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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 β-glycerophosphate 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 (Osдобy and Caplan, 1980; Tenenbaum and Heersche, 1985; Tenenbaum et al. 1986), mouse (Ecarot-Charrer et al. 1983), rat (Bellows et al. 1986; Maniatopoulos et al. 1988), and human (Auf'molk 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 Boyd, 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 refractory. 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 (Osдобy et al. 1982), these are not considered reliable markers of osteoclast differentiation (Hattersley and Chambers, 1989a) and in some species the demonstration...
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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 (Boyd 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 (Boyd 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 Maniopoulos et al. (1988). Briefly, for each rat, both femora were removed and washed four times with a 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 β-glycerophosphate (added as 1% of a 1M stock solution in DDH2O) 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 10⁻⁶M dexamethasone (DEX) in stage 1 cultures but DEX was not included in the stage 2 cultures described below. The DEX was prepared from a 10⁻⁶M stock solution in absolute ethanol diluted to produce a working solution of 10⁻⁶M 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 Maniopoulos et al. (1988). These rat bone marrow (RBM) cultures were maintained in a humidified atmosphere of 95% air with 5% CO₂.

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 10⁻⁶M 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 CO₂ (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
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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 DDH2O 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.
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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).

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.

to which they were spread on the underlying substratum (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-
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**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.

**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.

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
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**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.
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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.

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. Enzymatic 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 osteoclast-precursor survival. Indeed, during this culture period some of the matrix elaborated during the first stage was specifically resorbed by cells which created resorption patterns 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-
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associated with rather small cells (see Figures 12(b) and 12(c)). Although some larger resorptive cells were seen within resorption lacunae, which possessed the fimbriated 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 refractory 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)).
Sicher, 1951; who have found that silver staining cannot be used to stain afibrillar bone. Hence, we believe that the matrix found in the bone tissue was not stained by silver. Afibrillar matrix also occurs in thin layers, about 100 nm thick, over the enamel around the necks of teeth (Lister, 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 44-750 nm (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 vitro 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 resorptive cells agrees with recent observations by Heersche (personal communication) that osteoclasts of the posterior teeth are more active than the osteoclasts of the anterior teeth. 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 fimbriated borders and are lying in resorption lacunae. This fimbriated appearance has also been described by Horton et al. (1984) in human osteoclasts and Oursler et al. (1985) who combined morphological observations with
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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.

Acknowledgements

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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 Maniotopoulos 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 could 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 (Slakvin et al. (1990) Conn. Tiss. Res. 24(1) 41-51).

**P. Osdozy**  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 Maniotopoulos 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 occurred? 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.