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COLLAGEN FIBRES ARE NOT REQUIRED FOR INITIAL MATRIX MINERALIZATION BY BONE CELLS

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Abstract

Passaged primary cultures of young adult rat bone marrow cells were maintained in medium containing combinations of the supplements dexamethasone, ascorbic acid and Na-β-glycerophosphate. The cultures were examined by both light and scanning electron microscopy (with additional energy dispersive X-ray analysis). In cultures with fully supplemented medium, an extracellular matrix formed at the culture dish surface, beneath developing bone nodules; this matrix was mineralized but collagen-fibre free and corresponded to that which we have reported previously as the equivalent of the cement line *in vivo*. In selected samples this interfacial, cement line, matrix was analyzed by X-ray photoelectron spectroscopy. No evidence of this matrix was seen in the absence of dexamethasone, although collagen production was evident if ascorbic acid was present. No extracellular matrix of any type was visualized in cultures devoid of all three supplements. In the dexamethasone-containing cultures, collagen production was not evident in the absence of ascorbic acid. Addition of β-glycerophosphate augmented the amount of mineralized matrix. However, matrix was also seen in the absence of this supplement, demonstrating that Na-β-glycerophosphate was not essential to derive this biologically produced interfacial matrix. The results illustrate that the cement line matrix is the result of matrix production by differentiating osteogenic cells.

Key Words: Differentiating osteogenic cells, mineralized matrix, collagen, ascorbic acid, dexamethasone.

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Introduction

We have recently shown that differentiating osteogenic cells, derived from explants of young adult rat bone marrow, elaborate an interfacial matrix with the culture substratum which is collagen-free (Davies *et al.* 1991a,b). We hypothesized that this matrix was analogous not only to cement lines in natural bone tissue, but also constituted the baseline bone interface with endosseous implants. While we have shown that, indeed, morphological similarities can be observed between this *in vitro* matrix and that at cement lines in bony remodeling sites (Zhou *et al.*, 1994), we and others have also demonstrated that implant surfaces of various material types may demonstrate the same cement line-like matrix *in vivo* (Jarco, 1981; Pilliar *et al.*, 1991; Orr *et al.*, 1992; Baldan *et al.*, 1994; Davies and Baldan, 1997). These results have been confirmed by other workers (Sautier *et al.*, 1991; de Bruijn *et al.*, 1992a,b; Nanci *et al.*, 1996). Clearly, therefore, the culture model which we employ is capable of reproducing known *in vivo* morphological structures, and may thus be employed to dissect the mechanisms of bone formation at interfaces, in addition to determining the critical factors present in our culture medium which promote this particular osteogenic cell phenotypic expression. This application is of particular importance in unraveling the matrix elaboration events which culminate in mature osteoblastic phenotypic expression since, due to extensive work by others on gene expression in osteoblast differentiation, it is commonly held that collagen production precedes mineralization in bone (Stein *et al.*, 1989, 1990; Owen *et al.*, 1990; Lian and Stein, 1992; Quarles *et al.*, 1992; Liu *et al.*, 1994; Malaval *et al.*, 1994). The latter statement, while supported by the work of Aronow *et al.* (1990) and Owen *et al.* (1990), is not corroborated by either our morphological (Davies *et al.*, 1991a,b), or biolabeling, studies (Shen *et al.*, 1993) and thus raises a doubt concerning the congruence of mRNA expression and the elaboration of a bony extracellular matrix.

We believe that the interfacial matrix is initially laid down by differentiating osteogenic cells as a proteinace-

ous layer, which provides the nucleation sites for calcium phosphate crystallization and which increasingly mineralizes due to crystal growth, prior to the assembly of collagenous matrix. The latter then also undergoes mineralization.

In order to unravel the sequence of events in bone matrix assembly, we have conducted a series of experiments to block different stages of development in the formation of bone matrix by adding, or not adding, special components of the culture medium. The basic culture medium was α-MEM supplemented with fetal calf serum and antibiotics. The supplements used in different combinations were ascorbic acid, known to be important for collagen matrix assembly; β-glycerophosphate, important for mineralization as an organic phosphate source; and dexamethasone, known to stimulate osteogenesis by causing undifferentiated osteogenic cells to commit to the osteogenic differentiation pathway.

Materials and Methods

Cell culturing materials

The materials used in the experiments were obtained from various sources. The 35 mm polystyrene tissue culture dishes and the 25 cm^2 tissue culture (T25) flasks were obtained from Corning Glass Works, NY. The 70 μm nylon Cell Strainer was purchased from Beckton Dickinson Labware, Lincoln Park, NJ. The ascorbic acid free alpha minimum essential medium (α-MEM) was provided by the medium preparation laboratory of the Faculty of Medicine, University of Toronto. The fetal calf serum (FCS), trypsin and gentamycin were obtained from GIBCO BRL Life Technologies Inc., Gaithersburg MD. The penicillin G, amphotericin B, L-ascorbic acid (A), Na-β-glycerophosphate (β) and dexamethasone (D) were obtained from Sigma Chemical Company, St. Louis, MO. All other chemicals and materials were obtained from local suppliers.

Cell isolation

Rat bone marrow cells (RBMC) were obtained according to the method previously described (Davies *et al.*, 1991a). Briefly, femora derived from young adult (115-125 g) Wistar rats (Charles River) were excised and transferred into an antibiotic/fungizone solution {penicillin G (1670 units/ml), gentamycin (500 μg/ml), amphotericin B (3 μg/ml)}. After remaining 10 minutes in this solution, the femora were transferred into the same solution. This washing procedure was repeated a total of three times. Subsequently, the femora were transferred into ascorbic acid free alpha minimal essential medium (α-MEM). Epiphyses were removed and the marrow from each diaphysis was flushed out with 16 ml supplemented with 15% fetal calf serum (FCS), together with antibiotics and fungizone at one tenth the concentration used during excision. Marrow cells of both femoral diaphyses were collected in a sterile 50 ml centrifuge tube and cultured in the above medium containing all possible combinations of supplements of 100 μg/ml L-ascorbic acid (A), 10 mM β-glycerophosphate (β), 10^{-8} M dexamethasone (D). The resultant culture sub-groups are illustrated in Table 1.

Cell culture

Four ml of the cell suspension, supplemented with the specific components of each group, were cultured in T25 flasks and maintained in an incubator with a humidified atmosphere consisting of 95% air and 5% CO_2 at 37°C, and 100% relative humidity. The medium was changed three times a week. The first medium change occurred on day one; from then on the amount of medium per flask was increased to 5 ml. On day six the cells were subcultured, following which they were enzymatically released using 0.01% trypsin. These trypsinized cells were plated using, or not using, a cell strainer, onto 35 mm diameter polystyrene tissue culture dishes at approximately 10^4 cells per cm^2 and cultured for 14 days. Cell counts were carried out using a Coulter Counter® model ZM (Coulter Electronics Ltd., Luton, Beds., U.K.).

Light microscopy

Each culture dish was marked at several spots in order to take photomicrographs using phase optics of the same fields of view, each day, during the culture period. Furthermore, following fixation, representative dishes of each culture sub-group were photographed at low magnification (x 1.6 objective lens, x 10 eyepiece), employing dark field optics, to demonstrate variations in the morphology of the cultures.

Cell culture fixation

Cultures were washed 3 times in unsupplemented α-MEM and 3 times in 0.1 M Na-cacodylate buffer at pH 7.3, following which they were fixed using Karnovsky's solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M Na-cacodylate at pH 7.3) at room temperature for a minimum time of 2 hours. After fixation the cells were washed 3 times in 0.1 M Na-cacodylate and post-fixed in osmium tetroxide to preserve the cell membranes. Cultures were fixed in 2% OsO_4 in 0.1 M Na-cacodylate for 90 minutes at room temperature and then washed in 0.1 M Na-cacodylate.

Scanning electron microscopy (SEM)

Post fixed cultures were dehydrated through a graded ethanol series and critical point dried from CO_2 at a critical temperature of 31.1°C and a critical pressure of (7544 hPa; hPa = hecta-Pascal or 100 Newton per square meter) (Ladd Research Industries Inc.,

Burlington, VT). To examine the tissue at the cell-substratum interface the overlying cell-multilayers were removed using compressed air and the specimens were sputter coated with approximately 15 nm platinum (Polaron Instrument Inc., Doylestown, PA) or carbon (Edwards vacuum coater model 306, Edwards High Vacuum Ltd., Crawley, Sussex, U.K.) and examined in an SEM (Hitachi model 2500; Hitachi, Tokyo, Japan) at an accelerating voltage of 15 to 20 kV or an Hitachi field emission SEM model 4500 at an accelerating voltage of 5 kV furnished with a Link energy dispersive X-ray (EDX) analysis system (Oxford Instruments, High Wycombe, Bucks, U.K.).

X-ray photoelectron spectroscopy (XPS)

The elemental composition of the interfacial layer in selected groups was examined using XPS. 14 day cell cultures were Karnovsky fixed, critical point dried and treated with compressed air as described above. The spectra were obtained on a Leybold MAX 200 XPS system utilizing an unmonochromatized Mg Kα X-ray source operating at 12 kV and 25 mA. Presence of C, O, N, Ca, and P was detected on spectra run in a low-energy mode (pass energy = 192 eV) which were satellite subtracted. The energy scale was calibrated against Cu $2p_{3/2}$ and Ag $3d_{5/2}$ and scaled to place the main C peak at 285.0 eV. Elements were detected by peaks corresponding to their characteristic binding energies (O 1s : 531 eV, N 1s : 402 eV, Ca $2p_{3/2}$: 347 eV, C 1s : 285 eV, S 2p : 164 eV and P $2p_{3/2}$: 133 eV). Atomic ratios of Ca and P were determined on spectra that were normalized to unit transmission of the electron spectrometer and used sensitivity factors (Ca = 1.98, P = 0.61) which were empirically derived by the machine manufacturer.

Number of experiments

The entire experiment, including culture of the sub-groups and analyses, was repeated three times to generate the results reported herein.

Results

The explanted cells survived in all culture sub-groups, although the number of cells recovered from trypsinized primary cultures was different according to the supplements to which the cells were exposed. In the presence of ascorbic acid cells reached confluence 4-6 days after passage, whereas cultures in the absence of ascorbic acid reached confluence between days 6 and 8. Using the cell count numbers at subculturing, the yield was about 8-fold lower in the absence of ascorbic acid compared to the yield in the presence of ascorbic acid after 6 days of primary culture. The yield, at passage, was about 3-fold lower in the presence, than in the absence, of dexamethasone; whereas, the presence or absence of β-glycerophosphate appeared to have no influence on cell numbers, at this stage of the procedure. The use of cell strainers created a more even cell distribution which resulted in a more regular coverage of bone nodules on the culture surface, and in a more even and synchronized matrix deposition at the interface compared to the cell cultures that have not been strained at subculturing (not shown). Following fixation, low power dark field microscopy could be used to clearly demonstrate differences in the overall culture morphologies in the differing sub-groups (Figs. 1A-1D).

Table 1. The combinations of medium supplements used in the culture experiments.

Sub-group	(-)	A	β	D	Aβ	AD	βD	AβD
Ascorbic acid	-	+	-	-	+	+	-	+
β-Glycerophosphate	-	-	+	-	+	-	+	+
Dexamethasone	-	-	-	+	-	+	+	+

Because of morphological differences in cell shape and at the substratum interface, the other cultures have been divided into two groups depending on the presence of dexamethasone in the culture medium.

SEM observations: Cell-free control group

The cell free control group, containing fully supplemented medium kept under the same culturing conditions, and for the same time periods, as the other cultures showed no deposits on the culture dish surface (not shown).

SEM observations: Dexamethasone-containing cultures

This group was subdivided into four sub-groups depending on the additions of ascorbic acid and β-glycerophosphate, which are abbreviated herein to AβD, AD, βD and D (see Table 1).

Fully supplemented sub-group (AβD): In the fully supplemented AβD sub-group, which served as a positive control, after 14 days a bone like matrix had been elaborated in the culture dishes, consisting of an interfacial mineralized matrix approximately 0.5 μm in thickness and an overlying collagenous matrix which, in specific sites, had already mineralized and had begun to fuse with the interfacial matrix (not shown). Phase microscopy revealed that the cells within the developing bone nodules appeared mostly cuboidal.

Sub-group (βD): Ascorbic acid free: In the βD sub-group, lacking only ascorbic acid as a supplement, a similar interfacial matrix to the AβD sub-group was elaborated. After 14 days, a continuous layer of about

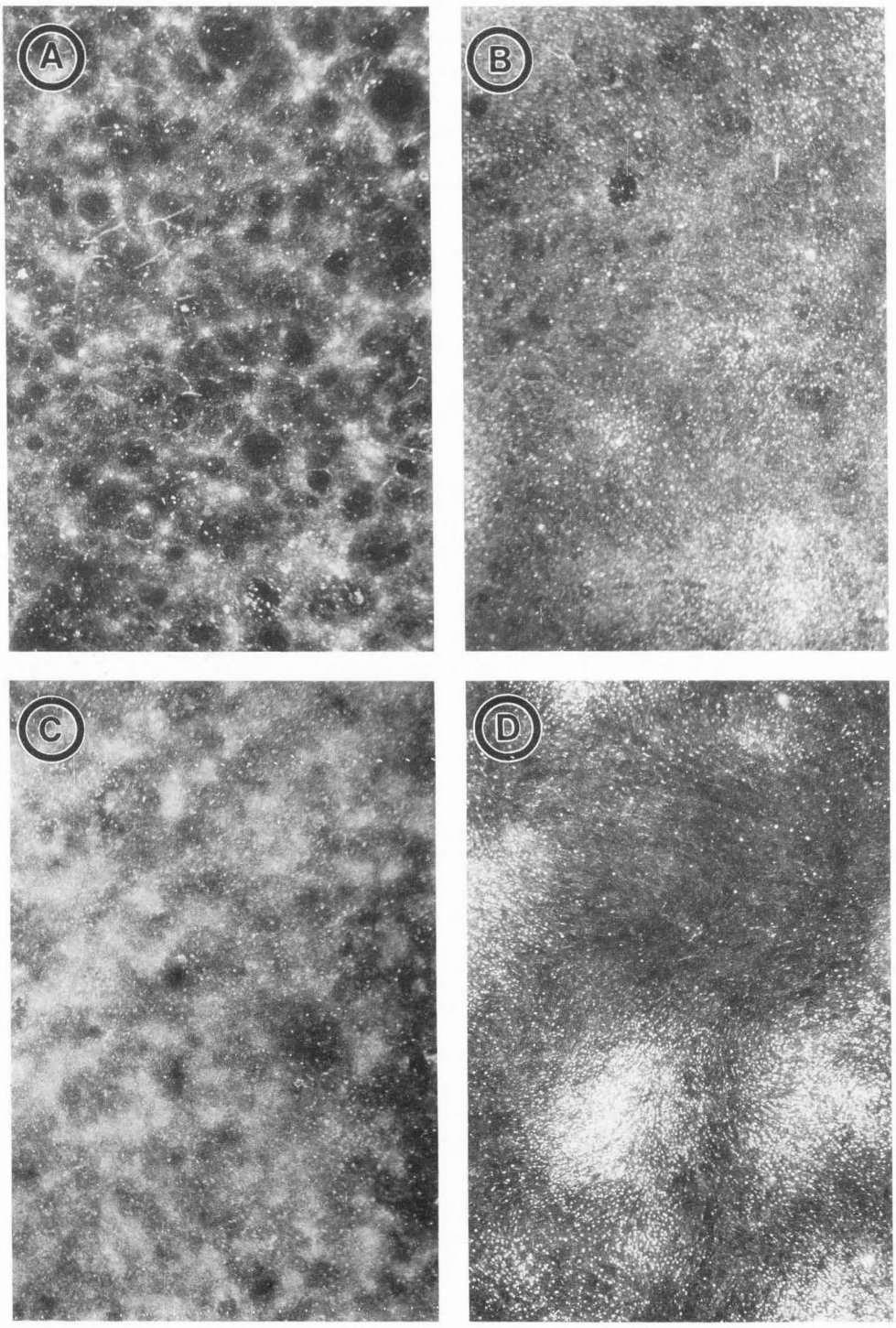


Figure 1. Dark field light microscopy of RBM-cultures: Cells were cultured in different supplemented media for 14 days and Karnovsky-fixed: (**A**) βD sub-group. Cells form a confluent layer with multilayered patches and a patchy-feathery overall appearance (field width, FW = 2 mm); (**B**) β sub-group. Cells are covering the dish in a confluent monolayer. No feathery or stringy appearance (field width 2 mm); (**C**) AβD sub-group. Cells form multilayered nodules with a feathery appearance (FW = 2 mm); (**D**) Aβ sub-group. Cells form confluent layer with a few multilayered areas. Note the stringy appearance, reminiscent of longitudinal fibroblastic cell populations (FW = 2 mm).

0.5 μm thickness with a morphology similar to that in the fully supplemented group was observed (Figs. 2A and 2B). However, no collagen fibre assembly could be detected (Figs. 2A and 2B). Interestingly, the fact that there was no collagen matrix assembly and hence no collagen fibre incorporation in the interfacial matrix, allowed a sufficient separation from the overlying cell layer, uncovering vast areas of interfacial matrix (Fig. 2B). The cell morphology was similar to the AβD sub-group.

Sub-groups (AD) and (D): β-glycerophosphate free: In the AD and D sub-groups both containing dexamethasone but lacking β-glycerophosphate globules could be seen at the substratum interface (Figs. 3A and 3B) and were seen fused together in the center of the nodules (Fig. 3A). The extent of the formation of the

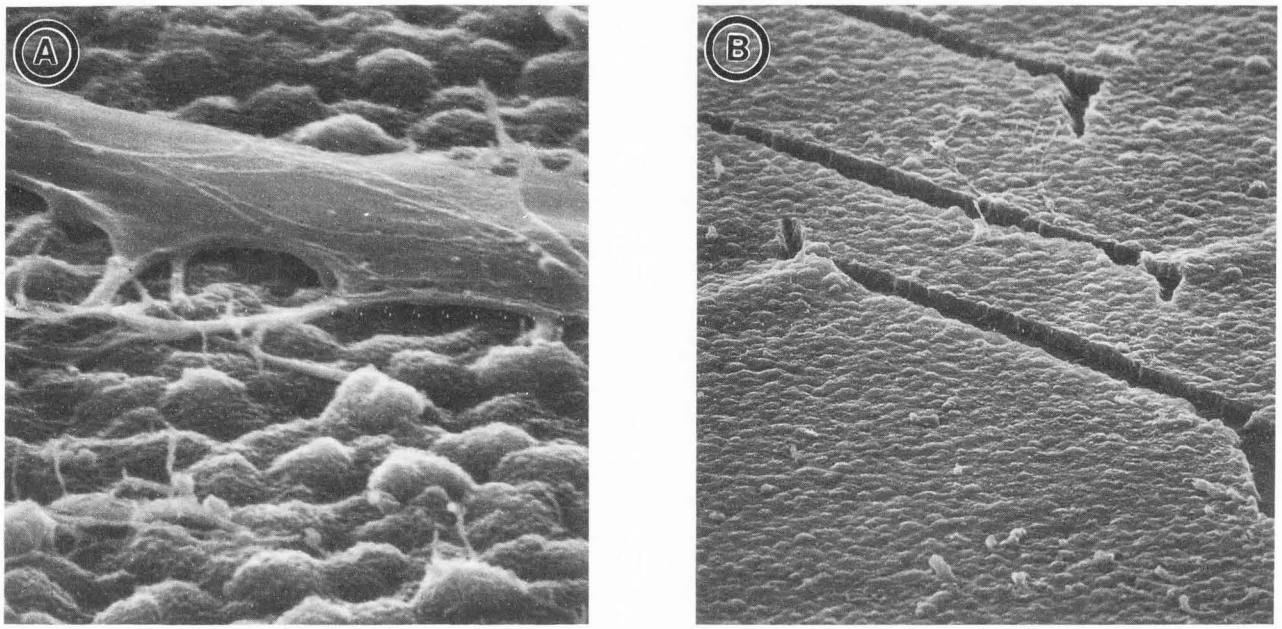


Figure 2. Scanning electron micrographs of the interface in the βD sub-group: (**A**) In this field of view, from a 14 day culture, the interfacial matrix can be seen to have an undulating appearance formed by the connected surfaces of fused globules. Note the cell remnant in the top half of the picture (FW = 4.3 μm). (**B**) After 14 days, the interfacial cement-line matrix is clearly seen, in the absence of collagen fibres in this culture, after removal of the overlying cellular sheet. The cracks visible in this field of view are the result of differential shrinkage, during processing for scanning electron microscopy, and facilitate measurement of the depth of this matrix ($\sim$0.5 μm) (FW = 17 μm).

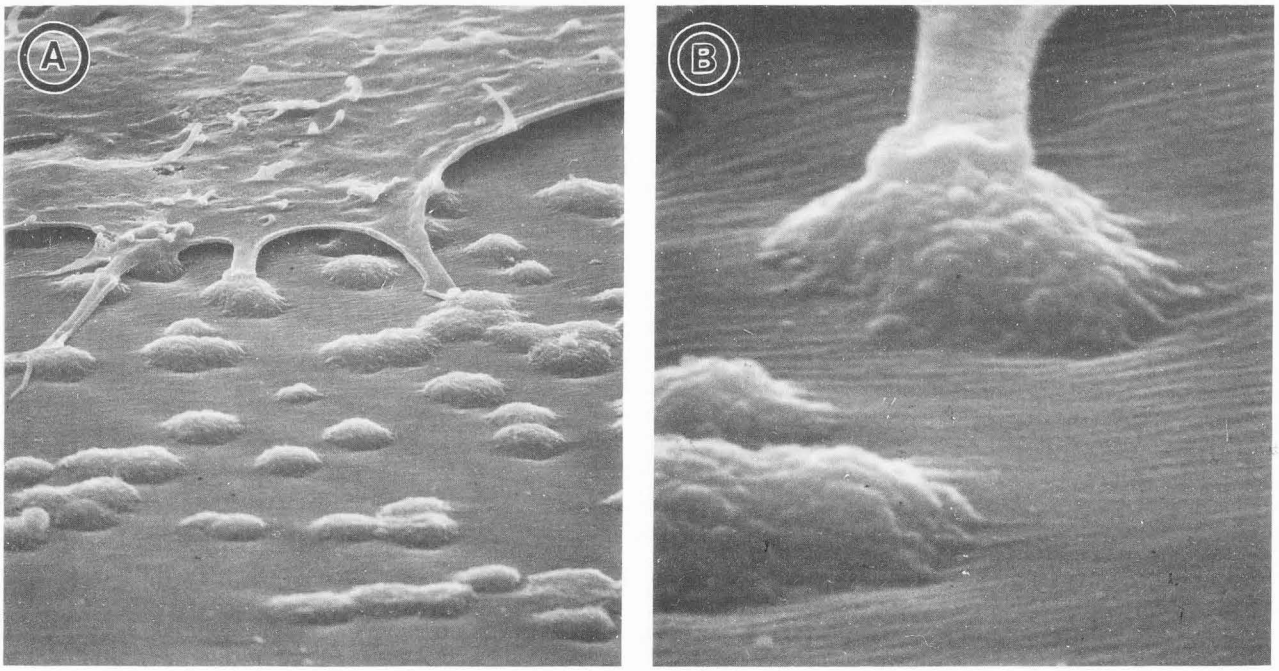
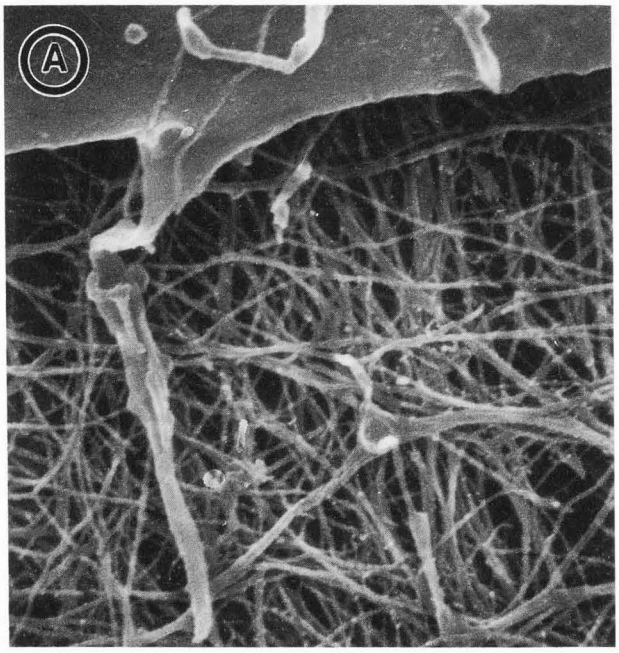
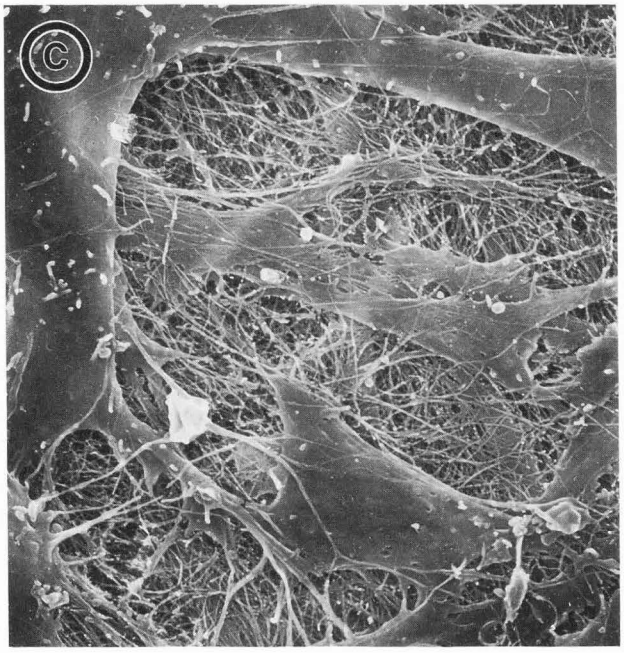


Figure 3. Scanning electron micrographs of the interface in the D sub-group: (**A**) In this scanning electron micrograph (tilted specimen) globular accretions are seen at the interface between the dish surface and the overlying cell layers. The globules are seen associated with cell processes. No collagen fibers are visible (FW = 8.5 μm). (**B**) The atherous appearance of this single globule (from Fig. 3A) is indicative of an heterogeneous structure (FW = 1.7 μm).





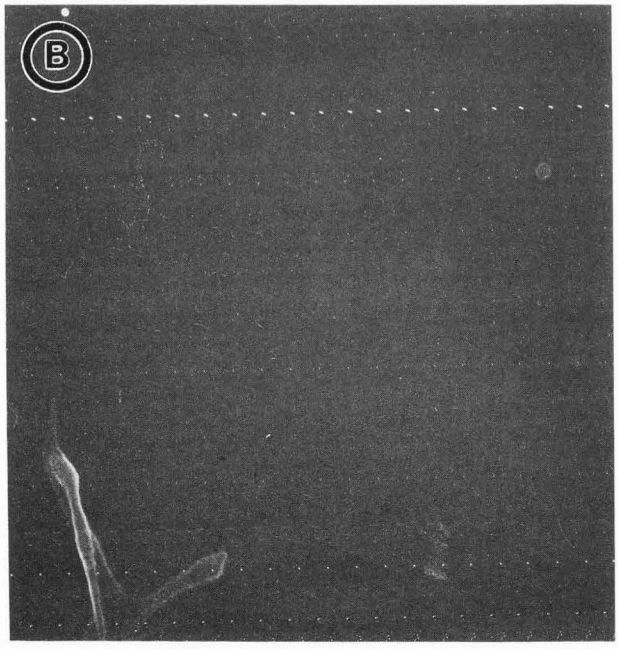


Figure 4. Scanning electron micrographs of the Aβ, β and AD sub-groups: **(A)** Within the cell layer of the Aβ sub-group, collagenous matrix can be seen. It shows no indication of mineralization. Note the part of a cell at the upper edge of the picture (FW = 4.2 mm). **(B)** At the interface underneath the cell layer in the β sub-group no matrix deposition is visible (FW = 17 μm). **(C)** Within the cell sheet of the AD sub-group collagenous matrix is seen. No indication of mineralization is visible (FW = 17 μm).

interfacial matrix was, however, not as advanced as in the previous β-glycerophosphate containing sub-groups. Only in the ascorbic acid containing AD sub-group was a collagenous matrix overlying the interfacial matrix (Fig. 4C). No evidence of mineralization of this collagenous matrix was visible. The cell morphology was similar to the sub-groups described above.

SEM observations: Cultures containing no dexamethasone

In parallel with the above cultures, this group was also subdivided into four sub-groups depending on the additions of ascorbic acid and β-glycerophosphate, which are abbreviated herein to Aβ, A, β and a negative control group (-) which contained no additions (see Table 1).

Sub-groups (Aβ) and (A): Containing ascorbic acid: After 14 days no afibrillar matrix deposition at the interface could be observed in either of these sub-groups. In both sub-groups a collagenous matrix was assembled (Fig. 4A) which, in neither case, appeared to be mineralized. Morphologically, the cells were spindle shaped, reminiscent of fibroblastic cell populations.

Sub-groups (β) and (-): Ascorbic acid free: After 14 days no interfacial or collagenous matrix deposition could be detected (Fig. 4B). The morphology of the cells was pleiomorphic including considerable amounts of spindle shaped cells.

EDX observations

EDX dot mapping of the interfacial matrix elaborated in the βD sub-group revealed the presence of P,

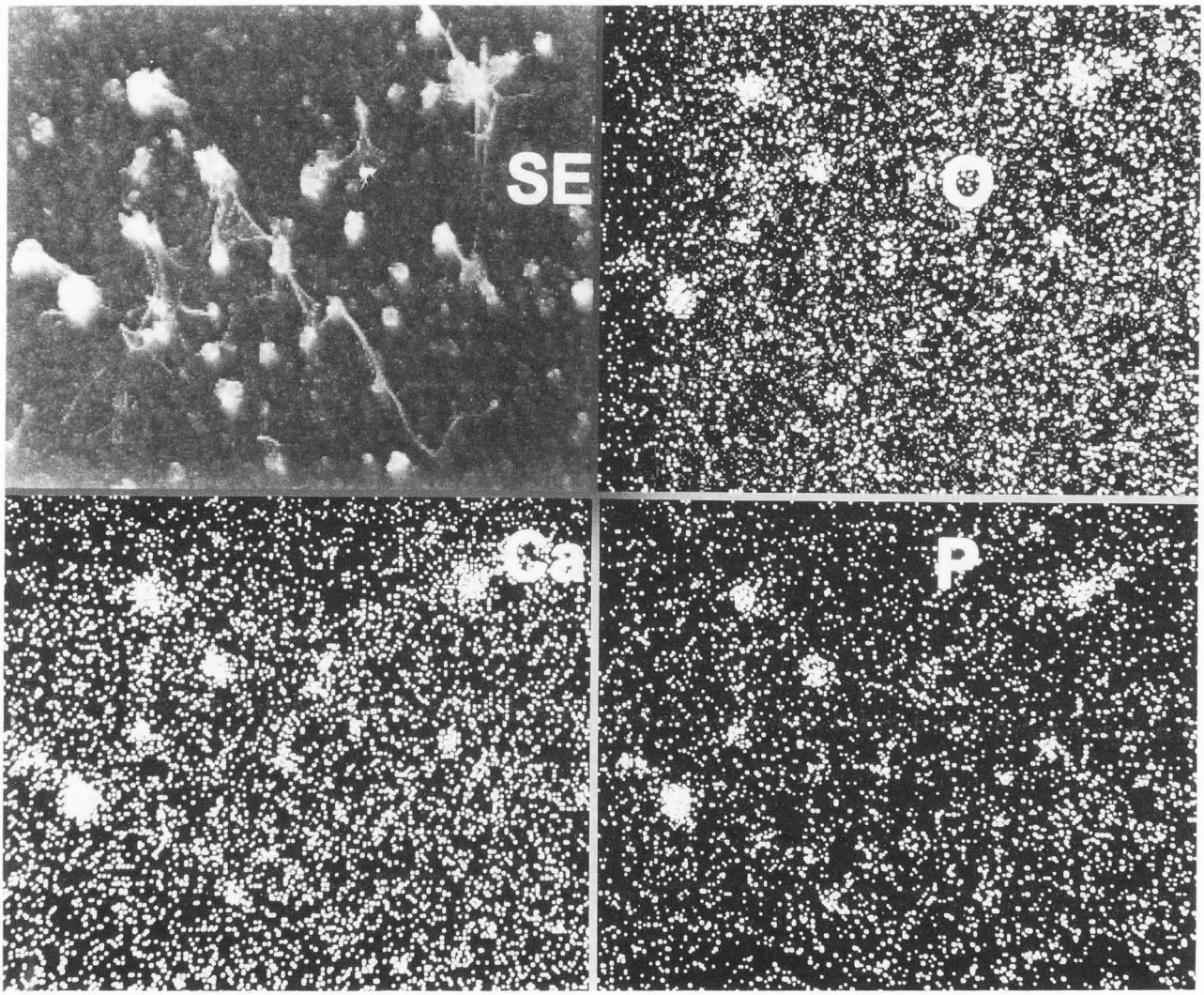


Figure 5. Energy dispersive X-ray dot plot images in the βD sub-group after 14 days of culture: EDX images have been obtained in a graphite-coated specimen of the βD sub-group, where the cellular layer had been removed using blasts of compressed air. The picture shows the scanning electron image (SE) and the secondary images of the same area obtained with signals selective for calcium and phosphorous. The secondary images of O, Ca and P are illustrated. The secondary images reveal the same feature as the scanning electron image (FW = 15 μm).

Ca and O with distributions congruent to that of the secondary image (Fig. 5). This distribution was similar to previous observations of the fully supplemented AβD sub-group, showing that the interfacial matrix was mineralized. EDX peak analysis also showed strong P and Ca signals along with a S signal (Fig. 6A). The atomic Ca/P ratio was calculated as 1.28. In order to examine whether the globules laid down at the interface in the β-glycerophosphate lacking cultures underwent mineralization, EDX peak analysis was also applied in the D sub-group (Fig. 6B). Only traces of P and Ca were detectable again along with a S trace. The atomic Ca/P ratio was with 1.32; in the range of the βD sub-group. A negative control measured at the interface in an area in between globules showed no significant peaks for any of these elements (Fig. 6C). Compared to the signals for P and Ca in the presence of β-glycerophosphate, the signals in the β-glycerophosphate lacking cultures were negligible, showing that the extent of mineralization in these cultures was very poor.

XPS measurements

In addition to the EDX measurements, XPS analyses in the different culture groups were carried out, after using compressed air to uncover the interfacial matrix, in order to determine the elemental composition of the

Table 2. Summary of the observations in the different culture sub-groups.

Matrix	Sub-group AβD	βD	AD	D	Aβ	A	β	(-)
Interfacial	+	+	+	+	-	-	-	-
Mineralized interfacial	+	+	-	-				
Collagenous	+	-	+	-	+	+	-	-
Mineralized collagenous	+		-		-	-		

latter. Interestingly, only in the βD sub-group were strong P and Ca signals measured along with O, N and C signals representing the organic components of this matrix (Fig. 7, upper plot). Traces of S were also detectable. The atomic Ca/P ratio was quantified as 1.17. In the fully supplemented AβD sub-group, no P and Ca peaks were detectable (Fig. 7, lower plot), suggesting that the interfacial matrix still was sufficiently covered by poorly mineralized collagenous matrix and cell debris trapped in this matrix. This assumption is supported by our SEM observations. In the other culture groups, also only very poor P and Ca levels in the range of background signals could be detected.

The SEM, EDX and XPS data are summarized in Table 2.

Discussion

The experiments reported here not only confirm the appearance of the interfacial matrix which has previously been described both *in vitro* (Davies *et al.*, 1990, 1991a,b; Lowenberg *et al.*, 1991; Qui *et al.*, 1993; Nanci *et al.*, 1996) and *in vivo* (Zhou *et al.*, 1994), but also dissect the structural and compositional components of this matrix, by manipulation of the culture medium.

The most striking observation from these experiments is that they provide clear evidence that collagen is not required for matrix mineralization, and that not only does a mineralized interfacial matrix precede collagen fiber assembly, but that this unique matrix may be elaborated even in the complete absence of collagen in the extracellular compartment. These findings would seem to contradict conventional wisdom which, based on the *in vitro* work of Owen *et al.* (1990) and Aronow *et al.* (1990), holds that collagen matrix assembly is an essential prerequisite for mineralization in bone. Nevertheless, the results of the present study do illustrate a pathway by which the collagen-free cement line interfacial matrix of bone, which has hitherto remained refractory to detailed compositional analysis, could be elaborated by differentiating osteogenic cells. Indeed, *de novo* bone formation occurs throughout life at remodeling sites, and also at the surfaces of endosseous implants.

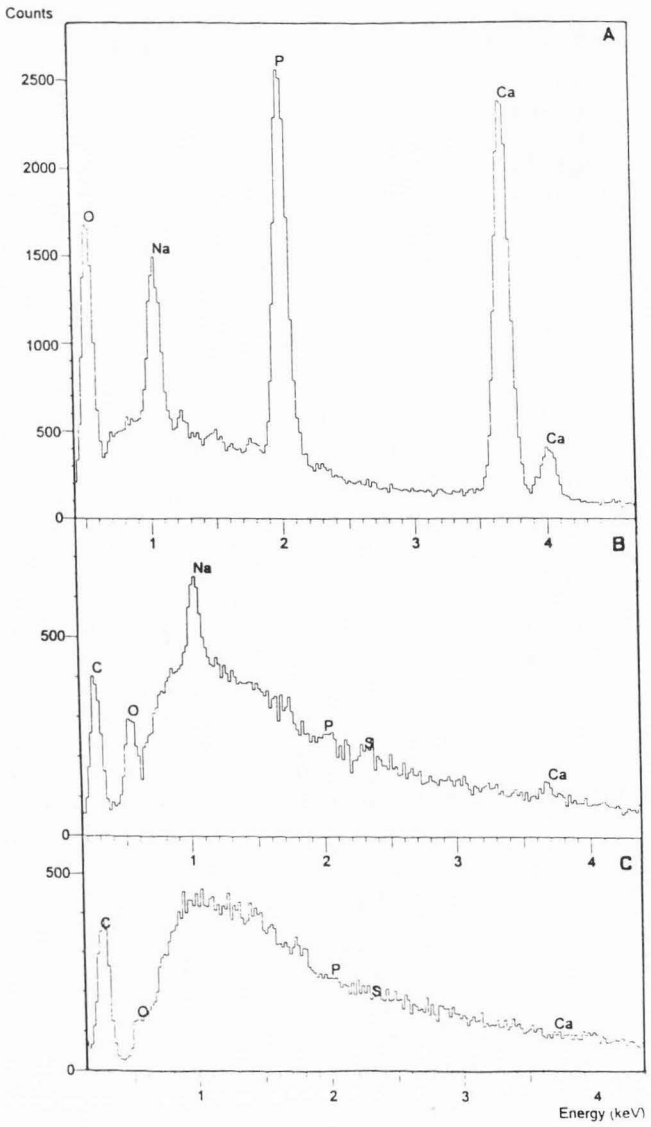


Figure 6. EDX peak analysis in the βD sub-group and D sub-group: EDX peak analysis has been applied in single globular accretions in the βD sub-group, the D sub-group and in an area without interfacial matrix, which served as a negative control. (**A**) EDX peak analysis shows strong signals for calcium phosphate (O, P and Ca) in the accretions of the βD sub-group. Sodium (Na) appears also to be entrapped in the globules. (**B**) In the D sub-group the globules only show weak signals for Ca, P and O, indicating that they are poorly mineralized. A trace of sulfur (S) can be detected. Sodium (Na) is again entrapped in globular accretions. (**C**) The negative control shows no signals for P, O, Ca or S.

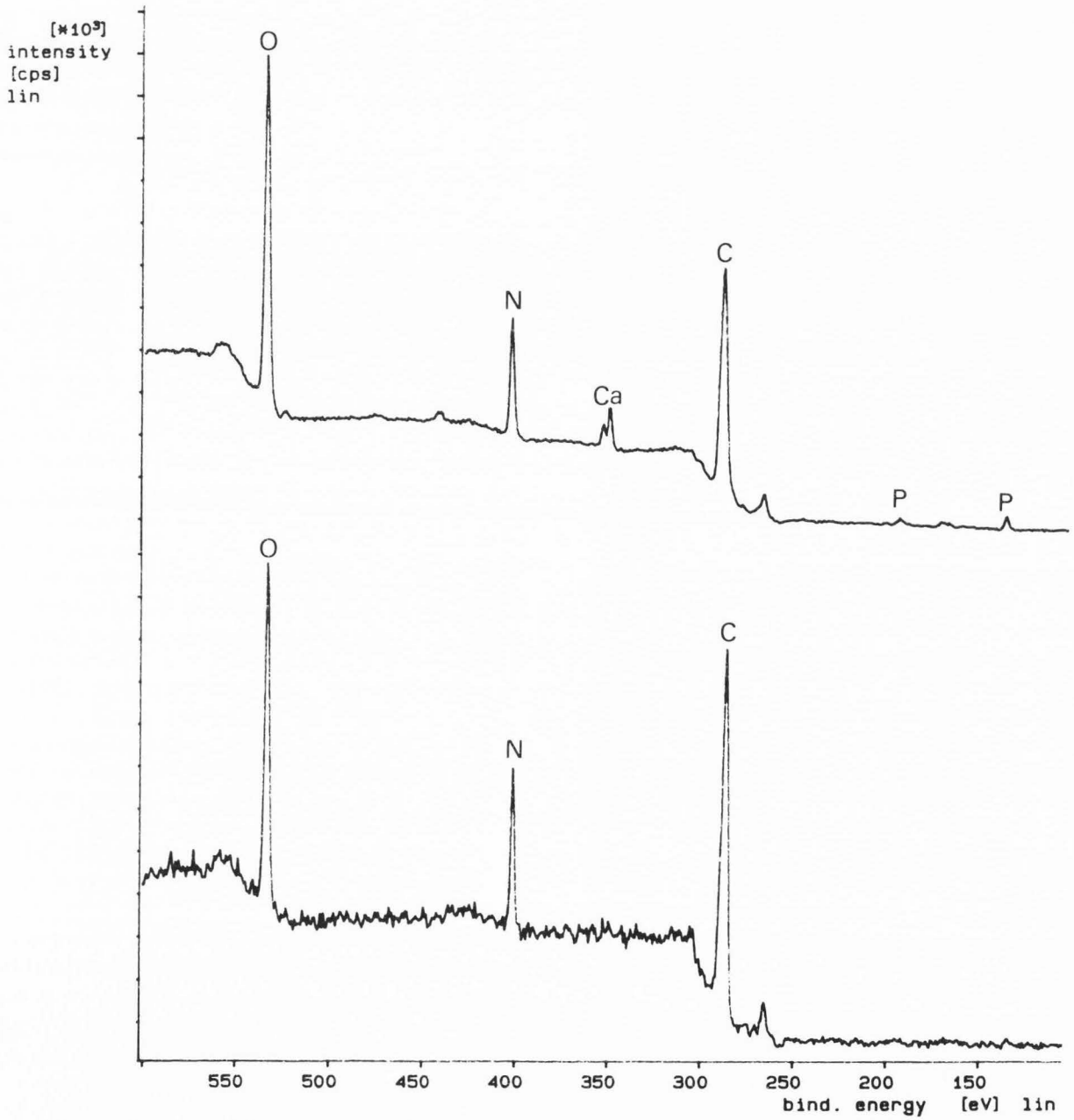


Figure 7. X-ray photoelectron spectroscopy plots in the βD sub-group and the AβD sub-group: XPS has been applied in the βD sub-group (represented by Fig. 2B) and the AβD sub-group in samples, where the cell layer has been retracted using blasts of compressed air. Upper plot: βD sub-group: Signals for Ca (347 eV) and P (133 eV and 190 eV) as well as signals for O (531 eV), N (402 eV) and C (285 eV), representing organic components are seen in this diagram. Lower plot: AβD sub-group: No signals for Ca and P are apparent. However, strong signals for the organic components (C, O and N) are seen, suggesting that the calcium phosphate is sufficiently covered by an organic matrix.

In bone cross-sections the borders, where new bone has initially been laid down on the surface created by the resorption of old bone can be seen as distinct lines, termed cement lines. These cement lines were first described by von Ebner (1875) and as collagen free by Weidenreich (1930).

That this culture system is able to produce an interfacial matrix which mimics that found *in vivo* is now indisputable. While early *in vitro* observations using this culture system first permitted speculation regarding the relevance of this cement line-like matrix, its cellular provenance, and the similarity to other mesenchymally

derived collagen-free hard tissue matrices (Davies *et al.*, 1991a), the first *in vivo* evidence of such a matrix was gained through examination of retrieved endosseous implant surfaces (Davies *et al.*, 1991b; Pilliar *et al.*, 1991; Orr *et al.*, 1992; Baldan *et al.*, 1994; Davies and Baldan, 1997). These results have been confirmed by other workers (Sautier *et al.*, 1991; de Bruijn *et al.*, 1992a,b; Nanci *et al.*, 1996). Interestingly, although largely ignored, several reports containing electron photomicrographs of bone cell culture systems also provide evidence of such a globular interfacial mineralized matrix (Pockwinse *et al.*, 1992, 1993; Gerstenfeld *et al.*, 1993; Pitaru *et al.*, 1993). More recently, this interfacial matrix has become evident at bone remodeling sites (Zhou *et al.*, 1994) indicating that the elaboration of this interfacial matrix is a general biological phenomenon, at sites where bone interfaces different substrata, whether an artificial implant material or bone itself. In fact, earlier observations indicated that the interfacial matrix is being laid down by differentiating osteogenic cells in the form of globules with a diameter in the range of 1 μm. These globules subsequently increase in size and number to form a confluent mineralized layer, the interfacial matrix. Biolabeling studies have shown that the organic component of the matrix is rich in both osteopontin (Shen *et al.*, 1993) and bone sialoprotein (BSP; Peel, 1995), which corroborates the work of McKee and colleagues in a variety of *in vivo* hard tissues (1992, 1993; Chen *et al.*, 1994; Nanci *et al.*, 1996). Collagen fibres appeared later in these cultures after the mineralized interfacial matrix was formed, suggesting that the mineralization of the interfacial matrix occurred prior to collagen matrix assembly.

Summarizing these findings with respect to the cement line, we suggested that this interfacial matrix is initially laid down by differentiating osteogenic cells as a proteinaceous layer, serving as the nucleation sites for calcium phosphate crystallization and which increasingly mineralizes due to crystal growth prior to the assembly of collagenous matrix which also subsequently undergoes mineralization (Davies, 1993, 1994). The fact, however, that in the culture dishes, the cells are not synchronized regarding their developmental stage in differentiation, did not allow us to strictly conclude that there was mineralization prior to collagen fibre appearance because the possibility still existed that the collagen, produced by more differentiated cells in the center of the nodule, might have an impact on the mineralization of the interfacial matrix at the margin of the nodule, where collagen was not yet assembled by the cells.

In the present experiments, the successful inhibition of collagen matrix assembly by ascorbic acid deprivation could be scrutinized by SEM observations in the ascorbic acid lacking sub-groups, which all showed no collagen matrix assembly. This observation is in agreement with previous studies of Anderson *et al.* (1984), who did not find any matrix accumulation without ascorbic acid supplementation in rabbit derived osteoblast-like cells and Aronow *et al.* (1990), who could not detect collagen using a hydroxyproline assay in rat calvaria osteoblast cultures in the absence of ascorbic acid.

Ascorbic acid is involved in many biochemical steps which require an antioxidant. In mesenchyme derived cells, ascorbic acid is known to enhance collagen production at multiple stages, such as transcription rate, mRNA stability, translation efficiency, hydroxylation and secretion. However, the post-transcriptional role of ascorbic acid appears to be of more importance regarding collagen matrix assembly (Chan *et al.*, 1990; Franceschi and Iyer, 1992). A lineage schema has been proposed for osteoblastic cells in which expression of several markers and bone formation are used to isolate distinct temporary differentiation stages (Aubin *et al.*, 1995; Aubin and Turksen, 1996). Applying a three stage model of proliferation, matrix maturation and mineralization, it has been stated that collagen matrix assembly is a requirement for, and therefore precedes, mineralization in bone (Aronow *et al.*, 1990; Owen *et al.*, 1990; Stein *et al.*, 1990; Lian and Stein, 1992).

The assumption that a collagenous matrix is required for mineral deposition is experimentally based on two studies of Owen *et al.* (1990) and Aronow *et al.* (1990). In the first study, fetal rat calvaria cells were cultured in MEM-medium until day 7 and then in BGJ_b-medium supplemented with 10% fetal calf serum and 10 mM β-glycerophosphate and ascorbic acid in three different concentrations of 0, 25, or 50 mg/l for different time periods up to day 30. Ca accumulation was measured by hydrolyzing the cell-layers and subsequently applying atomic absorption spectroscopy. No Ca accumulation was detected in any of the ascorbic acid lacking cultures and Ca levels appeared to increase with ascorbic acid concentration.

In the second study by Aronow *et al.* (1990), fetal rat calvarial cells were cultured for 13, 20 and 24 days in BGJ_b-medium and the less nutrient-rich MEM medium, supplemented with 10% fetal calf serum respectively, each medium in three groups with either no further supplement, 50 mg/l ascorbic acid or both, 50 mg/l ascorbic acid and 10 mM β-glycerophosphate. Ca accumulation was measured according to the first study. In both media there was an increase of Ca accumulation in the time course and related to the presence of β-glycerophosphate. However, only in BGJ_b-medium there was an increase in Ca accumulation related to ascorbic acid presence, whereas in MEM-medium there appeared to be no significant difference related to ascorbic acid presence. In both media only low levels

of Ca have been detected in the ascorbic acid lacking cultures. Nevertheless, it has to be taken into account that no β-glycerophosphate had been added to these cultures, and that in BGJ_b-medium there are somewhat higher calcium and phosphate ion concentrations. Collagen was present in all ascorbic acid containing cultures but in none of the ascorbic acid deprived cultures, regardless of which medium had been used.

The observations in the βD sub-group, reported herein, clearly show that, in the absence of ascorbic acid, an afibrillar interfacial matrix is formed showing every morphological feature, including mineralization, of the interfacial matrix in fully supplemented control medium first described by Davies *et al.* (1991a), demonstrating that neither ascorbic acid nor collagenous matrix is required for mineral deposition. In parallel, an interfacial matrix did form in the ascorbic acid lacking D sub-group according to that in the ascorbic acid containing AD sub-group showing again, that ascorbic acid had no influence on the formation of this particular matrix. Recent work by Franceschi and coworkers (1994) also indicate that in MC3T3-E1 cells, which are considered to mimic osteoblasts, ascorbic acid deficiency selectively reduces the secretion of procollagen rather than non-specifically affecting all extracellular matrix molecules. This is supported by other findings that bone proteins such as fibronectin (Berg *et al.*, 1983) and proteoglycans (Spindler *et al.*, 1989; Pacifici, 1990; Takeuchi *et al.*, 1993) are not affected by ascorbic acid. Another study by Abe *et al.* (1993) with fetal rat mandibular cells also reports of mineralization without adding ascorbic acid. However, since α-MEM, which contains a basic level of 50 mg/l ascorbic acid was the basic medium, it cannot be ruled out that the basic level of ascorbic acid did allow the mineralization as well as the collagen formation, observed in their study.

A possible explanation, of the studies of Aronow *et al.* (1990) and Owen *et al.* (1990), which found no mineralization in the absence of ascorbic acid, in contrast to the results reported herein could be the following. In the presence of collagenous matrix, there is an increased surface area available to which mineralization initiators could bind. On the contrary, in the absence of collagenous matrix, only the surface of the culture dish is available (the interfacial matrix being about 0.5 μm in thickness and the collagenous matrix being about 50 μm in thickness). Possible candidates for initiation of mineral deposition are noncollagenous bone proteins (Stein *et al.*, 1990; Gerstenfeld *et al.*, 1987) or proteoglycans which can initiate calcium phosphate mineralization, when immobilized to a surface (Linde and Lussi, 1989; Linde *et al.*, 1989). They are also found in higher concentrations at the mineralization front (Boskey, 1985). Studies of Hunter and Goldberg (1993) indicate that BSP initiates hydroxyapatite crystal formation. A nucleator mediated mineralization could, in part, explain why Aronow *et al.* (1990), and Owen *et al.* (1990) only measured very low mineralization levels in their ascorbic acid deprived cultures. However, it would not mean that biological mineralization phenomena could not occur in the absence of collagen. Mineralization could still have occurred in the absence of collagen matrix, however to a much lesser extent, due to its restriction to the afibrillar interfacial matrix.

Another possible explanation for the controversy between the present data and the data from Owen *et al.* (1990) and Aronow *et al.* (1990) is that osteoblast derived proteins, which are required for mineralization, can be also induced by other signal substances than ascorbic acid or collagen. This possibility is underlined by studies of Franceschi and Young (1990) who found that, in MG-63 human osteosarcoma cells, alkaline phosphatase levels are increased 4-fold by 1,25-dihydroxyvitamin D_3 in the absence of ascorbic acid, whereas ascorbic acid in the absence of 1,25-dihydroxyvitamin D_3 did not significantly increase alkaline phosphatase. However, this does not negate the influence of dihydroxyvitamin D_3 rather than ascorbic acid or collagen in playing a dominant role in committing the osteoprogenitor cells to the osteoblastic phenotype expression. In the present culture system the glucocorticoid dexamethasone is a similar candidate for such a signal substance (Shalhoub *et al.*, 1992), allowing further differentiation in the absence of ascorbic acid.

Glucocorticoids, including the synthetic hormone dexamethasone stimulate osteoprogenitor cells to follow the osteogenic pathway in fetal rat calvaria (Canalis, 1983; Bellows *et al.*, 1986, 1987, 1990; Pockwinse *et al.*, 1995), fetal rat mandible (Abe *et al.*, 1993), rat stromal cells (Maniatopoulos *et al.*, 1988; Kasugai *et al.*, 1991; Yao *et al.*, 1994; Herbertson and Aubin, 1995), chick (Owen, 1985; Tenenbaum and Heersche, 1985; McCulloch and Tenenbaum, 1986) and human (Cheng *et al.*, 1994), while they suppress the growth of other cells particularly fibroblasts (McCulloch and Tenenbaum, 1986; Tenenbaum *et al.*, 1986; Bellows *et al.*, 1990). This stimulation has been shown to be a receptor mediated regulation of gene expression (Green and Chambon, 1986), that is dose dependent (Canalis, 1983; Anderson *et al.*, 1984). The effects of dexamethasone have also been shown to be potentiated and modulated by the presence of other hormones such as parathyroid hormone or 1,25 dihydroxyvitamin D_3. The effects of dexamethasone on different stages of differentiation in the various cell populations present in rat bone marrow have been discussed by many authors (Howlett *et al.*, 1986; Maniatopoulos *et al.*, 1988; Bellows *et al.*, 1990; Aubin *et al.*, 1992). Comparing

alkaline phosphatase-positive and negative cell populations, Turksen and Aubin (1991) suggested that dexamethasone acts at the early osteoprogenitor-level, whereas late osteoprogenitor cells are already committed to become osteoblasts and do not need dexamethasone to form bone nodules. *In vivo*, however, dexamethasone decreases the number of osteoblasts and causes osteoporosis. This apparent contradiction has been explained by the inhibitory effect of dexamethasone on the generation of new osteoprogenitor cells (Heersche, 1989). Thus, although dexamethasone stimulates the early osteoprogenitor cells to proliferate, the osteoprogenitor reserves get depleted *in vivo*, because the stimulated cells divide only a limited number of times, while not being replaced from the stem cell population. *In vitro*, however, dexamethasone may be applied as a useful tool, to generate an adequate supply of differentiating osteogenic cells by stimulating the early osteoprogenitor population to proliferate.

In this culture system, dexamethasone has been shown to be of particular importance in its stimulative effects on osteoprogenitor cells rather than differentiated osteoblasts (McCulloch and Tenenbaum, 1986; Maniatopoulos *et al.*, 1988; Malaval *et al.*, 1994). This is consistent with the present findings that for the elaboration of the interfacial matrix, which is thought to be accomplished by the differentiating osteogenic cells and not by mature osteoblasts, dexamethasone is the sole component responsible. This assumption can be made because of the constant finding of the interfacial matrix in the dexamethasone containing sub-groups versus the complete absence of such a layer in all the dexamethasone deprived sub-groups.

Furthermore, this implies that only in the presence of dexamethasone are the proteins responsible for the formation of the interfacial matrix, which mineralizes in the presence of β-glycerophosphate, expressed. This conclusion is consistent with the observation in this study, that the collagenous matrix only mineralizes in the presence of dexamethasone, suggesting that proteins expressed by the dexamethasone stimulated osteogenic cells rather than the collagenous matrix itself can be held responsible for mineralization. Accordingly, in recent studies of Cheng and coworkers (1994) using human bone marrow cells, mineralization only occurred in the presence of dexamethasone, and dexamethasone was a requirement for bone matrix formation.

Another factor that has to be taken into consideration in comparing the studies of Owen *et al.* (1990) and Aronow *et al.* (1990) with the observations herein is the different behavior of fetal calvaria cells, used in the former studies and young adult bone marrow cells used in the present study. Fetal explants are enriched in osteoblasts and immediate precursors of osteoblasts that form bone-like tissue spontaneously, whereas adult bone marrow cells, which appear to be less differentiated require a specific stimulus such as dexamethasone for osteogenic differentiation. This is emphasized by studies of Turksen and Aubin (1991) who, applying alkaline phosphatase immunoselective flow cytometry, divided fetal rat calvarial cells into two osteogenic populations, alkaline phosphatase negative and alkaline phosphatase positive populations. Only the latter was capable of forming bone nodules in the absence of dexamethasone, suggesting that the non-alkaline phosphatase expressing, and therefore less mature, osteogenic population requires dexamethasone to differentiate into bone nodule-forming cells, while the more differentiated cells expressing alkaline phosphatase will form bone nodules in the absence of dexamethasone. Therefore, it is possible that the events involved in the elaboration of the interfacial matrix, which seem to occur in the very early stages of differentiation of osteogenic cells cannot be mimicked as adequately by using fetal rat calvarial cells in the absence of dexamethasone as they can be using the adult rat bone marrow system, since they might already be too differentiated to elaborate this early matrix. Recent studies by Nanci *et al.* (1996) indicate the existence of a similar interfacial matrix in rat calvaria cultures.

The finding that globules were present in non mineralizing β-glycerophosphate lacking sub-groups makes it unlikely that the deposition of the globules is a nonspecific, or ectopic, precipitation phenomenon. Second, it suggests that the production of the proteins forming the globules is not induced by β-glycerophosphate. In contrast to the observations reported herein, mineralization in rat bone marrow cultures has been reported in the absence of β-glycerophosphate (Satomura and Nagayama, 1991; Satomura *et al.*, 1991). These results, however, were achieved using only 3 week old rats, and mineralization occurred after culturing the cells for slightly longer time periods than in the culture system of the present study. The rationale for using β-glycerophosphate as an additional source of phosphate ions is 2-fold. First, whereas the inorganic phosphate levels in the culture medium are in the physiological range, the organic phosphate levels are well below the physiological levels without the addition of β-glycerophosphate (Hoffman, 1970). Second, by providing organic phosphate, phosphate ions are made available specifically in locations where alkaline phosphatase is present, thereby allowing a cellular control of mineralization and reducing the effects of possible nonspecific mineral precipitation. Another reason for using an organic phosphate source is that it may modulate phenotypic expression of osteogenic cells. Tenenbaum *et al.* (1992) suggested that osteogenic cells traverse an organic phosphate-sensitive phase after which they may be incapable of normal

mineralization if they have not been exposed to organic phosphate during this period.

In the present study, β-glycerophosphate was necessary for mineralization and seemed to enhance the extent of interfacial matrix production. This effect can be due to the amount of calcium phosphate incorporation into the interfacial matrix in the presence of β-glycerophosphate or due to an increased matrix protein production or a combination of both. The observation that the early accretions, prior to the globules, were not visible in the absence of β-glycerophosphate may as well be explained either by the poor mineralization of these proteins making them undetectable at the SEM-level, or by an enhancing effect of β-glycerophosphate on the production of proteins being present in these particular accretions. In other rat bone marrow (Kasugai *et al.*, 1991), chick (Gerstenfeld *et al.*, 1987) and MG-63 osteosarcoma cell studies (Franceschi and Young, 1990), it is shown that, with the exception of alkaline phosphatase, mRNA levels of other bone matrix proteins were not changed significantly by β-glycerophosphate. These results are confirmed by the studies of Lee *et al.* (1992) in rat calvaria in which they analyzed the synthesized proteins and their mRNA levels in mineralizing and non-mineralizing cultures in order to assess whether a negative feedback may exist between matrix mineralization on the one hand and the synthesis of bone matrix proteins on the other. Their results indicated, that matrix mineralization, with the exception of alkaline phosphatase, neither affected the mRNA levels for osteopontin, type I collagen, BSP and osteocalcin nor indeed the synthesis of total protein of collagen I and osteocalcin. However, in MC3T3-E1 cells, β-glycerophosphate has been reported to increase osteopontin mRNA (Franceschi and Iyer, 1992) and in hypertrophic chondrocyte cultures β-glycerophosphate has been reported to increase type X collagen mRNA as well as the specific activity of pp60c-src kinase (Coe *et al.*, 1992).

The effect of ascorbic acid and dexamethasone on cell proliferation was observed by monitoring cell counts at passage in the present study. β-Glycerophosphate appeared to have no effect on cell proliferation. However, it has to be considered, that the cell numbers obtained describe the resultant effect of these supplements on all of the populations present in the primary flush-out of bone marrow cells. Furthermore, the seeding density of proliferating cells may have varied from animal to animal and the effect of cell straining on the generated cell counts also has to be taken into account. The latter, however, would be expected to lower the cell number in sub-groups due to eradication of cell-clumps. This would favor those sub-groups, in which less cells are present, suggesting that the effects seen should have been more dramatic without straining.

The cell numbers obtained in the presence of ascorbic acid were about 8-fold elevated after 6 days of culturing as compared to those in the absence of ascorbic acid. These findings would be consistent with the results of Harada *et al.* (1991), who reported stimulation of MC3T3-E1 cell proliferation by ascorbic acid. They suggested that this was due to its effect on collagen synthesis in a way that collagen or related matrix proteins interact with cell surface receptors to cause the stimulation of proliferation in osteoblasts. Similar results were obtained by Senoo and Hata (1994), who showed that extracellular matrix upregulates cell proliferation and tissue formation. Denis *et al.* (1994) also reported a stimulation of cell proliferation by ascorbic acid, however, they could not confirm that the effect is coupled to the effect of ascorbic acid on collagen synthesis.

The observation of an inhibiting effect of dexamethasone on cell proliferation in primary cultures are in agreement with previous studies by Maniatopoulos *et al.* (1988). This effect, however, is heavily dependent on the composition of cell populations, as discussed in the **Introduction**. After subculturing, in a more homogeneous osteogenic population, light microscope observations indicated that dexamethasone stimulates cell proliferation, as would be expected for early osteoprogenitors.

Conclusions

Differentiating osteogenic cells derived from dexamethasone stimulated rat bone marrow cells express an afibrillar interfacial matrix, which mineralizes in the presence of β-glycerophosphate. Ascorbic acid enhances the proliferation of cells within the first six days of culturing. However, the formation and subsequent mineralization of the interfacial matrix is independent of ascorbic acid presence or collagen matrix assembly and cannot be explained by a non-biological precipitation phenomenon. Collagenous matrix which is assembled in all ascorbic acid containing sub-groups only mineralizes in the presence of both dexamethasone and β-glycerophosphate. These results illustrate that the cement line matrix, first described by von Ebner (1875), is the result of matrix production by differentiating osteogenic cells.

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Discussion with Reviewers

N. Katsura: I agree with your opinion that collagen fibers are not essential for the initial matrix mineralization, however, the evidence provided is insufficient to draw this conclusion. You must show that the removed overlaying cellular sheet has neither collagen nor calcium-phosphate clusters at higher magnification (at least 20,000-folds).

Authors: In the cell sheet overlaying the interfacial matrix, no collagen fibres could be seen at the SEM level in the ascorbic acid deficient culture groups. Recent results in our lab have confirmed this observation in TEM cross-sections. The observation, however, that collagen fibres are not assembled in cell culture under ascorbic acid deficiency is by no means a new one (Anderson *et al.*, 1984; Aronow *et al.*, 1990) as is indicated (at some length) in our **Discussion**.

With regard to calcium phosphate clusters, no such clusters were observed at the SEM level within the cell sheet of either the ascorbic acid deficient, or the dexamethasone deficient, cultures.

N. Katsura: In order to conclude that collagen fibres are not required for initial matrix mineralization by bone cells, you must also show a vertical cross section of nodules at high magnification and crystallographic analysis of the deposited mineral.

Authors: TEM cross sections of bone nodules showing mineral deposition within the collagenous matrix have been shown numerous times (see quoted text references to our own work and that of Nanci's group) in fully supplemented cultures. However, in the absence of dexamethasone no osteogenic cell population appears, which would be able to form nodules, as has been indicated in our **Discussion**. In the presence of dexamethasone, but absence of ascorbic acid, no collagenous matrix forms, in which the osteogenic cells become embedded to form the nodular multilayers. Thus, in neither dexamethasone nor ascorbic acid deficient cultures can cross sections of such bone nodules be shown, since nodules do not form in their absence.

Regarding the nature of the mineral in the interfacial matrix, we have recently confirmed the morphological similarity between the mineralized interfacial matrices of fully supplemented cultures and ascorbic acid lacking cultures in TEM cross sections. Using intermediate voltage field emission analytical transmission electron microscopy (200 kV FETEM) we have identified these mineral deposits as calcium phosphates (Davies *et al.*, 1993) by employing the sub-nanometer probe size of such an instrument to undertake both energy dispersive X-ray analysis and electron energy loss spectroscopy. Unfortunately, our limited access to such facilities which are essential to gain precise analytic data from such crystallites has not allowed us to complete the electron diffraction analysis which would render structural information to compliment the chemical information already reported. We are continuing to pursue this high resolution analytical microscopical approach to identify early crystallographic species. At later time points following crystal growth, analysis is of course much easier as we reported in 1991 (Davies *et al.*, 1991a).

J.D. de Bruijn: From the scanning electron micrograph in Figure 3a, it seems that the cell, with its globule associated cell processes, has a flat morphology. This would be in contrast to the morphology of the accretion (globule) producing osteoblasts as shown in the paper by Davies *et al.* (1991a), which exhibit a clear cell body and numerous membrane ruffles.

Is the cell in Figure 3a an osteoblast that has actually formed the globular accretions, and if so, could the absence of β-glycerophosphate and ascorbic acid in the culture medium be responsible for the altered cell morphology?

Authors: It is not possible to unequivocally conclude, from scanning electron micrographs, that a particular cell is responsible for the production of a specific globule. Therefore, it is very difficult to determine which particular cell morphology, or morphologies, may be related to globule production. Furthermore, we know that both the chemistry and topography of the culture substratum will affect cell morphology without necessarily influencing the ability of the cultured cells to produce this globular interfacial matrix. In this context, we would point out that the cells associated with such globules, which we first described in 1991 (see text references) were cultured on bacteriological grade polystyrene, whereas the substratum for the cultures of the current paper is tissue culture polystyrene. Cells cultured on tissue culture polystyrene generally assume a more flat morphology over cells cultured on bacteriological grade polystyrene due to better cell attachment.

With regard to the second part of your question, whether the cell in Figure 3a can be called an osteoblast,

the answer is "No". Classically, an osteoblast is described as a cell which is producing a mineralized collagenous extracellular matrix. We have maintained, since our original report of this culture system in 1991 (vide supra) that the mineralized cement line interfacial matrix precedes collagen assembly (as is the case in remodelling bone *in vivo*). Thus, the interfacial matrix is produced before the expression of the mature phenotype. For this reason, we have assumed that the cement line matrix, both *in vitro* and *in vivo*, is elaborated by cells of the osteogenic lineage which have not yet become osteoblasts. Our preferred term for these cells is "differentiating osteogenic cells." Of course, in the absence of ascorbic acid no collagen is assembled, as we have shown. In these circumstances, we believe that the formation of globules is indicative of the osteogenic potential of the cells as they are seen in ascorbic acid free conditions only when dexamethasone is employed.

E. Bonucci: The statement that non-collagenous proteins are possible candidates for the initiation of mineral deposition in the absence of collagen fibres is tenable. However, the globular appearance of the first deposited interfacial matrix calls attention to other structures that may be responsible for inducing calcification, i.e., matrix vesicles. Is there any evidence of the presence of these structures in your culture system and, above all, in the noncollagenous interfacial material?

Authors: We have found no evidence of matrix vesicles in these cultures. This was also asked of us in our original paper describing the interfacial matrix (Davies *et al.*, 1991a), but what is important to point out is that we often see membrane bound, circular bodies, particularly near the culture substrate. However, these have never demonstrated crystallite inclusions. Indeed, we believe that such circular bodies do not represent spherical matrix vesicles but cross-sections of cell processes.

Additional Reference

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