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DEPOSITION AND RESORPTION OF CALCIFIED MATRIX IN VITRO BY RAT MARROW CELLS

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Abstract

Rat bone marrow derived cells were cultured using α-Minimal Essential Medium supplemented with antibiotics, ascorbic acid and ß-glycerphosphate in the presence of 10^{-8}M dexamethasone, on polystyrene and hydrophilic fluorocarbon substrata for periods of 2 - 4 weeks. During this time, a large yield of bone nodules was achieved and the elaborated tissue was examined by both scanning and transmission electron microscopy. The matrix produced by the cells contacting the underlying substratum was an afibrillar, globular, calcified material which formed a layer approximately 0.5μm thick. The calcium and phosphorus content of this material was confirmed by energy dispersive X-ray dot mapping analysis. The collagenous matrix of the forming bone nodules was intimately associated with, and anchored to, this layer. The bulk of the bone nodule, above the interfacial zone, was of a normal appearance with osteocytes buried in a collagenous matrix exhibiting spheritic foci of mineralization. The cells, but not the extracellular matrix, of this culture were then removed using a trypsin citrate saline solution and the dishes containing these nodules reseeded with fresh bone marrow cells. These second stage cultures were maintained in supplemented medium, without dexamethasone. During this second period, osteoclasts resorbed both the afibrillar and collagen containing calcified matrices laid down in the first stage of the culture, producing characteristic scalloped osteoclast resorption lacunae.

KEY WORDS: Osteoblast, osteoclast, *in vitro*, afibrillar calcified matrix, bone, mineralization, resorption lacunae, scanning and transmission electron microscopy, energy dispersive X-ray analysis.

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Introduction

In vitro methods to investigate the behaviour of bone-derived cells are used extensively. In the case of osteoblasts and osteoblast-like cells these methods rely on the harvesting of cells or tissue from several species including chick (Osdoby and Caplan, 1980; Tenenbaum and Heersche, 1985; Tenenbaum et al. 1986), mouse (Ecarot-Charrier et al. 1983), rat (Bellows et al. 1986; Maniatopoulos et al. 1988), and human (Auf'mkolk et al. 1985; Robey and Termine, 1985). These researchers have been successful in demonstrating that such cells are capable of elaborating a collagen containing mineralized extracellular matrix *in vitro*, proving that osteoblast phenotypic expression may have been restored in spite of the transitory changes brought about by harvesting and culture procedures. While these experiments have been concerned predominantly with demonstration of (and factors which may affect) osteoblast phenotype, little attention has been paid to the interface formed between the bone derived cells and the culture substratum. An exception to this general rule are applications of bone-derived cell culture to bone biomaterials evaluation although, even there, few reports have emerged as has recently been reviewed (Davies, 1990).

Osteoclasts have also been extensively studied using *in vitro* methods (Jones and Boyde, 1977; Osdoby et al. 1982; Boyde et al. 1984; Chambers et al. 1984). However, while the demonstration of osteoblastic phenotype is relatively straightforward, by proof of production of bone tissue and/or specific responses to biochemical or humoral additions to the culture medium (*vide supra*), the identification of osteoclasts has been more refactory. While certain "classical" morphological and histochemical characteristics of osteoclasts such as multinuclearity and tartrate-resistant acid phosphatase activity provide supporting evidence for the osteoclast phenotype (Osdoby et al. 1982), these are not considered reliable markers of osteoclast differentiation (Hattersley and Chambers, 1989a) and in some species the demonstration

of cell surface calcitonin receptors provides a more specific marker (Hattersley and Chambers, 1989b). However, it is currently widely accepted that the ultimate identification of an active osteoclast can only be achieved by demonstration of the capacity of the cell to resorb a biological calcified matrix (Boyde et al. 1984; Hattersley and Chambers, 1989b). Histologically, hard tissue resorptive cells are provided with unique names depending on the tissue they resorb; for example osteoclasts, chondroclasts and odontoclasts (Sasaki et al. 1988), but *in vitro* methods of identification of "osteoclasts" rely most often on evidence of resorption of either bone (Chambers et al. 1984) or sperm whale dentine (Boyde et al. 1984), although Jones et al. (1984) have shown mammalian and avian osteoclasts to be capable of resorbing many different types of calcified tissues. Since these hard tissues have to be prepared as flat surfaces and usually thin slices from mature tissue, in order to be suitable as culture substrata, they preclude the study of interactions between vital bone matrix and a resorptive osteoclast population.

The aim of the work reported herein was to establish an *in vitro* method to allow both production and resorption of bone matrix which would, in the future, facilitate more detailed study of not only the individual activities of both osteoblasts and osteoclasts but also the interactions between these cells and the bone matrix with which they are both associated. Particular attention has been paid to initial matrix production by bone-derived cells on various polymeric substrata and the resorption of this matrix by osteoclasts.

Materials and Methods

Rat Marrow Cell Culture

Harvesting of Cells Bone marrow cells were obtained from young adult male Wistar rats (approximately 120 gm) according to the method developed by Maniatopoulos et al. (1988). Briefly, for each rat, both femora were removed and washed four times with α-Minimal Essential Medium (α−MEM) containing 1.0 mg/ml penicillin G, 0.5 mg/ml gentamicin and 3.0 μg/ml fungizone. The epiphyses were removed and the marrow washed out using α−MEM supplemented with 15% foetal bovine serum, 50μg/ml of freshly-prepared ascorbic acid (added as 1% of a 5mg/ml stock solution in phosphate buffered saline), 10mM Na ß-glycerophosphate (added as 1% of a 1M stock solution in DDH_2O) and antibiotics at 1/10th of the concentration described above. This medium, of total volume 30ml containing cells from two femora, was further supplemented with 10^{-8}M dexamethasone (DEX) in stage 1 cultures but DEX was not included in the stage 2 cultures described below. The DEX was prepared from a 10^{-4}M stock solution in absolute ethanol diluted to produce a working solution of 10^{-6}M in medium supplemented with serum and antibiotics as described above. This was then used as a 1% solution to achieve the final concentration. The specific concentrations of these culture medium additions employed were those reported by Maniatopoulos et al. (1988). These rat bone marrow (RBM) cultures were maintained in a humidified atmosphere of 95% air with 5% CO_2.

Stage 1 Culture Aliquots of the RBM cell suspension were cultured on one of the three substrata described below: 1ml per 35mm diameter dish and 3ml per 50mm diameter dish. The medium, containing 10^{-8}M DEX, was changed after the first 24 hours to remove non-adherent cells. Subsequently, the medium was renewed three times a week and the cultures maintained from 2 to 4 weeks. Some cultures were prepared for either scanning or transmission electron microscopy at this stage, while others were used for the second culture stage described below.

Stage 2 Culture The RBM cells were enzymatically released from their culture substratum, following a 1ml citrate saline wash, by incubation in 1ml of 0.01% trypsin citrate saline for a period of 1/2 hour at 37°C. Only the superficial cells, that is those not buried within the forming bone nodules, were susceptible to this enzymatic treatment. These cells were discarded and the culture substrata were washed twice with 2ml of standard supplemented medium (without DEX). These were then reseeded with fresh aliquots of RBM cells harvested in an identical fashion to that described above. Half of the medium, without DEX, was changed after the first 24 hours and the cultures maintained, with further thrice-weekly complete medium changes, for one to two weeks.

Cell Culture Substrata

Three substrata were employed for the cell cultures described above: 35mm diameter bacteriological grade and tissue culture polystyrene dishes were both supplied, in sterile form, by Falcon (Div. Becton Dickinson & Co., Cockeysville, MD); and 50mm diameter gas-permeable hydrophilic tissue culture fluorocarbon "Petriperm" dishes supplied by Heraeus GmbH (Hanau, FRG) but now manufactured exclusively by Bachofer GmbH (P.O. Box 70 89, Reutlingen, D7410 FRG).

Scanning Electron Microscopy

Cultures on both polystyrene and fluorocarbon substrata 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 either carbon or gold (approximately 10 nm) (Polaron Instrument Inc., Doylestown PA) and examined in an

Hitachi (model 2500) scanning electron microscope. On all culture substrata, tissue elaborated during the culture period presented some fractures due to the critical point drying procedure. This finding was utilized to further reflect the most dorsal tissue, using a laboratory compressed air supply, thus exposing the underlying cells and tissue which were again coated with either carbon or gold as described above. Energy dispersive X-ray (EDX) dot map analyses were carried out using a Link AN 10000 system (Link Analytical Ltd., High Wycombe, Bucks, U.K.).

Light and Transmission Electron Microscopy

Cultures on the fluorocarbon substrata were prepared for transmission electron microscopy (TEM) following thorough washing in 0.1 M Na cacodylate buffer (pH 7.2-7.4 at 37°C) and fixing in 1.5% paraformaldehyde/glutaraldehyde in the same buffer for 2h at 4°C. Samples were post-fixed in 1% osmium tetroxide in 0.1 M Na cacodylate buffer (pH 7.2-7.4) for 90 min at room temperature. All samples were thoroughly washed in the buffer, and additionally washed in DDH_2O before en bloc staining with 2% uranyl acetate in 50% ethyl alcohol for 2 hours at room temperature (to increase contrast).

The 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. The resin was polymerized at 40°C overnight and subsequently at 60°C for a further 3 days. 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 grids. The sections were double stained first with 3% uranyl acetate in 30% ethanol, and then with Millonig's lead tartrate acetate (Millonig, 1961). Samples were examined in a Phillips 400 T transmission electron microscope.

Results

Bone nodules were present in all cultures on the three substrata. The amount of calcified matrix, as demonstrated by von Kossa staining of whole dishes (not illustrated) clearly showed that, in the presence of DEX, mineralizing matrix continued to be formed throughout the culture period. Routine thick sectioning, prior to

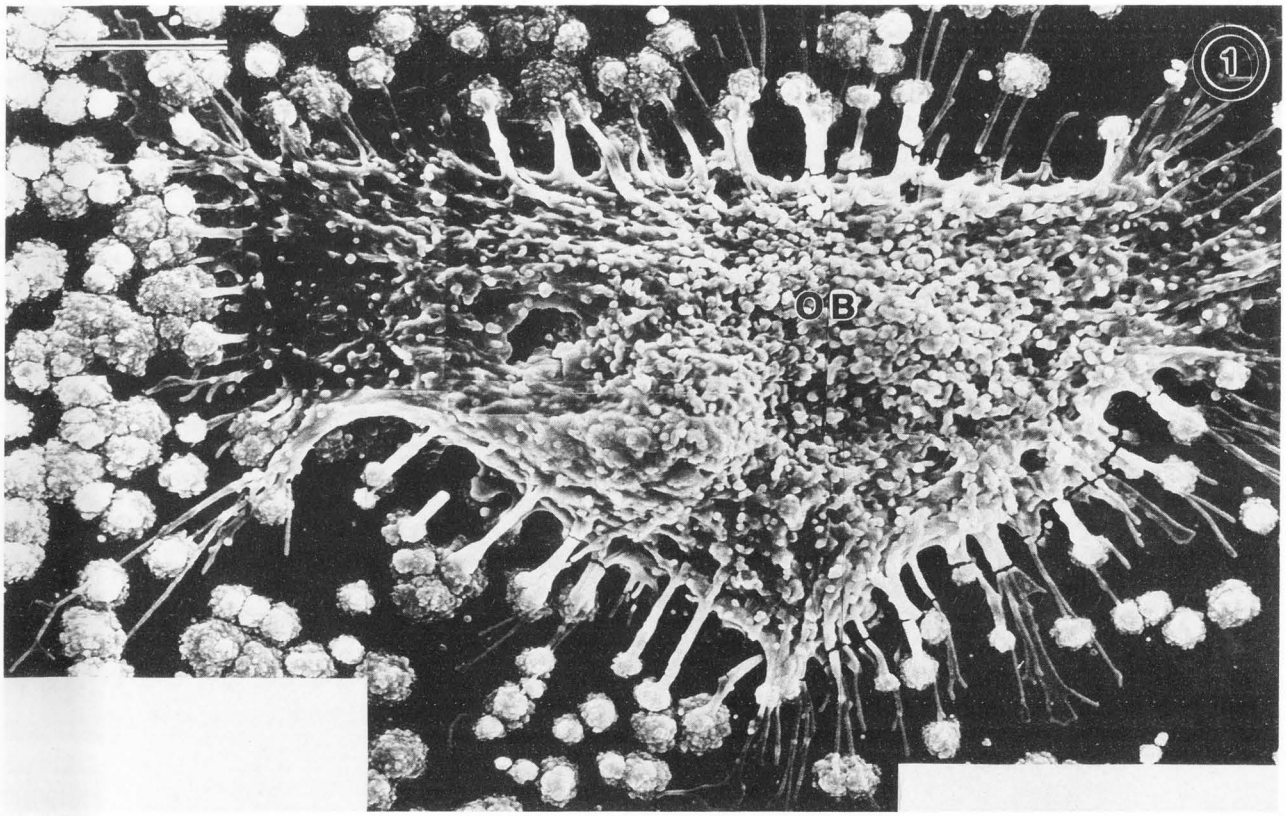


Figure 1 A single osteoblast (OB) anchored to a "Petriperm" tissue culture substratum via small accretions associated with the distal ends of individual cell processes. Bar = 6.0μm.

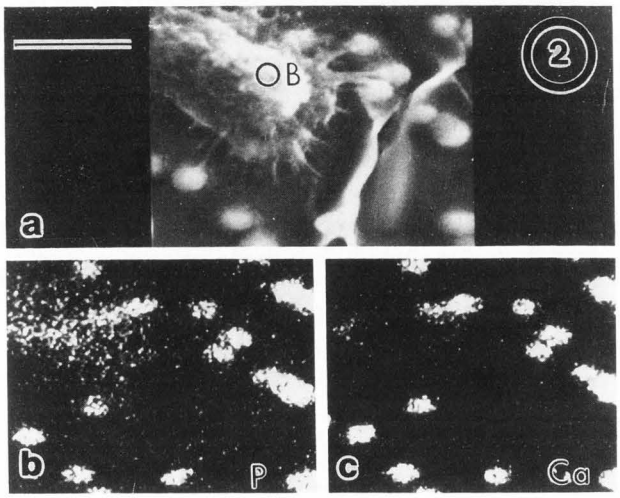


Figure 2 (a) Video captured secondary electron image of a single osteoblast (OB) possessing cell processes at the distal ends of which are accretions onto the underlying substratum. (b) Elemental dot map of image (a) for phosphorus (c) Elemental dot map of image (a) for calcium. Bar = 3μm.
NOTE: The congruence between the accretions in (a) and the P and Ca distributions in (b) and (c).

ultrathin sectioning of nodules on fluorocarbon substrata for TEM, demonstrated bone nodule histology similar to that described and illustrated by Maniatopoulos et al. (1988). Removing the most dorsal elaborated tissue, by blowing with compressed air as described above, and further sputter-coating with gold prior to examination by scanning electron microscopy (SEM), revealed individual cells toward the periphery of forming nodules which possessed numerous cell processes and a ruffled dorsal cell membrane surface. Many of the cell processes associated with such cells terminated, at their distal ends, in what appeared to be small globules of microcrystalline material of approximately 1μm in diameter. Such a cell is illustrated by Figure 1. These globular masses appeared to be attached to the underlying substratum and were thus designated as accretions. They were associated with larger diameter cell processes but, in some cases, smaller diameter processes radiated distally from the globular accretions to be attached to the substratum. Other small diameter cell processes did not terminate in globular accretions, although the total number of small diameter processes, of both types, comprised only about 30% of the total number of processes in the cell illustrated. Similar carbon coated preparations were then made to allow EDX analysis of these globular accretions. Figure 2 shows

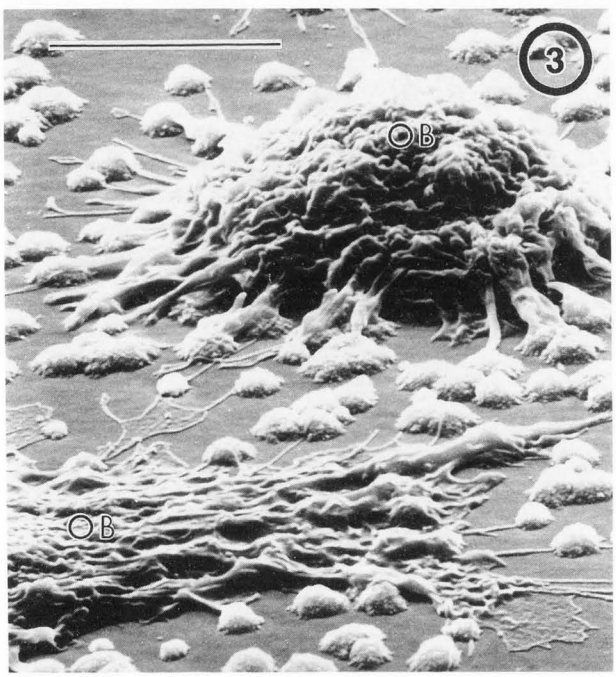


Figure 3 Detail of a field of osteoblasts (OB) with associated calcified, roughly hemispherical, accretions of approximately 1μm diameter. Bar = 6.0μm.

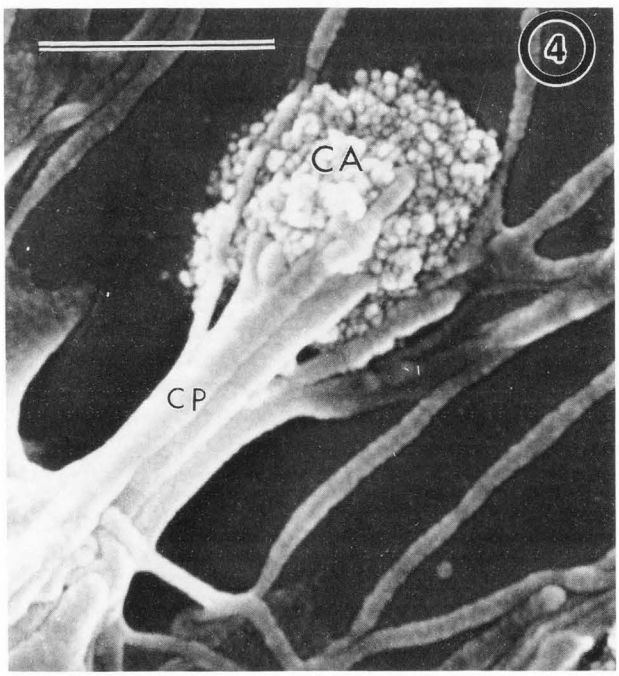


Figure 4 A plaited appearance seemingly generated by a small number of cell processes (CP) closely associated with a calcified accretion (CA) on the culture substratum. Bar = 1.20μm.

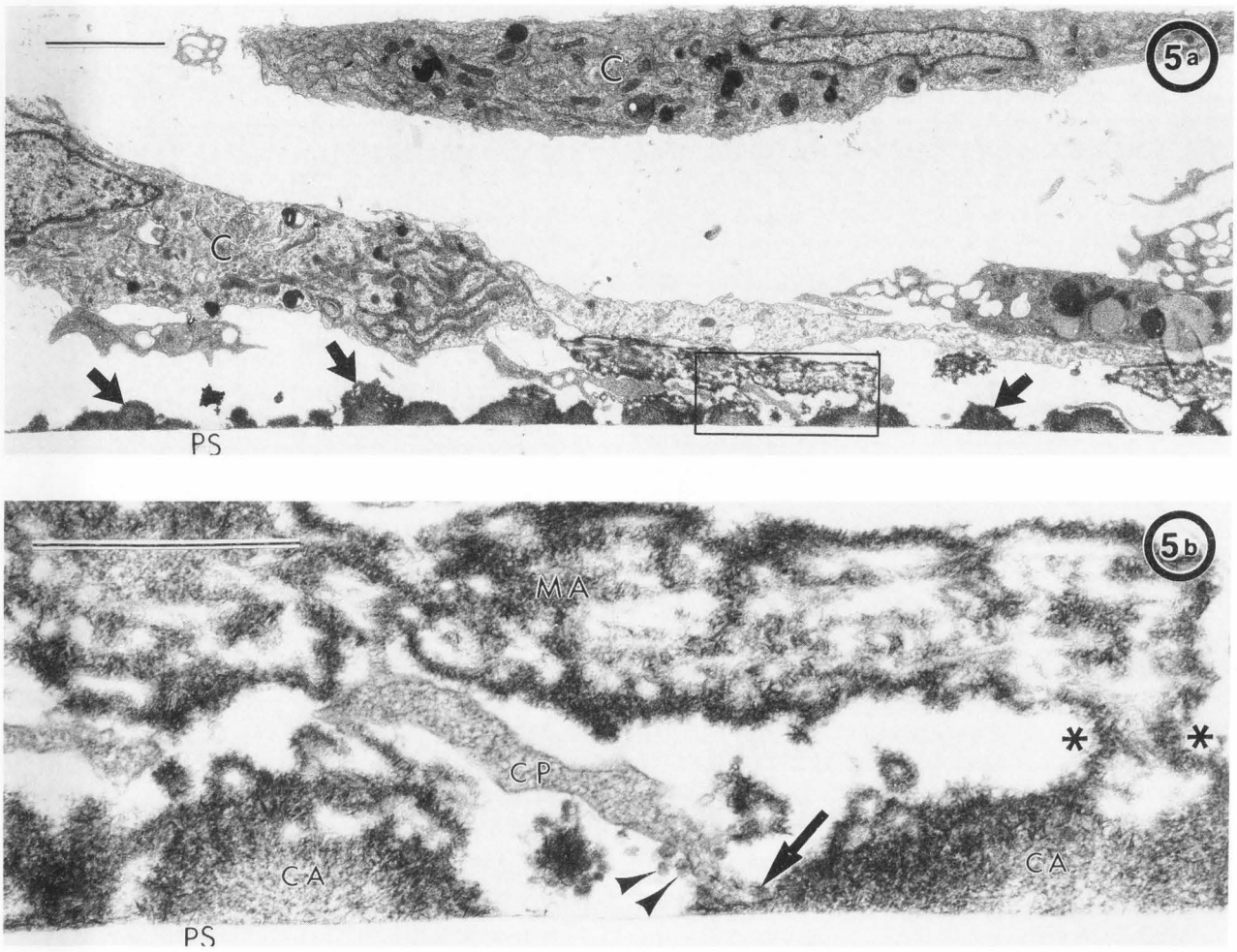


Figure 5 (a) This section of an early forming bone nodule clearly demonstrates that small accretions of approximately 1μm diameter (arrows) have been produced directly on the Petriperm substratum (PS). Neighbouring cells are rich in endoplasmic reticulum and also contain numerous lipid inclusions. Bar = 2.39μm. (b) This detail of the area outlined in Figure 5(a) shows that the calcified accretions (CA) abut directly onto the "Petriperm" substratum (PS). Intimate association with a cell process is shown (arrow) which may be actively secreting further mineral (arrow heads), while a connection is also demonstrated (*) with an overlying collagen containing mineralized area (MA). Bar = 0.86μm.

the result of such an analysis where the calcium and phosphorus dot-mapped signals were congruent with each other and also with the globular accretions which are seen in the video-captured secondary electron image of the same field of view, illustrated in Figure 2(a). This clearly demonstrated that the microcrystalline masses were calcium phosphate containing accretions by which the cell was attaching, in most part, to the underlying substratum. This was more clearly seen in tilted secondary images as illustrated in Figure 3 which demonstrated the attachment of thick cell processes to the almost hemispherical accretions. Although the cell in Figure 3 is approximately 12μm in diameter, considerable variation in the size of cells was seen which was related to the degree to which they were spread on the underlying subtstratum (compare Figures 1 and 3).

At higher magnification the relationship between the larger cell processes and the calcified accretions was more easily seen. Figure 4 shows a "single" cell process abutting a single calcified accretion of approximately 1μm diameter on a bacteriological grade polystyrene substratum, where the composite nature of the cell process is evident. The individual strands which make up this cell process separated on the accretion itself, which was seemingly composed of particulate matter of approximately 50nm diameter with no observable fibre component. Single cell extensions, exhibiting a somewhat cross-banded appearance, were also clearly seen in the imme-

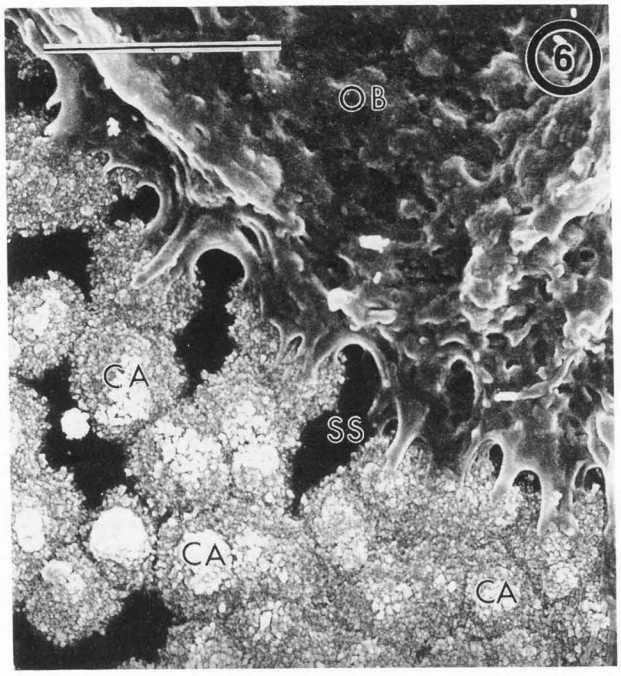


Figure 6 An osteoblast (OB) apparently leaving trails of calcified accretions (CA) on the substratum surface (SS). Bar = 4.3μm.

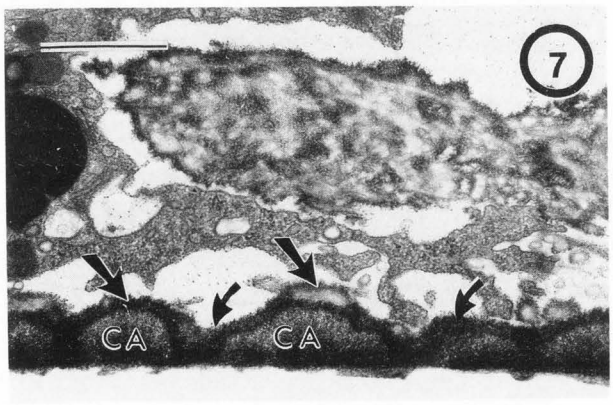
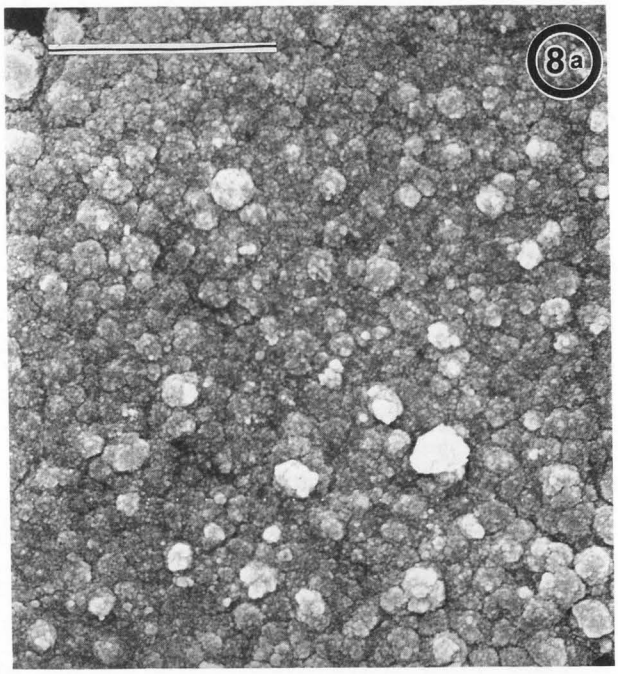


Figure 7 A more mature interfacial morphology than that shown in Figure 5 demonstrates that the calcified accretions (CA) are fusing to form a continuous sheet over the underlying substratum and creating an undulating appearance of hills and troughs (arrows).
Bar = 0.79μm. Compare with Figure 6.

diate vicinity of this afibrillar accretion.

Similar preparations of peripheral nodule regions were made from fluorocarbon substrata and processed for TEM, an example of which is illustrated in Figure 5. At low magnification the nodular appearance of the calcified accretions was again evident and no intervening tissue could be detected between these accretions and the underlying substratum. The cytoplasm of cells bearing cell processes adjacent to these accretions exhibited large quantities of rough endoplasmic reticulum. Figure 5(b) shows the interface more clearly but also demonstrates that these accretions were closely associated with both cell processes and also overlying collagen-containing mineralizing areas; no evidence of fibres was seen in these



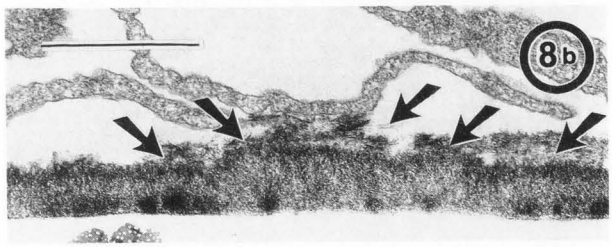


Figure 8 The maturation of the afibrillar calcified layer: (a) The substratum is now completely covered by fused individual calcified accretions. Bar = 6.0μm.
(b) This continuous layer is directly apposed to the underlying substratum and is losing the undulating surface contour seen in Figure 7. Immediately above this layer collagen fibres are now evident in the elaborated extracellular matrix (arrows) with which microcrystalline bodies are associated. Bar = 1.0μm.

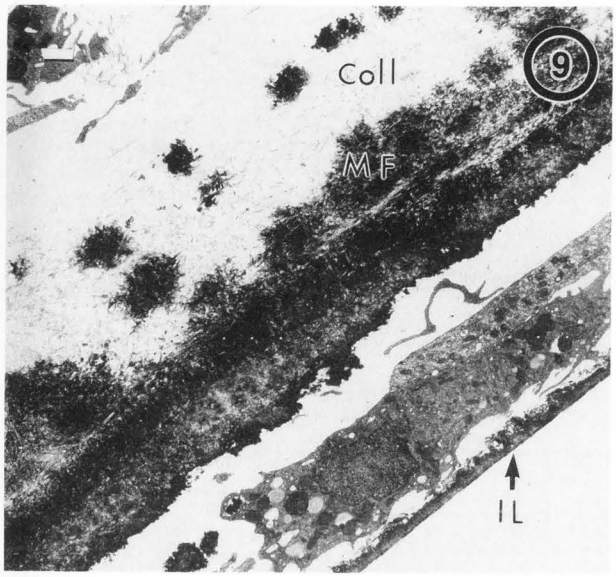


Figure 9 Vertical section through a forming bone nodule of approximately 25μm height at this site. The collagenous extracellular matrix (Coll) contains many spheritic foci of mineralization (MF). Mineral density increases ventrally in the nodule to a point above the single cell shown. The interfacial layer (IL) apposed to the "Petriperm" substratum is also clearly seen.
Bar = 2.0μm.

accretions confirming the SEM appearance in Figure 4.

Synthetically active cells appeared to lay down a series of these accretions from the same cell processes as illustrated in Figure 6, implying a movement of the cell with respect to the deposited accretions. As the density of the accretions increased so their borders seemed to fuse. The corresponding appearance was also seen by TEM as demonstrated in Figure 7. The interfacial zone being established between the forming bone nodule and the underlying substratum now developed into a continuous layer with a dorsal undulating contour resulting from the fusion of the individual accretions.

Figure 8(a) shows a continuous layer of such accretions on the surface of a tissue culture grade polystyrene substratum after blowing away the overlying tissue as described above. Clearly the accretions fused to completely cover the underlying substratum without evidence of a fibrillar component to this extracellular matrix. Again, corresponding TEM photomicrographs as illustrated in Figure 8(b) confirmed the afibrillar nature of this first-formed matrix and also demonstrated that, as the layer matured, the surface undulating morphology was transformed into a relatively smooth surface below which, with the staining regimes employed, the layer appeared to

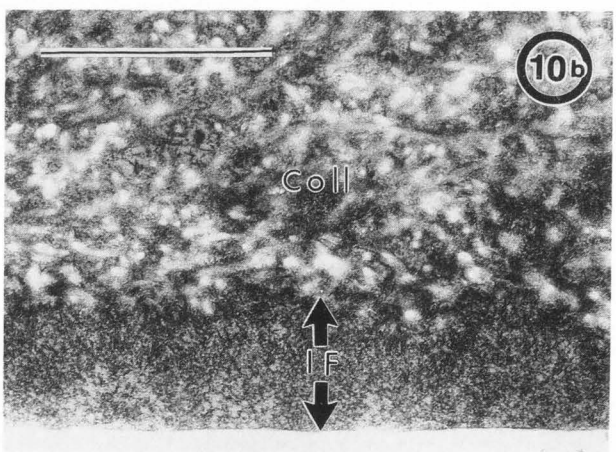


Figure 10 (a) A "blown" sample of the near-interfacial zone on a tissue culture polystyrene substratum which shows the intimate association of collagen fibres (arrows) with the underlying afibrillar material. Only the apices of the globular calcified accretions (CA) are visible protruding through the developing collagenous mat, the individual fibres of which are undergoing calcification.
Bar = 6.0μm. (b) A similar field now viewed by transmission electron microscopy demonstrates the distinction between the collagenous extracellular matrix which contains microcrystallites (Coll) and the afibrillar matrix of the interface (IF) with the substratum below.
Bar = 1.12μm.

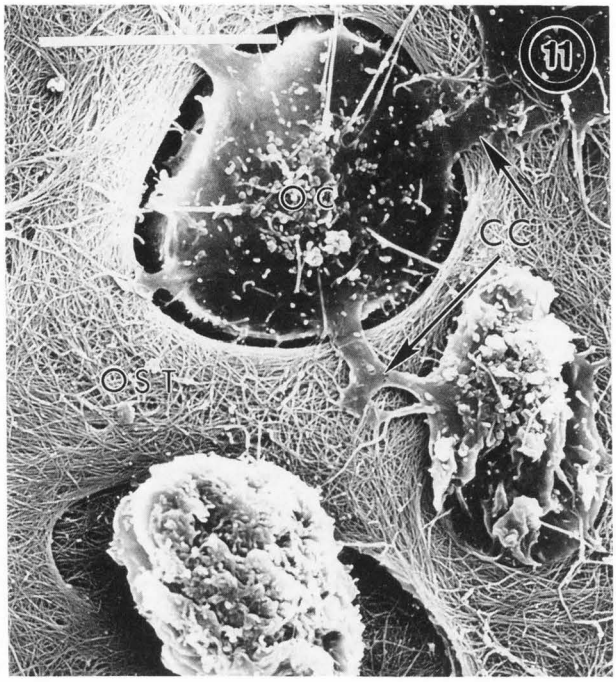
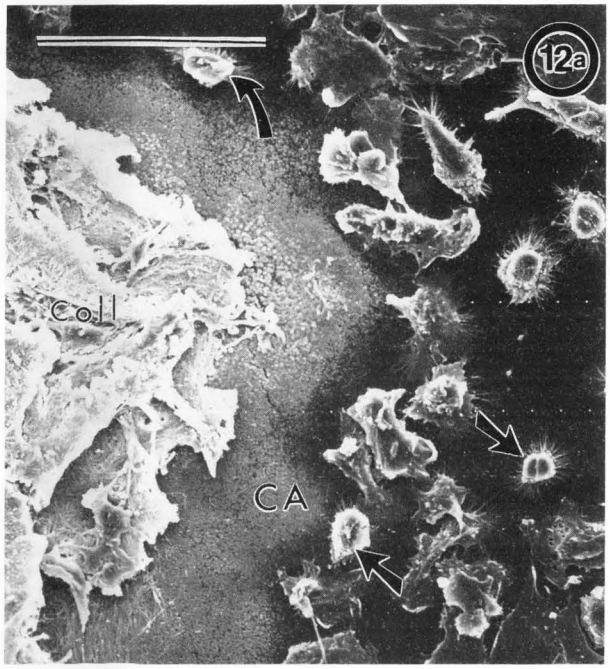


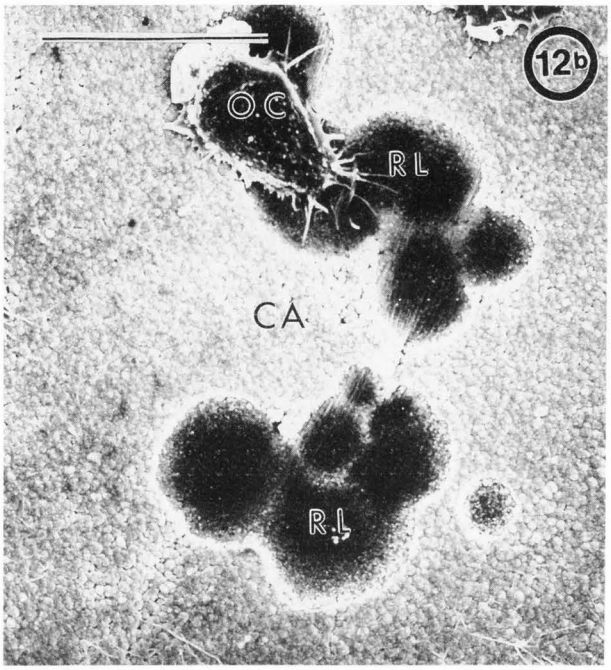
Figure 11 Nearer the dorsal surfaces of bone nodules this appearance was commonly seen. Here unmineralized collagen fibres comprise a dense mat of tissue containing individual lacunae for cells (OC) which communicate with their nearest neighbours by cell processes (CC). This appearance is typical of osteoid (OST). Bar = 10μm.

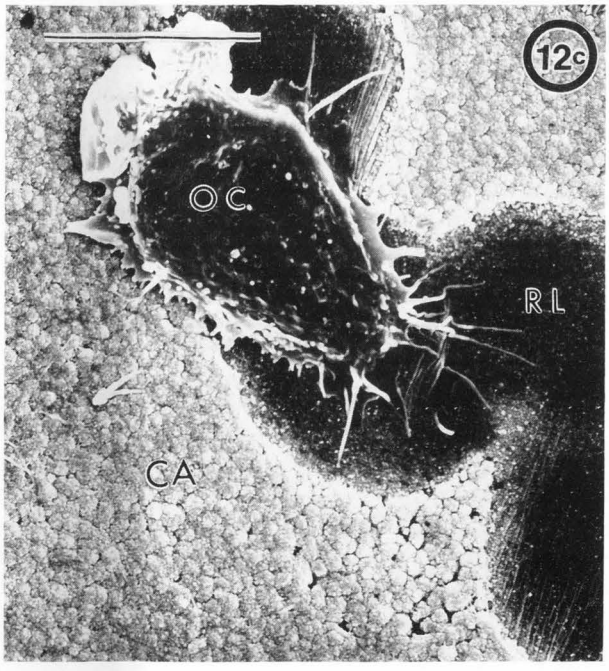
comprise an homogeneous fine microcrystalline structure. Attached to the dorsum of this interfacial layer fine collagen fibrils could be discerned which were themselves undergoing calcification. The afibrillar layer was approximately 0.5μm in thickness (see Figure 8(b)) and quite distinct from the spheritic foci of calcification associated with the rich collagenous extracellular matrix in the main volume of the forming bone nodule (Figure 9). However, in some preparations it was evident that the afibrillar layer of fused globular accretions did act as a substratum for collagen fibre attachment. Figure 10(a) shows the "blown" appearance of the near-interfacial region of a forming bone nodule on a polystyrene tissue culture substratum. The collagen fibres were interwoven in the troughs created by the globular accretions. The distinction between afibrillar and collagen-containing extracellular matrix was seen even more clearly in transmission electron photomicrographs such as that in Figure 10(b). At other planes towards the dorsal surfaces of forming bone nodules cells could be seen partially surrounded by a non-mineralized collagen matrix which possessed smooth surfaced lacunae to contain the cells which themselves contacted their immediate neighbours by cell processes (Figure 11).

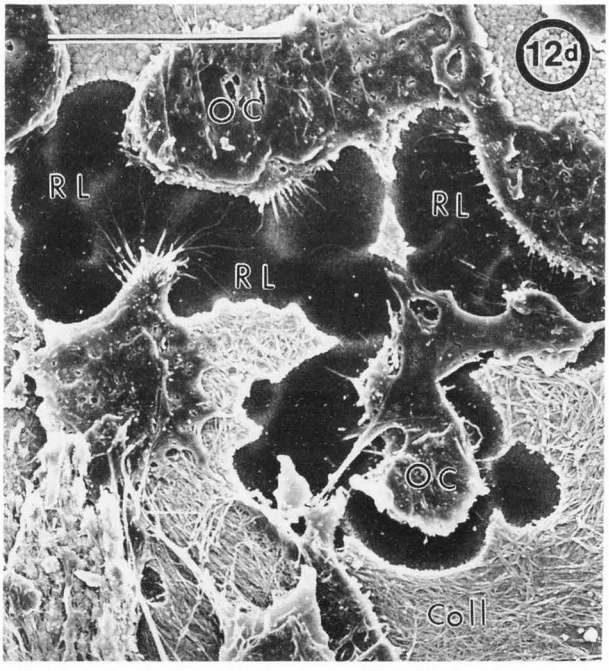
The density of the afibrillar globular accretions diminished at the periphery of forming bone nodules as is shown in Figure 12(a). It was also clear that the trypsin citrate saline treatment at the end of the first culture stage was removing cells, including those partly buried in the forming collagenous matrix of bone nodules, but not removing the collagen matrix of the nodules. This matrix was then used as a substratum for the second culture stage in medium not containing DEX. Since during this stage the bone marrow cell culture was not driven toward os-

Figure 12 (a) Both the remains of the first culture stage and the cells of the second culture stage are seen in this "blown" sample on a tissue culture grade polystyrene substratum. Enymatic digestion has removed the cells of the first culture stage to expose at the periphery of this bone nodule the first formed afibrillar calcified matrix (CA) which is covered, towards the centre of the nodule, by a dense collagenous extracellular matrix (Coll). It is clear that the trypsinization has also removed some of the cells which were partially buried in the surface of this matrix. On the right of the field of view, cells adhering to the culture substratum are from the second culture stage and grown in medium without dexamethasone (arrows). Bar = 60μm. (b) In some areas clear evidence of matrix resorption was found as illustrated here. The cell designated as an osteoclast (OC) is small, being approximately 18μm in length. It occupies part of a resorption lacuna (RL) within the afibrillar substratum apposed calcified layer of extracellular matrix (CA), which displays the classic appearance of resorption lacunae seen in mature bone slices. A second resorption lacuna of approximately 25μm diameter is seen at the bottom of the field of view, at the right side of which a resorption pit of 4.5μm diameter is also visible. Bar = 20μm. (c) Higher magnification of (b). The fused individual calcified globular accretions are more easily seen (CA) but their typical structure is lost around the periphery of the resorption lacuna (RL). The latter exhibits a sharp surface delineation and a bevelled lower margin approaching the culture substratum to give an impression of the 3-dimensional structure of these resorption lacunae seen in (b), (c) and (d). Bar = 10μm.
(d) Larger osteoclasts, up to 65μm in length, are seen in this field of view which also demonstrates the capacity of these cells to resorb not only the afibrillar calcified accretions (top) but also the matted collagenous extracellular matrix (Coll) laid down on the former. This resorption lacuna is at least 200 μm across. Bar = 25μm.









teogenesis, there was an increased likelihood of osteoclast and osteoclas-precursor survival. Indeed, during this culture period some of the matrix elaborated during the first stage was specifically resorbed by cells which created resorption paterns in the matrix similar to those found in mature bone slices (Boyde et al. 1984; Chambers et al. 1984). However, while tartrate-resistant acid phosphatase (TRAP) staining of whole cultures at this stage demonstrated the presence of both large multinucleate TRAP-positive giant cells and small mono- or bi-nuclear TRAP-positive cells (not illustrated), SEM examination revealed that, generally, the resorption lacunae were as-

sociated with rather small cells (see Figures 12(b) and 12(c)). Although some larger resorptive cells were seen within resorption lacunae, which possessed the fimbrillated border typical of actively resorbing osteoclasts (Jones and Boyde, 1977) (Figure 12(d)), the nuclearity of these cells could not be discerned from the scanning electron micrographs since the preparation method for these samples included removal of the more superficial tissue. While this did not necessarily disrupt the dorsal cell membranes of individual cells (see Figures 1, 3, 6 and 12), the cell surface was flattened due to the previous presence of an overlying layer and nuclear-created bulges in the dorsal cell membrane surface associated with some isolated osteoclast culture systems could not be observed. However, it was clear that the resorptive cells were capable of removing both the underlying afibrillar layer of calcified accretions and the mineralized collagenous matrix which was subsequently deposited on the former during the first culture stage.

Discussion

The two stage culture procedure described is based on both the capacity of DEX to drive a bone marrow cell culture towards osteogenesis and our previous observations (unpublished) that bone nodules are refactory to even prolonged trypsinization procedures.

The effect of DEX on the stimulation of osteogenesis, by stimulation of progenitor cell populations, was first described by Tenenbaum and Heersche (1985) following earlier work (Dietrich et al. 1979, Canalis, 1983 and Hahn et al. 1984) which showed that glucocorticoid stimulation increased collagen synthesis, alkaline phosphatase activity and bone formation *in vitro*. The specific effect of DEX on progenitors, rather than differentiated cells (McCulloch and Tenenbaum, 1986), is of importance in the young adult bone marrow cell culture described as previously discussed by Maniatopoulos et al. (1988). Very recent evidence has emerged that DEX has a specific activity on the alkaline phosphatase gene (Green et al. 1990).

The relatively mild enzymatic digestion with 0.01% trypsin citrate saline for 1/2 an hour permitted removal of both the cells adhering to the culture substratum and those partly buried in the superficial layer of the collagenous extracellular matrix of forming bone nodules. The appearance of these digested samples, shown in Figure 12(a), clearly illustrated that, after removal of the overlying cell layer, the peripheries of bone nodules comprised only the afibrillar calcified globular accretions described herein and that the latter were resistant to the digestion procedure. Resorptive cells subsequently found in these areas after the second culture stage produced resorption lacunae clearly demarcated from the surrounding afibrillar matrix (Figure 12(b)).

The rationale for the use of three culture substrata was that, while regular tissue culture polystyrene would act as a standard surface, the gas-permeable hydrophilic fluorocarbon substratum is easily processed and embedded to produce tissue sections for TEM. Furthermore, we knew from previous studies that bacteriological grade polystyrene (especially if sterilized by gamma irradiation - unpublished) would support the adhesion, spreading and extracellular matrix synthesis of primary bone marrow derived cells and calvarial cells (Shelton, 1989). However, as these cell activities are less pronounced on bacteriological grade when compared to tissue culture polystyrene the former substratum facilitated the observation of synthetic activities of individual cells described below. Therefore, while the three substrata were employed for differing reasons, the results clearly demonstrated that the matrix production phenomena were similar in each case. We have recently shown that similar osteoblast matrix elaboration events are common to metal surfaces of interest as bone-substitute materials (Davies et al. 1990; Lowenberg et al. 1991) and that cultured dental papilla cells can also produce a similar extracellular matrix (Andrews et al. 1990). However, since each culture dish contains many bone nodules at differing stages of maturity and within each nodule the periphery is less developed than the central area, we are unable at the present time to provide quantitative temporal information on the appearance of the afibrillar matrix. However, an overall sequence of events can be constructed from the morphological appearances illustrated in Figures 5, 7 and 8(b).

Two critical questions which have to be addressed with respect to the observed afibrillar matrix are: First, whether the afibrillar layer represents a normal synthetic activity of osteoblasts and second, whether the initial calcium phosphate containing deposits represent an extracellular precipitation which is a result of the culture conditions employed. There is little doubt from our experimental findings as represented in Figures 1, 3, 4 and 6 that an intimate relationship exists between the calcified accretions and cell processes. This is confirmed in the TEM photomicrographs in Figures 5, 7 and 8(b). As the deposits did not occur ubiquitously in our culture systems, but were associated with cell processes, one can assume that their production was cell mediated. Furthermore, we have reported other experiments where these deposits did not occur in the absence of RBM (Lowenberg et al. 1991). In addition, since the deposits were associated only with the forming bone nodules in culture and not the other cell populated areas of the culture dishes, it is reasonable to assume that the cells responsible for elaborating the bone nodules, osteoblasts, also produced these initial afibrillar calcified accretions. The presence of large numbers of lipid inclusions in these cells as noted in Figure

5(a), which would contain phospholipids, may also be associaed with active mineralization. Although we do not show direct evidence of the secretion of mineral by the cells near the substratum interface, the relationship between cell process and extracellular mineral illustrated in Figure 5(b) would support this possibility. Of course, the possibilty also exists that this extracellular mineral was produced by precipitation resulting from interaction between akaline phosphatase, assumed to be expressed on the membranes of the cell processes, and the constituents of the culture medium. However, not only would the above argument negate this, but the appearance in Figure 6 would also suggest that the cells are responsible for actively laying trails of accretions. This was frequently observed in our culture systems and could be the result of either cell migration or changes in cell shape. We believe the former is unlikely, due to the restrictions imposed by the maturing matrix itself, while the latter is more probable. Indeed, as the cells became surrounded by matrix they tended to adopt a round, short columnar appearance (not illustrated). Since the cells are initially quite spread on the substratum, as shown in Figure 1, it may be that their production of the initial afibrillar matrix allows adoption of a more compact morphology which would then be associated with changes in their secretory activity to include the production of collagen. This would create a histomorphology not unlike the classical cuboidal appearance of polarized osteoblasts on bone surfaces *in vivo*.

A final possible explanation of the production of the accretions would be as a pathological calcifying activity of the cells most closely in contact with the culture substratum which would result in cell death. This was clearly not the case as witnessed by the condition of the cell membranes in the SEM photomicrographs herein and the signs of active synthetic activity. The latter is illustrated by the abundant rough endoplasmic reticulum in the cytoplasm of cells providing the accretion associated cell processes (Figure 5(b)). Even so, this cellular activity could still be one associated with the reaction of osteoblasts to the foreign surfaces represented by the culture substrata. Indeed, in parallel experiments we have reported the formation of similar calcified accretions directly on the titanium oxide surface covering passivated bulk commercially pure titanium metal substrata (Lowenberg et al 1991). However, there are several corollaries to this afibrillar matrix found *in vivo* in naturally forming hard tissues. Resting and reversal lines are naturally occuring linear arrangements of basophilic cement substance which reflect the continual remodelling of bone tissue. They remain entirely unstained in silver-impregnated sections and thus contain no fibrils (Weinmann and Sicher, 1955). A similar cement-like substance has recently been reported by Beertsen and van den Bos (1990) who reported the production in culture of a mineral phase, of unknown origin, comprising "fine needle-shaped crystallites embedded in a granular matrix of moderate electron density" into which collagen fibrils were embedded. Afibrillar cement also occurs in thin layers, about 100nm thick, over the enamel around the necks of teeth (Listgarten, 1966). Another dental example of afibrillar calcified matrix is that of intratubular dentine, the hypermineralized dentine which is slowly laid down within the dentinal tubules and ranges in thickness from 44-750nm (Ten Cate, 1989).

From these observations it can be concluded that the afibrillar matrix elaborated during the culture period was not only produced by the same cells which could then proceed to lay down collagen, mineralize this collagen and produce bone nodules, but also that the appearance of this matrix is analogous to afibrillar matrices produced *in vivo* by hard tissue synthetic cells. We believe that this is the first report of such matrix production *in vitro*; although numerous studies have reported the elaboration of mineralized matrix by bone cells *in vitro* (*vide supra*), they have not considered the interfacial matrix that we report here which is laid down on the culture substratum. It should be emphasized that this matrix was only associated with forming bone nodules and was not seen elsewhere in the culture vessels. Furthermore as the nodule matured, as judged by the morphology of the extracellular matrix passing from the periphery to the more central areas, this thin initial afibrillar matrix layer was used to anchor collagen fibres and was continuous with the calcified collagenous matrix of the bone nodule proper.

Whether the afibrillar layer should be called bone, in the same way as the matrix comprising resting and reversal lines in bone tissue is considered as part of the tissue as a whole, is open to some debate. However it is evident that, like these bony landmarks (Reid, 1986), the afibrillar material is resorbed by osteoclasts. The size of the resorptive cells agrees with recent observations by Heersche (personal communication) that osteoclasts of low nuclearity are most actively resorptive in culture and that such cells often demonstrate small cytoplasmic "tails" as is seen in Figure 12(c). Jones and Boyde (1977) described the morphological distinction between actively resorbing osteoclasts and motile non-resorbing cells and the larger cells seen in this study agree with their description of actively resorbing cells. It is therefore interesting to compare the morphology of the cells in Figures 5 and 6 of their paper with those designated as osteoclasts, herein, in Figure 12(d). In each case the large cells possess fimbrillated borders and are lying in resorption lacunae. This fimbrillated appearance has also been described by Horton et al. (1984) in human osteoclasts and Oursler et al. (1985) who combined morphological observations with

the existence of specific chick osteoclast antigens to identify osteoclasts both *in vivo* and *in vitro*. It is also worthy of note that the osteoclasts, observed in the experiments reported herein, were capable of resorbing both calcified and collagenous components of the extracellular matrix elaborated during the first culture stage.

We feel that these experimental observations are of potential value in providing a new method to investigate the interactions of both osteoblasts and osteoclasts with one another as well as the matrix with which they are both associated, which has hitherto not been possible. Furthermore, the ability of the osteoclasts to resorb the afibrillar calcified matrix may be employed to investigate not only the resorptive activity of osteoclasts but also the properties of the substratum physiochemistry which permits resorption by osteoclasts. Gaining this information may be of value to those who wish to produce an artificial substratum to investigate osteoclast function in culture.

Conclusions

Primary non-enzymatically digested rat bone marrow cells, cultured in the presence of dexamethasone, are capable of producing bone nodules which interface with the underlying culture substratum by forming an afibrillar calcium phosphate containing extracellular matrix layer. This layer is less than 1μm thick and may resemble afibrillar mineralized matrix known to occur *in vivo* at bone reversal lines, in osteocyte lacuna linings and other sites.

This afibrillar calcified extracellular matrix, together with the mineralized collagenous extracellular matrix comprising the major volume of cultured bone nodules, can be resorbed by osteoclasts to produce scalloped resorption lacunae which expose the underlying culture substratum.

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Discussion with Reviewers

S.C. Miller Why was dexamethasone added to some of the cultures?

Authors As described, we have chosen to adopt the method developed by Maniatopoulos et al. (1988) for the first culture stage. The effect of dexamethasone on the stimulation of osteogenesis is discussed, at some length, in the second paragraph of our Discussion.

S.J. Jones Can the calcified accretions form in the absence of serum?

Authors It is interesting to speculate whether this would be possible but we have not carried out such experiments. Certainly, in other culture systems it has been shown that mineralizing hard tissue matrices may be elaborated in chemically defined media which contain no serum (Slavkin et al. (1990) Conn. Tiss. Res. 24(1) 41-51)).

P. Osdoby Is there any indication that the structures observed have any relationship to matrix vesicles? In this context can you localize alkaline phosphatase to these structures?

Authors We have seen no evidence of matrix vesicles in these cultures and therefore have not attempted alkaline phosphatase localization. However, the nodules themselves do contain cells which are alkaline phosphatase positive, as originally described by Maniatopoulos et al. (1988) and which we have routinely confirmed.

S.J. Jones Did the authors see any similarity of accretions with calculus formation? Was an adsorbed protein layer present before mineralization occured? Have the authors identified any cell attachment proteins (by immunolocalization)?

Authors We have not examined calculus formation using these preparatory techniques and therefore cannot comment on this possibility. Yes, a protein layer will have been present as adsorption from the serum takes place as soon as the culture medium is added to the culture dish and before the first cells attach. We are currently undertaking work to localize specific non-collagenous bone proteins in these afibrillar calcium phosphate accretions.