastructurally, the bone tissue is separated from the underlying metal oxide surface by an interface described as a proteoglycan-glycosaminoglycan-rich zone approximately 20-1000 nm wide (Albrektsson et al. 1983). The chemical composition of the metal implant is considered to play an important role in the morphology of this interface. Johanssen et al. (1989) compared the ultrastructural character of bone tissue interfaces with titanium and titanium alloy (Ti6Al4V) implants. However, the value of their comparative observations was compromised not only by the difficulties in preparation of the retrieved implant interfaces for ultramicrotomy, including the need to partially decalcify the specimens, but also the requirement to employ titanium or titanium alloy magnetron sputtered polycarbonate implant plugs rather than solid, surface passivated, metal implants.

Clearly, to understand both the differential tissue reactions which may be generated by metal implants and the events which result in the establishment of the bone tissue/metal interface, it is necessary to overcome the difficulties associated with ultrastructural examination of retrieved implants. One approach is to use *in vitro* systems to model these phenomena but, until recently, solid metal substrata have only been used *in vitro* to measure cell attachment up to 12 hours (Walter et al. 1989). However, far longer culture periods are required to observe the

effect of biomaterials on, for example, cell secretions and the development of a bonding zone between biological tissues and implants. We have developed an *in vitro* model system (Davies et al. 1990) using discs of commercially pure titanium (cpTi) upon which rat bone marrow cells were cultured for periods of two to three weeks. This procedure enables the establishment of both bone cell and bone tissue interfaces *in vitro* resulting in an interfacial morphology which reflects that reported in *in vivo* experiments.

We report herein the use of this culture technique to elucidate the composition and structure of the immediate interfacial region established between cpTi and forming bone tissue.

Materials and Methods

Titanium Discs

Solid rod cpTi was used in the preparation of these 5 mm diameter and 1 mm thick discs; the surface was hand finished to 600 grit. They were sonicated for 1 h in 2% Decon, washed, and passivated for 1 h in 28% laboratory grade nitric acid. Finally these discs were sonicated for 5 min in each of 5 separate washes of double-distilled water (DDH$_2$O) and then autoclaved for 30 min at 121-132°C.

Cell Culture

Femora obtained from young adult male Wistar rats (120 gm) had their epiphyses removed and the marrow then washed out, using supplemented medium (SM), according to the method developed by Maniatopoulos et al.(1988).

These explanted rat bone marrow cells (RBM) were maintained in tissue culture flasks (Falcon, Div. Becton Dickinson & Co., Cockeysville, MD), in a humidified atmosphere of 95% air with 5% CO$_2$ and refed twice with SM before being used 5 d following explantation.

The RBM cells were enzymatically released from their tissue culture flasks using 0.01% trypsin citrate saline, following a citrate saline wash. The cells were then counted in a Coulter Counter (model ZM, Coulter Electronics, Hialeah, FL) and resuspended in SM after centrifugation and aspiration of the trypsin supernatant, at a cell concentration of 1×10^5/ml.

Individual cpTi discs which had been placed separately in flat-bottomed wells of bacteriological grade plastic 96 microwell plates (Nunc Plasticware, Gibco, Burlington, Ont.) each received a 100μl aliquot of this cell suspension. The cells were allowed to attach during overnight incubation and a further 100μl of SM was then added to each well on the following day. The cultures were refed 3 times a week and maintained for 2-3 weeks, then fixed and processed as described below for examination by either scanning (SEM) or transmission electron microscopy (TEM).

Some discs were incubated for similar periods of time in SM without any cells, to monitor the appearance of the surface of the disc under these conditions.

Scanning Electron Microscopy

Disc cultures were fixed in 1.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.3), then dehydrated in graded alcohols, critical-point dried from CO$_2$ (Ladd Research Industries Inc., Burlington VT), sputter-coated with gold (approx. 10 nm) (Polaron Instrument Inc., Doylestown PA) and examined in a Hitachi (model 2500) scanning electron microscope. Energy dispersive X-ray (EDX) dot map analysis (Link AN 10000 system, Link Analytical Ltd., High Wycombe, Bucks, U.K.) was carried out on selected portions of the surface.

Transmission Electron Microscopy

Other disc cultures (following thorough washing in 0.1 M Na cacodylate buffer (pH 7.2-7.4 at 37°C) were fixed in 1.5% paraformaldehyde/glutaraldehyde in the same buffer for 2 h at 4°C; 1 mg/ml ruthenium red (RR) (Luft, 1971) was added to the fixation solution of some specimens. Further washing in this buffer preceded postfixation by 1% osmium tetroxide in the same cacodylate buffer for 90 min at room temperature (R.T.) except for those samples which had previously been exposed to RR; these were post-fixed for 3 h at R.T. in 1% osmium tetroxide to which RR had been added. All samples were thoroughly washed in the buffer, and the remaining (i.e. non-RR-stained) samples were additionally washed in DDH$_2$O before en bloc staining with 2% uranyl acetate in 50% ethyl alcohol for 2 h at R.T. (to increase contrast).

All specimens were dehydrated through graded alcohols, cleared in propylene oxide, infiltrated first with a mixture of propylene oxide and epon resin and then with several changes of pure epon resin.

These cultures, still *in situ* on their cpTi disc substrata, were then placed in beam capsules and additional pure epon resin mix was added; they were polymerized at 40°C overnight and subsequently at 60°C for a further 3 days. Excess epon was trimmed away from the edge of the disc. Brief immersion in liquid nitrogen caused fracture at the tissue/metal interface due to the differences in coefficient of thermal expansion between polymer and metal. The epon surface produced in this manner showed a mirror image of the 600 grit polishing lines on the cpTi disc thus confirming the presence of the interface within the resin. These embedded cultures were now re-embedded following orientation.

Silver to pale gold thin sections were cut on an ultra-microtome (LKB) and mounted on 1.5% Pioloform (in chloroform) coated single slotted and 200 mesh copper
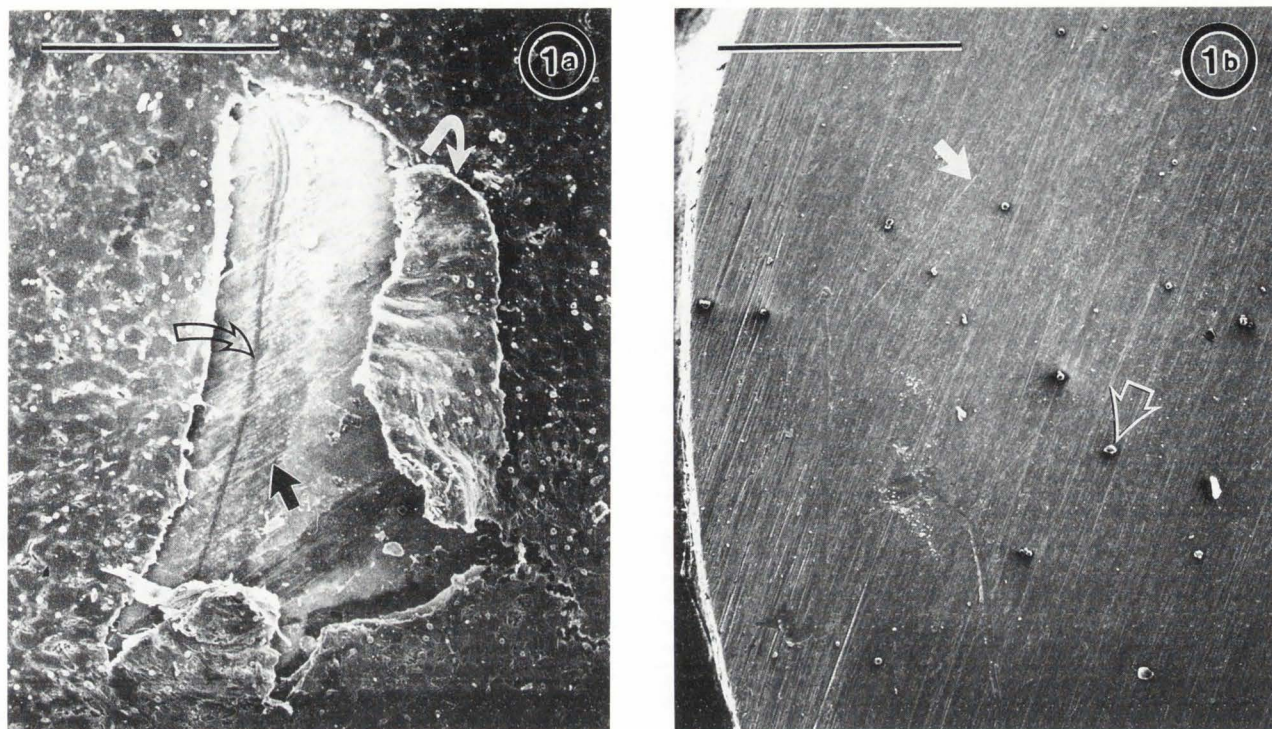
Figure 1 (a) SEM photomicrograph of a cpTi disc inoculated with RBM and maintained as described in the text. The resulting thick deposit of ECM ruptured during processing, a portion of which (curved arrow) withstood elevation and retraction, as shown here. The 600 grit grooves of the underlying cpTi substratum can be identified in the bared area (straight arrow). The hollow arrow indicates a post-culture scratch. Bar = 0.38mm. (b) This cpTi disc was incubated in the same medium but without cells for 11 days. The SEM shows the original 600 grit marks (arrow) and some surface irregularities (hollow arrow). Bar = 0.50mm.

grids. Some sections were double stained first with 3% uranyl acetate in 30% ethanol, and then with lead tartrate acetate (Millonig, 1961). Samples were examined in a Phillips 400 T transmission electron microscope.

Results

During the culture period the bone marrow cells adhered to the cpTi discs and also elaborated an extracellular matrix. The critical point drying procedure caused this matrix to rupture in some areas (Figure 1(a)) and the tissue flaps so created were sufficiently coherent to withstand elevation and retraction. This allowed examination of the sub-surface extracellular matrix which, in some areas, was more tightly adherent to the underlying cpTi substratum than to the overlying tissue. In contrast, the appearance of discs incubated for a similar period in fully supplemented culture medium, but containing no cells, exhibited only some surface irregularities superimposed upon the 600 grit finishing scratches (Figure 1(b)). By comparison of Figures 1(a) and 1(b) it is clear that the extracellular matrix which remained on the cpTi substratum was sufficiently thin to enable the underlying 600 grit scratch marks to still be visualized. The reflected tissue comprised a densely packed collagenous matrix containing both mineralized and unmineralized layers (Figure 2).

In some areas the cpTi-adherent extracellular matrix seemed to be composed of granular crystalline accretions of diameters up to approximately 1μm. These clearly covered the substratum more thickly in some areas than others (Figure 3). Fibrous material was attached to these accretions (Figure 4). On closer examination, fine fibrils (approximately 40 nm diameter) could be seen in intimate association with the crystalline accretions both connecting directly from one accretion to the next and also supporting slightly larger fibrils of approximately 80 nm diameter (Figure 5). EDX dot mapping of this substratum adherent matrix revealed a calcium phosphate composition. Figure 6(a) shows that the most intimate coverage of the titanium substratum by calcium phosphate was relatively homogeneous but as the matrix
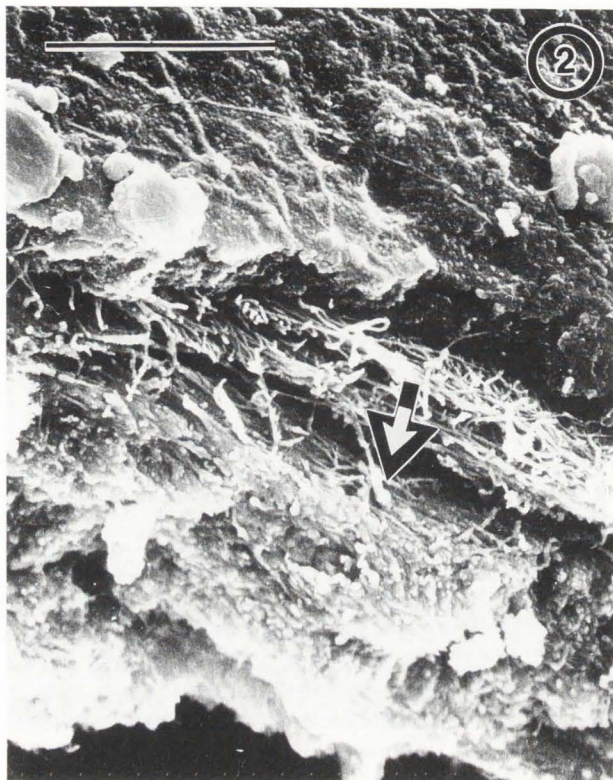
Figure 2 The exposed edge of a portion of multi-layered ECM. A collagen fibre (arrow) can be seen connecting a layer below having a preponderance of globular masses to a more superficial layer which appears to be more fibrous. Bar = 4.3µm.
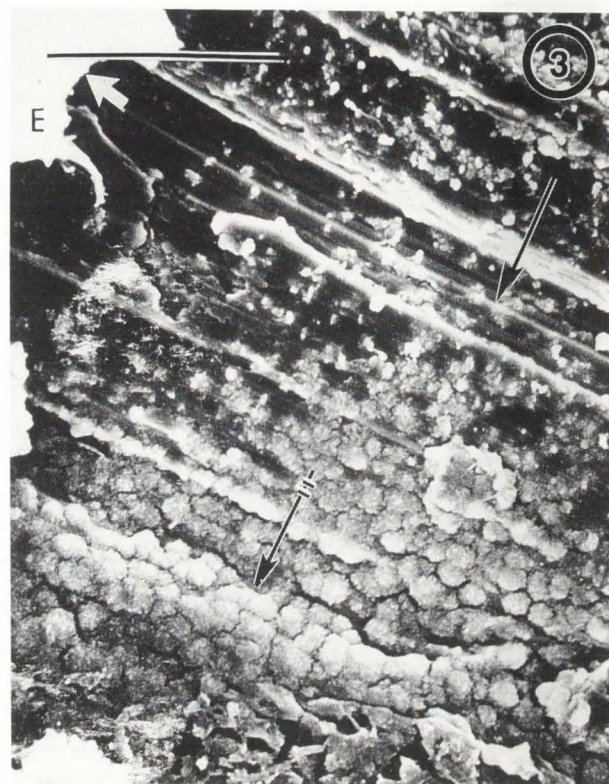

Figure 3 A remnant (E) of the thick-layered ECM which originally covered the field shown in this SEM micrograph can still be discerned in the upper left corner (arrow); the remainder has been mechanically removed in order to reveal the most tenaciously adherent component of this bone cell-generated matrix. Clusters of globular masses can be seen (hatched arrow) which resisted dislodgement in this area. Towards the top of this field, the grooved cpTi substratum is partly visible (arrow) in a region where much smaller and more widely dispersed spherical bodies can be seen. Bar = 6.0µm.

production increased, titanium, calcium and phosphorus dot mapping demonstrated that the titanium signal was diminished by the increasing densities of calcium and phosphorus (Figure 6(b)).

In areas where the substratum was covered by an apparently thicker calcified layer, larger fibres (approximately 0.4 - 0.5µm diameter) were clearly seen to be buried within the matrix and spanning the troughs in the matrix created by the underlying 600 grit scratches on the titanium substratum (Figure 7). In more mature regions of the extracellular matrix the collagen fibre network was far more complex although still associated with the initial calcified accretions (Figure 8).

Figure 9 shows a vertical cross-section through the tissue elaborated during the culture period. On the surface of the tissue a single mononuclear cell is seen with abundant and polarized rough endoplasmic reticulum. Below this cell is a tissue layer approximately 6-7µm thick comprising mainly a collagenous extracellular matrix exhibiting focal areas of mineralization. Dispersed through the extracellular matrix are somewhat flattened cells, resembling osteocytes, possessing radiating cell processes. The degree of mineralization increases ventrally towards the cells most closely apposed to the underlying titanium substratum. However, these cells are separated from the underlying substratum by a layer of apparently amorphous darkly staining material, in the upper part of which unstained collagen fibres can be discerned. In areas where the elaborated extracellular matrix is less developed, this initial substratum-apposed matrix (IF) appeared to be composed of a micro-crystalline material (Figure 10) in which few fibrillar components were evident.
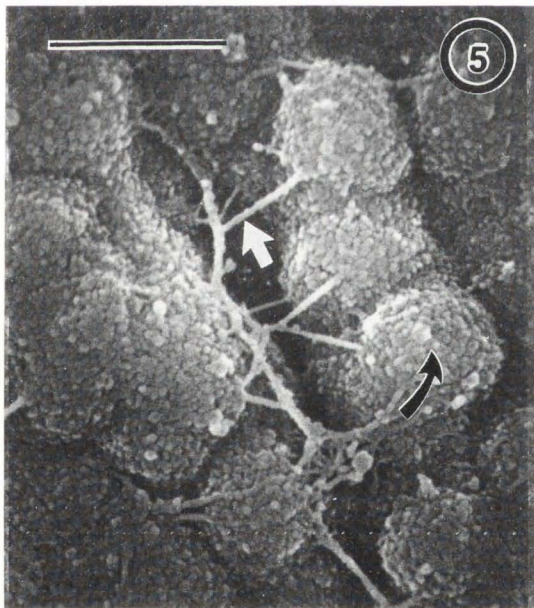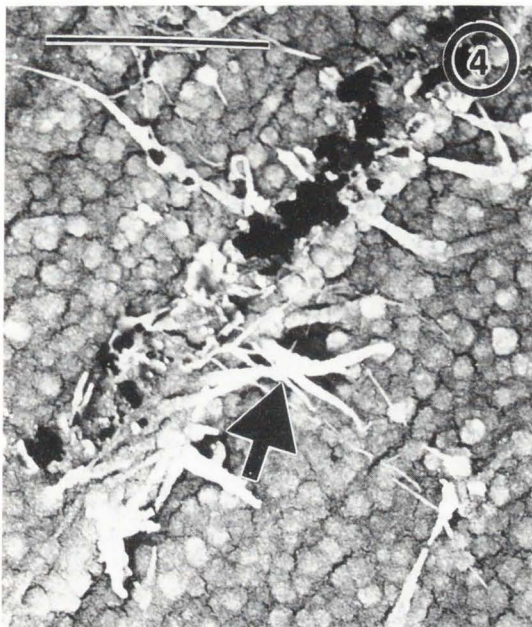
Figure 4 Dense aggregations of globular bodies can be identified. Bundles of fibres can also be seen (arrow) whose thickened ends frequently appear to merge with the mass of globules. Bar = 6.0µm.

Figure 5 Delicate fibre bundles (white arrow) connect some of the globular aggregations of smaller spherical bodies (black arrow) to slightly thicker fibres or to one another. Bar = 1.2µm.
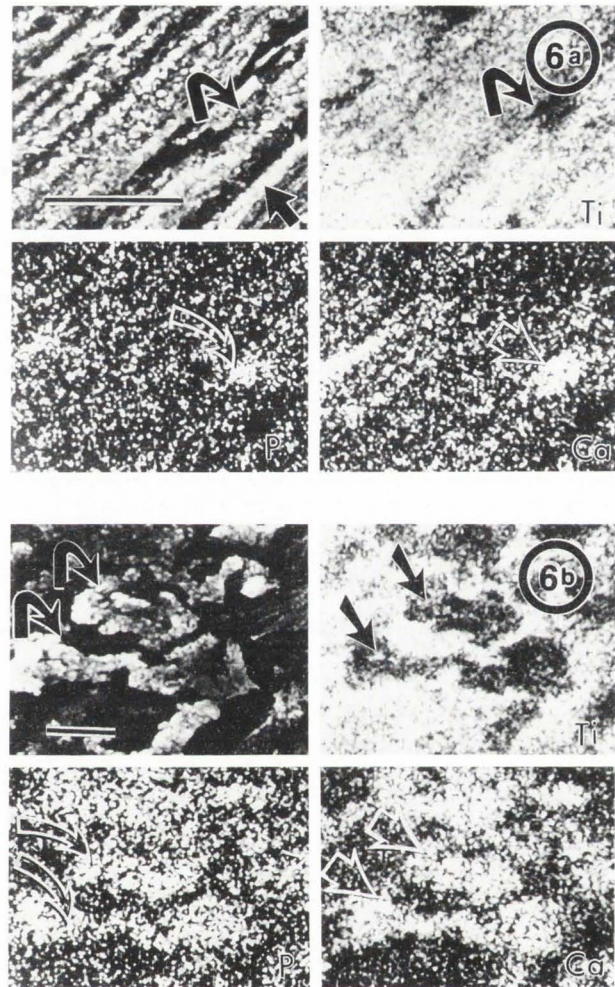
Figure 6 (a) A portion of cpTi substratum had most of its ECM removed and EDX dot-map analysis carried out; its video-captured SEM image is shown in the upper left quadrant. The parallel ridges resulting from polishing can still be detected (arrow) and small thickened portions of ECM can be identified (curved arrow). EDX confirmed the presence of Ti, whose signal was diminished by the thicker ECM remnants (curved arrow, upper right quadrant). Conversely, the Ca and P signals were more evident in those particular areas (hollow arrow, lower right; and curved hollow arrow, lower left quadrant respectively). Bar = 10µm. (b) An EDX dot-map produced in the same manner used for the specimen shown in Fig. 6(a). In this case however, thicker ECM deposits remain (curved arrows) which impede the Ti signal more extensively (straight arrows), thus also providing larger areas for Ca and P signals (hollow straight and curved arrows respectively). Note the corresponding shapes in all four quadrants. Bar = 2µm.

Figure 7 Collagen fibres (arrows) can be seen connecting ridges of aggregated globular bodies (arrowheads) with which the fibres appear to be intimately integrated. Bar = 5.0μm.

Figure 8 The fibre bundles apparent in this SEM (arrow) are more plentiful but less uniform in size and orientation than those seen in the previous Figures. Spherical bodies are again obvious closely associated with the bundles of fibres. Bar = 3.0μm.

There is a striking resemblance between the mineralizing area (M) shown here and the IF in this section. Both regions exhibit linear electron-dense patterns (arrows) as well as a similar degree of granularity of the ground substance.

The matrix of the specimen shown in Figure 11 contains more extensive areas of mineralization than seen in Figure 10 and is therefore more mature. When the ruthenium red-stained interfacial zone (see also Figure 12) is compared to the sample shown in Figure 10 which was not exposed to RR staining, it is apparent that the known affinity of RR for proteoglycans reveals additional features in the interfacial region. Small unstained collagen fibre fragments are widely dispersed in an amorphous RR positive ground substance immediately next to the substratum (bonding zone, BZ) while in the cell adjacent layer (CA) the unstained fibre fragments are more tightly packed and also much larger. In addition, the ground substance here reacts much more positively with RR. The similarity in staining reaction of the interfacial zone and the mineralizing areas depicted in the sample (not stained by RR) illustrated in Figure 10, is also noticeable in the RR-stained specimen illustrated in Figure 11

where the circumferential zone (arrow) of the sectioned mineralizing globules and the deeper, cell-adjacent layer of IF share a similar strongly positive response to RR and an analogous distribution of unstained fibre fragments. Likewise the substratum-adjacent bonding zone (hollow arrow in Figure 11 and BZ in Figure 12) and the central areas of the mineralizing globules have the same lower degree of RR affinity, unstained fibre fragment distribution, and ground-substance appearance.

Discussion

Our results clearly demonstrate that the most intimate extracellular matrix apposed to solid titanium and secreted by osteoblasts is an afibrillar mineralized layer less than 1μm in thickness. This layer evolves by the fusion of individual calcium phosphate-containing globular accretions of approximately 1μm diameter produced at the distal ends of osteoblast cell processes attaching to the underlying substratum. Evidence for the production of these accretions has been discussed, in detail, elsewhere (Davies et al. 1991). In the present context it is assumed
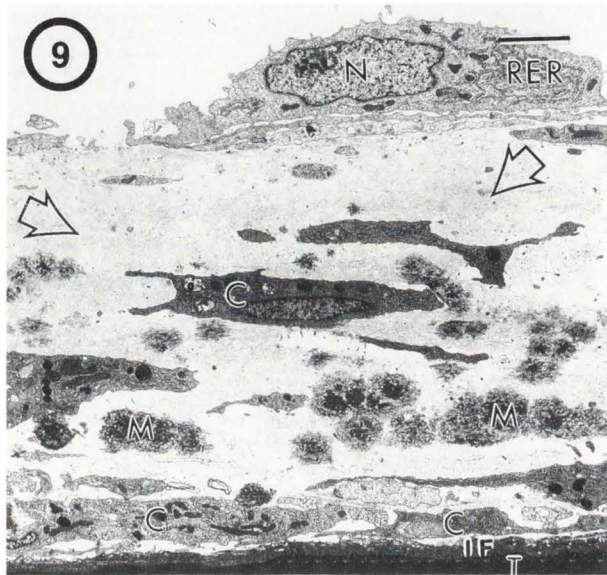
Figure 9 This transmission electron micrograph depicts a vertical cross-section of an RBM culture processed as described in the text. This specimen has been post-stained with uranyl acetate and Millonig's lead tartrate acetate. Part of a cell with well-developed rough endoplasmic reticulum (RER) and a large well defined nucleus N can be seen at the top of the field. Other more flattened cells (C) with extended processes are dispersed throughout the thickness of the ECM, where extensive regions containing massed fine collagen fibre fragments (hollow arrows) and - nearer the substratum (T) - rounded areas of mineralization (M) can be identified. Adjacent to the space previously occupied by the cpTi substratum, in the interfacial zone (IF), is a layer of amorphous ground-substance containing unstained collagen fibre fragments. Bar = 0.98μm.
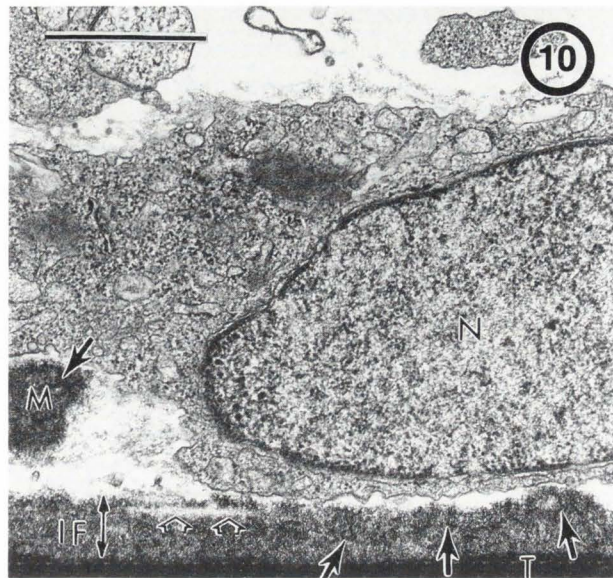
Figure 10 The sample shown in this transmission electron micrograph was processed and stained in the same manner used for that in Fig. 9 (compare with Fig. 12). Part of the nucleus (N) and cytoplasm of a cell can be seen close to the interfacial zone IF, which lies adjacent to the place (T) formerly occupied by the cpTi substratum. A mineralizing area (M) can be seen towards the left side of this field. The granular or microcrystalline appearance of IF and M are very similar, and both of these structures also exhibit linear electron-dense lines (arrows). A collagen fibre fragment (double hollow arrows) can be discerned in the deeper, cell-adjacent part of the IF. Bar = 1μm.

that these calcium phosphate containing accretions would also contain a proteinaceous ground substance that may include specific non-collagenous bone proteins, although we have not attempted protein compositional analyses to date. These accretions would seem to be directly attached to the oxide layer which covers cpTi bulk metals. Interesting evidence has recently emerged to suggest that TiO_2 may act as an epitaxial substratum for calcium apatite growth on both titanium and titanium alloy (Ti6Al4V) solid surfaces (Hanawa et al. 1990).

Using other culture substrata which permit easier sectioning for transmission electron microscopy, we have reported that these accretions only occur on the ventral surface of forming bone nodules and not ubiquitously in our culture systems (Davies et al. 1991). We have also noted that the interfacial morphology is variable on the

solid titanium substrata which we employ herein and the accretions were also limited to the ventral surfaces of the thicker areas of tissue which permitted elevation of flaps. The adherence of this afibrillar calcified layer to the underlying titanium oxide when the tissue flaps were raised could, in part, be due to the conditioning of the titanium oxide surface by calcium phosphate ions as proposed by Hanawa et al. (1990). The relevance of the demonstration of these globular calcified accretions to clinical implantology is supported by recent findings of a similar appearance on both hydroxyapatite ceramic and titanium implants retrieved following implantation studies (C.A. van Blitterswijk, Leiden and L. Sennerby, Gothenburg respectively - personal communications). Furthermore, it is known that osteoblasts may produce afibrillar calcified matrices both at the beginning of their active functional state, as seen in the formation of cement lines at the peripheries of Haversian systems, and when their phase of active extracellular matrix production has
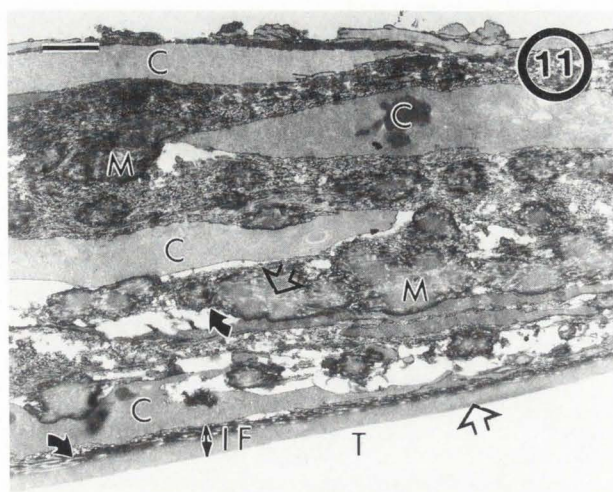
Figure 11 This specimen, which has been treated with RR only, has more mineralizing areas (M) than seen in the previous samples. The interfacial zone (IF) can here be seen to consist of two layers: the upper portion (solid arrow) next to the cell C staining much more heavily with RR and also having a much higher proportion of unstained fibre fragments than the lower part (hollow arrow) lying next to the region previously occupied by the cpTi substratum (T). This duality in degree of response to RR is shared by the mineralizing globules seen in this section, which also display a much more positively reacting circumferential zone (solid arrow) containing more fibre fragments than seen in the internal portions (hollow arrow). Compare this parallel reaction between IF and M with the analogous situation for a specimen not exposed to RR shown in Figure 10. Bar = 1.02μm.

Figure 12 This transmission electron micrograph, of a specimen treated with ruthenium red (RR) and also post-stained with uranyl acetate and Millonig's lead tartrate acetate, is taken at the same magnification used for the less developed sample shown in Figure 10 and shows the interfacial zone in greater detail. Densely packed collagen fibres (closed hollow arrow) in a strongly RR positive ground substance can be seen in the cell-adjacent layer (CA) while in the bonding zone (BZ), next to the space previously occupied by the cpTi substratum T, is a layer whose amorphous ground substance has been less deeply stained and which displays fewer and much smaller collagen fibre fragments (open hollow arrow). The degree of contrast in Figure 12 is greater than in Figure 11 because of post-staining with uranyl acetate and lead tartrate acetate in the former. Part of a cell body containing rough endoplasmic reticulum (RER) can be seen deep to the IF. Bar = 1.27μm.

sharply declined, as witnessed by the mineralized lining of osteocyte lacunae. Thus it is reasonable to assume that the initial afibrillar matrix which we report here on titanium is analogous to similar material formed *in vivo* both in bone itself and on some implant surfaces. However, it should be pointed out that this possible similarity between tissue reactions to dense hydroxyapatite ceramic and titanium implant surfaces is in sharp contrast to the interface established with bioactive calcium phosphate-based materials which demonstrate bone-bonding by collagen interdigitation with their chemically reactive surfaces (see Davies, 1990 for review). Indeed, it is interesting to note that in our previous experiments using this culture technique on solid titanium, we reported that the interfacial mineral layer was reflected intact with the tissue flap and we were able to visualize the ventral surface which provided a mirror image of the troughs and ridges of the 600 grit surface markings (Davies et al. 1990).
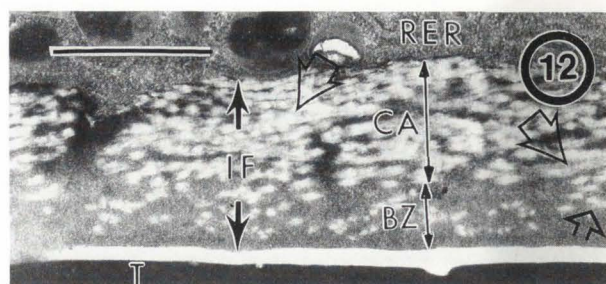
This was not the case in the present experiments since the overlying tissue was less mineralized.

In other words, if the forming bone nodule matures sufficiently to enable the overlying bone matrix to fuse with the underlying afibrillar layer then raising a tissue flap will create a division between the afibrillar mineralized layer and the underlying substratum. Alternatively, if fusion of these matrices has not occurred, which is the case in the work reported herein, division occurs above the afibrillar layer in the zone containing collagen fibres (see Figures 3 and 12). Thus, the *in vitro* method described is capable of examining the temporal establishment of the bone/titanium interface. Furthermore, the elucidation of the bi-phasic response of osteoblasts (afibrillar matrix preceding mineralizing collagenous matrix) would explain in a functional environment, in spite of initial mineralized deposits on the metal surface, how mature bone may approximate to a metal implant surface but be separated from it by both ground substance and a collagenous extracellular matrix (Johansson et al. 1989).

Factors affecting cellular responses to implant materials continue to be intensively investigated (French et al. 1984; Kasemo and Lausmaa, 1988). The influence of

the biological environment on the implant material (Mc-Queen et al. 1982, Sundgren et al. 1986, Michel, 1987), the effect of surface topography on cell behaviour (Lowenberg et al. 1987; Brunette, 1986) and the host tissue response to ion release from implant metal (Finnegan, 1989) are some of the many parameters which have been examined. The *in vitro* model used in this study is clearly suitable for the examination of interfacial reactions between bone tissue and solid metal substrata. In particular, the capacity to examine that portion of the mineralized matrix immediately adjacent to the substratum is of importance since the early response of host cells and tissues to implanted materials greatly influences the eventual clinical prognosis.

This investigation has therefore provided further evidence that, under the conditions described here, normal bone and a normal interfacial zone analogous to those formed in comparable *in vivo* situations can be produced in culture; thus establishing *in vitro* assays with which candidate implant materials can be assessed.

Conclusion

The extracellular matrix secreted by osteoblasts onto titanium, *in vitro*, is an afibrillar calcified layer less than 1μm thick.

Acknowledgements

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Discussion with Reviewers

T. Hanawa: Why did the authors passivate the specimen in nitric acid? After passivation a titanium nitrate layer must surely have formed on the titanium. Doesn't this layer affect the result?

H.-A. Hansson: Different procedures have been used to produce the interface titanium surfaces in studies *in vivo* and *in vitro*. How critical is that for the formation of bone matrix and for ossification?

Authors: The present paper only refers to *in vitro* work and the preparatory procedures involved followed ASTM F86 guidelines which specify passivation treatment of metal alloy implant surfaces with nitric acid. Survey scans of the surfaces employed in our experiments using X-ray photoelectron spectroscopy (XPS) have not demonstrated a nitrogen peak if rinsing, following passivation, is sufficiently thorough. It is true that other groups use different procedures which are developed locally and do not necessarily follow established standard protocols. The question therefore is a central and critical one in the biomaterials field. Until now it has been impossible to satisfactorily judge the biological reaction to subtle differences in surface chemistry produced due to variations in preparatory procedures of implant surfaces because sufficiently sensitive biological probes have not been available. We believe that the type of culture system which we are describing herein and elsewhere (Davies et al. 1991) are beginning to provide such biological tools and hopefully this important question will be addressed in the near future.

H.-A. Hansson: Is it possible to detect leakage of titanium ions from the metal discs? Degradation of the implant and detachment of metal fragments are important questions as implants are supposed to stay for the life time of the patient.

Authors: It would be foolhardy of us to suggest that cell culture experiments lasting days or weeks can mimic the long-term degradation problems associated with implant prostheses placed in biological milieux for the life time of the patient. In the particular system described herein we have not attempted to measure titanium ion leaching.

J.C. Keller: What are the specific components, if any, of the culture system which allow cells to express an osteoblast phenotype?

Authors: This question has been dealt with in some detail in text references Davies 1990, Davies et al. 1990, Davies et al. 1991.

J.C. Keller: In Figure 1b what is the nature of the irregularities present on the surface of titanium? Since the specimen was incubated under the same culture conditions (but devoid of cells) as the other specimens, was there any evidence for deposition or attachment of protein or other serum components on the surface? Could the deposition of such material be a precursor to the epitaxial formation or mineralized particles as suggested by Hanawa? Is there any evidence that mineralization may have, in part, been initiated by such events rather than solely by cellular activities as suggested here?

Authors: The surface irregularities referred to could be due to many things including the relatively rough surface preparation using six hundred grit paper, crystallization of components during sample preparation or simply particulate matter which has contaminated the sample during storage. Since the specimen was incubated under the same culture conditions as the other specimens, we would indeed expect protein adsorption to have taken place but it is impossible to image protein adsorption at this magnification using SEM. The deposition of serum proteins inevitably precedes the deposition of proteins produced by cells and thus will form a component of the interface. Since the work of Hanawa et al.(1990, text reference) used non-protein containing solution we cannot concur that Hanawa suggested deposition of serum proteins being precursors to epitaxial formation of mineralized particles. The present experiments and those reported in Davies et al. (1991) clearly demonstrate that the mineralization observed occurs only beneath forming bone nodules and not ubiquitously in the culture system. If protein adsorption or any other non-bone cell generated pathway was responsible for this mineralization it would be seen elsewhere as well as beneath the developing bone nodules.

J.C. Keller: Are the differences in any interfacial organization depicted in Figures 10 and 12 due to the actual temporal maturation or perhaps localized differences on the surface of the substrate? Were they produced under different culture conditions or the same specimen?

Authors: We believe that these images do represent different stages of time dependent development of the interface. They were produced under the same culture conditions.

S.H. Ashrafi: How do you know the material shown in Figure 9 is a mineralized material? What is the evidence to assume that the calcium phosphate containing accretions contain proteinaceous substances?

H.-A. Hansson: Is the SEM picture of the "granular crystalline secretions" per se sufficient characteristic to allow statements about calcification or must other methods be used in addition?

J.C. Keller: Concerning Figures 6a and b, what where the instrument conditions which were used to prepare the dot maps shown? These conditions could significantly affect the interpretation of the results. Do the calcium phosphate accretions in Figure 6 compare in size,

composition and morphology to those shown in 3 or 4? There is currently no specific information which allows the authors to make the sole conclusion presented.

Authors: The material in Figure 9 is morphologically similar to the mineralized tissue found by all authors who report the formation of bone nodules in culture. We have characterized this using not only routine histological staining such as Alizarin Red and von Kossa but also EDX and electron diffraction at TEM. The EDX information (20 kV, scan time 10-15 mins and approximately 2000 counts/sec) which we report is of course additional to the morphological information gained from SEM. The analysis clearly shows the presence of calcium and this is the basis of our argument. However, we have discussed these calcified globular accretions, of which we provide an example below (Figure 13), in considerably more detail elsewhere (Davies et al. 1991). At the time of writing the original manuscript for this paper we made the assumption that the mineralized non-collagenous matrix was preceded by an organic, probably proteinaceous, secretion because all other naturally formed hard tissues of which we are aware exhibit this trend. We have since, experimentally, shown this to be true in these cultures. Indeed, the initial organic matrix is sulphated which would correlate well with the fact that several of the non-collagenous bone proteins and proteoglycans are highly sulphated.

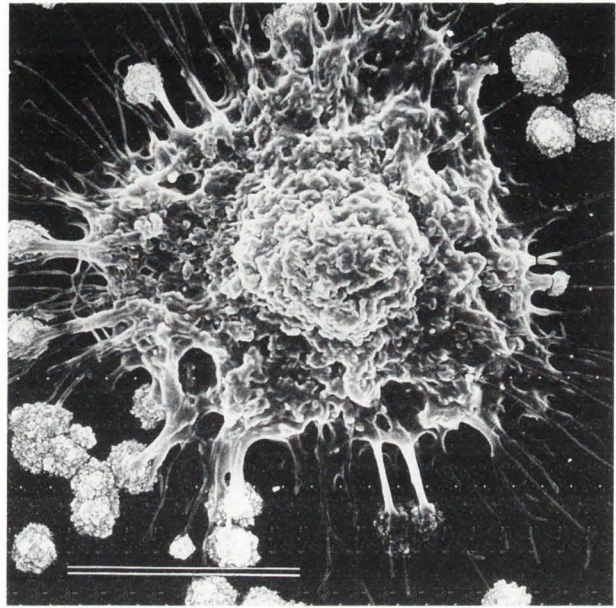We feel the evidence cited provides considerable information which justifies the conclusion reached.

Figure 13: Shows a single bone-derived cell cultured on a polystyrene surface. The distal ends of the cell processes are associated with globular masses of an extracellular material of micro-crystalline form. Bar = 7.5 μm.