Formation of Cement Lines, But Not Laminae Limitantes, Requires Contact of Differentiating Osteogenic Cells to Solid Surfaces

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FORMATION OF CEMENT LINES, BUT NOT LAMINAE LIMITANTES, REQUIRES CONTACT OF DIFFERENTIATING OSTEOCGENIC CELLS TO SOLID SURFACES

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Abstract

We designed in vitro experiments to address the hypothesis that cement lines and laminae limitantes are structurally distinct as a result of cell contact, or absence of cell contact, during their elaboration. We subcultured young adult primary rat bone marrow cells in well inserts with polycarbonate membranes of 0.45 µm pore size, which allowed protein but not cell passage. The cells, extracellular matrix, lower side of the membrane and the surface of the polystyrene culture well were examined by scanning and transmission electron microscopy. The cells elaborated a bone-like matrix on top of the membrane. In such cultures the cell/substrate interface, beneath developing bone nodules, is occupied by a collagen-free mineralized cement line-like matrix about 0.5 µm thick. Matrix was also observed on the cell-free surfaces on the lower side of the membrane, and the well-surface. This matrix morphologically resembled laminae limitantes and did not occur in cell-free control cultures. On the lower side of the membrane the matrix varied in thickness from 20-100 nm, while on the polystyrene well bottoms it was of a more uniform thickness (about 40 nm). Calcium phosphate mineralization, in the presence of osteopontin and bone sialoprotein, was detected at both cell-free surfaces by X-ray photoelectron spectroscopy (XPS) and immunofluorescence. These data suggest that the key factor which results in the structurally divergent features of these bony extracellular architectures, lamina limitans and cement line, is direct cell contact to osteogenic cells at solid surfaces.

Key Words: cement lines, lamina limitans, differentiating osteogenic cells, cell culture, mineralized matrix, β-glycerophosphate, well insert cultures.

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Introduction

It has recently been shown that differentiating osteogenic cells derived from primary explants of young adult bone marrow elaborate an interfacial matrix with the culture substratum, reaching a typical thickness of 0.5 µm. Collagenous bone matrix which itself mineralizes fuses with this initial interfacial matrix (Davies et al., 1991a,b). This matrix, elaborated in vitro, shows conspicuous ultrastructural morphological similarities with cement lines found at bony remodeling sites in vivo (Zhou et al., 1994), which were first described histologically by von Ebner (1875). A similar cement line-like matrix is formed in vivo at endosseous implant surfaces of various types (Davies et al., 1991c), and thus these observations indicate that the in vitro model may provide an important insight into both initial matrix formation at bony remodeling sites and the bone response to various implant materials (for review of the model see Davies, 1996). However, some authors have equated cement lines with the laminae limitantes, first described by Scherft (1972), as demarcating the mineralizing front during ossification. In fact, these thin (20-50 nm) electron dense features can be seen ubiquitously on many calcified tissue surfaces including calcified cartilage (McKee et al., 1992) and osteocyte lacunae and canaliculi (McKee and Nanci, 1993). As a result, discussion has arisen in the literature concerning the basis for the synonymous use of these two terms in both the bone implant (van Blitterswijk et al., 1992; Nanci et al., 1994) and bone biology (McKee and Nanci, 1993, 1995, 1996) contexts.

Our previous studies (Davies et al., 1991a,b) have shown that this interfacial matrix, which appears beneath forming bone nodules, is initiated in the form of globular accretions that fuse to form a continuous layer of about 0.5 µm thickness. The observation that these globules are typically associated with cells and cell processes along with the observation that these globules often appear to be localized along trail-like lines (Davies et al., 1991a Fig. 6; 1991b Fig. 20.4 C) could be indicative of a relationship between cellular contact and the
formation of these globules and hence the cement line matrix. However, while the structural dimensions of cement lines and laminae limitantes differ by an order of magnitude, these two morphological features of bone are compositionally similar (McKee and Nanci, 1993). Thus, we hypothesized that cement lines and laminae are structurally distinct as a result of osteogenic cell contact, or absence of osteogenic cell contact, during their elaboration respectively. We describe experiments herein, employing young adult rat bone marrow cultures cultured on polycarbonate membrane well inserts with 0.45 μm pore size, which test our hypothesis.

Materials and Methods

Materials
The materials used in the experiments were obtained from various sources. The 6 well 35 mm style polystyrene tissue culture dishes (Cell Wells™) were obtained from Corning Glass Works, Corning, NY. The 75 cm² tissue culture flasks (Falcon®) and the gamma irradiated 0.45 μm pore size Cyclopore™ membrane culture inserts (Falcon®) were purchased from Beckton Dickinson Labware, Lincoln Park, NJ. The alpha minimum essential medium (α-MEM), 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, Na-β-glycerophosphate and dexamethasone were obtained from Sigma Chemical Company (St. Louis, MO). Monoclonal hybridoma (MP III B10,(VD 12)) anti-osteopontin and (WV I D1(9C5)) anti-BSP antibodies were obtained from the Developmental Studies Hybridoma Bank, John Hopkins University, Baltimore, MD. Fluorescein (FITC) conjugated secondary antibodies, FITC-goat anti-mouse IgG and FITC-rabbit anti-goat IgG were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). All other chemicals and materials were obtained from local suppliers.

Methods

Cell isolation. Rat bone marrow cells (RBMC) were obtained according to the method previously described (Davies et al., 1991a). Briefly, femora were excised from young adult Wistar rats (115-125 g) and transferred into an antibiotic/fungizone solution [penicillin G (1670 units/ml), gentamycin (500μg/ml), amphotericin B (3μg/ml)]. After remaining 10 minutes in this solution the femora were transferred into a fresh aliquot of the same solution. This washing procedure was repeated a total of three times. Subsequently, the femora were transferred into alpha minimal essential medium (α-MEM). Epiphyses were removed and the marrow from each diaphysis was flushed out with 15 ml of α-MEM supplemented with 15% (v/v) fetal calf serum (FCS), 100 mg L-ascorbic acid, 10mM β-glycerophosphate, 10⁻⁸ M dexamethasone, together with antibiotics and fungizone at one tenth the concentration used during excision. Marrow cells of both diaphyses were collected and cultured in the above medium.

Cell culture. Fifteen ml of the cell suspension were cultured in a T 75 flask maintained at 37°C in an incubator with an atmosphere consisting of 95% air, 5% CO₂ and 100% relative humidity. The medium was changed on days one and four. On day five the cells were subcultured by enzymatic release using 0.01% trypsin. The trypsinized cells were plated on polycarbonate membrane well inserts and on separate 35 mm tissue culture polystyrene wells not furnished with a well insert (positive control) at a seeding density of approximately 10⁴ cells per cm² and cultured for a further 14 days in the same conditions as listed above, with three medium changes/week. Cell counts were carried out using a Coulter Counter® model ZM (Coulter Electronics Ltd., Luton, Beds. England).

In order to distinguish between deposition events at the substratum interface due to the presence of osteogenic cells and events occurring spontaneously in the culture medium, two groups of negative control specimens were prepared. The first group comprised of the dry untreated membrane and the underlying surface of the floor of the well, the second group of the membrane and the well surface, which were maintained in identical culture medium, and under the same conditions including media changes, as that used in the cell cultures, but without cells.

Cell culture fixation. Cultures and negative control specimens 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 in Karnovsky 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 2% OsO₄ in 0.1 M Na-cacodylate for 90 min at room temperature, to preserve the cell membranes, and then again 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 CO₂ at a temperature of 31.1°C and pressure of 1073 psi (Ladd Research Industries Inc., Burlington, VT). To examine the tissue at the cell-substratum interface the overlying cell-multilayers were removed using a compressed air jet and the specimens were sputter coated with approximately 15 nm platinum (Polaron Instrument Inc., Doylestown, PA) and examined in an SEM (Hitachi model 2500; Hitachi, Tokyo, Japan) at an accelerating voltage of 15 to 20 kV.
Transmission electron microscopy (TEM). Samples for TEM were fixed with Karnovsky's fixative and post fixed with osmium tetroxide as described above. All samples were thoroughly washed prior to enbloc staining with uranyl acetate in 50% ethanol for 2 hours at room temperature. Specimens were dehydrated in graded alcohols. Samples were infiltrated with a series of alcohol-epoxy mixtures and finally with 2 changes of pure epon. The epon was polymerized at 40°C overnight and then at 60°C for a further 3 days. The embedded tissue was separated from the base of the polystyrene dishes. Selected areas of the tissue were re-embedded in beam capsules.

Silver to pale gold thin sections were cut on an ultra-microtome and mounted on copper grids. The sections were double stained first with 3% uranyl acetate in 30% ethanol, and then with Reynold's lead citrate. Samples were then examined in a Phillips 400T transmission electron microscope.

X-ray photoelectron spectroscopy (XPS). The elemental composition of the interfacial layer was analyzed using XPS. 14 day cell cultures were Karnovsky fixed, critical point dried and treated with compressed air as described above. The spectrum was obtained on a Leybold MAX 200 XPS system utilizing an unmonochromatized Mg Kα X-ray source operating at 12 kV and 25 mA. The presence of C, O, N, Ca, and P was detected by spectra run in a low-energy mode (pass energy = 192 eV) and normalized to unit thickness of the electron spectrometer. The characteristic peaks for the different atoms correspond to their binding energy (O 1s: 531 eV, N 1s: 398 eV, Ca 2p: 349 eV, C 1s: 250 eV and P 2p: 130 eV).

Immunofluorescence (IF). The presence of osteopontin and bone sialoprotein on the floor of the well beyond the membrane was investigated using immunofluorescence. For these assays, glass coverslips were introduced into the polystyrene culture dishes to facilitate handling and avoid the autofluorescence of polystyrene, thus immunolabeling of the polystyrene surface itself was not undertaken. Immunofluorescence staining methods were based on those described previously (Shen et al., 1993). Briefly, samples were rinsed with unsupplemented α-MEM, fixed with 4% paraformaldehyde in PBS at pH 7.4 for 30 minutes and then rinsed with PBS. The samples were then incubated with the primary antibody for 30 minutes. After rinsing with PBS the samples were incubated with an appropriate secondary antibody, conjugated with FITC, for 30 minutes. A negative control was prepared with the secondary antibody only. The specimens were washed in PBS again, prior to being mounted with anti-bleach medium [90% glycerol, 10% PBS containing p-phenylenediamine (10mg/ml), pH 8.0]. Samples were examined using a light-microscope equipped with a selective filter for fluorescein.

**Results**

Cell free control surfaces (negative controls)

Both negative control samples, the polystyrene dish and the membrane were examined with SEM and showed only a small amount of debris at the membrane surface and well floor. No differences could be observed between the untreated specimens and those which had been cultured without cells. The polystyrene surface appeared smooth whereas the polycarbonate membrane showed more surface structure (Figs. 1 A-D).

Control cultures (positive controls)

The positive control cultures, cultured under standard conditions on the tissue culture polystyrene wells reached confluence around day 5 after subculturing and the development of multilayered bone nodules could be observed in the light microscope (not shown). Two week cultures were examined by both SEM and TEM. SEM observations showed the elaboration of a bone like matrix, consisting of a continuous globular interfacial matrix and an overlying collagenous matrix which, in some areas, appeared to have undergone mineralization and had started to fuse with the interfacial matrix. TEM also showed an electron dense interfacial layer of about 0.5 μm thickness underlying, in specific sites, a mineralizing collagenous matrix in which cells were embedded, all of which we have shown previously (Davies et al., 1991a,b; Davies, 1996).

Appearance of membrane inserts following culture

In parallel with the control cultures the cells on the membrane reached confluence at about day 5 after subculturing and nodules were visible by light microscopy (not shown). The 14 day cultures were examined by SEM, TEM and with XPS. SEM was employed to observe both sides of the membrane. On the side where the cells were seeded similar observations to the positive control group were made with the cells forming a bone like tissue (Fig. 2A). Again the typical globular interfacial matrix accompanied by an overlying collagenous matrix was observed (Fig. 2B). The pores in the membrane were still visible and were not occluded after the 14 days of culturing. On the lower side of the membrane, facing the floor of the well no cells or cell-processes were visible, but the membrane surface was covered with a thin continuous layer (Figs. 2C,D). XPS measurements on this cell free side of the membrane revealed the presence of P and Ca0, indicating that this layer was mineralizing, along with C, O and N representing the organic components of this matrix. Traces of sulphur could also be detected (Fig. 3).
Figure 1. Scanning electron micrographs of the untreated and medium incubated surfaces of polystyrene dish and polycarbonate membrane. Note the sparse deposits on all samples. No differences between untreated (A, B) and medium incubated (C, D) samples are apparent. The polystyrene surface (A, C) appears smoother whereas the polycarbonate membrane (B, D) has a more textured structure. (A) untreated polystyrene culture dish (field width 4.3 μm); (B) untreated polycarbonate membrane (field width 4.3 μm); (C) polystyrene dish after culturing for two weeks in cell free conditions (field width 4.3 μm); (D) polycarbonate membrane after culturing for two weeks in cell free conditions (field width 4.3 μm).

TEM examination showed similar features, on the cell side of the membrane, to the observations in the positive control cultures. Again an interfacial electron dense layer of about 0.5 μm in thickness was present covered by a collagenous matrix (Figs. 4A,B). Foci of mineralization were seen throughout the collagenous matrix within which cells were embedded (Fig. 4A). A cross section through the membrane revealed a thin
Figure 2. Scanning electron micrographs of the polycarbonate membrane after 14 days of culture. (A) In this field of view bone-like tissue can be seen on the polycarbonate membrane (field width 172 μm); (B) The interfacial cement-line matrix with its undulating appearance is clearly seen on the cell side of the polycarbonate membrane. Note the pores in the membrane (arrows) (field width 8.6 μm). (C) Image of the lower side of the polycarbonate membrane. No cells are visible. A confluent matrix layer can be seen. Note the pores in the membrane (arrows) (field width: 86 μm); (D) This picture shows a higher magnification of the lower side of the membrane. The overlying layer can be seen in the bottom part of the picture. Note the pore in the membrane (arrow) (field width 4.3 μm).

electron dense layer, varying in thickness from 20-100 nm, covering both the insides of the pores of the membrane and also the lower side of the membrane (Fig. 4A,C). No cells were seen within or beneath the membrane (Fig. 4A).

Appearance of the floor of culture wells, and glass coverslips, following culture

The floor of the culture wells was examined, by
Figure 3. X-ray photoelectron spectroscopy (XPS) plot of the lower side of the membrane after 14 days of culture. In this XPS-plot characteristic peaks for Ca and P can be detected on the lower side of the membrane. Also traces of S are detected. The elements O, C and N are indicative of proteins.

Discussion

Differentiating osteogenic cells, derived from primary explants of young adult rat bone marrow, elaborate an interfacial matrix to a variety of solid substrata, such as polystyrene (Davies et al., 1991a; b), titanium (Davies et al., 1990; Lowenberg et al., 1991) and calcium phosphate ceramics (de Bruijn et al., 1992a; b). This interfacial matrix has been shown to be morphologically similar to the interfacial matrix elaborated in vivo at both, endosseous implant surfaces (Davies et al., 1991c; Pilliar et al., 1991; Orr et al., 1992) and at cement lines in bone remodeling sites (Zhou et al., 1994). We have shown that this interfacial matrix develops as individual globules, that are quite typically seen associated with cell-processes (Lowenberg et al., 1991), and which fuse to form a continuous layer at solid surfaces (Davies et al., 1991a). There is also biochemical, and biolabelling, evidence for the similarity between the interfacial matrix formed in these in vitro and in vivo situations since both contain the same non-collagenous proteins (Shen et al., 1993; Ingram et al., 1993; McKee et al., 1993). Yet both in vitro and in vivo observations have generated considerable debate concerning the distinction between cement lines and laminae limitantes. McKee and Nanci (1995) have suggested an operational distinction between cement lines and laminae limitantes according to which cement lines are found at matrix-matrix interfaces whereas laminae limitantes appear at cell-matrix interfaces. While this definition points out the importance of the stage in the life cycle of the cells for the respective interfacial matrices, it is not consistent with the terms cement line and lamina limitans as they were originally introduced to the literature by von Ebner (1875) and Scherft (1968) respectively and their use thereafter. According to the definition of McKee and Nanci (1995) a resting line at a matrix-matrix interface would be classified as a cement line, however, we prefer to use the term cement line strictly in the spirit of the term when it was first introduced by
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Figure 4. Transmission electron micrographs of the polycarbonate membrane after 14 days of culture. (A) The lower part of the picture is a cross section through the membrane whereas the upper part is a cross-section through the overlying cell-layer. The border is demarcated by the dark electron dense interfacial matrix. Overlying this is the collagenous matrix with embedded cells and electron dense foci of mineralization. Within the membrane a pore can be seen which is covered with an electron dense layer which is continuous with that on the lower side of the membrane (field width 18 µm); (B) This image shows a higher magnification of the cement line matrix on the polycarbonate membrane. This matrix is approximately 0.5 µm thick and seen below a cell process (P) and the collagenous extracellular bone matrix (C) (field width: 2.3 µm); (C) This photomicrograph shows a high magnification of the electron dense layer on the lower side of the membrane (field width 1.4 µm).

von Ebner in 1875 as a line which cements a secondary osteon to the surrounding cortical bone. Similar situations occur at reversal sites in trabecular bone, at the cartilage-bone interface when bone is cemented to cartilage during endochondral ossification or, indeed at bone-implant sites, where new bone has been formed on the implant surface. These situations have in common that bone is formed *de novo* on a solid surface which is in contact with the bone tissue. We believe that the expression of the cement line which is consistently in the order of 0.5 µm regardless of the underlying solid surface represents the secreted matrix of osteogenic cells in a specific stage of their early differentiation before their differentiation into osteoblasts resulting in the assembly of a collagenous matrix (Davies, 1996; Hosseini *et al.*, 1996). According to the proposed definition of McKee and Nanci (1995), a cement line would initially be classified as a lamina limitans during its elaboration while it is still in contact to differentiating osteogenic cells that assemble the cement line. It appears to be rather ungainly, however, to attribute two different names to the very same matrix, when all that has changed is not the matrix itself but the neighboring environment from an osteogenic cell to collagenous matrix. Furthermore, such classification of laminae limitantes would not be consistent with the understanding of a lamina limitans as a matrix created due to cessation of mineralization as introduced by Scherft (1978) (see below), since the developing cement line represents a stage of initiation of mineralization (Hosseini *et al.*, 1996) rather than cessation. It has to be pointed out in this context, however, that the morphological feature of a cement line only appears at sites, where *de novo* bone formation occurred on a solid surface, such as artificial
substrata of polystyrene, polycarbonate, titanium and hydroxyapatite as well as the physiological substrata of resorbed bone (reversal line) or cartilage during endochondral ossification. In the case of de novo bone formation in the absence of a solid surface as in intramembranous ossification no cement line forms, since the matrix components secreted during de novo bone formation that otherwise comprise the cement line will not adhere at a spatially distinct plane but rather mingle with the already existing 3-dimensional collagenous matrix to form foci of mineralization (McKee and Nanci, 1993) that in size and morphology indeed resemble the globules that are formed on solid surfaces. Summarizing our past experience with regard to cement lines, we would consider the most striking morphological features of the cement line to be the presence of mineral but lack of collagen fibers within the cement

Figure 5. Electron micrographs of the polystyrene dish beyond the membrane after 14 days of culture. (A) Irregular shaped particles ranging from 20 to 100 nm in diameter that tend to coagulate are seen on the dish surface. No signs of cells are visible (field width: 4.3 \( \mu m \)); (B) This transmission electron photomicrograph shows a cross-section through the matrix on the polystyrene well surface (field width: 1.3 \( \mu m \)).

Figure 6. Light microscopic immunofluorescence images of glass cover slips underneath the membrane 14 days of culture. (A) A rather even distribution of osteopontin can be seen. An intense label can be seen where the proteinaceous layer has been peeled back using forceps (field width: 3.5 mm); (B) A more patchy pattern can be seen for BSP. Again parts of the layer have been retracted using forceps (field width: 3.5 mm); (C) Negative control. No stain can be seen. At the scratch site (arrow) this is no significant difference in image contrast (field width: 3.5 mm).
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line and the globular origin of the cement line as well as the requirement for a solid underlying surface.

Laminae limitantes on the other hand can best be seen in demineralized sections of bone. While according to the operational definition of McKee and Nanci (1995) laminae limitantes are found at cell-matrix interfaces the term was originally introduced (Scherft, 1968) and continuously used to describe matrix-matrix interfaces (Scherft, 1972, 1973, 1978). Laminae limitantes as originally described by Scherft (1968) in mouse radii surround foci of mineralization within cartilage or bone as electron dense layers of organic constituents. The term lamina limitans, however, has been extended to other sites such as osteocytic lacunae including their connecting canaliculi (Scherft, 1972) and resting lines (Scherft, 1978), making laminae limitantes rather ubiquitous features in bone. The commonality at these sites is that mineralization has ceased. Scherft (1978) suggested that the lamina limitans forms in a cessation phase of calcification possibly through adsorption of organic molecules. We have consistently held that while cement lines and laminae limitantes may comprise the same organic and inorganic components, their dimensions - an order of magnitude difference in thickness, as seen in non-demineralizing sections - indicate distinctly different morphological entities which must reflect differences in cell function (published discussions following van Blitterswijk et al., 1991 as well as McKee and Nanci, 1993). Since cement lines represent highly localized, rather than ubiquitous, morphological features in bone, we considered it important to design experiments to deconvolute the provenance of these compositionally similar, but structurally divergent, features of hard tissue and the specific role of cells in the elaboration of these two extracellular architectures. Thus the hypothesis underlying this study was that cement lines will only form at sites of cellular-substratum contact, while cell contact is not a pre-requisite for the formation of laminae limitantes.

Our results show that an interfacial matrix consistent with the dimensions of a cement line was elaborated on the cell side of the membrane whereas a significantly thinner matrix, which appears electron dense by TEM was formed on the cell-free surfaces. This matrix was also mineralized on the lower side of the membrane.

The assumption that all these matrix layers form due to the presence of the cells in the culture can be made, because in the negative control wells, which were cultured without cells, no such features were observed. Furthermore this is supported by the immunofluorescence and XPS observations which indicate that this mineralized non-cell-contacting matrix contained the cell-secreted matrix proteins osteopontin and BSP. That these proteins are secreted by osteogenic cells in this adult rat bone marrow system has been demonstrated by ourselves and others (Kasugai et al., 1991; Shen et al., 1993; Yao et al., 1994). Furthermore, the fact that both negative control groups, the untreated membrane and well surfaces as well as the membrane and well surfaces incubated with serum containing medium under cell free conditions, showed no morphological differences suggests that adsorbed serum proteins are not involved in the elaboration of these thin layers.

On the lower side of the membrane the matrix was confluent but of varying thickness from 20-100 nm. SEM examination of the polystyrene well surface showed a non-confluent layer with individual patches of matrix in the range of a 1μm diameter. This matrix, when sectioned for TEM examination demonstrated a relatively uniform layer of approximately 40 nm thickness.

Other studies have reported the formation of mineralized layers on thin slices of bovine dentin incubated in culture medium supplemented with 10 mM β-glycerophosphate and containing alkaline phosphatase immobilized on agarose beads (Beertsen and van den Bos, 1991). Their study showed that the mineralization of the demineralized organic matrix, which may provide the initiation sites for the mineralization requires alkaline phosphatase. The relationship between β-glycerophosphate, alkaline phosphatase and mineralization has been shown by Tenenbaum (1987), who demonstrated that mineral deposition in the presence of β-glycerophosphate was stopped in bone cultures when alkaline phosphatase activity was inhibited by levamisol. It therefore appears likely that if there is an initiating matrix present, mineralization can occur in the presence of β-glycerophosphate supplemented bone cultures. Non-collagenous proteins have been suggested as possible candidates for initiation of mineral deposition (Gerstenfeld et al., 1987; Stein et al., 1990). Nagata et al. (1991) showed that BSP and osteopontin are the major osteoblast derived proteins to bind to the bone mineral. The importance of BSP for biological mineralization has been shown by Chen et al. (1991) and Hunter and Goldberg (1993, 1994). This would be consistent with our observations, that proteins secreted by the osteogenic cells into the medium, particularly BSP, become adsorbed to surfaces, where they may initiate mineralization. This assumption may provide a mechanistic explanation for the formation of the thin mineralized layers observed. The different morphological appearances of the mineralized thin layers on the lower side of the polycarbonate membrane and the polystyrene well surface may be indicative of a combination of different surface topographies and availability of the requisite proteins.

However, not only are the mineralized layers formed in the absence of cell/substratum contact very
m much thinner (40 to 100 nm) compared to the 0.5 μm thick cement line matrix formed on the cell side of the membrane, but the morphology of these layers is also very different. The cement line appears as an undulating interfacial matrix, reminiscent of its origin as initial globules, typically 1 μm in diameter, while the thinner layers exhibit no such structure. This implies that there could be a fundamentally different mechanism involved in the elaboration of these two interfacial matrices. The fact that the cement line matrix only forms on the cell side of the membrane suggests that the osteogenic cells possess a locally specific matrix assembly mechanism. On the contrary a non-specific mechanism could be involved in the lamina limitans elaboration. This would correlate with the known histological and ultrastructural findings that laminae limitantes are ubiquitous features of many hard tissue surfaces whereas cement lines are morphological features specifically localized to remodeling sites where differentiating osteogenic cells colonize a resorbed bone surface to initiate the reversal phenomenon.

Conclusions

Cement lines which are approximately 500 nm thick only assemble in direct contact with differentiating osteogenic cells at sites of de novo bone formation on solid surfaces. However, laminae limitantes which are thin by comparison (40 to 100 nm) form on any surfaces which are exposed to the secretion products of osteogenic cells.

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References


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Discussion with Reviewers

Reviewer I: The hypothesis offered is that "cement lines will only form at sites of cell-substratum contact, while cell contact is not a pre-requisite for the formation of laminae limitantes". Surely the authors realize that all cells in bone are in contact with a substratum - they are not floating in the "aether". Under what physiological conditions, then, would a lamina limitans form?

Authors: As has been outlined in the Discussion there are many matrix-matrix interfaces where cell contact is not present. Under physiological conditions a lamina limitans can attain its characteristic features as a result
of exposure of a surface to the secretion product of cells without being in contact to the cells itself.

**Reviewer I:** The authors attempt to model their hypothesis by means of a trans-filter approach. This is a classical method of distinguishing biological phenomena that require cell-cell contact from those that only require a diffusible mediator. It is not clear how one can apply this approach to a cell-substratum interaction, particularly since the cells are making the substratum. This is a classical method of distinguishing biological phenomena that require a diffusible mediator. It is not clear how one can apply this approach to a cell-substratum interaction, particularly since the cells are making the substratum. Therefore, although it is correct that all extracellular matrix substrata and their interfaces are ultimately formed by cells it is not correct to assume that all extracellular matrices have to be formed in direct juxtaposition to the cells responsible for their elaboration. While some of the interfacial matrices are formed at a time when cells had formerly been present at the location (e.g. cement lines) and have lost their immediate cell contact after being separated by extracellular matrix secreted by the cells thereafter, others (e.g., laminae limitantes) are formed at a time when cells are at a distance from that location by adsorbing diffusible proteins which are secreted by the cells into the extracellular matrices.

**Authors:** The fact that a trans-filter approach has been used by others to study cell-cell interactions does not negate the possibility that it can be used to address other questions. Therefore, although it is correct that all extracellular matrix substrata and their interfaces are ultimately formed by cells it is not correct to assume that all extracellular matrices have to be formed in direct juxtaposition to the cells responsible for their elaboration. While some of the interfacial matrices are formed at a time when cells had formerly been present at the location (e.g. cement lines) and have lost their immediate cell contact after being separated by extracellular matrix secreted by the cells thereafter, others (e.g., laminae limitantes) are formed at a time when cells are at a distance from that location by adsorbing diffusible proteins which are secreted by the cells into the extracellular matrices.

**S.C. Miller:** Do you or can you make any distinction between the cement lines, such as occur between the lamellar sheets in osteons and reversal lines. It seems that the cement lines that are described in the last sentence of the manuscript are "reversal" lines. Are there any differences at the ultrastructural level?

**Authors:** The boundaries between the lamellar sheets within secondary osteons are not cement lines. SEM and TEM studies by Marotti (1993) have shown that lamellar bone is made up of alternating collagen-rich (dense lamellae) and collagen-poor (loose lamellae) layers, all having an interwoven arrangement of fibres. Furthermore, no interlamellar cementing substance was observed between the lamellae, and collagen bundles were shown to form a continuum throughout lamellar bone (Marotti, 1993). Ultrastructurally, the thickness of the interlamellar boundaries is in the order of 100 nm. Cement lines, on the other hand, which in lamellar bone only appear at the outer margins of the secondary osteons, and cement the secondary osteon to the surrounding bone, do not contain collagen fibres and their thickness is in the order of 0.5 μm.

With regard to the second part of the question, the terms "cement line" and "reversal line" are often used interchangeably. We prefer to use the term reversal line to describe the solid surface which is created by bone resorption and the term cement line to describe the interfacial matrix which is laid down on the reversal surface.

**A. Nanci:** The surface immediately below the cells is clearly most advantageous for accumulation of material. Proteins are released against that surface and diffuse to eventually establish a concentration gradient. An equilibrium state may, however, not be attained if proteins interact with mineral. In this case the mineralization site closest to the site of protein release would likely be favored resulting in a preferential accumulation as compared to more distant sites, particularly when these are separated by a physical barrier. Indeed, the thickness of the layer coating the pore illustrated in Figure 4A is somewhat in between that of the top and bottom layers, consistent with the gradient hypothesis. Thus the experimental design does not allow to reach the conclusion that any difference in the thickness of accumulated material is related to cell contact.

**Authors:** It is correct that the surface immediately below the basal cell membrane is the most advantageous for the accumulation of secretory products. Indeed, the establishment of a concentration gradient, along with the high affinity of the secreted proteins for mineral, would result in the precipitation of these proteins before the equilibrium state of the diffusion process will be reached, particularly since alkaline phosphatase as an ectoenzyme is cell-membrane-bound and provides inorganic phosphate in direct cell proximity and therefore superimposes another concentration gradient for mineralization. Such establishment of multiple overlaying concentration gradients in combination with reaction kinetics of higher order results in the multiplication of effects and could have a spatial reaction profile at which virtually the entire chemical reaction that leads to precipitation of mineral and protein would be completed in direct proximity to the cell membrane. Thus the gradient hypothesis provides a potential mechanistic explanation of the observed results and does not contradict them. However, it has also to be noted that differentiating osteogenic cell contact alone is not sufficient for the formation of a cement line. Recent experiments in our lab have shown that no cement line forms at the cell-substratum interface if the substratum is a gel-like matrix (unpublished results). This observation emphasizes that a solid surface substratum is also essential for the formation of a cement line. Therefore, the underlying essential condition for the elaboration of a cement line may not primarily be the presence of concentration gradients but rather the establishment of a sealed environment on the basal side of the differentiating osteogenic cells, allowing locally high concentrations. The observation that the pores, which are only
0.45 μm in diameter, were not occluded by the surrounding 0.5 μm thick cement line (Fig. 2B) provides support for the hypothesis of the requirement of a sealed environment because at such a pore no sealed environment could be established. With regard to the thickness of the layer covering the pore (Fig. 4A) it has to be considered that the thickness of this layer in cross section is dependent on the position of the cross-section to the center of the pore. Only if the cross section runs through the center of the pore the thickness in the cross section will reflect the true thickness of the covering layer. In any other cross section through that pore, which is the more likely event, the thickness in the cross section will appear larger than the actual thickness of the covering layer. Therefore, it cannot be concluded that the thickness of the covering layer inside the pore is thicker than the layer on the bottom side of the membrane. It can only be concluded that it is thinner than the cement line on the top side of the membrane.

A. Nanci: Does the cement line cover entirely and uniformly the cell-substratum interface? Is the presence of such a layer a prerequisite for mineralized bone nodule formation?

Authors: The answer to the first question is yes if the surface at the cell-substratum interface is solid, such as the polycarbonate membrane used in this experiment. However, no cement line forms at the cell-substratum interface in the absence of a solid substratum. Such situations occur in vivo during intramembranous ossification, as pointed out in the Discussion or in vitro if cells are seeded on gel-like substrata, as recent experiments in our lab have shown (see above). With respect to the second question the answer is no, since bone nodules also form on the gel-like substrata, where no cement line forms at the cell-substratum interface.

R.S. Tuan: It is interesting to note that the matrices found on the bottom, non-cell contacting surface of the polycarbonate membrane and on the bottom of the culture well are structurally similar. However, it was also pointed out that the former is confluent, whereas the latter is patchy and non-confluent. If the "lamina limitans" is the product of bone matrix components deposited on substratum surfaces, one would expect that the bottom of the dish, located a distance away from the secreting bone cells, should receive a uniform deposition, given the random diffusion that must take place in the fluid phase of the culture medium. Could the authors elaborate some more on the possible mechanisms responsible for this phenomenon? This reviewer suggests placing a piece of polycarbonate filter on the bottom of the dish in the cultures to provide the same substratum material as the underside of the filter may well give some insights as to whether this is due to the intrinsic difference between polystyrene and polycarbonate.

Authors: It should be noted that the patchy appearance of the layer at the bottom of the dish underneath the well insert was only observed in the SEM preparation (Fig. 5A). In the TEM preparation of the same location a more confluent appearance of this layer could be seen (Fig. 5B). The more patchy appearance in the SEM preparation could be due to collapsing of the protein content of the layer after dehydration and critical point drying, leaving only mineralized particles uncollapsed, since they contain less water. The TEM preparation with embedding in Epon may have structurally preserved the organic content of that layer and therefore resulted in a more confluent appearance, as expected for random diffusion phenomena. The reason for the patchy appearance of the mineral content may be caused by preferential deposition of mineral onto a pre-existing mineral phase, if the actual initiation of mineralization is thermodynamically less favorable than crystal growth.

With regard to the second part of the question, we believe that the thicker and even at the SEM level confluent appearance of the deposited layer on the polycarbonate membrane is due to improved protein adsorption of this substratum over polystyrene, resulting in an earlier onset of mineralization and earlier confluence of the mineral particles. An experimental design as suggested by the reviewer will certainly be helpful in testing this hypothesis and is worthwhile pursuing in future studies.

R.S. Tuan: If indeed cement line formation requires cell contact, would plating osteoblasts onto a surface decorated with bone matrix protein (i.e., either the under surface of the polycarbonate membrane or the bottom of the well) and elaborating a lamina limitans-like material then result in the appearance of a cement line?

Likewise, would osteoblast conditioned medium placed into a culture well produce a coating of lamina limitans-like structure on the bottom of the well? Authors: With regard to the first question the answer is yes, provided the plated bone cells are undifferentiated enough to express the osteogenic phenotype of producing a cement line, such as the demethasone stimulated adult rat bone marrow cultures used in this study. Actual osteoblasts which, per definition, already assemble collagenous matrix may not express this osteogenic phenotype anymore, and a cement line may not form. Differentiating osteogenic cells, however, always deposit a cement line on any solid surface that they can adhere to, before they start producing collagen containing bone matrix.

Addressing the second question, it would appear
likely that under the described conditions a lamina limitans-like structure would form on the bottom of the dish, since the factors responsible for the formation of that matrix have to be transferred by the medium. It has to be noted, however, that after the cells create a sealed environment at the substratum side, the proteins which are released into that sealed environment may not be the same as those released into the overlying medium, since the secretory cells responsible for the elaboration of this matrix are polarized and thus will most likely show a different secretory behavior at their apical and basal sites respectively. Therefore, only conducting the proposed experiment could answer this question.

Additional Reference


Editorial questions

1. In the de Bruijn et al. (1992) reference, we need names and initials of all editors.

2. Note that Abstract has been slightly condensed to make it fit into the space available.

3. See cover message regarding halftones in this paper.