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ORGANIC EXTRACELLULAR MATRIX COMPONENTS AT THE BONE CELL/SUBSTRATUM INTERFACE

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Abstract

We have recently shown that differentiating bone cells are capable of elaborating, *in vitro*, a calcified matrix at the interface with several non-biological substrata which is similar to cement lines found in remodelling bone tissue. From previous morphological observations, this matrix appeared free of assembled collagen fibres which indicated that osteogenic cells produce an initial mineralized matrix before overt collagen fibre production. In the work reported herein, we used six antibodies to extracellular matrix components and two conjugates of secondary antibody, fluorescein and colloidal gold, to initiate a preliminary characterization of the organic components at the bone cell substratum interface at early periods of culture. The labels were visualized by transmitted fluorescence light and immuno-gold scanning electron microscopy, respectively. Both secondary and backscattered modes were employed in the latter.

The results showed that while chondroitin sulphate was ubiquitous in this culture system, osteopontin labeled discretely at the ends of cell pseudopodia, initially, following which the substratum surface was strongly labeled. The distribution of fibronectin was significantly different to that of osteopontin and indicated that this protein was primarily involved in cell/cell rather than cell/substratum adhesion. Although Type I collagen was not present in the extracellular matrix at early periods of culture, it was evident intracellularly at 3 days and extracellularly after the formation of the initial cement-like matrix. However, Type III collagen displayed the inverse sequence. In less mature cultures, it exhibited a ubiquitous distribution, while it was un-detectable at later stages of culture. Finally, osteocalcin was the only antibody employed which showed no labeling at early culture periods while at later stages, both the cellular compartment and the previously formed interfacial matrix labeled positively.

Key Words: Bone-substratum interface, osteopontin, fibronectin, collagen I, collagen III, osteocalcin, chondroitin sulphate.

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Introduction

The formation of bone depends upon the metabolic and secretory activities of only one cell type: the osteoblast. While this statement emphasizes the activity of phenotypically mature osteoblasts, it does not negate the importance of the cascade of biological phenomena which comprise osteogenic cell maturation. When osteogenic cells reach the site of imminent bone tissue elaboration, matrix secretion events which precede expression of the mature osteoblastic phenotype may be of critical importance not only in the elaboration of the bone proper but also in influencing the further differentiation of the osteogenic cells, as is seen with other hard tissue matrices (Magloire *et al.*, 1992; Thesleff and Vaahtokari, 1992). Indeed, at remodelling bony sites, two distinct matrix formation events occur before the onset of collagen mineralization. First: a cement line, comprising a mineralized but collagen free matrix, acts as the interfacial layer between old and new bone proper. Second: the latter is elaborated as an unmineralized collagenous matrix, osteoid, which after a further delay becomes calcified. Although little is known concerning early matrix synthesis by differentiating bone cells, osteoblast differentiation is, currently, considered to be accompanied by the expression of several non-collagenous bone proteins prior to the onset of both collagen assembly and mineralization (Stein *et al.*, 1990; Lian and Stein, 1992). Clearly, the implications of unravelling this cascade of bone cell secretory activity is of importance not only in understanding bone tissue metabolism but also the nature of the interface which may be established between bone tissue and artificial materials.

To avoid the complexity of the *in vivo* environment, we have used an *in vitro* method to investigate the earliest matrix elaboration, by rat bone marrow derived differentiating osteogenic cells which have been reported to elaborate high yields of morphologically and biochemically identifiable bone matrix in short experimental periods (Maniatopoulos *et al.*, 1988). These *in vitro* experiments have demonstrated that the initial matrix elaborated by differentiating osteogenic cells is analogous to the cement line, found *in vivo*, which forms the natural interface between old and new bone (Davies *et al.*,

1991a). Indeed, we have recently shown that the globular mineralized matrix, approximately half a micrometer thick, which comprises this interfacial layer can also be morphologically identified in natural bone tissue at remodeling sites (Zhou *et al.*, 1993). This *in vitro* approach, and its critical dependence upon the presence of differentiating cells at the interface with the culture substratum, has provided not only new information on the formation of bone on natural and foreign surfaces, but also a re-interpretation of ultrastructural descriptions of bone biomaterial interfaces previously available in the literature (Davies *et al.*, 1991b,c; Lowenberg *et al.*, 1991; Pilliar *et al.*, 1991). Specifically, since the cement line structure comprises a layer of individual globular accretions, we postulated that an organic matrix, secreted by the differentiating bone cells, must play an important role in the initial cement line mineralization events (Davies *et al.*, 1991a).

In the work reported herein, we used six antibodies to extracellular matrix components and two conjugates of secondary antibody, fluorescein and colloidal gold, to initiate a preliminary characterization of the organic components at the bone cell substratum interface at early periods of culture. The labels were visualized by transmitted fluorescence light and immuno-gold scanning electron microscopy (immuno-SEM) respectively.

Materials and Methods

Cell harvesting and culture

Rat bone marrow (RBM) cells were obtained from young adult male Wistar rats (approximately 120 gm) according to the method described previously (Davies *et al.*, 1991b). Briefly, for primary cultures, femora were removed and washed 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% fetal bovine serum, 50 μg/ml of freshly-prepared ascorbic acid [added as 1% of a 5 mg/ml stock solution in phosphate buffered saline (PBS)], 10 mM Na ß-glycerophosphate (added as 1% of a 1 M stock solution in double distilled water) and antibiotics at 1/10th of the concentration described above. This medium was further supplemented with 10^{-8} M dexamethasone (DEX). The specific concentrations of these culture medium additions employed were those reported by Maniatopoulos *et al.* (1988).

Aliquots of the RBM cell suspension, at a cell density of 0.4 x 10^5 cells/coverslip, were cultured on 22 mm square glass coverslips, maintained in a humidified atmosphere of 95% air with 5% CO_2. The medium, containing 10^{-8} M DEX, was changed after the first 24 hours to remove non-adherent cells. The cultures and the medium was renewed up to three times. These cultures were maintained from 3 to 12 days and then prepared for fluorescence microscopy.

Other primary cultures, prepared as described above but in T75 tissue culture flasks (Falcon) were passaged at Day 5 at 10^5 cells/ml into 35 mm dishes (1 ml/dish). These sub-cultures were maintained for 11 days in identical conditions to those described above and processed for immuno-SEM.

Primary and secondary antibodies

Six antibodies were included in the present study: **(i)** CS-56 mouse monoclonal anti-chicken chondroitin sulphate [Sigma Immuno Chemicals, St. Louis, MO; working dilution (wd) 1:200] which has been shown to react with chondroitin sulfates of types A and C (not with type B) but shows no species or cell specificity (Abnur and Geiger, 1984; 1985; Takeuchi *et al.*, 1990); **(ii)** affinity purified goat polyclonal anti-human (and bovine) Type III collagen (Southern Biotechnology Associates, Inc., Birmingham, AL; wd 1:200) which reacts with type III collagen from most other species; **(iii)** mouse monoclonal anti-rat osteopontin (University of Iowa Hybridoma Bank, Iowa City, IA; wd 1:800); **(iv)** goat polyclonal anti-rat osteocalcin (Biomedical Technologies Inc., Stoughton, MA; wd 1:20); **(v)** mouse monoclonal anti-chicken cellular fibronectin (Sigma Immuno Chemicals; wd 1:400) which reacts with most species; **(vi)** affinity purified rabbit polyclonal anti-rat Type I collagen (Biodesign International, Kennebunk, ME; wd 1:100).

Secondary FITC (Fluorescein isothiocyanate)-conjugated antibodies (Jackson Immunoresearch Laboratories, Inc.; wd 1:50) for fluorescence microscopy while secondary 20 nm and 30 nm colloidal gold-conjugated antibodies (Biocell Research Laboratories, wd: 1:20) were used for scanning electron microscopy (SEM).

Microdissection of cultures

In order to facilitate access, by the antibodies employed, to the extracellular matrix at the culture substratum, it was necessary to undertake a simple microdissection procedure following fixation. Briefly, the tissue culture dishes were viewed with a dissecting microscope (Wild M3) and areas of greater cell density, which represented the easily discernable developing bone nodules, were identified. Using a scalpel (number 11 blade) parallel lines, approximately 2 mm apart, were cut from the centre to the periphery of a nodule and into the surrounding extra-nodular cell layer. A perpendicular incision was then made to transect these parallel cuts in the central portion of the nodule. Using the tip of the scalpel blade and fine forceps, a flap of cells and tissue was raised from the central area of the nodule which extended beyond the periphery of the nodule, and detached from the remaining tissue in the culture dish. Several flaps were created in any one culture dish to allow labeling of multiple developing bone nodules.

Fluorescence microscopy

The cells cultured on coverslips were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS for 1 hour, then incubated with primary antibody for 30 minutes at room temperature, washed in PBS and incubated again with secondary FITC-conjugated antibody. The cells were washed again with PBS before being

mounted with anti-bleach medium. Controls for labeling specificity included omission of the primary antibody, normal serum, collagen Type II and pre-saturated primary antibody with correlated antigen. The cultures were then observed in Leitz microscope equipped with a selective filter for fluorescein.

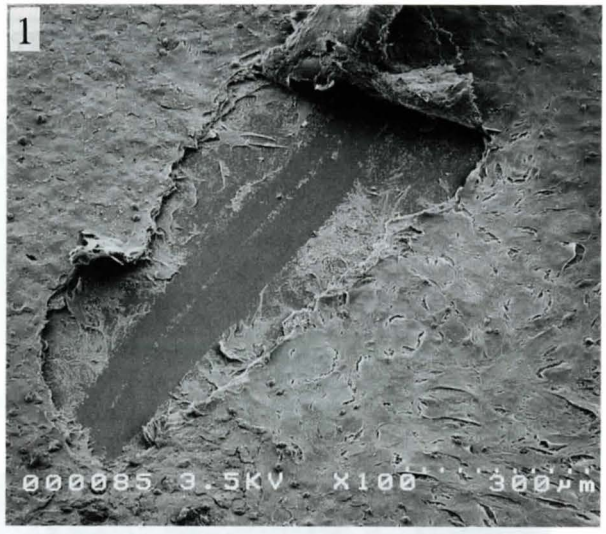
Scanning electron microscopy

The immunogold labeling method for SEM was adapted from the procedure described by de Harven *et al.* (1984). Following rinsing in α-MEM and PBS, first passage subcultured rat bone marrow cells were prefixed in 4% paraformaldehyde in PBS, pH 7.4, for 30 minutes at 4°C and then washed with 0.1% glycine in PBS to quench free aldehyde groups. The cell multilayer was retracted, as described above, to expose the interface structure and facilitate antibody access to the interfacial extracellular matrix. To reduce non-specific labeling, the cells were placed in 5% normal serum (from the same species as the host used to raise the secondary conjugated antibody) in 0.5% bovine serum albumin/PBS for approximately 30 minutes. After this, they were incubated with the primary antibody overnight at 4°C following which a further incubation was carried out, for 2 hours at room temperature, with 20 or 30 nm secondary gold-conjugated antibody. After thorough rinsing in PBS, the cells were post-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 hours at 4°C. Ethanol dehydration was followed by critical point drying from CO_2 and mounting on aluminum stubs. The preparations were coated with evaporated carbon to facilitate detection of the gold particles by backscatter electron imaging (BSEI). Specimens were observed with an Hitachi S-570 scanning electron microscope, operated at 20 kV, in both the secondary electron imaging (SEI) and BSEI modes. Photomicrographs were recorded on Polaroid P/N 55 film.

Results

Initial attempts to label the components of the extracellular matrix at the interface with the underlying culture substratum failed due to lack of penetration of primary antibody through the dense surface cell layers. To circumvent this problem, the microdissection procedure, described above, was employed. An example of the result of the dissection procedure is seen in Figure 1. While damaging the tissue in this way clearly exposed the underlying interfacial matrix, it also led to the scratching of the culture dish surface. We refer to such scratches in the description of several figures below, since these provided convenient markers of the culture substratum surface and therefore, the level of the interface. In older cultures, the removal of the cell layer allowed clear visualization of the globular interfacial matrix as illustrated in Figure 2.

It should be noted that while the cultures examined varied in age from 3 to 11 days, these were not monolayer cultures. As described in the original report by Maniatopoulos *et al.* (1988), the cells start to multilayer, prior to reaching confluence, as a prelude to bone nodule formation. Thus, a cell nodule exists before discernable mineralized matrix production and, as the nodule develops, the periphery of a nodule represents a less mature region than the central area. Each developing bone nodule therefore represents a time-capsule of development and an eight-day culture, for example, will contain nodules which comprise central regions which represent eight days of development while other, more peripheral, regions of the same nodule are representative



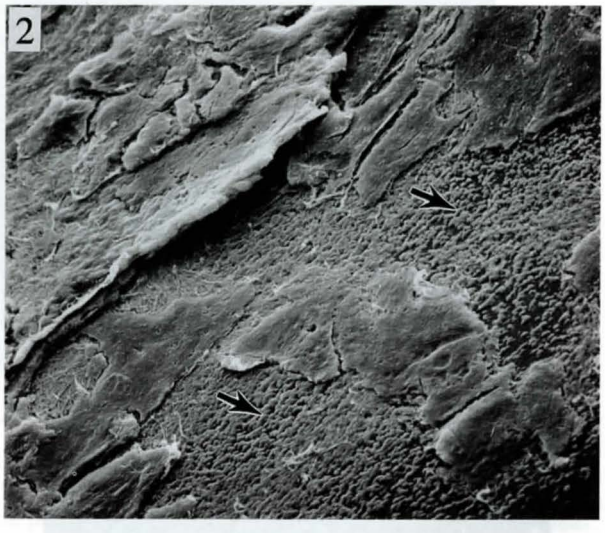
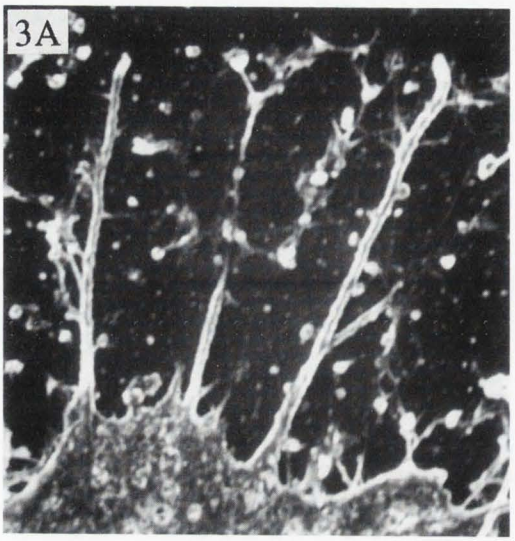


Figure 1. Low magnification scanning electron micrograph to illustrate the microdissection of a rat bone marrow cell culture. The multilayered geometry is clearly seen as are the scratch marks in the interfacial tissue which have exposed the underlying culture dish surface. Field Width [FW] = 1.04 mm.

Figure 2. The cell layer has been removed from part of this culture to reveal the underlying globular interfacial matrix (arrows). FW = 104 μm.



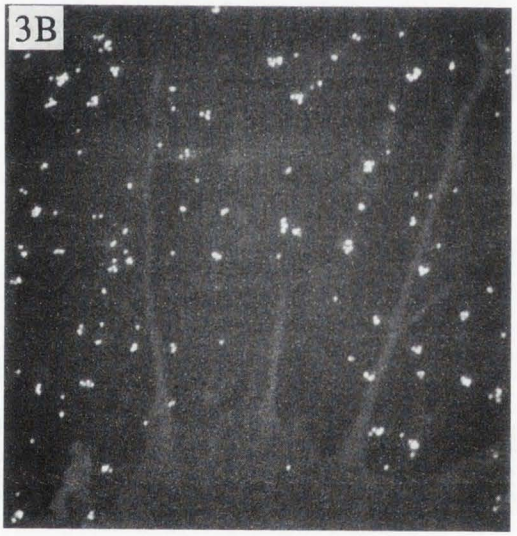
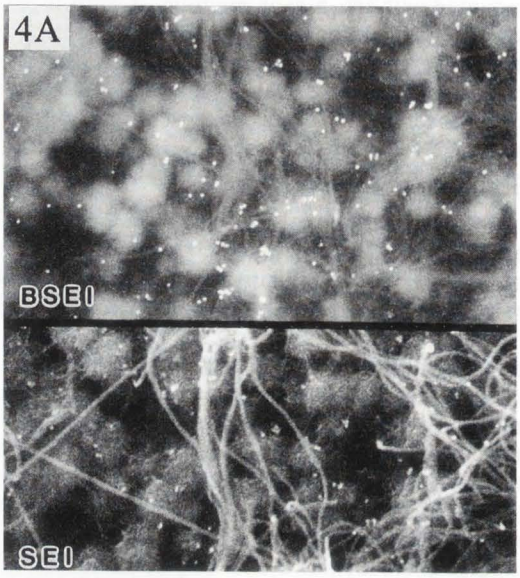
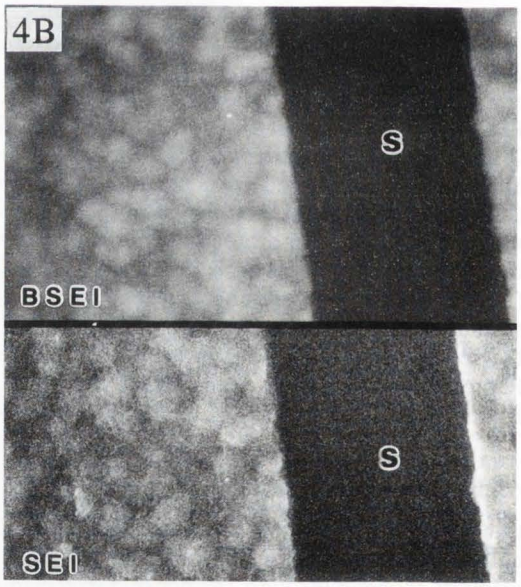


Figure 3 (above). Immuno-SEM of CS-56 at an early culture stage. (**A**) Secondary electron image (SEI) and (**B**) backscattered electron image (BSEI) of the same field of view. FW = 6 μm.





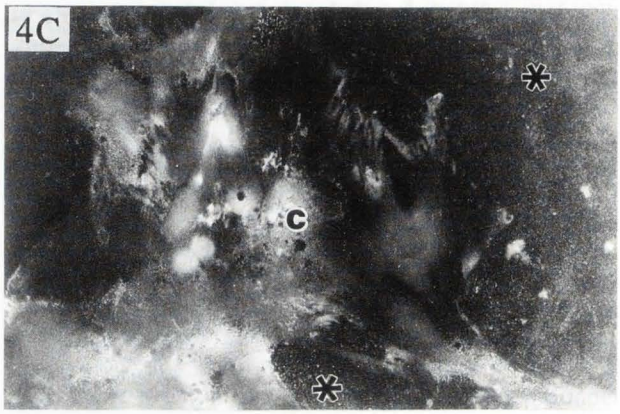
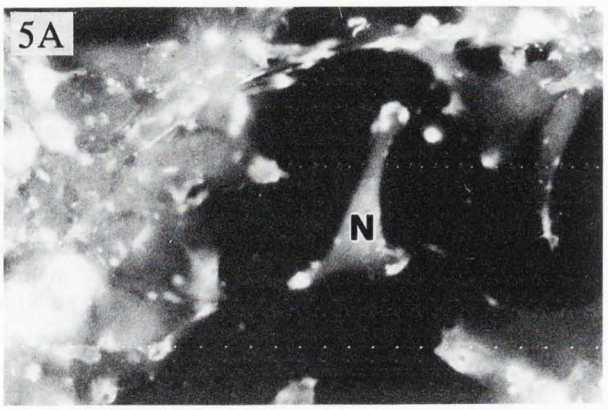
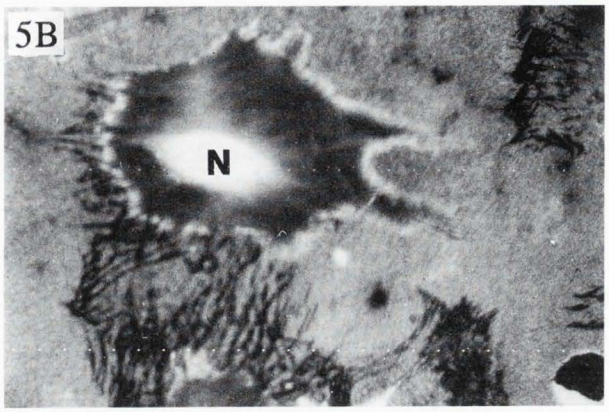
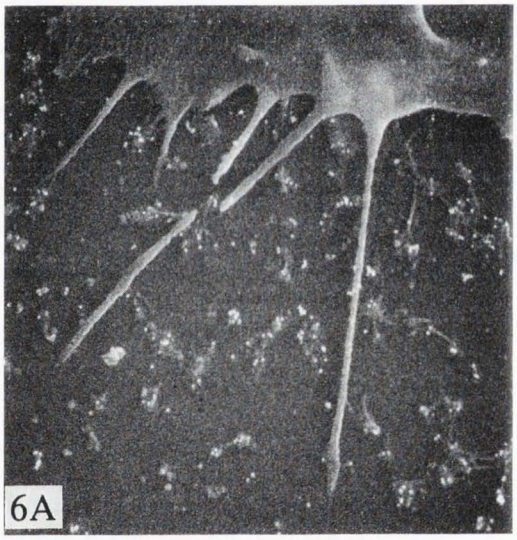


Figure 4 (at right). Immuno-SEM of CS-56 at a later culture stage. (**A**) SEI [bottom] shows collagen fibres overlying the previously formed globular accretions. The BSEI [top] clearly demonstrates the presence of the label, but no association with the collagen fibres is apparent. (**B**) SEI [bottom] and BSEI [top] of a control sample incubated without the primary antibody. The parallel sided dark band running vertically through the field of view is a scratch mark (S) exposing the underlying culture substratum. (**C**) FITC image of CS-56 labeled sample showing ubiquitous distribution of this antibody. Note: the punctate appearance of the label on the culture substratum (*) as well as the cell labeling (c). FW = 6 μm (A, B); 225 μm (C).







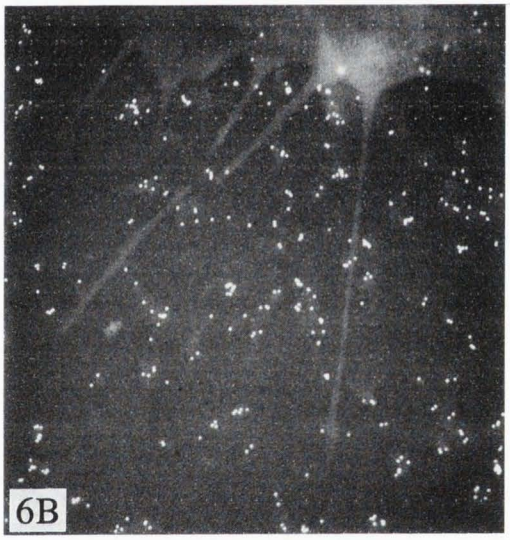


Figure 5. Osteopontin signals at two developmental stages of this culture system. (**A**) At an early stage the label is strongly expressed at the ends of cell pseudopods associated with attachment to the underlying substratum. At a later stage (**B**) the substratum itself is strongly labeled together with the periphery of the cell membrane. Note: 1) the linear striations in the substratum label indicative of re-distribution of the protein as a result of change in cell shape in (B); and 2) the strong signal from the nuclear area of the cell (N) is due to autofluorescence resulting from the glutaraldehyde fixation. These black and white images were reproduced from original Ektachrome 400 colour slides. FW = 225 μm (A); 140 μm (B).

Figure 6. Osteopontin visualized at an early development stage in culture is associated with sub-micrometer fragments of extracellular matrix which are clearly seen (**A**) in the SEI. The immuno-gold conjugate of the same area seen in BSEI mode (**B**) is congruent with the matrix seen in (A). FW = 6 μm.

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of the least mature stages of development. For this reason, the majority of observations we make herein are referred to as originating from less or more mature developmental stages rather than quoting the age of the culture in days.

In all cases, the antibodies employed demonstrated reproducible differences between experimental, negative and positive control specimens although the intensity of fluorescence or density of gold labeling varied within each sample group as a function of the site within a particular culture being viewed and the morphology of the attendant tissue.

Chondroitin sulphate was observed at the earliest time periods in this culture system and displayed a ubiquitous distribution both on the culture substratum and within the multilayered cell nodules. Figures 3A and 3B show the secondary and backscattered images of deposits on the culture substratum which are labeled with CS-56. At later time periods, CS-56 was still detectable (Fig. 4A); again the distribution was random without obvious association with collagen fibres (Fig. 4A). A control sample, without the primary antibody clearly demonstrated the specificity of the label (Fig. 4B). The immunofluorescent staining for CS-56 was observed

from 3 days primary culture. The fluorescence exhibited a fine dotted pattern both on the substratum and over the cell surface with an increased density along the margin of cells as shown in Figure 4C.

The distribution of osteopontin was quite different as visualized using the FITC-conjugated antibody. At early culture stages the label was demonstrated predominantly at the ends of cell pseudopodia which were clearly associated with areas of attachment to the underlying substratum (Fig. 5A). With increasing culture maturity, this distribution changed significantly to exhibit strong immuno-labeling on the substratum surface as well as the cell periphery. Also, the distribution on the substratum surface was marked by linear areas which appeared devoid of label. These linear patterns were directed towards the individual fimbriae of the neighbouring cell as illustrated in Figure 5B. Immuno-SEM revealed that osteopontin was, indeed, detectable at the culture surface at very early stages of extracellular matrix development (Fig. 6).

The other major adhesion protein assayed was cellular fibronectin which showed a distribution quite different to that of osteopontin. In less mature cultures, the label was visible only in direct association with cells, as shown in Figure 7A. While no apparent signal was detected on the substratum surface, it was not possible to judge if the cell-related label was internal or external to the cell membrane. Nevertheless, labeling was prominent at points of cell attachment, associated with the classical appearance of intracellular filaments, which suggested some involvement in attachment mechanisms. This became clearer with the maturity of the culture where the cellular fibronectin antibody labeled inter-cellularly, possibly in association with some extracellular matrix (Figure 7B). In the spaces between cells there was, again, no evidence of labeling on the substratum surface as had been so easily seen in the case of osteopontin. The association of this label with extracellular matrix was more clearly seen by immuno-SEM. Figures 8A and 8B show the SEI and BSEI of a more mature culture with an evident and abundant extracellular fibre matrix. Here, the fibronectin label was specifically associated with this fibre compartment.

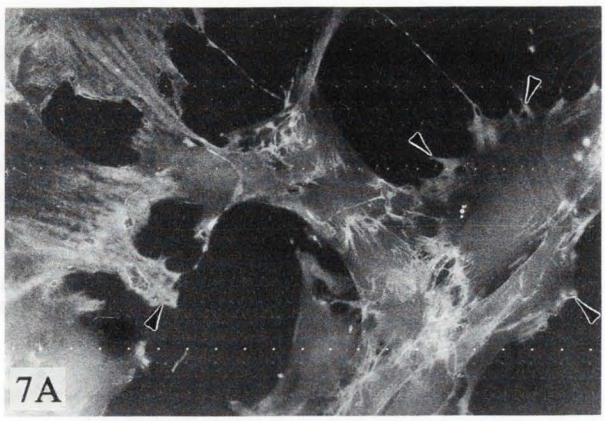
No evidence of the presence of Type I collagen in the extracellular matrix was found at early periods of culture, although intracellular labeling was positive (Fig. 9A). Only in more mature cultures, following the formation of the initial cement-like matrix, was Type I collagen evident as assembled fibres (Fig. 9B) which labeled specifically as illustrated in Figures 10A to 10F. Even in samples demonstrating the specificity of the Type I antibody employed (Figs. 10B, 10D) no evidence was seen of labeling of the underlying calcified accretions of the cement line matrix. However, Type III collagen displayed the inverse sequence. In less mature cultures, it exhibited a ubiquitous distribution, somewhat similar to chondroitin sulphate (Fig. 11), while it was undetectable at later stages of culture. Immuno-SEM demonstrated the apparent association of the Type III antibody with the fibrous extracellular matrix of mature cultures, although the specificity of labeling, as seen with Type I collagen, was absent (Figs. 12A and 12B).

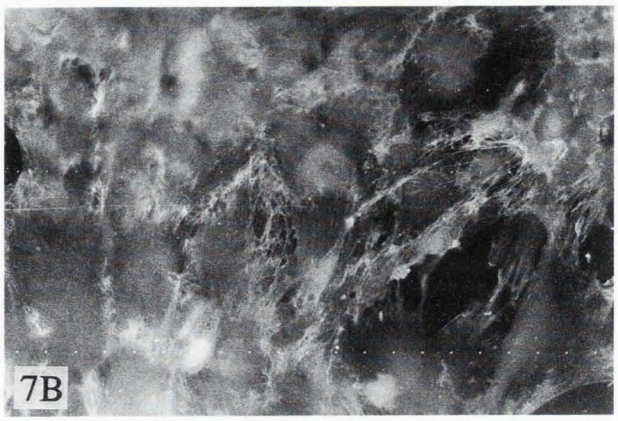
Finally, osteocalcin was the only antibody employed which showed no labeling at early culture periods while at later stages both the cellular compartment and the previously formed interfacial matrix labeled positively. Indeed, using the fluorescently labeled antibody, the labeling clearly demarcated scratches in the interfacial layer caused by sample preparation, in a similar manner described for osteopontin (Fig. 13A). Immuno-SEM confirmed the attachment of the label to the previously formed calcified accretions (Figs. 13B and 13C).

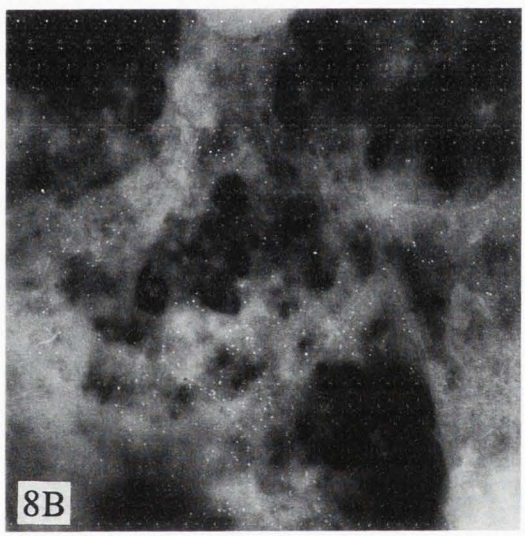
Discussion

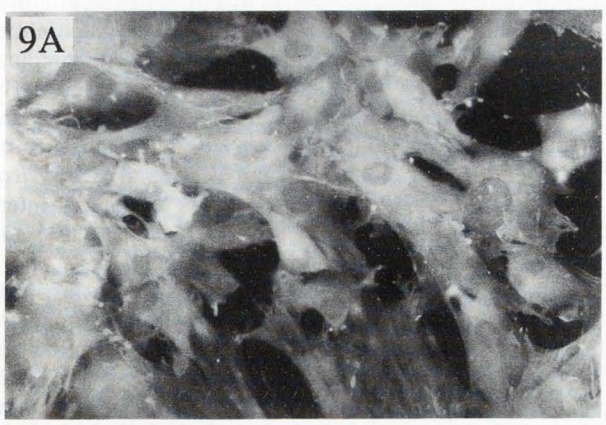
The purpose of the experiments reported herein was to characterize the initial, interfacial, extracellular matrix laid down by the differentiating osteogenic cells in our culture system. We know from the original description (Maniatopoulos *et al.*, 1988) that this culture system will produce a high yield of bone nodules that exhibit morphological, ultrastructural and biochemical characteristics of bone tissue. Specifically, Maniatopoulos *et al.* (1988) demonstrated that the mineralized, calcium hydroxyapatite, extracellular matrix of such nodules comprised collagen (predominantly type I, although type III was also detectable), osteonectin and osteocalcin. We have also shown, in previous studies, that the cells at the substratum surface will produce an organic matrix, apparently free of assembled collagen fibrils, which becomes seeded with small crystallites to form globular accretions approximately 1 μm in diameter and approximately 0.5 μm in height (Davies *et al.*, 1991a). Fusion of these globules on the culture surface creates a linear calcified layer which separates the developing bone nodule from the underlying substratum. We have compared this initial extracellular matrix with the cement line found in natural bone tissue and have shown the structural similarities of this early *in vitro* matrix with extracellular matrix elaborated at reversal lines in both mature and immature bone. The purpose of the work reported herein was twofold: first, to identify the presence of bone specific organic components in the early interfacial matrix which could be implicated in providing seeding sites for calcium phosphate crystals; and second, to confirm, using immunohistochemistry, our lack of morphological evidence of a collagen component to this early matrix.

Initial characterization of the earliest organic matrix secreted by the differentiating osteogenic cells in our culture system has been achieved using fluorescent or gold labeled antibodies to six known matrix components. Clearly, even a comprehensive approach including a large number of specific and non-specific bone proteins could exclude proteins which may play important roles in these early matrix assembly events but which have not yet been identified. However, our initial









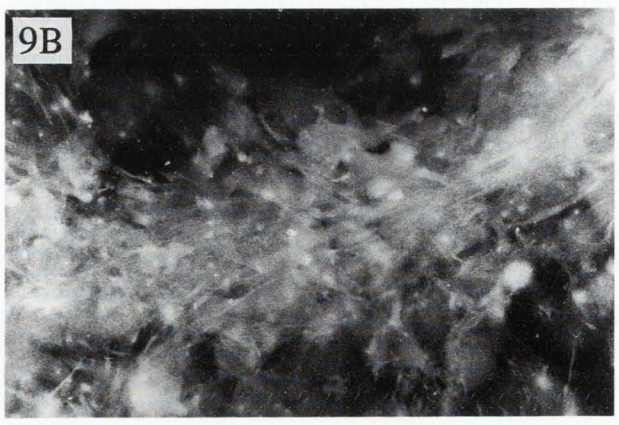


Figure 7. Cellular fibronectin visualized at an early stage (**A**) and more mature stage (**B**) of culture. At neither stage was there labeling of the culture substratum as noted for osteopontin. While there is early evidence of label at sites of focal contacts (arrowheads), as the culture developed, it became evident that the fibronectin was associated with a fibrillar extracellular compartment. FW = 225 μm (A, B).

Figure 8. Immuno-SEM of cellular fibronectin: (**A**) SEI and (**B**) BSEI. The antibody label is clearly associated with the fibrillar extracellular compartment. FW = 15 μm.

Figure 9. Labeling for Type I collagen at early (**A**) and later (**B**) stages of culture. Note that in (A), only an intracellular signal is seen. FW = 225 μm (A, B).

studies have yielded interesting results which correlate both with our previous morphological observations and other reports in the literature concerning expression of osteoblast phenotype related gene products. We have shown temporal changes in interfacial protein synthesis. Fibronectin, collagen type III and osteopontin were all present at the interface before collagen type I, although the latter was expressed by cells at early time points, while osteocalcin was only seen in the later stages of culture. These differences were consistent between the two techniques used.

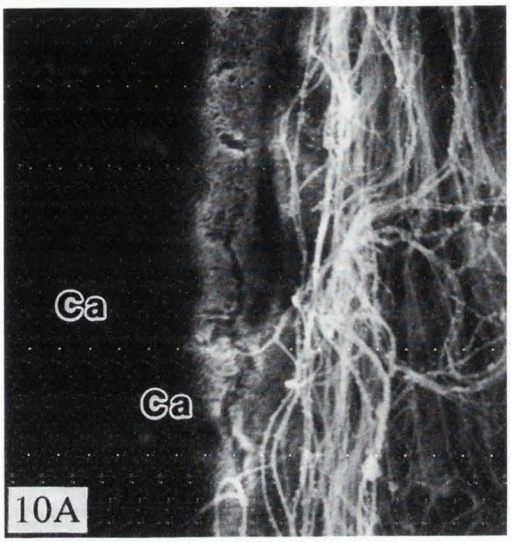
The secretion of an organic matrix prior to calcification is to be expected since this forms the basis of all biomineralization systems where macromolecules containing acidic groups, sulphates, carboxylic acids or phosphates may be involved (Alper, 1992). The roles of non-collagenous bone proteins have been thoroughly reviewed, in the implant context, by Sodek *et al.* (1991). Of these osteopontin, which is highly sulphated, deserves special attention as it is implicated, by the presence of an arginine-glycine-aspartic acid (RGD) sequence, in osteoblast attachment (Oldberg *et al.*, 1986; Sommerman *et al.*, 1987; Butler, 1989) as well as hydroxyapatite nucleation and could thus serve both functions at the interface.

The presence of osteopontin was expected based on its localization at cement lines *in vivo* (McKee *et al.*, 1992, 1993; Chen *et al.*, 1993), and that studies on the developmental appearance of osteopontin have demonstrated that 'preosteoblasts', as well as osteoblasts and osteocytes, expressed osteopontin mRNA (Nomura *et al.*, 1988, Weinreb *et al.*, 1990) and were strongly stained immunohistochemically (Mark *et al.*, 1987). This would appear to contradict the results Ibaraki *et al.* (1992) who reported that *in vitro* osteopontin levels were initially low, and increased coordinately with the expression of type I collagen and alkaline phosphatase, and those of Yoon *et al.* (1987) who reported that *in vivo* osteopontin expression occurs late, after collagen I and alkaline phosphatase but coordinately with osteocalcin, which suggests that osteopontin expression occurs only in fully differentiated osteoblasts. This difference may be explained by the results of Owen *et al.* (1990, 1991) and Strauss *et al.* (1990) who reported that there was an initial peak of osteopontin mRNA levels early in culture which declined and was followed by a second peak as the cultures began to mineralize. Indeed, Owen *et al.* (1990) observed that osteopontin gene expression peaked at 25% maximal levels on day 7, was no longer detectable on days 10 and 12, and finally reached maximal levels on day 20 in rat fetal calvarial cultures. It has been suggested that the initial induction of osteopontin may be involved in controlling the relationships between cells and extracellular matrices (Lian and Stein, 1992). It was therefore not surprising that osteopontin was localized at the ends of cell pseudopodia at early culture stages, implying a role in cell attachment, and strongly expressed on the substratum surface where calcification ensued to produce the cement-like globular layer.

Figure 10 (on the facing page). SEI (**A**, **C** and **E**) and the corresponding BSEI (**B**, **D** and **F**) of Type I Collagen. In (A), bundles of collagen fibres are seen running vertically on the right of the field of view. Little can be discerned of the underlying calcified globular accretions (Ca) which are seen more easily in the BSEI in (B). The colloidal gold marker is only seen in the collagen fibre containing compartment. The specificity of the antibody to these fibres is illustrated in higher magnifications of a similar field of view (C, D). Control specimens, without the primary antibody are shown in (E) and (F). FW = 6 μm (A, B, E, F); 3 μm (C, D).

Owen and co-workers (Owen *et al.*, 1990, 1991; Barone *et al.*, 1991; Lian and Stein, 1992) have examined the expression of several matrix proteins *in vitro*. Fibronectin mRNA levels are initially high and decline throughout the culture period, osteopontin as already discussed peaks early, declines and then rises to its maximal level. Type I collagen mRNA rapidly reaches its maximal level and then declines. Osteocalcin levels are undetected until late in culture at which point they rapidly increase to maximal levels. Although type I collagen mRNA levels were initially 50% maximal and reached maximal levels by day 10 of culture type I collagen did not begin to accumulate in the cell/matrix layer until after the mRNA levels were declining (Gerstenfeld *et al.*, 1988; Barone *et al.*, 1991). Examination of collagen secretion and accumulation into the matrix of chick osteoblast cultures revealed that although collagen was being synthesized and secreted into the medium from the earliest time point examined (day 3), collagen accumulation into the matrix was initially negligible, rapidly increasing between days 6 and 12 (Gerstenfeld *et al.*, 1988). This was thought to occur due to incomplete processing of the procollagen molecules in the early stages of culture. Our results are not only in agreement with these reports, but also corroborate the absence of assembled collagen in the interfacial matrix *in vitro* (Davies *et al.*, 1991b) as well as at bone implant interfaces (Davies *et al.*, 1991a,c; Pilliar *et al.*, 1991) and cement lines *in vivo* (Zhou *et al.*, 1993).

The early intercellular matrix was rich in fibronectin, as were focal contacts, although no fibronectin was observed at the substratum interface. Similar results were obtained by Gronowicz and DeRome (1992) who examined the differential expression of fibronectin, osteonectin and collagen I during calvarial development. Utilizing northern blot analysis, *in situ* hybridization and immunofluorescence, they demonstrated that fibronectin synthesis preceded type I collagen expression and was predominantly seen around the condensed preosteoblasts. Majmudar *et al.* (1991) demonstrated that the matrix synthesized by chick osteoblasts *in vivo* and *in vitro* contained fibronectin and type III collagen as well as type I collagen. It is known that one adsorbed protein species can be replaced by another of higher affinity for a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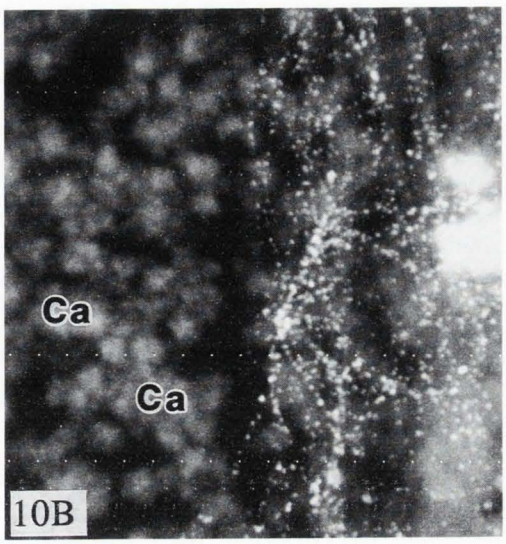
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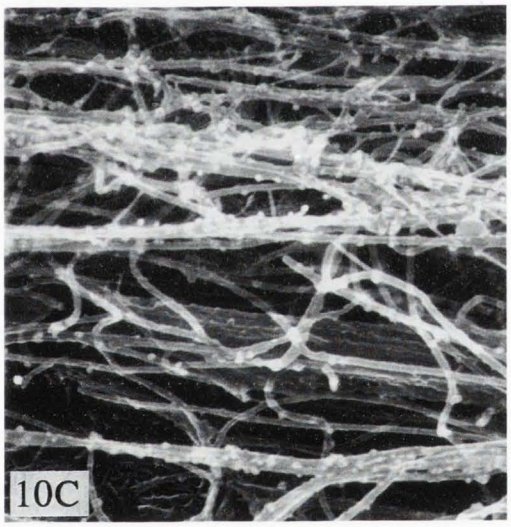
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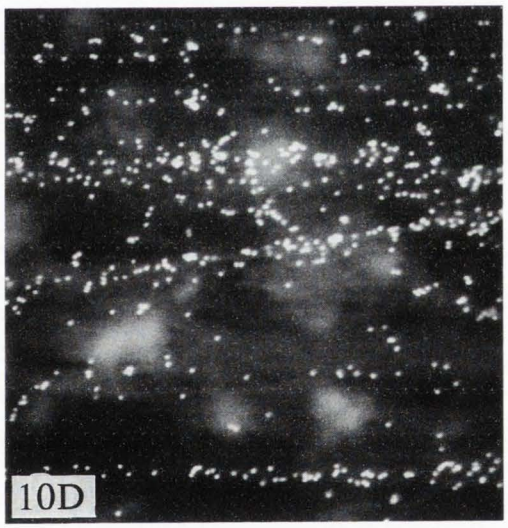
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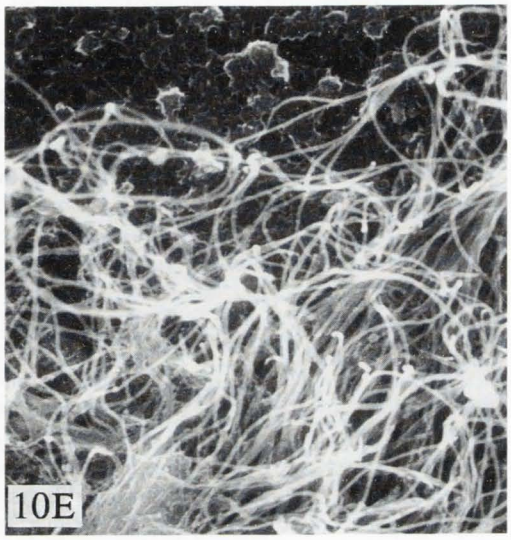
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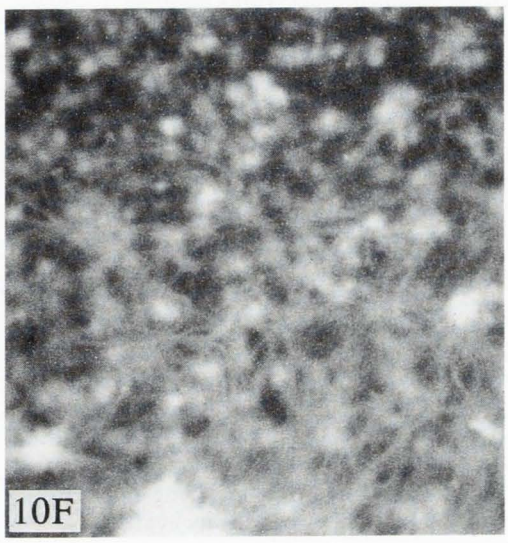
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surface (Norde, 1986). Thus, it is possible that fibronectin was competitively replaced by osteopontin at the substratum surface.

Numerous studies have reported that bone cells *in vitro* synthesize collagen type III as well as type I (Maniatopoulos *et al.*, 1988; Wranna *et al.*, 1988; Collin *et al.*, 1992) although it was not clear whether this was synthesized by osteogenic cells or by contaminating fibroblasts. Multimaki *et al.* (1987) examined collagen gene expression during endochondral fracture repair and demonstrated that, during the first week, type III collagen mRNA increased to the greatest extent which was correlated with the development of granulation tissue and primitive mesenchymal callus. By 28 days, the predominant collagen mRNA was type I with very little type II or III. Collagen type III has also been identified in mesenchymal condensations prior to endochondral ossification (Reddi *et al.*, 1977; Lane *et al.*, 1986; Page *et al.*, 1986) and recently has been identified in intramembranous bone (Carter *et al.*, 1991; Majmudar *et al.*, 1991). These results suggest that type III collagen is expressed during the initial stages of osteogenesis. We have observed that type III collagen is present in the early extracellular matrix and that as the culture matures, collagen III becomes undetectable.

We observed that osteocalcin did not appear in the early cultures, but was seen at later stages, being associated with both the cellular compartment and the previously formed calcified accretions of the cement line-like layer. This observation would suggest that while osteocalcin was not synthesized during the formation of the cement like matrix, this bone specific protein, once secreted, did attach to the underlying mineralized matrix. Our results agree with previous studies on the expression of osteocalcin which have invariably demonstrated that it is not expressed until late in the developmental cascade (Bronkers *et al.*, 1987; Yoon *et al.*, 1987; Owen *et al.*, 1990, 1991; Strauss *et al.*, 1990; Weinreb *et al.*, 1990). Recently there have been reports that osteocalcin was localized at reversal lines (McKee *et al.*, 1992, Carlson *et al.*, 1993). This does not necessarily mean that osteocalcin was synthesized as the cells elaborated the cement line matrix. Osteocalcin is present in serum and rapidly accumulates in mineralized tissues due to its high affinity for hydroxyapatite (Hauschka *et al.*, 1989). Delmas *et al.* (1984) demonstrated that bone contains many serum proteins which are not derived from osteogenic cells. This suggests that osteocalcin found at reversal lines could derive from neighbouring osteoblasts which were in the process of synthesizing and secreting matrix, including osteocalcin, or could have been released into the extracellular fluid from bone matrix during resorption. A similar process may be occurring in our model system where osteocalcin appears at both the interface and in the intercellular compartment. After matrix mineralization is initiated, mature osteoblasts, present in the culture, would be synthesizing and secreting osteocalcin which could adsorb to any available mineralized matrix.

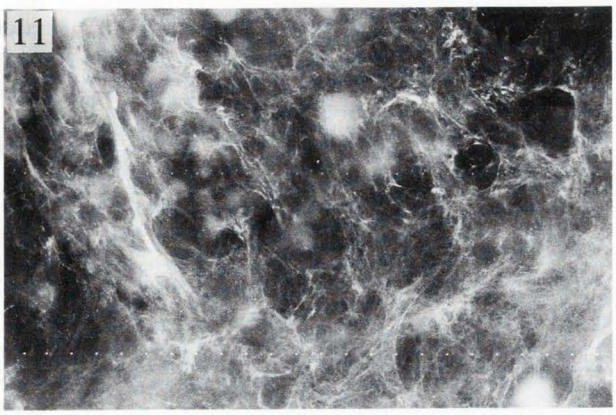
Figure 11. Immuno-fluorescence of Type III collagen at an early culture stage demonstrates the ubiquitous distribution of this protein. FW = 225 μm.

Figure 12. Immuno gold labeling of Type III collagen (**A**) SEI and (**B**) BSEI, shows preferential distribution in the fibrous extracellular matrix rather than the cell surface. FW = 6 μm (A, B).

Figure 13. (**A**) Immuno-fluorescence of anti-osteocalcin antibody at a more mature culture stage. No signal for this protein was detected in early cultures. Nevertheless, the calcified interfacial extracellular matrix is strongly positive and illustrates scratch marks (s) on the substratum surface. (**B**) SEI and (**C**) BSEI of calcified accretions. Note that each gold particle is associated with an accretion rather than the underlying culture substratum. FW = 225 μm (A); 6 μm (B, C).

One question which must be addressed is: Does an *in vitro* assay bear any relevance to *in vivo* reality? This is especially important in the context of modeling bone/implant interfaces in view of the expressed belief that such experiments "reflect the assumed reality of the test tube" (Albrektsson, 1992). This issue has been addressed previously (Davies, 1990), and we have also published experimental evidence which indicates the similarity of the morphological appearance of the cement line-like extracellular matrix, at retrieved implant surfaces, to that which we have described for the first time in culture (Davies *et al.*, 1991a,b,c). However, the cement line structure is not always recognized from *in vivo* studies. Some confusion has arisen with the linking of this morphological descriptor with the term lamina limitans both in the bone and implant literature (McKee *et al.*, 1993; Davies, 1991). The lamina limitans is a feature produced in demineralized sections only, and represents a concentration of proteins which is not visualized in sections which are un-decalcified, while cement lines are distinct, and normal, morphological structures in bone.

Finally, the change in cell shape, evidence for which we have now observed in both scanning electron microscopy (Davies *et al.*, 1991b) and the osteopontin labeling at the substratum surface illustrated herein, is worthy of note. Cell morphology is maintained by the cytoskeleton, and the latter is linked to intranuclear filaments. Thus, the cytoskeleton and associated changes in cell shape are implicated in important cell functional roles (Fey *et al.*, 1984), since it is through this fibre network continuum that gene regulation by extracellular agents has been hypothesized (Ben-Ze'ev, 1991). Thus, observations of change in shape of the differentiating osteogenic cells in our culture system may well be related to changes in gene expression linked to the continued differentiation of these cells. The correlative light and scanning electron microscopy demonstrated similar cell morphology on coverslip glass and tissue culture polystyrene. However, from the perspective of bone cell/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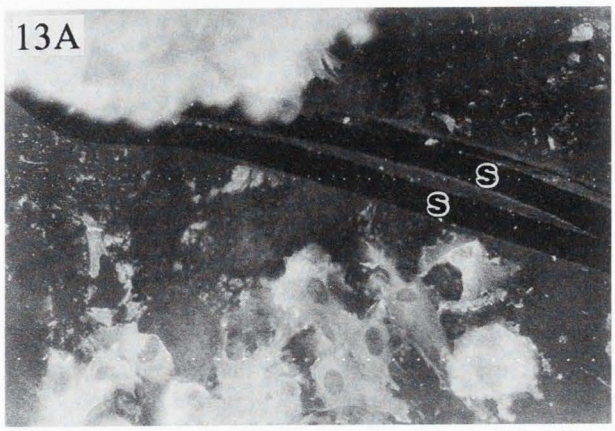
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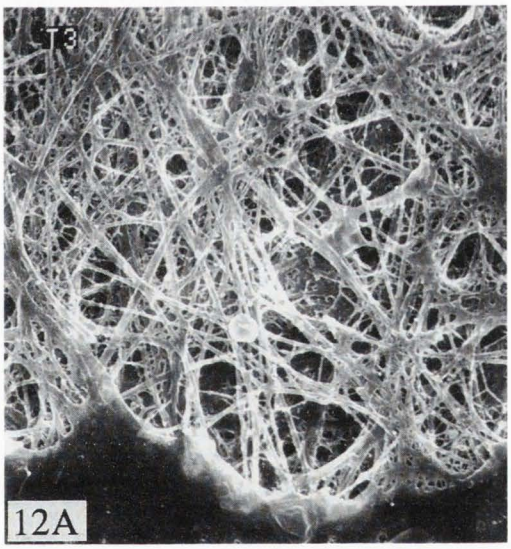
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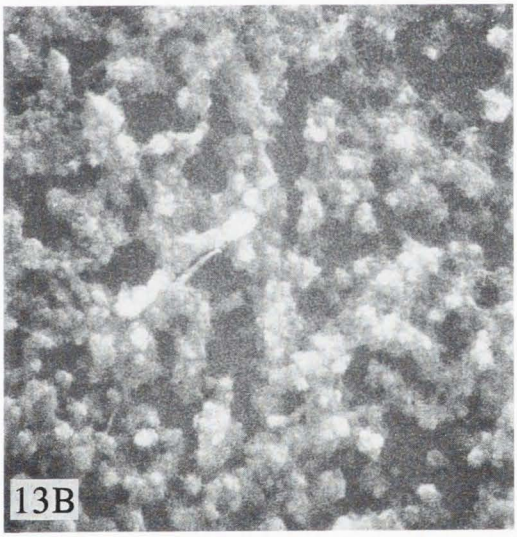
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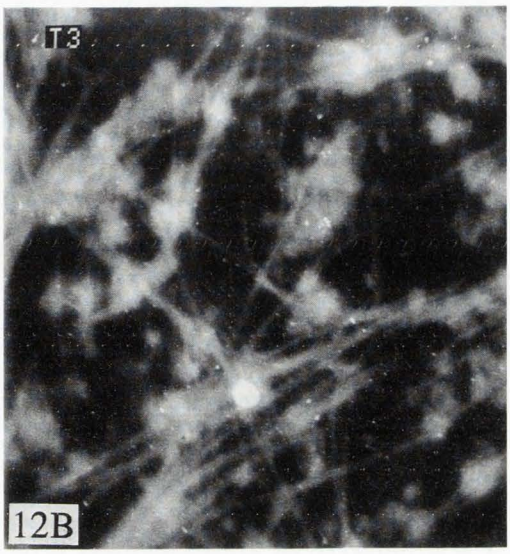
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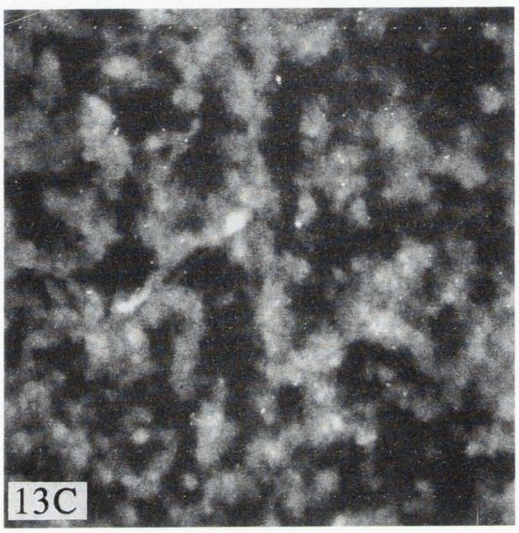
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biomaterial interactions, the observations that bone-derived cells can adopt different morphological appearances on substrata of different chemical (Davies *et al.*, 1986) or physical (Bowers *et al.*, 1992) properties may have important implications in bone formation on candidate implant materials.

Conclusions

The experimental approach described has been employed to deconvolute the temporal sequence of events which characterize organic matrix secretion by differentiating osteogenic cells at the surface of a culture substratum. In particular, the presence of osteopontin implies a dual role for this protein in both cell attachment and mineralization of an organic matrix which is devoid of assembled type I collagen fibres and, we consider, reflects the structure and composition of cement lines found at remodeling sites in bone. The appearance of both type I collagen and osteocalcin at later developmental stages in culture not only agrees with previously published data, but also indicates that the cement line-like matrix is elaborated, and mineralized before these osteogenic cells express their mature, osteoblastic phenotype.

Acknowledgments

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References

Abnur Z, Geiger B (1984) Immunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. Cell **38**, 811-822.

Abnur Z, Geiger B (1985) Spatial interrelationships between proteoglycans and extracellular matrix proteins in cell cultures. Experimental Cell Res. **158**, 321-332.

Albrektsson T (1992) Current Issues Forum. Int. J. Oral & Maxillofac. Implants **7**, 416-417.

Alper M (ed.) (1992) Biology and materials synthesis. Part 1. MRS Bulletin, Volume **XVII** (October, 1992), 24-59.

Barone LM, Owen TA, Tassinari MS, Bortell R, Stein GS, Lian JB (1991) Developmental expression and hormonal regulation of the rat matrix Gla protein (MGP) gene in chondrogenesis and osteogenesis. J. Cell. Biochem. **46**, 351-365.

Ben-Ze'ev A (1991) Animal cell shape changes and gene expression. BioEssays **13**, 207-212.

Bowers KT, Keller JC, Randolph BA, Wick DG, Michaels CM (1992) Optimization of surface micromorphology for enhanced osteoblast responses *in vitro*. Int. J. Oral & Maxillofac. Implants **7**, 302-310.

Bronckers ALJJ, Gay S, Finkelman RD, Butler RD (1987) Developmental appearance of Gla proteins (osteocalcin) and alkaline phosphatase in tooth germs and bones of the rat. Bone Min. **2**, 361-373.

Butler WT (1989) The nature and significance of osteopontin. Conn. Tissue Res. **23**, 123-136.

Carlson CS, Tulli HM, Jayo MJ, Loeser RF, Tracy RP, Mann KG, Adams MR (1993) Immunolocalization of noncollagenous bone matrix proteins in lumbar vertebrae from intact and surgically menopausal Cynomolgus monkeys. J. Bone Min. Res. **8**, 71-81

Carter DH, Sloan P, Aaron JE (1991) Immunolocalization of collagen types I and III, tenascin, and fibronectin in intramembranous bone. J. Histochem. Cytochem. **39**, 599-606.

Chen J, McKee MD, Nanci A, Sodek J (1993) Ultrastructural localization and mRNA expression of bone sialoprotein (BSP) and osteopontin (OPN) in fetal porcine calvarial bone. Histochem. J. (in press).

Collin P, Neffussi JR, Wetterwald A, Nicolas V, Boy-Lefevre M-L, Fleisch H, Forest N (1992) Expression of collagen, osteocalcin, and bone alkaline phosphatase in a mineralizing rat osteoblastic cell culture. Calcif. Tissue Int. **50**, 175-183.

Davies JE (1990) The use of cell and tissue culture to investigate bone cell reactions to bioactive materials. In: CRC Handbook of Bioactive Ceramics. Yamamuro T, Hench LL, Wilson J (eds.). CRC Press, Boca Raton, FL. pp. 195-225.

Davies JE (ed.) (1991) The Bone-Biomaterial Interface. Univ. Toronto Press, Toronto, Canada. p. 226.

Davies JE, Causton B, Bovell YP, Davy K, Sturt CS (1986) The migration of osteoblasts over substrata of discrete surface charge. Biomaterials **7**, 231-233.

Davies JE, Ottensmeyer P, Shen X, Hashimoto M, Peel SAF (1991a) Early extracellular matrix synthesis. In: The Bone-Biomaterial Interface. Davies, JE (ed.), University of Toronto Press, Toronto, pp. 214-228.

Davies JE, Chernecky R, Lowenberg B, Shiga A (1991b) Deposition and resorption of calcified matrix *in vitro* by rat bone marrow cells. Cells and Materials **1**, 3-15.

Davies JE, Pilliar RM, Smith DC, Chernecky R (1991c) Bone interfaces with retrieved alumina and hydroxyapatite ceramics. In: Bioceramics, Volume 4. Bonfield W, Hastings GW, Tanner KE (eds.) Butterworth-Heinemann Ltd, London. pp. 199-204.

de Harven E, Leung R, Christensen H (1984) A novel approach for scanning electron microscopy of colloidal gold-labeled cell surfaces. J. Cell Biol. **99**, 53-57.

Delmas PD, Tracy RP, Riggs BL, Mann KG (1984) Identification of the noncollagenous proteins of bovine bone by two-dimensional gel electrophoresis. Calcif. Tissue Int. **36**, 308-316.

Fey EG, Wan KM, Penman S (1984). Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: three-dimensional organization and protein composition. J. Cell Biol. **98**, 1973-1984.

Gerstenfeld LC, Chipman SD, Kelly CM, Hodgens KJ, Lee DD, Landis WJ (1988) Collagen expression, ultrastructural assembly, and mineralization in cultures of chicken embryo osteoblasts. J. Cell Biol. **106**, 979-989.

Gronowicz G, DeRome M (1992) Differential expression of bone matrix proteins during early mineralization of rat calvaria *in vivo*. J. Bone Min. Res. **7** (Suppl. 1), S270 (Abstract 711)

Hauschka PV, Lian JB, Cole DEC, Gundberg CM (1989) Osteocalcin and matrix Gla protein: Vitamin K-dependent proteins in bone. Physiol. Rev. **69**, 990-1047.

Ibaraki K, Termine JD, Whitson SW, Young MF (1992) Bone matrix mRNA expression in differentiating fetal bovine osteoblasts. J. Bone Min. Res. **7**, 743-754.

Lane JM, Suda M, von der Mark K, Timpl R (1986) Immunofluorescent localization of structural collagen types in endochondral fracture repair. J. Orthop. Res. **4**, 318-329.

Lian JB, Stein GS (1992) Concepts of osteoblast growth and differentiation: Basis for modulation of bone cell development and tissue formation. Crit. Rev. Oral Biol. Med. **3**, 269-305.

Lowenberg B, Chernecky R, Shiga A, Davies JE (1991) Mineralized matrix production by osteoblasts on solid titanium *in vitro*. Cells and Materials **1**, 177-187.

Magloire H, Bouvier M, Joffre A. (1992) Odontoblast response under carious lesions. Proc. Finn. Dent. Soc. **88** (Suppl. 1), 257-274.

Majmudar G, Bole D, Goldstein SA, Bonadio J (1991) Bone cell culture in a three-dimensional polymer bead stabilizes the differentiated phenotype and provides evidence that osteoblastic cells synthesize type III collagen fibronectin. J. Bone Min. Res. **6**, 869-881.

Maniatopoulos C, Sodek J, Melcher AH (1988) Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats. Cell Tiss. Res. 254: 317-330.

Mark MP, Prince CW, Oosawa T, Gay S, Bronckers ALJJ, Butler WT (1987) Immunohistochemical demonstration of a 44-KD phosphoprotein in developing rat bones. J. Histochem. Cytochem. **35**, 707-715.

McKee MD, Glimcher MJ, Nanci A (1992) High resolution immunlocalization of osteopontin and osteocalcin in bone and cartilage during endochondral ossification in the chicken tibia. Anat. Rec. **234**, 479-492.

McKee MD, Farach-Carson MC, Butler WT, Hauschka PV, Nanci A (1993) Ultrastructural immunolocalization of noncollagenous (osteopontin and osteocalcin) and plasma (albumin and a_2HS-glycoprotein) proteins in rat bone. J. Bone Min. Res. **8**, 485-496.

Multimaki P, Aro H, Vuorio E (1987) Differential expression of fibrillar collagen genes during callus formation. Biochem. Biophys. Res. Comm. **142**, 536-541.

Nomura S, Wills AJ, Edwards DR, Heath JK, Hogan BLM (1988) Developmental expression of 2ar (osteopontin) and SPARC (osteonectin) RNA as revealed by *in situ* hybridization. J. Cell Biol. **106**, 441-450.

Norde W (1986) Adsorption of proteins from solution at the solid-liquid interface. Adv. Colloid Interface Sci. **25**, 267-340.

Oldberg A, Franzen A, Heinegard D (1986) Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. Proc. Natl. Acad. Sci. USA **83**, 8819-8823.

Owen TA, Aronow M, Shaloub V, Barone LM, Wilmig L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS (1990) Progressive development of the rat osteoblast phenotype *in vitro*: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J. Cell. Physiol. **143**, 420-430.

Owen TA, Aronow MS, Barone LM, Bettencourt B, Stein GS, Lian JB (1991) Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: Dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. Endocrinology **128**, 1496-1504.

Page M, Hogg J, Ashurst DE (1986) The effects of mechanical stability on the macromolecules of the connective tissue matrices produced during fracture healing. I. The collagens. Histochemical J. **18**, 251-265.

Pilliar RM, Davies JE, Smith DC (1991) The bone-biomaterial interface for load-bearing implants. MRS Bulletin **Volume XVI** (September, 1991), 55-61.

Reddi AH, Gay R, Gay S, Miller EJ (1977) Transitions in collagen types during matrix-induced cartilage, bone, and bone marrow formation. Proc. Natl. Acad. Sci. USA **74**, 5589-559.

Sodek J, Zhang Q, Goldberg HA, Domenicucci C, Kasugai S, Wrana JL, Shapiro H, Chen J (1991) Noncollagenous bone proteins and their role in substrate-induced bioactivity. In: The Bone-Biomaterial Interface. Davies JE (ed.). University of Toronto Press, Toronto, pp. 97-110.

Sommerman MJ, Prince CW, Sauk JJ, Foster RA, Butler WT (1987) Mechanism of fibroblast attachment to bone extracellular matrix: role of a 44-Kilodalton bone phosphoprotein. J. Bone Min. Res. **2**, 259-265.

Stein GS, Lian JB, Owen TA (1990) Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB **4**, 3111-3123.

Strauss PG, Closs EI, Schmidt J, Erfle V (1990) Gene expression during osteogenic differentiation in mandibular condyles *in vitro*. J. Cell Biol. **110**, 1369-1378.

Takeuchi Y, Matsumoto T, Ogata E, Shishiba Y (1990) Isolation and characterization of proteoglycans synthesized by mouse osteoblastic cells in culture during the mineralization process. Biochem. J. **266**, 15-24.

Thesleff I, Vaahtokari A (1992) The role of growth factors in determination and differentiation of the odontoblastic cell lineage. Proc. Finn. Dent. Soc. **88** (Suppl. 1), 357-368.

Weinreb M, Shinar D, Rodan GA (1990) Different pattern of alkaline phosphatase, osteopontin and osteocalcin expression in developing rat bone visualized by **in situ** hybridization. J. Bone Min. Res. **5**, 831-842.

Wrana JL, Maeno M, Hawrylyshyn, Yao K-L, Domenicucci C, Sodek J (1988) Differential effects of transforming growth factor-β on the synthesis of extracellular matrix proteins by normal fetal rat calvarial bone cell populations. J. Cell Biol. **106**, 915-924.

Yoon K, Buenaga R, Rodan GA (1987) Tissue specificity and developmental expression of rat osteopontin. Biochem. Biophys. Res. Comm. **148**, 1129-1136.

Zhou H, Chernecky R, Davies JE (1993) Deposition of cement at reversal lines in rat femoral bone. J. Bone Min. Res. (in press).

Discussion with Reviewers

M.D. McKee: Could you elaborate on what you consider to be the distinction between cement lines and laminae limitantes in the context of bone remodeling?

Authors: As has been discussed in detail elsewhere (see index entries in Davies, 1991), cement lines are distinct morphological features of normal bone which are seen at specific sites (e.g., the outer margins of Haversian systems), whereas laminae limitantes are features only observable in decalcified sections. A useful analogy may be drawn here with the basophilia associated with many linear structures in bone, resting and reversal lines, interlamellar lines etc. Such common staining patterns indicate some common compositional features of many structures, which depend on the use of decalcified specimens (a true complication when interpreting hard tissues), but are not themselves structural markers. This is important since, on the one hand staining bands (sic. laminae limitantes) may occur at diverse sites in tissue which (in the normal state) may be either calcified or not calcified while, the linear structures stained may be of widely different thicknesses and, perhaps, represent entirely disparate functional roles.

Thus, the term lamina limitans can be applied to any linear structure which separates one area, or type, of tissue from another, e.g., the relatively broad electron dense borders of variable width which can be seen in decalcified sections of focal areas of mineralization in bone, or the fine linear arrangements seen on the walls of osteocyte canaliculi. Such staining reactions, may be as generalized as basophilia itself, or within one tissue may be due to the presence of similar groups of proteins at various sites within, for example, bone matrix. On the other hand cement lines which share both the basophilia and non-collagenous bone protein composition with other linear features in bone are structurally quite distinct. Unlike the lamina limitans of the osteocyte canaliculus, which is of the order of 2 nm wide and (probably) not mineralized, the cement line is about 500 nm wide and of the same mineral composition as neighbouring bone although of relatively greater density due to the absence of assembled collagen fibres. While the osteocyte canaliculus marks the limit of a hard tissue mass, the cement line forms the interface between old and new bone tissue. No other linear basophilic structure in bone is of this dimension. Interlamellar lines, while basophilic and marking the junction between older and newer bone, do not represent the discontinuum of the collagen containing bony compartment seen at cement lines but simply a change in direction of collagen fibres produced by the same population of osteogenic cells.

Thus, in terms of remodeling, cement lines are the morphologically distinct boundary tissue which provides the interface between old (resorbed) bone and new bone being laid down by the new population of osteogenic cells which occupy the resorption site. Since cement lines contain bone-associated proteins such as osteopontin and bone sialoprotein and appear at a time when the osteogenic cell population, which will form the first bone lamella, is differentiating, we believe that the cement line represents the earliest bone matrix produced by differentiating bone cells and is thus of central importance in the remodeling cascade. We believe that our *in vitro* results, reported and cited herein, attest to this interpretation of the relevance of cement lines.

H. Lesot: What is known about the molecular composition of cement and what is involved in the mineralization of this specific matrix?

J.-R. Nefussi: It is not clear what the authors mean by cement like matrix *in vitro*. Is it (1) the first material synthesized by a bone cell during its attachment and spreading, or (2) the first deposit preceding the matrix bone formation, or (3) both of them. Knowing that all bone cells attached to a surface will not express a bone matrix until a three dimensional environment is formed (as accepted by most of the authors in the literature, ourselves included, Nefussi et al., 1985). We do not believe in the first and third hypothesis. Therefore, results concerning the very early times where no nodules have yet been formed are, in that respect, questionable.

Authors: Comparatively little is known about either the composition or mineralization of cement lines. We have recently provided a review of the available literature in Zhou *et al.* (1993). What is becoming clear from both our work (Davies *et al.*, 1991b) and that of McKee *et al.* (1992, 1993) is that the cement line comprises an organic matrix, devoid of assembled collagen fibres (confirming the original histological work of Weidenreich, 1930) but rich in other proteins characteristic of bone matrix. Even less is specifically known about the mechanisms of mineralization of this matrix, although both osteopontin and bone sialoprotein have been implicated in providing seeding sites for calcium phosphate crystal nucleation (Butler, 1989; Sodek *et al.*, 1991). However evidence from Goldberg *et al.* (1993) have implicated only BSP in this role. With regard to the cement like matrix *in vitro*, we have described this in detail elsewhere (Davies et al., 1991a, b) and believe it to be an integral, although morphologically distinct, part of normal bone extracellular matrix; just as is the case *in vivo*.

H. Lesot: How does the appearance-disappearance of antigens occur when following the immuno-staining from the periphery to the center of individual nodules? Are there overlapping zones? Are there specific areas where cells transitorily co-express antigens?
Authors: The first point to make is that as the center of individual nodules is approached, the extracellular matrix, both at the interface and within the bulk of the nodule, calcifies. This, as is well known in hard tissue biology, compromises the penetration of antibodies and also blocks binding sites which would have been available before mineralization. For this reason, we have not said collagen III disappears but rather that it is undetectable. Nevertheless, in the maturation of enamel matrix, it is now believed that proteins are specifically removed from the matrix by ameloblasts. Thus, there may be a possibility that analogous behaviour may be found in osteoblasts.

H. Lesot: According Fig. 4C, one might expect chondroitin sulphate to be closely associated with focal contacts; is this true when using scanning electron microscopy? Is there a correlation between the two techniques as far as antigens potentially involved in the formation of the focal contacts are concerned?
Authors: It is not possible, with the SEM technique which we have employed, to visualize the molecules involved in cell membrane binding at focal contacts, and we are thus unable to address this question.

H. Lesot: It has been reported that use of intact immunoglobulins instead of Fab fragments could lead to negative results because of steric problems. Could the authors comment on this point based to their experience?
Authors: We have no experience with the use of Fab fragments in this specific experiment. However, since we exposed the substratum surface by stripping away the overlying cell layers, we do not believe that our results would be compromised by the lack of penetration of immunoglobulins which, in intact tissue, can lead to false negative results.

H. Lesot: If osteopontin does play a specific role in controlling the relationships between cells and extracellular matrices in this precise case, does it mean that osteopontin has functions essentially different from fibronectin (i.e., in the mediation of matrix-matrix interactions) which would also be a good candidate for such a function or does it mean that differential expression of integrins might indeed play a key role?
Authors: Although we do not know the functions of these proteins, it has been observed that cell attachment and spreading persists on osteopontin coated substrata while it is only transitory on fibronectin coated substrata (Sauk *et al.*, 1991). This along with the observation that osteopontin expression is differs from that of fibronectin (Owen *et al.* 1990; Stein *et al.* 1990) would suggest that they have different functions in osteogenesis. We have no information concerning the expression of integrins in our model system. From the literature (Sauk *et al.*, 1991) it is evident that fibronectin binds to a variety of different integrins, including two which are specific for fibronectin. Osteopontin is thought to bind to the $a_v\beta_3$ receptor, which itself binds several other matrix proteins, including fibronectin. Thus, it is possible that differential expression of these integrins plays a role in the interaction between the cells and the matrix.

H. Lesot: Is it known whether the replacement of fibronectin by osteopontin is accompanied qualitative changes in the expression of integrins?
Authors: No, we are suggesting replacement of fibronectin by osteopontin may happen but have no experimental data to propose this idea as an hypothesis.

J.-R. Nefussi: The authors should not exclude to indicate the time of the observations reported when pictures are related to early time points (before nodule formation). This will avoid any confusion as the absence of collagen fibers at early periods of culture which is normal before 24 hours but which would be surprising after.
Authors: We discuss, in some detail, why we believe it is misleading to attach specific temporal values to our cultures. However, we should perhaps emphasize that the earliest cultures were 3 days and therefore, we are not in a position to comment on pre-24 hour cultures.

J.-R. Nefussi: How do the authors explain detectable osteopontin protein "at very early stages" at areas where no cells are present as mentioned in the Results? Where is this protein coming from?
Authors: The protein is coming from cells which have been removed to expose the underlying culture surface.

R.M. Albrecht: Have isotype controls been performed to insure that nonspecific binding of the primary monoclonal antibody is also minimal?
Authors: Yes, we performed such controls for the fluorescence labelling and found no signal detectable other than the autofluorescence due to glutaraldehyde fixation. We did not perform equivalent controls for the immuno-SEM.

R.M. Albrecht: In some instances, the level of gold label seems to be in line with the extent of deposition of the antigenic species (Fig. 6 for example). In other instances the level of label appears to be less dense (Fig. 3 or 4). Does this reflect a reduced number of antigenic species or a reduced efficiency of labeling?
Authors: Given the extent of both matrix and labelling in Figures 3 and 6, we feel that it would be unreasonable to state that these images represent significant differences in either the number of antigenic species or the labeling efficiency. In the case of Fig. 4, a later stage of culture in which calcification of the interfacial matrix is already clearly evident, the apparent reduction in labeling could be due to either an absolute reduction of

antigenic species, or a masking of the latter due to the mineralization.

R.M. Albrecht: Have the authors seen any significant differences in the patterns of deposition and sequence of deposition on the different substrates they have used to date?
Authors: Yes, we have seen considerable differences in patterns of deposition on modified polystyrene surfaces (Peel *et al.* 1992, Callen *et al.*, 1993), but have not yet compared this morphological information with the immuno-labeling which we report herein.

Additional References

Callen BW, Sodhi RNS, Shelton RM, Davies JE (1993) Behavior of primary bone cells on characterized polystyrene surfaces. J. Biomed. Mater. Res. **27**, 851-859.

Goldberg HA, Benedictson JA, Hunter GK (1993) Nucleation of hydroxyapatite by bone sialoprotein. J. Dent. Res. **72** (suppl. 120), abstract 135.

Nefussi JR, Boy-Lefevre ML, Boulekbache H, Forest N (1985) Mineralization *in vitro* of matrix formed by osteoblasts isolated by collagenase digestion. Differentiation **29**, 160-168.

Peel SAF, Sodhi RN, Duc TM, Davies JE (1992) Polymer surface modification causes change in phenotypic expression of primary bone cells. In: Tissue-Inducing Biomaterials. Cima LG, Ross ES (eds.). Mat. Res. Soc. Symp. Proc. **252**. Materials Research Society, Pittsburgh, PA. pp. 71-77.

Sauk JJ, van Kampen CL, Somerman MJ (1991) Role of adhesive proteins and integrins in bone and ligament cell behaviour at the material surface. In: The Bone Biomaterial Interface, Davies JE (ed.). University of Toronto Press. pp. 111-119.

Weidenreich F (1930) Das Knochengewebe (Bone tissue). In: Handbuch der Mikroskopischen Anatomie des Menschen (Handbook of Microscopic Human Anatomy). Volume 2. von Mollendorf W (ed.). Verlag Julius Springer, Berlin. pp. 391-520.