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# Ultrastructural, Cytochemical, and Immunocytochemical Studies on Bone and its Interfaces

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# ULTRASTRUCTURAL, CYTOCHEMICAL AND IMMUNOCYTOCHEMICAL STUDIES ON BONE AND ITS INTERFACES

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## Abstract

Bone cells possess the ability to synthesize, secrete and direct the assembly and maintenance of extracellular matrix to form a functionally rigid and/or weight-bearing mineralized tissue complex, the skeleton. The skeleton not only supports and protects the organs and tissues of the body, but also acts as a mineral ion reservoir for maintaining systemic calcium balance (calcium homeostasis). The remarkable biological precision necessary for the formation, turnover and constant adaptation of bone to external mechanical forces is a dynamic process requiring a coordinated cellular effort relying on a variety of cell-cell and cell-matrix/mineral interactions. Indeed, cell-matrix interfaces found in a variety of locations in bone are sites potentially rich in signalling mechanisms related to bone modeling and remodeling. Detailed examination of the structure, composition and function of bone cells, and the extracellular matrix they produce and continuously remodel, is a complex task for such a mineralized tissue and requires integration and correlation of data from numerous sources. Various morphological approaches have added significantly to our understanding of bone form and function, and have allowed us to partly unravel some of the complex cell-matrix-mineral associations that occur during osteogenesis. In this context, the present article reviews some of our recent ultrastructural, cytochemical and immunocytochemical data on mammalian and avian intramembranous and endochondral bone formation (modeling) and turnover (remodeling).

**Key Words**: Bone, osteoblast, osteoclast, osteocyte, bone-lining cell, immunocytochemistry, osteopontin, non-collagenous bone proteins, cytochemistry, biomineralization, microscopy.

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## Introduction

Bones are mineralized connective tissues having the ability to protect and support the various organs and tissues of the body. Furthermore, in conjunction with the calcium handling activities of the gastrointestinal tract and kidneys, bone acts as a reservoir through which calcium homeostasis is maintained (reviewed by de Bernard, 1992). To perform these functions, the tissue-specific events of intramembranous and endochondral ossification rely on the coordinated efforts of bone cells and their cell-cell and cell-matrix interactions. Together, these osteogenic processes produce a mineralized bone matrix whose supramolecular structure not only provides the three-dimensional skeletal architecture capable of withstanding the daily demands of vertebrate life (Frost, 1990a-d), but also allows great versatility in regulating systemic calcium balance (discussed by Parfitt, 1990). In addition to its mechanical and metabolic properties, bone matrix may, in turn, also regulate the activities of cells in contact with it. Thus, a cell's ability to recognize components of the extracellular matrix may constitute a phenotypic hallmark (Majeska *et al.*, 1993), and may provide a means to influence some of the homeostatic mechanisms classically associated with the skeleton and bone turnover.

Appreciation of such functions for bone clearly involves a need to understand the interactive principles and mechanisms necessary to develop these important attributes of the skeleton. One approach towards elucidating these mechanisms is to take advantage of the resolving power of the electron microscope in order to describe the structural relationships that ultimately, and by necessity, allow biological interactions to take place. It is of paramount importance in bone, as well as in other mineralized tissues, to understand the meaning of the precise structural relationships among the cellular and organic and inorganic extracellular matrix components. Both conceptually and experimentally, it is reasonable to predict that the physical proximity and molecular interactions among these components guide bone development, processes that can be, at least in part, resolved using

different morphological approaches.

Morphological techniques using electron microscopy allow high-resolution, cytochemical characterization of cellular and matrix compartmentalization with respect to, for example, enzyme activity (e.g., phosphatase cytochemistry), glycoconjugate localization (lectin cytochemistry) and sulfated glycoconjugate distribution (high-iron diamine staining), or with respect to the distribution of specific molecules within the structures of a tissue (immunocytochemistry). Furthermore, the introduction of various colloidal-gold probes (reviewed by Leunissen and De Mey, 1989) has added to our ability to identify molecules *in situ* with high resolution, and in the case of bone, has led to a better understanding of matrix-mineral relationships in this tissue. Fine structural detail in bone, heretofore only described in morphological terms, can now be addressed with regard to precise composition, information not currently obtainable by traditional biochemical methods. Indeed, it can be said that many of these approaches represent a form of 'biochemistry on section', and as such, offer the distinct advantage of allowing correlation of biochemical reactions between molecules with tissue ultrastructure. Such powerful techniques clearly fulfill a fundamental need to relate form to function in the mineralized tissues. Here, we present a review and compilation of our recent data utilizing these methodologies to describe relationships among the cells, matrix and mineral of bone.

## Materials and Methods

### Animals and tissue processing

For morphological, cytochemical and immunocytochemical analyses of bone from embryonic chickens and fetal pigs, tibiae and calvariae were dissected, cut into segments, and fixed by immersion for 18-24 hours at 4°C with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) or with 4% paraformaldehyde in 0.034 M phosphate buffer containing 0.1 M lysine and 0.01 M sodium metaperiodate, pH 6.8 (McLean and Nakane, 1974). Similar studies in the rat, male Wistar rats ranging from newborn to 2 months-old were anesthetized with chloral hydrate (Sigma Chemical Co., St. Louis, MO) and perfused through the heart and into the aorta for approximately 20 seconds with lactated Ringer's solution (Abbott Laboratories, Montreal, QC, Canada) followed by 1% glutaraldehyde, or 4% paraformaldehyde plus 0.1% glutaraldehyde, in 0.08 M sodium cacodylate buffer containing 0.05% calcium chloride, pH 7.3, for 20 minutes. The tibiae, ribs, calvariae and mandibular alveolar bone were dissected and additionally fixed by immersion in the same fixative for another 3 hours at 4°C. Some samples were left calcified, while others

were decalcified in 4.13% disodium ethylenediaminetetraacetic acid (EDTA) (Warshawsky and Moore, 1967), with or without 0.1% glutaraldehyde, for 21 days followed by extensive washing with 0.1 M sodium cacodylate buffer containing 5% sucrose, pH 7.3. All specimens were either post-fixed with potassium ferrocyanide-reduced osmium tetroxide for 2 hours at 4°C prior to dehydration through a graded acetone series or were left non-osmicated and immediately dehydrated through graded methanol or ethanol at room temperature or -30°C. Osmicated samples were embedded in Epon 812 epoxy resin substitute (E.F. Fullam, Latham, NY or Marivac, Halifax, NS, Canada) and the resin was polymerized at 60°C. Non-osmicated tissues were embedded in Lowicryl K4M (Chemische Werke Lowi GmbH, Germany) and the resin was polymerized with ultraviolet light (360 nm wavelength) at -30°C [embedding procedures were as detailed in (Nanci *et al.*, 1990)]. Osmicated and non-osmicated samples were additionally embedded in LR White (Mecalab, Montreal, QC, Canada) and polymerized at 58°C.

Thick sections (1-2 $\mu$m thick) were cut with a glass knife on a Reichert Ultracut E or LKB Nova microtome and either stained with toluidine blue and observed by light microscopy, or stained with uranyl acetate and lead citrate and examined using the backscatter mode in a JSM-840 scanning electron microscope as described previously (Nanci *et al.*, 1990). Light micrographs were taken with a Zeiss Axiophot optical microscope. For electron microscopy, thin sections (80-100 nm) were cut with a diamond knife and conventionally stained with uranyl acetate and lead citrate. Sections were examined with a JEOL JEM 2000FX-II transmission electron microscope (TEM) operated at 80 kV. Other sections were left unstained and processed for immunocytochemistry as described below.

### Cytochemistry

Mandibles were obtained from 100 gm rats that had been perfusion-fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, containing 0.05% calcium chloride. After fixation, the mandibles were washed for several hours in 0.1 M sodium cacodylate buffer, pH 7.4, containing 4% sucrose (CS buffer) and prepared for cytochemistry according to Smith (1981) and Nanci *et al.* (1990). Briefly, the mandibles were partly decalcified for 5 days in a solution of 4.13% ethylene-bis(oxy-ethylenenitrilo) tetraacetic acid (EGTA), pH 7.2. After washing in CS buffer, segments of alveolar bone were embedded in agar (7% solution) and sectioned at a 60 $\mu$m thickness using a Sorvall TC-2 tissue chopper. The sections were washed several times in cold CS buffer and then rinsed twice at room temperature in 0.1 M sodium acetate buffer, pH 5.0. Sections

were then incubated at pH 5.0 for 1 hour at 37°C in a medium containing 2 mM orotidine 5'-monophosphate (OMP, sodium salt, Sigma), 0.1 M sodium acetate buffer (pH 5.0), 5% sucrose and cerium as capture ion. Controls for all experiments consisted of incubating sections for 1 hour at 37°C in duplicate media which did not contain the substrate. After incubation, sections were washed several times with sodium acetate buffer (pH 5.0) and then rinsed with several changes of CS buffer. Sections were then osmicated, dehydrated in alcohol followed by propylene oxide, and embedded in Epon for thick and thin sectioning. For TEM, Epon sections were stained only with lead citrate.

For cytochemical demonstration of sulfated glyco-conjugates, some samples of 1% glutaraldehyde-fixed rat bone were processed for pre-embedding, high-iron diamine thiocarbohydrazide-silver proteinate (HID-TCH-SP) staining (Spicer et al., 1978; Sannes et al., 1979; Kogaya et al., 1990). Briefly, the bone tissue was incubated en bloc for 18 hours at room temperature in HID solution [120 mg N,N-dimethyl-p-phenylenediamine HCl (Sigma Chemical Co., St. Louis, MO) in 50 ml distilled water]. They were then osmicated and conventionally processed for embedding in Epon. Thin sections were mounted on stainless steel grids and reacted with 2% thiocarbohydrazide (Merck, Darmstadt, Germany) in 10% acetic acid for 20-40 minutes, washed and treated with 1% aqueous silver proteinate (Merck) for 15-30 minutes in the dark. Sections were left unstained prior to viewing by TEM.

For lectin-gold cytochemistry (reviewed by Benhamou, 1989), thin sections of Lowicryl-embedded bone were incubated with Limax flavus agglutinin (LFA; courtesy of P.-M. Charest), Ricinus communis I agglutinin (RCA; Sigma) or Helix pomatia agglutinin (HPA; Sigma). RCA and HPA were directly coupled to gold particles of approximately 14 nm while LFA was revealed indirectly by a second incubation with fetuin-gold (Roth, 1983; Roth et al., 1984). The lectins LFA, RCA and HPA show specificities for N-acetyl-neuraminic acid (sialic acid), galactose and N-acetyl-D-galactosamine, respectively. For the direct method, tissue sections were floated at room temperature on a drop of lectin-gold complex for 1 hour and washed with 0.01 M phosphate-buffered saline (PBS, pH 7.2) followed by distilled water. For the indirect method, sections were first incubated with LFA for 1 hour followed by fetuin-gold for 30 minutes, and then washed with PBS and distilled water. Controls consisted of tissue sections incubated with the lectin or lectin-gold complexes in the presence of 0.2 M of the corresponding competing saccharide. Sections were then routinely stained with uranyl acetate and lead citrate and viewed by TEM.

## Immunocytochemistry

The protein A-gold immunocytochemical technique (reviewed by Bendayan, 1989) was applied to thin sections of bone as described previously (McKee et al., 1990). Briefly, grid-mounted tissue sections were floated on a drop of sodium metaperiodate (osmicated tissues only) for 1 hour at room temperature. They were washed with distilled water, floated for 5 minutes on a drop of 0.01 M phosphate-buffered saline (PBS) containing 1% ovalbumin (Sigma) and then transferred and incubated for 1 hour at room temperature on a drop of one of the following polyclonal antibodies: rabbit anti-chicken osteopontin (courtesy of Drs. L.C. Gerstenfeld and Yozo Gotoh), rabbit anti-chicken osteocalcin (courtesy of Dr. J.B. Lian), goat anti-rat osteopontin (courtesy of Drs. M.C. Farach-Carson and W.T. Butler), goat anti-rat osteocalcin (courtesy of Dr. P.V. Hauschka), rabbit anti-pig bone sialoprotein (courtesy of Dr. J. Sodek), rabbit anti-rat fibronectin (Chemicon, Temecula, CA), goat anti-rat fetuin ($\alpha_2$HS-glycoprotein; courtesy of Drs. M.C. Farach-Carson and W.T. Butler) or rabbit anti-rat albumin (Cappel, Scarborough, ON, Canada). After incubation, sections were rinsed with PBS, placed again on PBS-1% ovalbumin for 5 minutes, followed by incubation for 30 minutes at room temperature with protein A-gold complex (gold particles of approximately 14 nm). Tissue sections were then washed thoroughly with PBS, rinsed with distilled water, and conventionally stained with uranyl acetate and lead citrate prior to examination by TEM.

## Results and Discussion

Figures are presented so as to first show the cellular features of the various bone cells (Fig. 1-6) and then to illustrate ultrastructural and compositional characteristics of the extracellular bone matrix (Fig. 7-11).

### Bone cell structure

The structure of the various cells of bone has recently been reviewed (see: Matthews and Davis, 1985; Marks and Popoff, 1988; Holtrop, 1990, 1991; Bonucci, 1990; Scherft and Groot, 1990; Miller and Jee, 1992). Emphasis here will be given to those cells primarily found directly apposed to bone surfaces (i.e., cell-matrix interfaces) and distinguishable by morphological criteria, namely, osteoblasts, osteocytes, bone-lining cells and osteoclasts. The following reviews contain examples of discussions concerning osteoprogenitors and osteoclast precursors: Mundy and Roodman (1987), Marks and Popoff (1988), Vaes (1988), Tenenbaum (1990), Burger and Nijweide (1991), Aubin et al. (1992) and Nijweide and de Grooth (1992). Likewise, recent reviews detailing the effects of local (e.g., growth factors, cytokines)
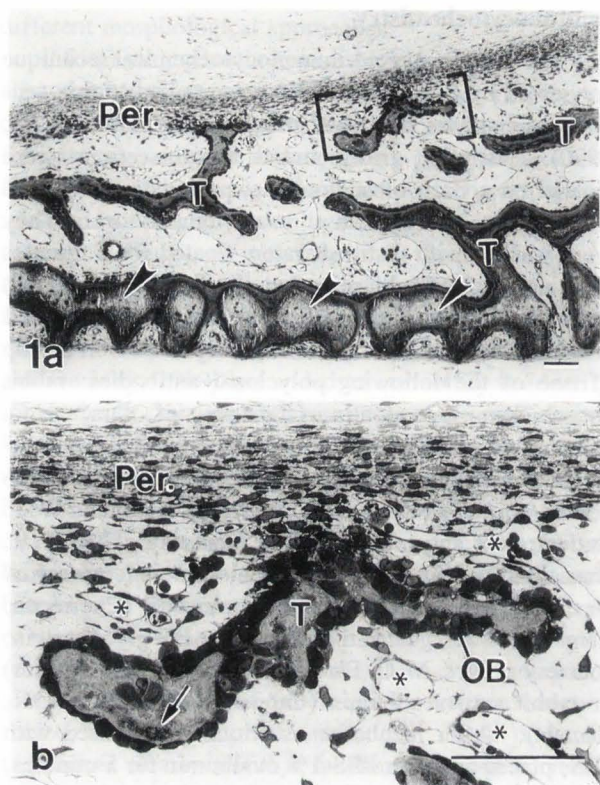
**Figure 1.** Intramembranous ossification in fetal pig calvaria. (a) In close proximity to the periosteum (Per), newly-forming trabeculae (T) of bone begin as isolated plates of osteoid lined by a contiguous layer of osteoblasts. The addition of nascent trabeculae, together with the continued expansion, mineralization and coalescence of pre-existing trabeculae (arrowheads), ultimately define the three-dimensional architecture and integrity of this intramembranous bone. (b) Higher magnification of the bracketed area shown in Figure 1a illustrates an early site of mineralization (arrow) within a trabecula lined by osteoblasts (OB) and surrounded by loose connective tissue containing numerous small blood vessels (asterisks). Epon sections of calcified calvariae stained with toluidine blue. Bars = 100 μm.

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and systemic (e.g., hormones) factors on bone cells are: Hauschka (1990), Buchanan and Preece (1992), Centrella *et al.* (1992), and Korhonen and Väänänen (1992).

Osteoblasts are generally cuboidal or slightly elongated cells that line a large percentage (depending on age) of bone surfaces and are primarily responsible for the production of the organic matrix of bone (Figs. 1-4, 11). The organic matrix produced by osteoblasts consists predominantly of type I collagen (reviewed by Miller, 1985; van der Rest, 1991) and various other non-collagenous bone proteins (reviewed by Boskey,

1989; Gehron Robey, 1989; Simmons and Grynpas, 1990; Rodan and Noda, 1991; Sodek *et al.*, 1991; Gorski, 1992; Young *et al.*, 1992). Osteoblasts are of mesenchymal origin, and when fully differentiated and actively secreting bone matrix, are 'plump-' looking, post-mitotic cells containing a cytoplasm rich in synthetic and secretory organelles such as rough endoplasmic reticulum), Golgi apparatus, and secretory granules (Figs. 3, 4). Osteoblasts also contain a variety of other organelles normally associated with cell metabolism such as mitochondria, microfilaments, microtubules and endosomal/lysosomal elements. Regarding the production of collagen by these and other cells (reviewed by Marchi, 1988), the stages of secretory granule formation and maturation have been well-documented for the major collagen-producing cells - namely, osteoblasts (Weinstock, 1975), fibroblasts (Trelstad and Hayashi, 1979; Cho and Garant, 1981; Marchi and Leblond, 1983), and odontoblasts (Weinstock and Leblond, 1974; Garant and Cho, 1985). A particularly intriguing question that remains unanswered, however, concerns the 'packaging' site and exocytotic route taken by the non-collagenous proteins known to be secreted by osteoblasts. Indeed, it is still not clear whether these proteins are packaged into the same secretory granules as collagen and secreted in tandem via the same pathway, or whether a separate population of secretory granules and a separate exocytotic pathway exist specifically for these proteins. Here, it should be emphasized that the often misquoted early radioautographic studies by Weinstock and colleagues (Weinstock *et al.*, 1972; Weinstock and Leblond, 1973; Weinstock, 1979) on bone and teeth demonstrated phosphoprotein and/or glycoprotein **deposition** into pre-bone (osteoid) and pre-dentin, followed by its subsequent **accumulation** at the mineralization front in these tissues. Thus, the release of non-collagenous proteins, like collagen, appears to occur primarily at the base of osteoblast (and odontoblast) cell processes and from the cell body itself. These proteins then migrate (diffuse) through the osteoid to accumulate at the mineralization front. Some secretion probably also occurs deeper in the matrix since osteoblast cell processes sometimes are observed to contain secretory granules.

Regarding the secretion of collagen in bone, collagen filaments released by osteoblasts assemble extracellularly into fibrils to form the osteoid, a light microscopic term for the seam of unmineralized bone matrix closest to the osteoblast (Figs. 1, 2), although some small foci of mineralization can be observed within the osteoid layer by electron microscopy (Figs. 3a, 7). Ultrastructurally, certain non-collagenous and plasma proteins co-localize with these early foci, although the vast majority appear to accumulate at the mineralization front and throughout the mineralized bone matrix (McKee *et*
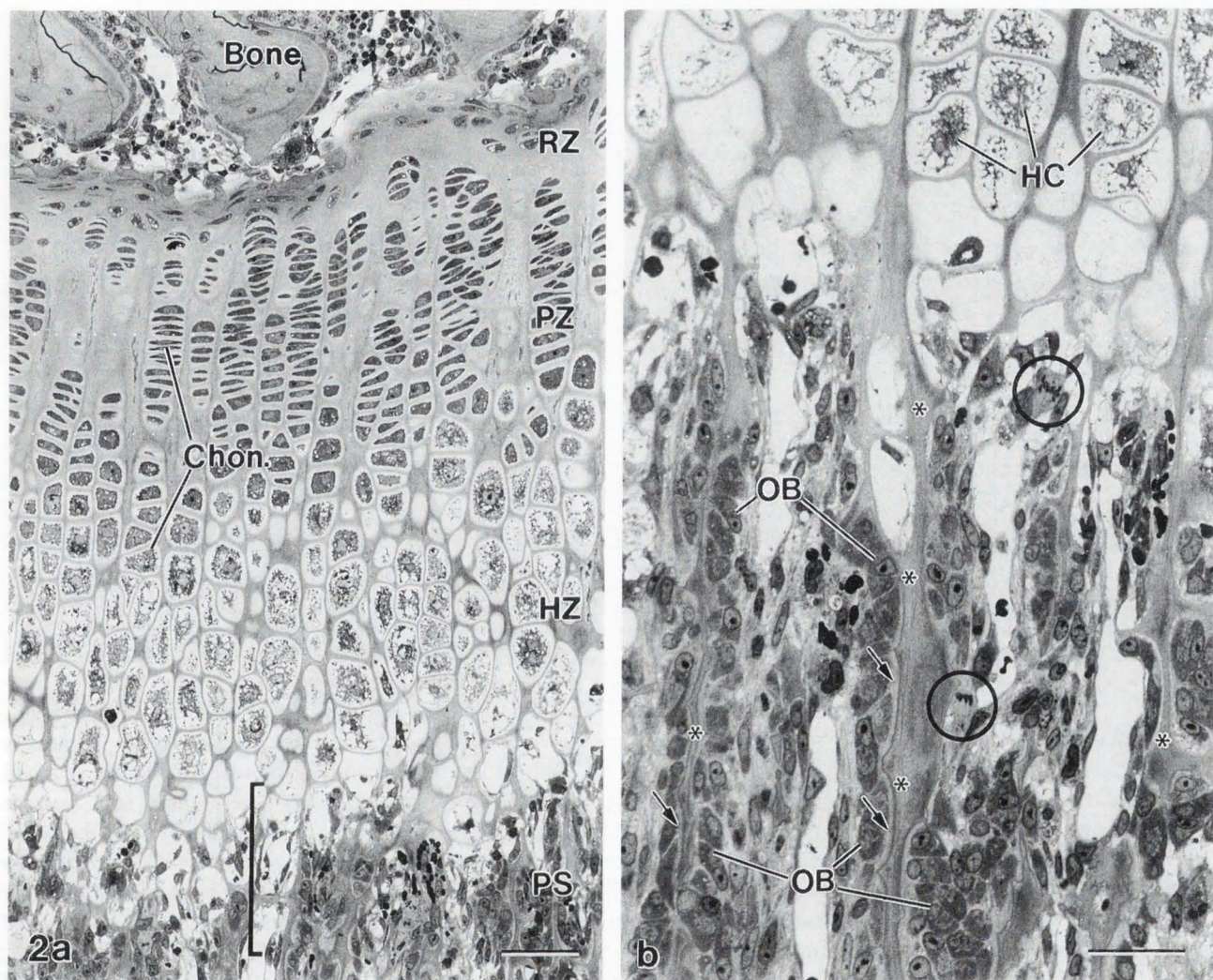
**Figure 2.** Endochondral ossification in the rat tibia. (a) During lengthening of a long bone, bone matrix is first deposited onto a scaffolding of calcified cartilage created by a series of cellular and extracellular matrix events involving the cartilaginous growth plate. The growth plate can be broadly divided into a resting zone (RZ), proliferation zone (PZ) and hypertrophic zone (HZ) within which chondrocytes (Chon) proliferate, differentiate and hypertrophy and regulate the extracellular calcification of the surrounding cartilage. The growth plate is then invaded by vascular elements (region indicated by bracket) to form the primary spongiosa (PS). (b) In the primary spongiosa, numerous osteoblasts (OB) line extensions of calcified cartilage (asterisks) and deposit a thin layer of bone (arrows) onto the cartilage to formed the so-called 'mixed spicules.' Throughout this region, occasional mitotic figures (circles) can be observed. HC, hypertrophic chondrocytes. Epon sections of decalcified tibiae stained with toluidine blue. Bars = 100 μm (a) and 50 μm (b).

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al., 1990, 1992, 1993; Romanowski *et al.*, 1990; Hultenby *et al.*, 1991; Sodek *et al.*, 1992; Bianco *et al.*, 1993; Chen *et al.*, 1993). Morphological indications for synthesis, post-translational modification and packaging of proteins for secretion by osteoblasts can be readily appreciated by examining the prominence and extent of rough endoplasmic reticulum and the Golgi apparatus by routine TEM (Fig. 3b). Likewise, the Golgi apparatus

is clearly delineated following cytochemical incubation with orotidine 5'-monophosphate (OMP), for example, a substrate hydrolyzed by an intracellular, acid pH-dependent phosphatase(s) (Nanci *et al.*, 1990) to give reaction product specifically localized to lysosomes, multivesicular bodies and the trans aspect of the Golgi apparatus (Fig. 4).
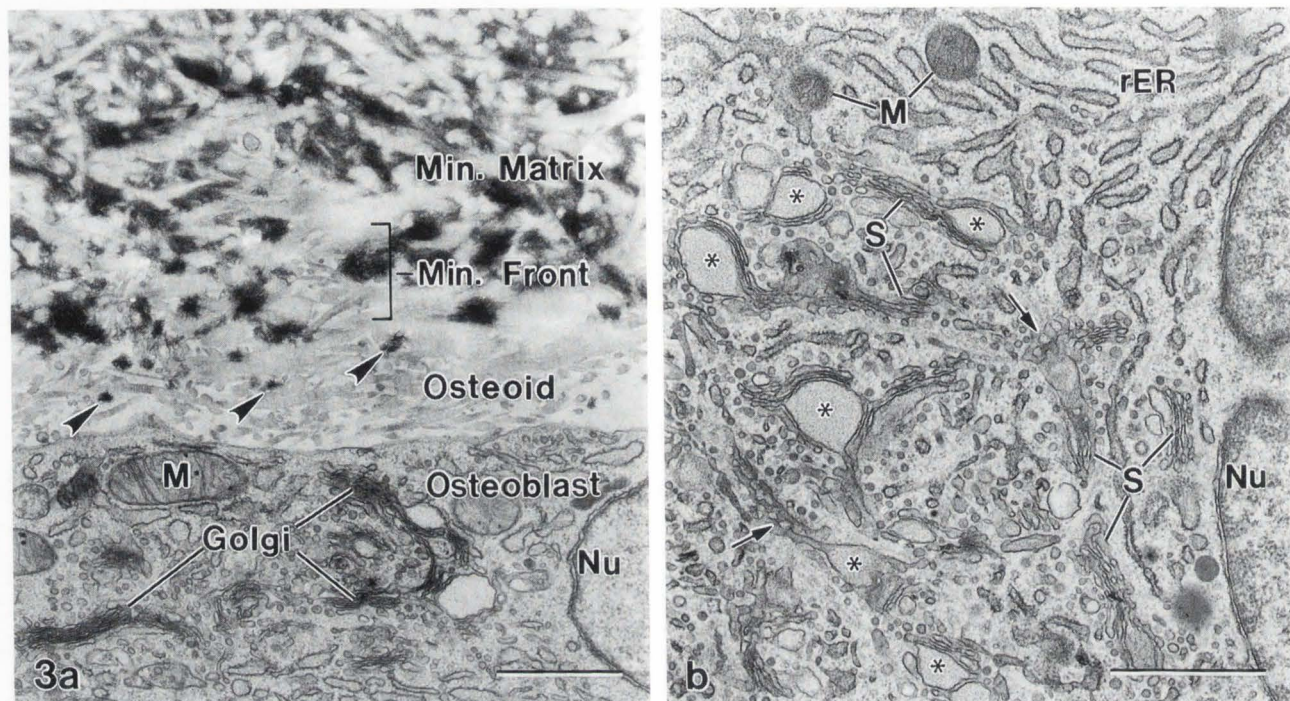
**Figure 3.** (a) Electron micrograph from the mid-diaphysis of a chicken tibia illustrating an osteoblast and the major extracellular compartments related to bone formation. Here, an osteoblast containing an extensive Golgi apparatus is directly apposed to a collagenous extracellular matrix that can be broadly divided into (i) the osteoid containing small foci of mineralization (arrowheads), (ii) the mineralization front (Min. Front, bracket) where there is a massive deposition of apatitic mineral, and (iii) the mineralized matrix proper (Min. Matrix) that forms the bulk of the bone. (b) Higher magnification of the Golgi region of a rat tibial osteoblast showing the extensive development of intracellular organelles involved in protein synthesis and secretion. Stacks of saccules (S) comprising the Golgi apparatus often show spherical (asterisks) and cylindrical distensions characteristic of collagen-producing cells, and are usually surrounded by rough endoplasmic reticulum (rER). Arrows indicate the fenestrated nature of some of the Golgi saccules. M, mitochondria; Nu, nucleus. Epon sections of calcified (a) and decalcified (b) tibiae stained with uranyl acetate and lead citrate. Bars = 1 $\mu$m.

**Figure 4 (on the facing page).** Cytochemical preparations of rat alveolar bone incubated with orotidine 5'-monophosphate (OMP) and showing intracellular, acid pH-dependent phosphatase activity in osteoblasts. (a) Backscattered electron image of a 1 $\mu$m-thick Epon section showing cerium-captured reaction product in the Golgi region of osteoblasts (OB) lining the alveolar bone. (b) Transmission electron micrograph of an osteoblast with acid pH-dependent phosphatase activity in various elements of the Golgi apparatus (G), multivesicular bodies (MVB) and lysosomes (Lys, inset). BV, blood vessel; rER, rough endoplasmic reticulum; M, mitochondria. Epon sections of decalcified alveolar bone stained with uranyl acetate and lead citrate (a) and lead citrate alone (b). Bars = 10 $\mu$m (a), 1 $\mu$m (b), and 0.5 $\mu$m (inset, b).

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Osteocytes occupy spaces (lacunae) in bone and are defined as cells surrounded by bone matrix (see recent reviews by Bonucci, 1990; Marotti et al., 1990), whether mineralized or still part of the osteoid seam (Fig. 5a). Although a sub-classification of osteocyte development has been proposed based on their progressive encasement first into the osteoid, and then into mineralized matrix (Jande and Bélanger, 1973; Yeager et al., 1975; Bonucci, 1990), the formation of osteocytes should be viewed as a continuum involving a change in the surrounding extracellular environment with accompanying cellular metabolic changes. Osteocytes have a decreased quantity of synthetic and secretory organelles, and indeed are smaller cells than osteoblasts (Marotti, 1977), with the nucleus occupying a significantly larger proportion of the cell (Fig. 5a). Although diminished in size, it should be recognized that these cells have the full complement of organelles capable of effecting protein secretion (Baylink and Wergedal, 1971; Tonna, 1973), a feature perhaps reflected by the variable appearance of
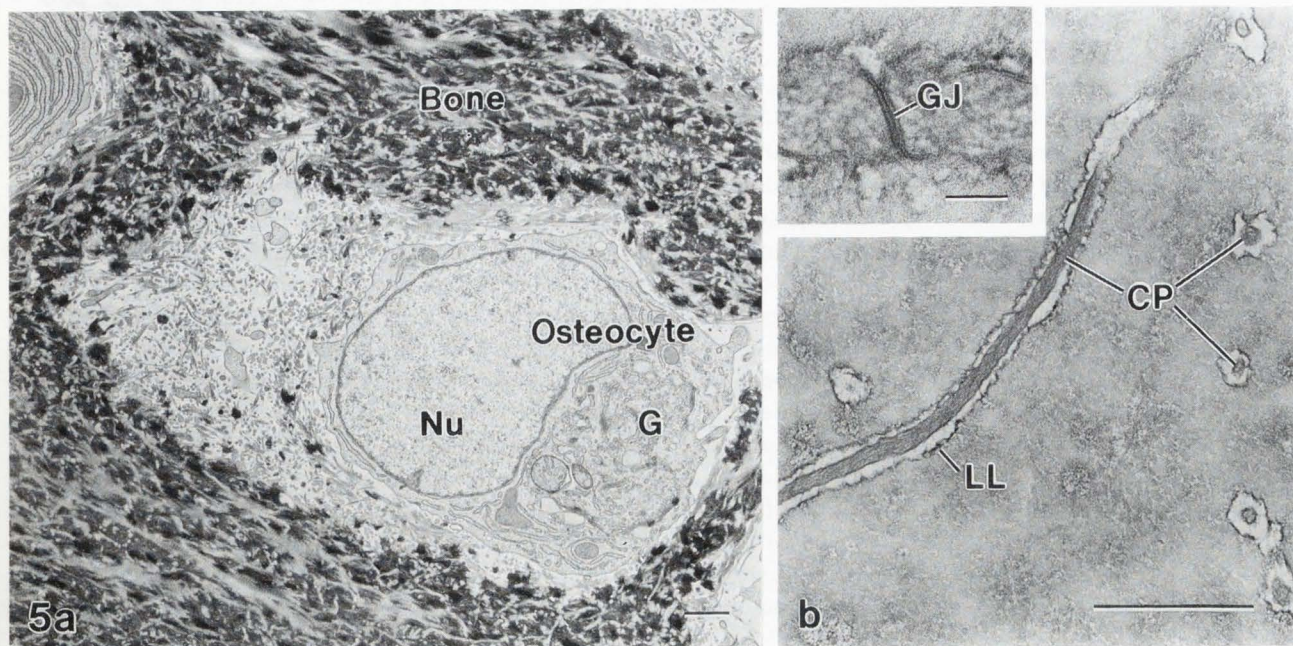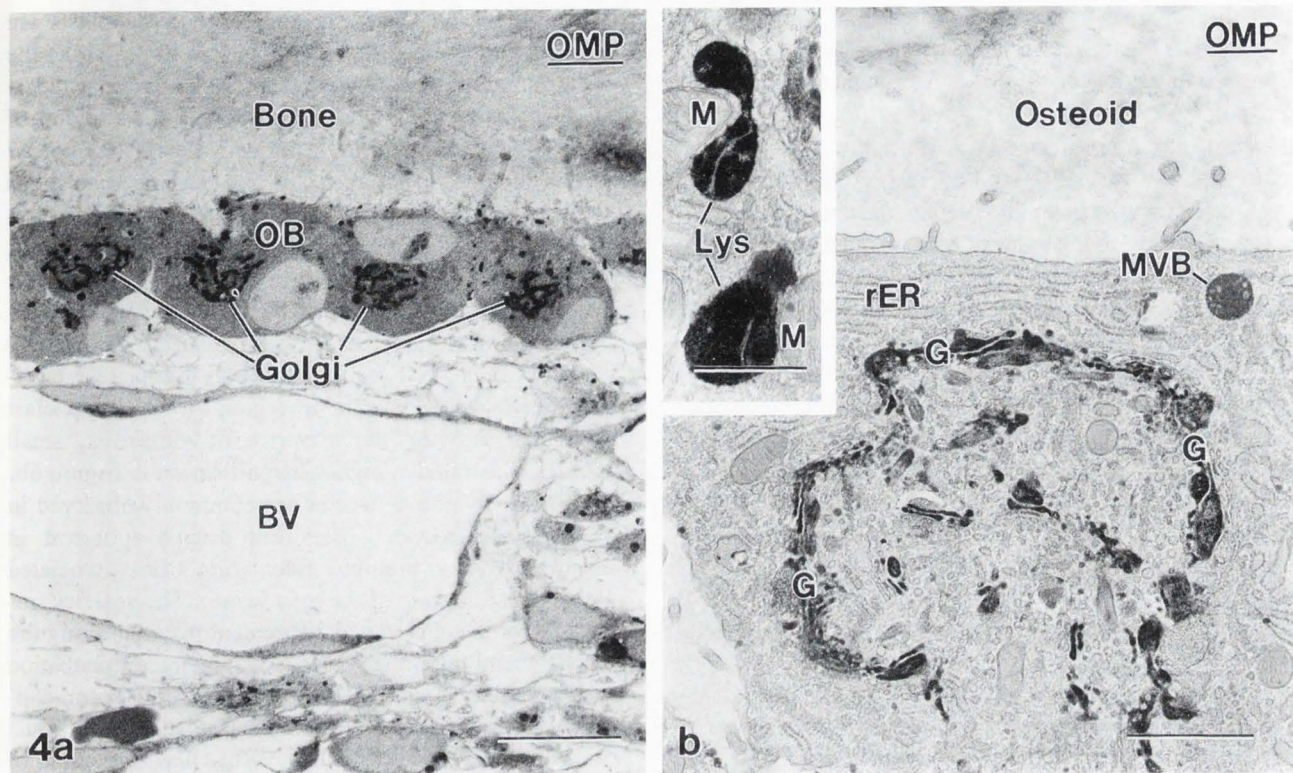
**Figure 5.** (a) Osteocytes encased within bone matrix are found throughout bone and typically show less cytoplasm and organelles than their predecessors, the osteoblasts. (b) Numerous cell processes (CP) deriving from osteoblasts, osteocytes and bone-lining cells course throughout the bone matrix, make contact with each other frequently through gap junctions (GJ, inset) and are contained within canaliculae usually lined by an electron dense lamina limitans (LL). Nu, nucleus; G, Golgi apparatus. Epon sections of calcified chicken tibial bone (a) and decalcified rat alveolar bone (b) stained with uranyl acetate and lead citrate. Bars = 1 $\mu$m (a,b) and 0.1 $\mu$m (inset, b).

the perilacunar matrix frequently observed surrounding these cells (Jande, 1971; Jande and Bélanger, 1971; discussed by Bonucci, 1990). Although controversial (Boyde, 1972; Parfitt, 1977; Boyde et al., 1982; Jones et al., 1985; Marotti et al., 1990), it is believed by some that the formative and/or resorptive activity of these cells may vary under certain metabolic requirements, resulting in the concept of 'osteocytic osteolysis' (Baylink and Bernstein, 1967; Baud, 1968; Bélanger, 1969, 1971; Tonna, 1972). Finally, a major feature of osteocytes is the presence of numerous and extensive cell processes that ramify throughout the bone and make contact, frequently via gap junctions, with processes from other osteocytes or with similar processes extending from osteoblasts or bone-lining cells at the surface of the bone (Fig. 5b; and Doty, 1981; Palumbo, 1986; Palumbo et al., 1990). Thus, considerable potential exists for communication among these cells (Doty, 1988), a factor of great importance considering the spatial isolation of osteocytes within a dense, rigid mineralized matrix.

Bone-lining cells cover most, but not all (Chole and Tinling, 1993; McKee and Nanci, unpublished data), bone surfaces in the adult skeleton (Luk et al., 1974a, b; VanderWeil et al., 1978; Miller et al., 1980; Miller and Jee, 1987, 1992). The transition between osteoblasts and bone-lining cells clearly involves a series of gradual morphological and functional changes (Miller et al., 1980; Menton et al., 1984) that culminate in decreased protein secretion. The relative paucity of organelles in these cells indicate that they are less involved, if at all, in classical protein secretion of bone matrix, although not precluding the ability of these cells to produce local regulatory substances and/or modify the composition of the underlying lamina limitans. Transformation into bone-lining cells may represent the final phenotype of the osteoblast lineage prior to activation of the bone remodeling sequence at sites occupied by these cells (reviewed by Parfitt, 1990). The ultimate fate of bone-lining cells is presumably death by apoptosis (Wyllie et al., 1980; Kardos and Hubbard, 1982; Schwartzman and Cidlowski, 1993). In adult birds, however, bone-lining cells appear to retain osteogenic potential, proliferating in response to estrogen and contributing to the formation of woven medullary bone (Bowman and Miller, 1986). However, there is little direct evidence that bone-lining cells have the ability to regain a synthetic and secretory capacity to produce lamellar bone matrix in mammals. Finally, the large percentage of bone covered by a contiguous layer of bone-lining cells, and their extended, flattened morphology at bone surfaces, potentially allows for an important homeostatic role in compartmentalizing the bone matrix and influencing calcium and phosphate metabolism, substance exchange and/or the initiation of

**Figure 6 (on the facing page).** (a) Osteoclasts are large, multinucleated bone cells capable of resorbing the extracellular matrix of hard tissues such as bone and cartilage (Cart). This process is mediated via a region of plasma membrane infolding termed the ruffled border (RB) which is surrounded by cytoplasmic extensions termed the clear (sealing) zone (CZ). Osteoclasts typically possess many mitochondria (M), a limited amount of rough endoplasmic reticulum (rER) and a perinuclear system of Golgi apparatuses (G) with fenestrated saccules (asterisks). Nuclear pores (arrowheads) are clearly visible in tangential sections through the nuclear envelope. Brackets indicate a region of the osteoclast cytoplasm showing the presence of numerous, small vesicles illustrated at higher magnification in Figure 6b. (b) Small, indented vesicles are commonly observed in osteoclasts (arrows). They are usually spherical in shape and show a single indentation often associated with a dense coating of the membrane. The vesicles frequently contain a clumped, flocculent material along the vesicle membrane. (c) After cytochemical incubation with orotidine 5'-monophosphate (OMP), cerium-captured, acid pH-dependent phosphatase reaction product can be observed within various vesicular structures of the osteoclast. Epon sections of decalcified rat tibial (a,b) and alveolar (c) bone stained with uranyl acetate and lead citrate (a,b) and lead citrate alone (c). Bars = 1 $\mu$m (a,c) and 0.5 $\mu$m (b).

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events leading to activation of the bone remodeling sequence (Matthews et al., 1978; Miller and Jee, 1987, 1992; Parfitt, 1990). Together with osteocytes, bone-lining cells and their connecting cell processes (Fig. 5b) appear to form an extensive homeostatic network of cells capable of regulating plasma calcium concentration through mechanisms partly independent of those related to the bone remodeling system (Canas et al., 1969; Talmage, 1970; Parfitt, 1976, 1987, 1990; Matthews and Talmage, 1981). Indeed, quiescent surfaces are known to be the primary site of mineral ion exchange between blood and adult bone (Parfitt, 1981, 1989).

Of central importance in the ability of bone to respond to biological regulatory factors and the external forces of daily use is the capacity of the large, multinucleated osteoclasts to resorb bone. Indeed, the coupling of bone resorption with bone formation constitutes one of the fundamental principles by which bone is necessarily remodeled throughout life (Harris and Heaney, 1969; Howard et al., 1981; Baylink et al., 1982; reviewed by Puzas and Ishibe, 1992). Apart from its multinucleation, the most striking feature of the osteoclast is the presence of an actin-, vinculin- and talin-containing clear (sealing) zone (Lakkakorpi et al., 1989; reviewed by Teti and Zambonin-Zallone, 1992; Aubin, 1992) in the
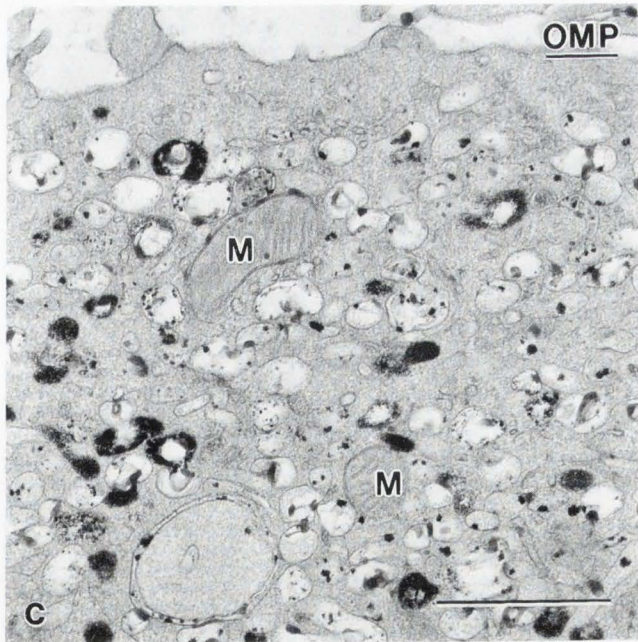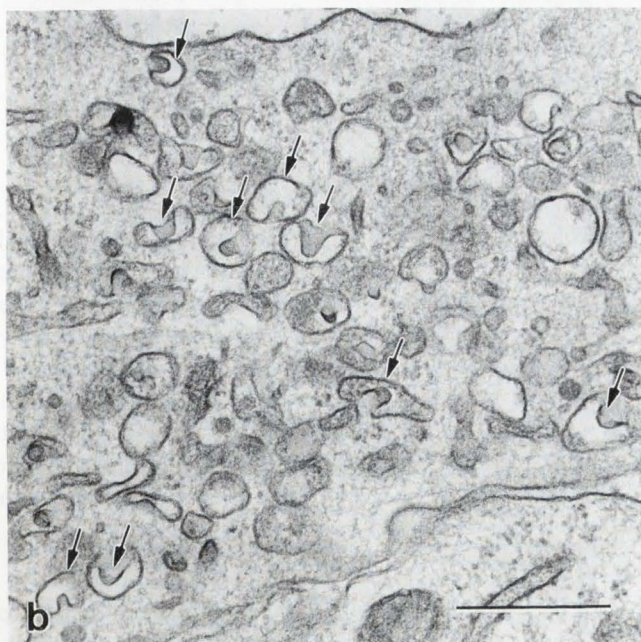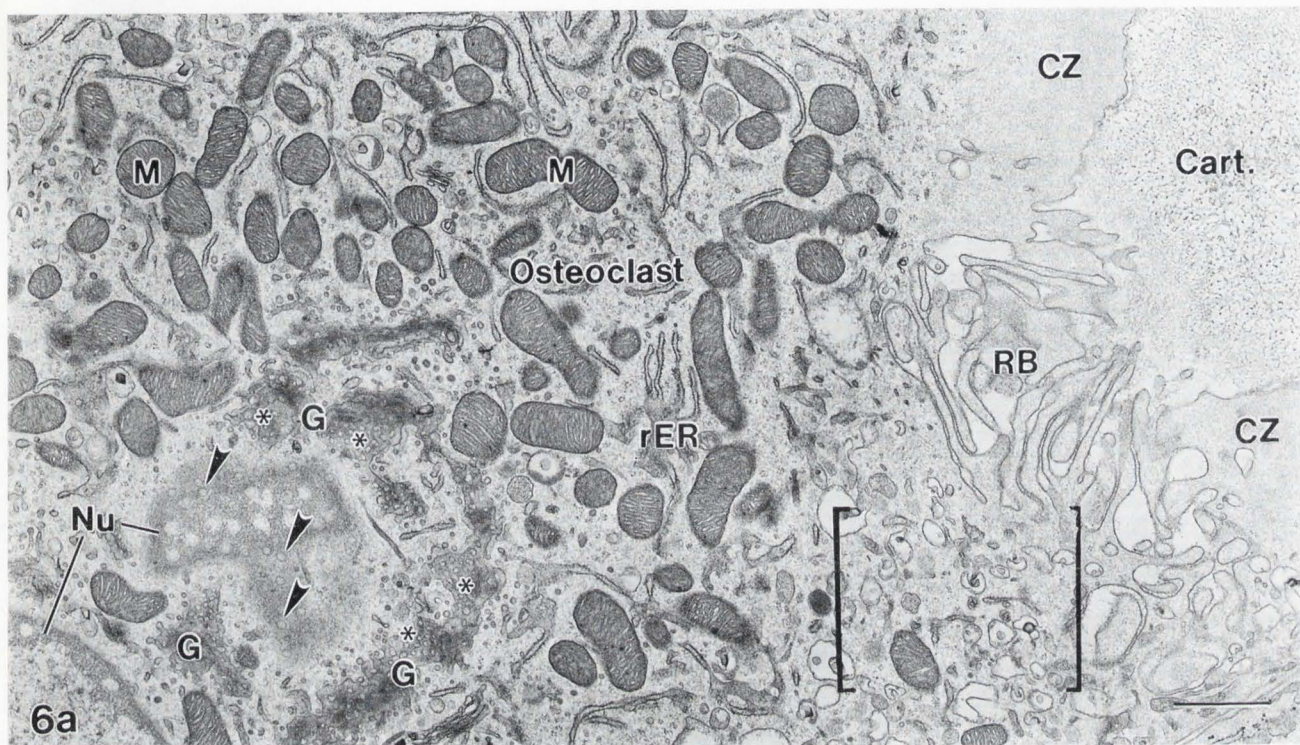
peripheral cytoplasm of this cell which delineates a more central region of membrane infoldings (plates) and finger-like processes termed the ruffled border (Fig. 6a; and Domon and Wakita, 1989; reviewed by Holtrop, 1991). Resorption of bone occurs in an acidified extracellular matrix compartment as a result of the combined actions of a variety of ruffled border membrane-associated enzymes including a tartrate-resistant, vanadate-sen- sitive acid ATPase (Andersson et al., 1984, 1986), carbonic anhydrase isozyme II (Gay et al., 1983; Väänänen and Parvinen, 1983; Marie and Hott, 1987) and proton-pumping ATPases (Baron et al., 1985; Akisaka and Gay, 1986; Väänänen et al., 1990; reviewed by Baron, 1989). Although numerous states of osteoclast differentiation can be observed and are reflected by different osteoclast morphologies (Fukushima et al., 1991), it is
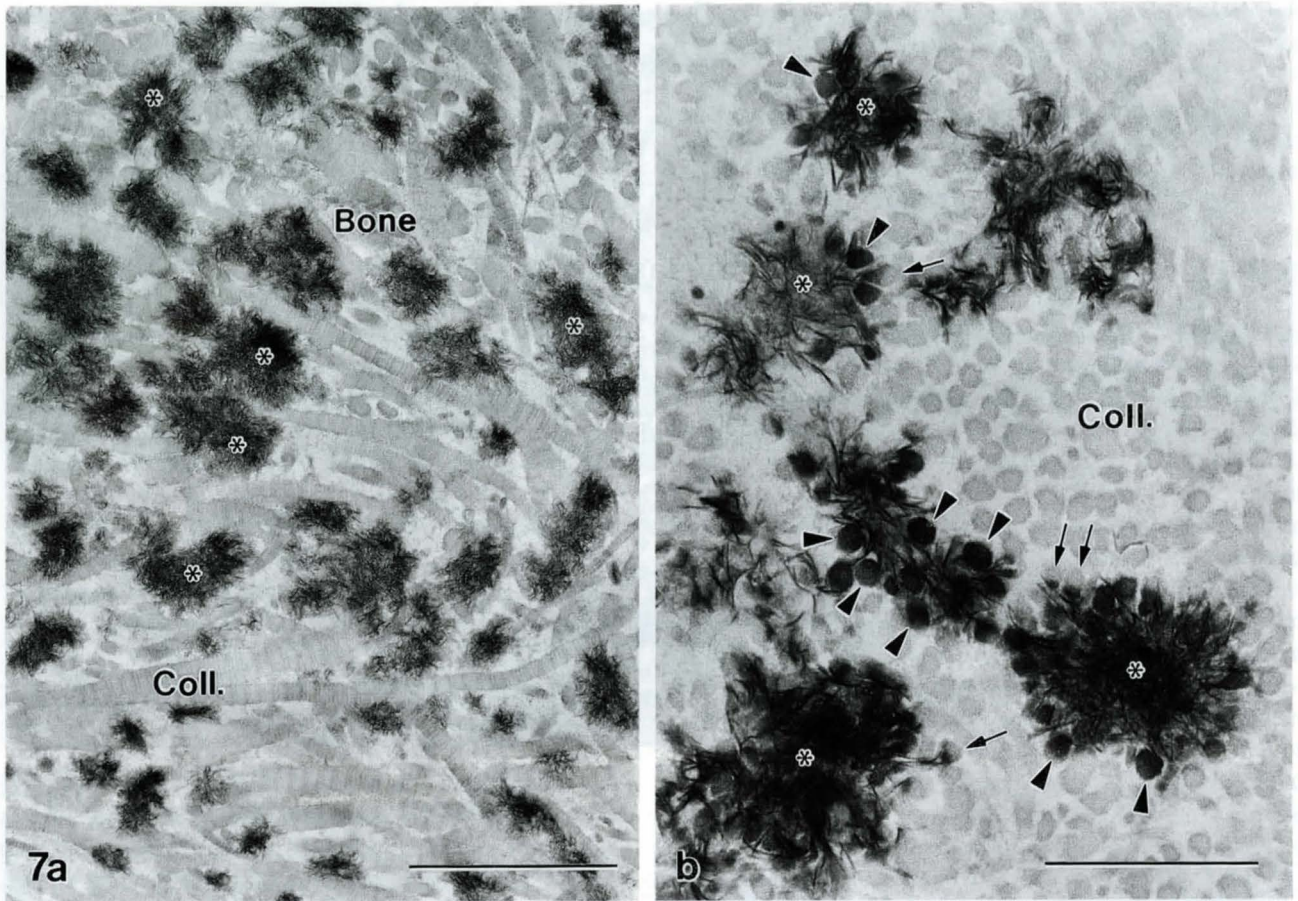
**Figure 7.** (a) Tibial bone near the mineralization front showing impregnation of the collagenous matrix with the solid, inorganic mineral phase of bone. Here, the striated collagen (Coll.) fibrils are cut predominantly longitudinally and show a close spatial association with the mineral phase (asterisks). (b) Where collagen fibrils of bone matrix are cut mainly in transverse section, foci of mineralization (asterisks) appear as radially-oriented clusters of relatively large crystals frequently surrounded by electron-dense circular profiles of cross-cut collagen fibrils (arrowheads). The density of these structures presumably results either from an intrafibrillar mineral phase or from a high concentration of mineral ions at these sites. The electron-dense, circular profiles of cross-cut collagen are usually found in close association with the larger crystal aggregates of the foci. Occasionally, images are observed where only a portion of the fibril closest to the foci is electron-dense (arrows). These types of images suggest a spatial and temporal propagation of interfibrillar mineral into the collagen fibrils. Epon sections of calcified chicken tibial (a) and rat alveolar (b) bone stained with uranyl acetate and lead citrate. Bars = 1 $\mu$m (a) and 0.5 $\mu$m (b).

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thought that the presence of small and large cathepsin-containing (Goto *et al.*, 1992; Sasaki and Ueno-Matsuda, 1992; Page *et al.*, 1993) cytoplasmic vacuoles/vesicles (Lucht, 1972; reviewed by Holtrop, 1991) often in the immediate vicinity of the ruffled border are indicative of resorptive activity by these cells (Fig. 6; and reviewed by Marks and Popoff, 1988; Gay, 1992). Among these membrane-bound structures, there exists a population of small, spherical vesicles having a single indentation of the membrane at one site, a region that appears to be associated with an electron-dense coating (Fig. 6b). A definitive role for these vesicles has not

yet been established, although some of them appear to contain lysosomal (phosphatases; e.g., using orotidine 5'-monophosphate as substrate, Fig. 6c) and plasma membrane (Fukushima *et al.*, 1991) enzymes, and thus may participate in certain degradative activities performed by the osteoclast (Doty and Schofield, 1972) and/or plasma membrane recycling (Fukushima *et al.*, 1991).

**Bone matrix structure and composition**

Bone consists of an organic matrix which houses the solid, inorganic calcium-phosphate mineral phase of

bone. A detailed discussion of matrix-mineral relationships in calcified tissues has recently been given by Christoffersen and Landis (1991), Traub *et al.* (1992) and Landis *et al.* (1993) and will not be dealt with here. The present article will focus on describing the distribution of specific proteins in bone which, apart from collagen, constitute a significant and potentially important arsenal for directing events related to bone formation and remodeling.

The extracellular matrix of bone can be arbitrarily and broadly divided into several spatially-distinct compartments that conceptually allow for a useful description of the sequence of events comprising the synthesis, secretion, accumulation and mineralization of bone matrix. Following synthesis and release of organic molecules by the cellular compartment (osteoblasts), a collagenous stroma, called the osteoid, is formed which ultimately acts as a scaffolding for apatite mineral deposition and the accumulation of non-collagenous and plasma proteins (Fig. 3a). These latter events occur predominantly at what is known as the mineralization front, a site where mineralization propagates extensively throughout and between the collagen fibrils. Although the first mineral to appear may be found at small, discrete foci within the osteoid seam (Fig. 3a), the precise nucleation sites of this, and the subsequent deposition of more confluent mineral at the mineralization front (Fig. 7) of the mineralized bone matrix proper (the mineralized bone compartment), remain controversial (discussed by Christoffersen and Landis, 1991).

With regard to the precise location and distribution of non-collagenous proteins in bone, it has long been known that, at the light microscopic level in decalcified sections of this tissue, certain general protein stains (e.g., toluidine blue) show a particularly strong affinity for bone matrix commencing at the mineralization front and extending throughout the mineralized bone matrix compartment. This contrasts with a relatively light staining of the adjacent osteoid. Likewise, using heavy metal stains such as uranyl acetate in conjunction with TEM, there is an increased electron density associated with originally mineralized portions of the matrix in decalcified sections (discussed by McKee *et al.*, 1990). [A similar phenomenon can be observed as predentin is converted to dentin in the developing tooth (Weinstock and Leblond, 1973)]. This is currently interpreted as representing the accumulation of various non-collagenous proteins at these mineralized sites (see below).

Although not fibrillar in nature like collagen, and therefore, less easily discernible as distinct entities within the tissue section, the structure of these generally globular, non-collagenous proteins, and the nature of their accumulation to impart density to the tissue section, is apparent at higher magnifications. What is less clear,

however, is the exact relationship of these organic moieties to the collagen fibrils themselves, or to the mineral within and between these fibrils. This problem is further complicated by the fact that the aqueous procedures generally used during tissue processing of bone for electron microscopy solubilize mineral, and depending on the nature of a particular matrix-mineral relationship, may lead to protein translocation and other potential artifacts. In this regard, various cryotechniques such as cryofixation and particularly freeze-substitution hold great potential for future studies on matrix-mineral relationships in bone and other mineralized tissues. Regardless of the conditions necessary for mineral to nucleate between, at the surface of, or within collagen fibrils, the definitive and massive accumulation of bone proteins at the mineralization front strongly implicates them in the mineralization process. Their appearance, as observed by TEM of calcified sections (Figs. 3a, 5a, 10b), is as accumulations of amorphous or finely granular material located apparently between the fibrils of collagen, and, depending on the three-dimensional packing of the fibrils at a particular site, may form a variety of polymorphic outlines called the 'grey patches' (McKee *et al.*, 1990). After decalcification in acids or in EDTA at neutral pH prior to embedding, these 'patches' appear somewhat reticulated and filamentous (Fig. 11b), indicating a disturbance in the combined architecture of these proteins following extraction of the mineral at these sites.

Having visually described by electron microscopy the non-fibrillar organic component and its distribution in bone matrix, the natural question arises as to the identity of this material. A detailed ultrastructural and histochemical examination of this interfibrillar organic material has been performed by several investigators; in particular, Groot (1982a, b) and Takagi *et al.* (1983) performed a careful analysis of "mineralization nodules" or "focal spherical profiles" both in the osteoid and in mineralized bone following different fixation, histochemical staining and decalcification protocols. It was concluded by the authors that these sites are rich in acid groups and sulfated glycoconjugates. These data, for the most part, are considered as demonstrating the presence and focal concentration of sulphated glycosaminoglycans (proteoglycans) in bone. Based on HID-TCH-SP staining of organic 'patches' in bone for sulfated glycoconjugates (Fig. 8), and on the immunocytochemical co-localization of individual acidic, sulfated glycoproteins at these same sites (see below), it is our contention that sulfated glycoproteins, such as osteopontin and bone sialoprotein, for example, contribute significantly to the aforementioned cytochemical data. Additional recent studies (Sauren *et al.*, 1991, 1992) have used cuprolinic blue and polyethyleneimine to visualize proteoglycans throughout mineralized bone and in other more specialized bone matrix
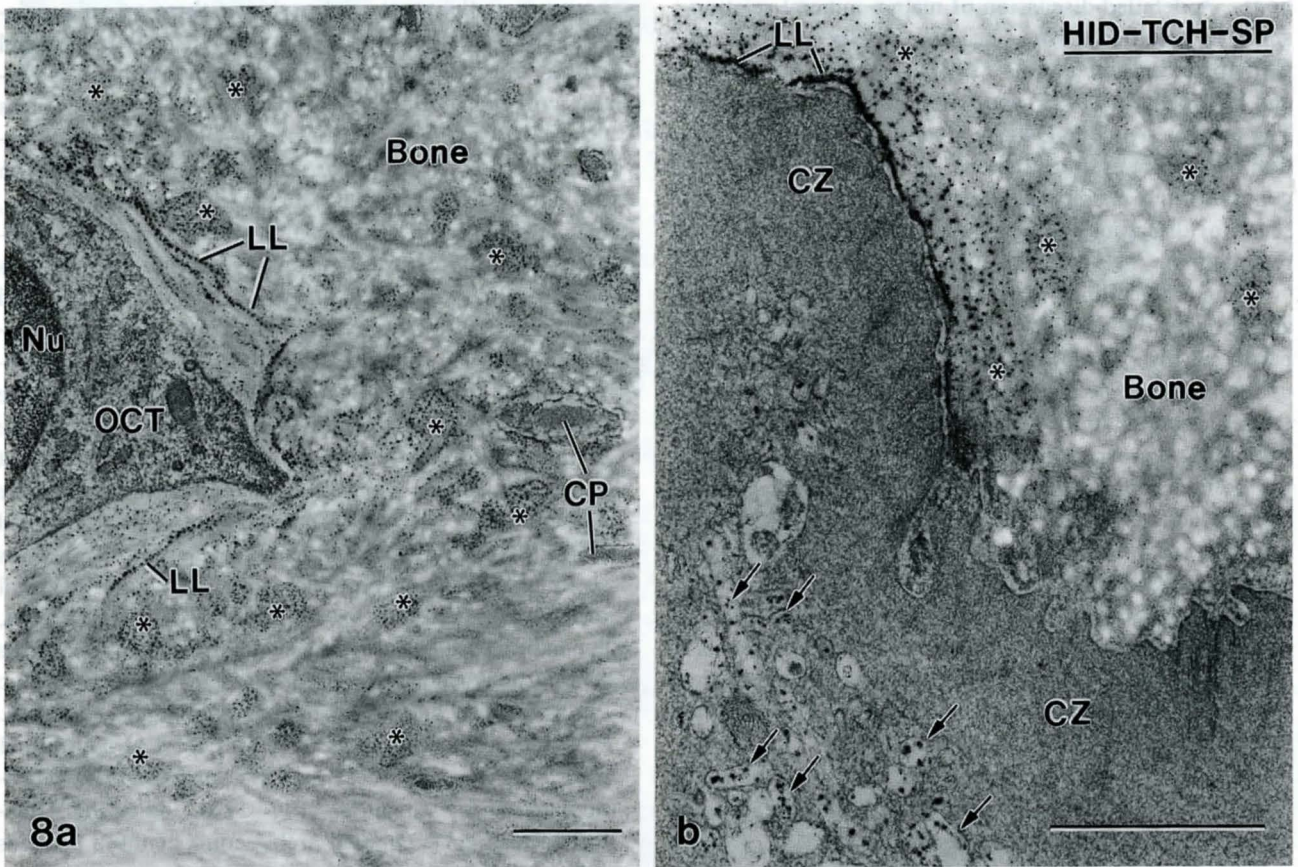
**Figure 8.** High-iron diamine thiocarbohydrazide-silver proteinate (HID-TCH-SP) cytochemical staining for sulfated glycoconjugates in bone. (**a, b**) Reaction product is observed predominantly over 'patches' of organic matrix in bone (asterisks) and over laminae limitantes (LL) delineating osteocyte (OCT) lacunae, cell process canaliculae and bone surfaces. In Figure 8b, an osteoclast clear zone (CZ) is directly apposed to a sulfated glycoconjugate-containing lamina limitans at the bone surface. Small vesicles (arrows) in the osteoclast cytoplasm are also stained by this technique. Nu, nucleus. Epon sections of decalcified rat alveolar bone without uranyl acetate or lead citrate staining. Bars = 1 μm.

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compartments such as the perilacunar matrix of osteocytes and the laminae limitantes associated with osteocytes and cell processes.

As a further refinement to these initial characterizations, the availability of a variety of lectins has allowed ultrastructural demonstration of the presence and concentration of specific carbohydrate moieties within bone (reviewed by Benhamou, 1989). Cytochemical application of colloidal-gold conjugates of these lectins to thin sections of bone indicates that numerous sites within bone cells and the bone matrix label positively for a number of specific sugar residues. Figure 9a shows such an example, where a concentration of sialic acid residues (as detected by *Limax flavus* agglutinin followed by fetuin-gold complex) can be observed at sites of bone matrix previously shown to be rich in sulfated glycosaminoglycans and now known to contain an abundance of noncollagenous and plasma proteins (see below). Other

plasma membrane (Fig. 9b), intracellular (Fig. 9c) and matrix reactions can be observed using lectins specific for galactose (*Ricinus communis I* agglutinin) and N-acetyl-D-galactosamine (*Helix pomatia* agglutinin). Such labeling patterns and specificities again raise the issue as to whether proteoglycans are the principal component responsible for the data generated by earlier histochemical work describing acidic and sulfate groups at these sites. It is evident that the extensive carbohydrate content of the acidic, bone matrix glycoproteins (reviewed by Gorski, 1992), and other post-translational modifications of these proteins, may contribute significantly to the staining and labeling patterns obtained from these diverse cytochemical techniques.

With recent developments in the biochemical isolation and characterization of individual bone proteins, and with the production of antibodies to these purified molecules, it has now become possible to identify, with high
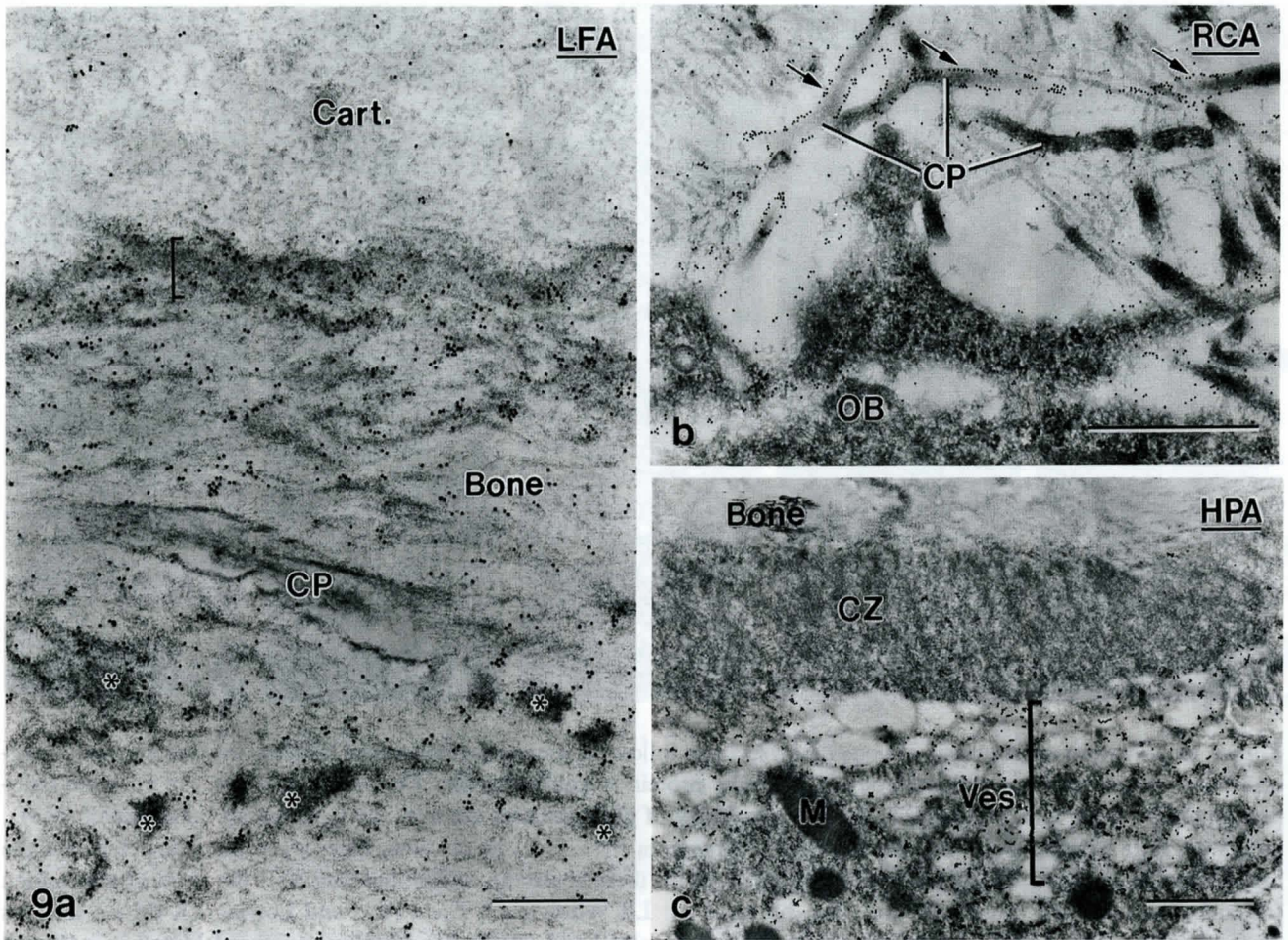
**Figure 9.** Lectin-gold labeling of bone cells and matrix. (a) *Limax flavus* agglutinin (LFA) labeling, specific for *N*-acetyl-neuraminic acid (sialic acid), is primarily concentrated over the bone matrix at the interface between calcified cartilage (Cart) and bone in the growth plate (bracket), and over electron-dense 'grey patches' (asterisks) throughout the bone matrix. (b) *Ricinus communis I* agglutinin (RCA) labeling for galactose residues can often be found over some (arrows), but not all, osteoblast (OB) cell processes (CP). (c) Cytoplasmic vesicles (Ves. and bracket) in osteoclasts label intensely with *Helix pomatia* agglutinin (HPA), thus indicating the presence of *N*-acetyl-D-galactosamine residues in these structures. CZ, clear zone; M, mitochondria. Lowicryl K4M sections of decalcified rat (a,c) and chicken (b) tibial bone stained with uranyl acetate and lead citrate. Bars = 0.5 $\mu$m (a) and 1 $\mu$m (b,c).

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resolution, their localization *in situ*. Thus, several recent immunocytochemical studies have been able to identify the composition of the organic material described above and to describe its ultrastructural association with sites of mineralization throughout the tissue (Camarda *et al.*, 1987; McKee *et al.*, 1990, 1992, 1993; Sodek *et al.*, 1992; Bianco *et al.*, 1993; Chen *et al.*, 1993). What is more difficult to achieve, however, is a determination of the spatio-temporal sequence by which these proteins accumulate and associate with the mineral phase; that is, whether the proteins accumulate prior to, concomitant with, or after mineral nucleation. Nevertheless, the co-localization of certain non-collagenous

proteins with mineral is a necessary prerequisite for determining their participation in the mineralization process. Conversely, proteins not found at these sites can be considered as not having a **direct** effect on mineralization. Thus, electron microscopic immunocytochemical classification of protein distribution in bone can be considered as an initial step in elucidating the regulatory role of these molecules during the mineralization process. These recent ultrastructural co-localization studies have been made possible only through the use of the **post-embedding** colloidal gold methodology, since the density of bone matrix precludes the ability of antibodies to penetrate homogeneously throughout the tissue during
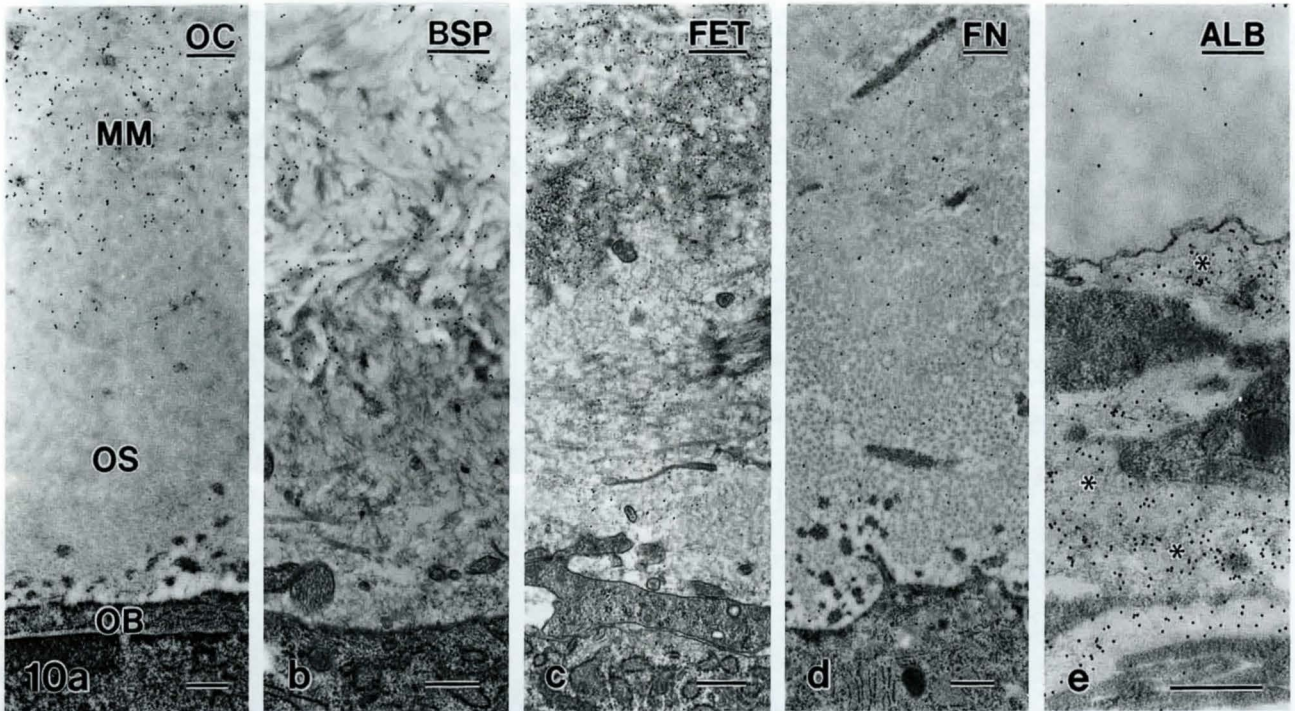
**Figure 10.** Electron micrographs of bone tissue sections following immunocytochemical labeling for various non-collagenous and plasma proteins with antibody followed by protein A-gold complex. For osteocalcin (OC, in **a**), bone sialoprotein (BSP, in **b**), and fetuin (FET, in **c**), the majority of gold particles are over the mineralized bone matrix (MM), with very little labeling over the osteoid (OS). Fibronectin (FN, in **d**) incubations show some gold particles over the osteoid and mineralized matrix. For albumin (ALB, in **e**), there is also an intense labeling over the extracellular milieu (asterisks) surrounding osteoblasts and bone-lining cells. LR White (a,d), Epon (b,c) and Lowicryl K4M (e) sections of decalcified [a (rat costal); c,d (rat tibial); and e (rat alveolar)] and calcified [b (fetal pig calvarial)] bone stained with uranyl acetate and lead citrate following immunocytochemistry. OB, osteoblast; Nu, nucleus; LL, lamina limitans. Bars = 0.5 μm.

**Figure 11 (on the facing page).** Electron micrographs of bone following immunocytochemical labeling of tissue sections with anti-osteopontin (OPN) antibody and the protein A-gold complex. **(a,b)** In the bone matrix, cement lines (CL and inset) and 'patches' of filamentous matrix [asterisks and inset; amorphous and evenly electron-dense ('grey') in calcified sections] among the collagen fibrils are intensely labeled. **(c)** Bone section showing portions of an osteocyte and its cell processes (CP), and the clear zone (CZ) of an osteoclast, apposed to bone surfaces labeled by the anti-osteopontin antibody. Laminae limitantes (LL) frequently surround osteocytes and their cell processes and show numerous gold particles (arrowheads, inset). Both clear and ruffled zones of osteoclasts directly appose osteopontin-containing bone matrix which appears either as a distinct lamina limitans (arrows) or as an area of more diffusely-labeled bone matrix (bracket). Note the heavy labeling of the mineralized bone matrix. LR White and Lowicryl K4M (inset, c) sections of decalcified rat calvarial and alveolar bone stained with uranyl acetate and lead citrate following immunocytochemistry. Bars = 1 μm (a,b,c), and 0.5 μm (insets).

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pre-embedding immunocytochemical incubations. Hereafter, utilizing such ultrastructural approaches, we summarize the matrix localization of some of the major non-collagenous and plasma proteins found in bone.

Using electron microscopy, several investigators have examined the ultrastructural localization of various non-collagenous bone proteins. Osteocalcin, a small, vitamin K-dependent gamma-carboxyglutamic acid-containing protein (reviewed by Hauschka, 1986; Cole and Hanley, 1991), was shown by Groot et al. (1986), Camarda et al. (1987), Boivin et al. (1990), and McKee et al. (1992, 1993) to be primarily associated with mineralized bone matrix. Figure 10a shows osteocalcin immunolabeling over the mineralized bone after application of the protein A-gold immunocytochemical approach. Osteopontin (see below and references therein) and bone
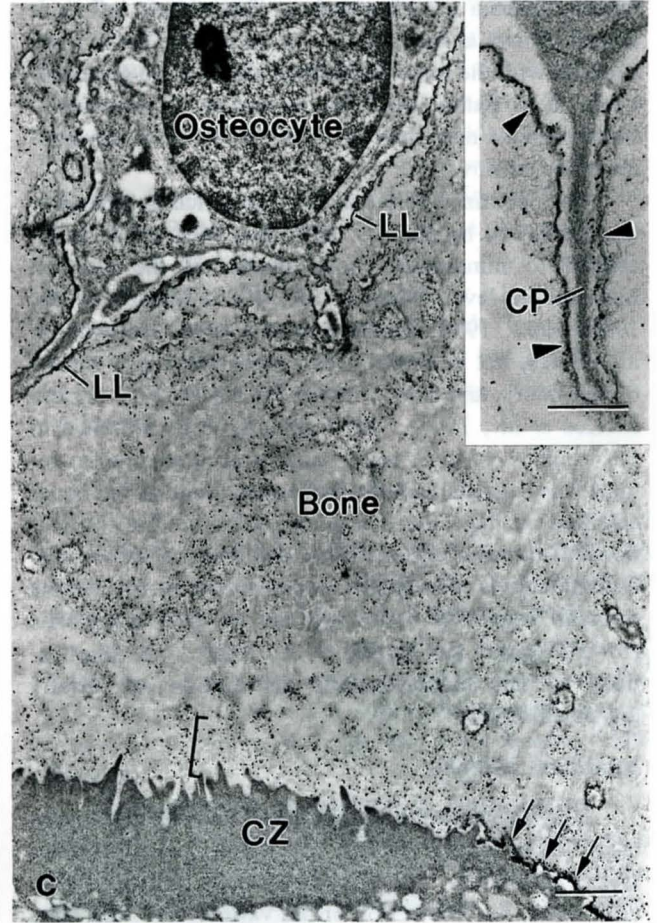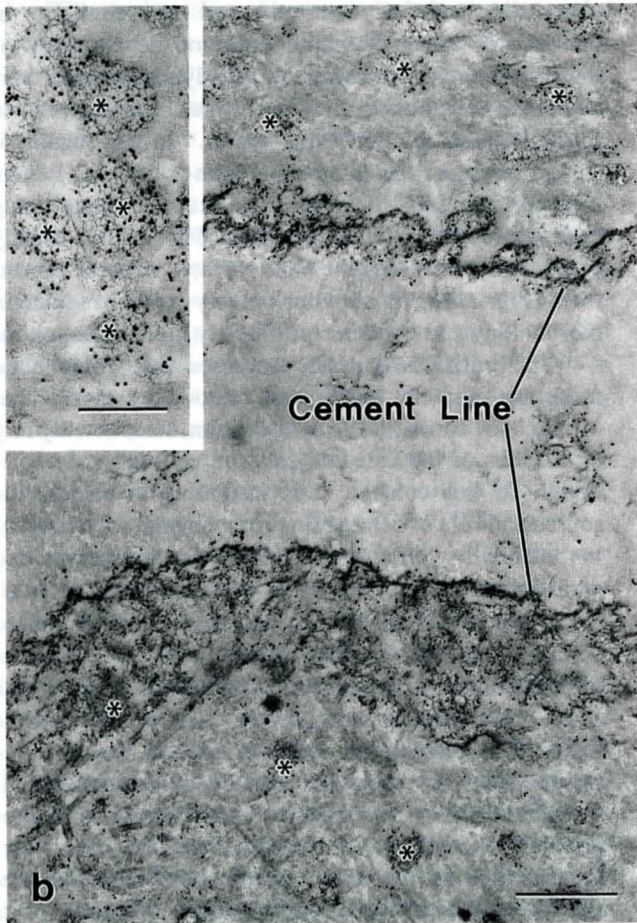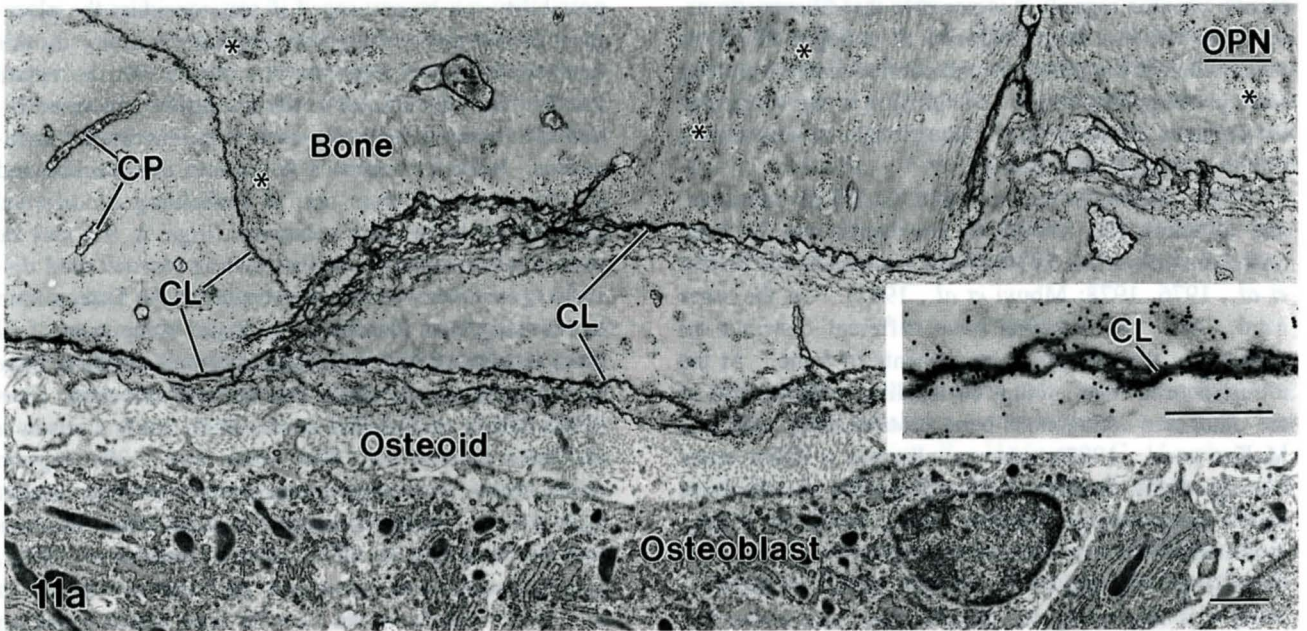
sialoprotein (BSP, Fig. 10b), both sulphated, glyco-sylated phosphoproteins found in bone (reviewed by Butler, 1991; Gorski, 1992; Sodek *et al.*, 1992), also co-localize with mineral in bone (BSP, Sodek *et al.*,

1992; Bianco *et al.*, 1993; Chen *et al.*, 1993). Fibro-nectin (Fig. 10d), on the other hand, appears to be more diffusely dispersed throughout the bone matrix and is not particularly concentrated in any of the bone compart-

ments. In this regard, it is possible that fibronectin associates with the large majority of collagen fibrils in bone, whereas the other aforementioned mineral-binding bone proteins may reside and function at the surface of the collagen fibrils or in the interfibrillar spaces during mineralization at these sites.

Two plasma proteins, fetuin ($\alpha_2$HS-glycoprotein) and albumin, are known to be sequestered in bone (Ashton et al., 1974, 1976; Dickson et al., 1975; Triffitt et al., 1976, 1978; Mbuyi et al., 1982). On the other hand, these two proteins have different distributions throughout the various compartments of bone. At the ultrastructural level, fetuin co-localizes with several of the other non-collagenous bone proteins (Fig. 10c; and McKee et al., 1993), whereas albumin is either more homogenously distributed throughout the various bone matrix compartments (McKee et al., 1993), or is concentrated in the extracellular milieu surrounding osteoblasts (Fig. 10e) and in the osteoid. Based on these results, it is interesting to consider that certain mineral-binding plasma proteins such as fetuin may influence crystal growth and dissolution by competing for mineral surfaces with the mineral-binding, acidic bone glycoproteins. If so, pathological fluctuations in serum levels of these proteins may thus adversely affect bone development and turnover.

Osteopontin, a sulfated, sialic acid-rich phosphoprotein (reviewed by Butler, 1989), has been similarly localized to mineralized bone matrix at the electron microscopic level (Mark et al., 1987; McKee et al., 1990, 1992, 1993; Reinholt et al., 1990; Hultenby et al., 1991), but in addition, is concentrated in a variety of cell-matrix and matrix-matrix interfaces (Fig. 11; and McKee et al., 1993). Most striking is its presence in the 'patches' of bone matrix and its accumulation in cement lines and the laminae limitantes found at the bone/calcified cartilage interface during endochondral ossification and surrounding osteocytes and bone-cell processes. Osteopontin is also highly concentrated in the lamina limitans frequently found adjacent to bone-lining cells (for illustration see McKee et al., 1992). The implications of this highly-specific distribution are briefly discussed below.

## Interfaces in bone

Interfaces in bone are numerous and can be broadly classified as being either cell-matrix interfaces or matrix-matrix interfaces. As such, the former represents predominantly the interaction of the various bone cell lineages with surfaces of bone matrix, and the latter represents the situation where newer bone is deposited onto older bone (or calcified cartilage in the case of endochondral bone growth) to create an interface between two spatially- and temporally-distinct matrices. [A third

possibility can be envisaged if one considers the placement of dental or orthopaedic implants in bone. In this case, the host bone response to create a matrix-implant interface may be similar to the osteogenic processes required to create a normally-occurring, matrix-matrix interface. Indeed, a comparison between this scenario and the deposition of bone onto a scaffolding of calcified cartilage in the primary spongiosa (Figs. 2, 9a) may be particularly relevant and illuminating (for detailed discussion see Nanci et al., in preparation)]. Osteopontin has recently been identified as a major component of all these interfaces, and with other molecules such as bone sialoprotein is particularly concentrated in cement lines (Figs. 11a, b; and McKee et al., 1992, 1993; Carlson et al., 1993; Chen et al., 1993; Frank et al., 1993; Ingram et al., 1993). Although a commonality between cement lines and the laminae limitantes found at other locations in bone [including the titanium-bone (Nanci et al., 1993, in preparation) and hydroxyapatite-bone (Kawaguchi et al., 1993) interface] can now be appreciated based on observations of their continuity and on a similarity in osteopontin composition, the temporal development of these structures with regard to cell dynamics, and their additional constituents, may be different and will require further studies to clarify this matter. In this regard, it should be noted that the osteopontin-containing lamina limitans found in the matrix adjacent to bone-lining cells, osteocytes and cell processes (Fig. 11c) is usually consistently thin along the bone surface, whereas cement lines (Figs. 11a, b) are considerably more irregular in morphology and contain significantly more osteopontin.

The initial osteogenic processes that guide bone modeling, and the subsequent remodeling events that couple bone formation with resorption, undoubtedly rely on a series of intricate cell-cell and cell-matrix interactions. In this context, it is intriguing to discuss the source and role of cement line components, for example, in potentially acting as signalling or attachment and spreading factors for the variety of osteogenic cells directly apposed at one time or another to this specialized form of bone matrix. Despite reports of the expression of osteopontin by osteoclasts (Tezuka et al., 1992; Frank et al., 1993; Merry et al., 1993), it is unlikely that the majority of osteopontin found at cement lines in bone results from the secretory activity of osteoclasts, although there may be some small contribution in this regard. The osteoblast, on the other hand, has been clearly identified as the major source of osteopontin (reviewed by Butler, 1989) and most likely accounts for its appearance at this interface where newer bone is appositionally deposited against older bone, thus perhaps reflecting the reversal event that spatio-temporally separates bone resorption from formation during remodeling. In support of this notion is the observation in vitro of an

initial, similar deposition of an osteopontin-containing, cement-like substance against various substrata by cultured rat osteoblastic cells derived from bone marrow (Shen et al., 1993). Temporally, cement lines and the lamina limitans at the bone-calcified cartilage interface in vivo could be considered to contain secretory products such as osteopontin and bone sialoprotein produced early in the life cycle of the osteoblast, whereas the other laminae limitantes described above most likely contain osteopontin produced relatively late in the history of the osteoblast or osteocyte and just prior to their becoming either a bone-lining cell or a fully-differentiated osteocyte, respectively. Furthermore, regional differences in cellular and matrix labeling observed for osteoblast-derived proteins imply that discrete cohorts of osteoblasts function asynchronously, in accordance with current theory on modeling and basic multicellular unit (BMU)-based remodeling (Frost, 1990a-d), and thus may express and secrete organic matrix at times and rates completely distinct from those of neighboring cells.

The implications of a concentration of osteopontin at these interfaces is intriguing from the point of view of the biochemical structure of osteopontin and its functional properties (Prince, 1989). This well-characterized molecule contains a number of amino acid motifs which make it a strong candidate for regulating mineralization and/or mediating local cell dynamics (reviewed by Butler, 1989; Gorski, 1992). A nine amino acid, polyacidic domain appears to be responsible for the strong binding of osteopontin to mineral. Furthermore, the potential ability of phosphoserine residues present in this molecule to bind calcium and form ternary complexes with the inorganic phosphate of the solid, mineral phase of bone may act as a nucleation mechanism for the biological precipitation of mineral (Glimcher, 1984). On the other hand, the presence of the cell adhesion, tri-peptide sequence Arg-Gly-Asp (RGD) (Oldberg et al., 1986) points to a role for this protein in cell attachment and spreading (Oldberg et al., 1986; Somerman et al., 1987, 1988, 1989). A third possibility for the role of osteopontin, based on its distribution at matrix-matrix and matrix-implant (Nanci et al., 1993, in preparation; Kawaguchi et al., 1993) interfaces in bone, may be to act as a biological adhesive for retaining the overall integrity of spatio-temporally distinct matrices (or implants) during endochondral bone growth, bone remodeling and tissue repair. Indeed, its accumulation among the collagen fibrils in mineralizing bone may reflect a similar adhesive phenomenon on a finer scale. Despite a tendency to attribute specific functions to particular domains within a molecule, it may be useful to emphasize that it is probably the coordinate activities and interactions between molecules that elicit particular cellular responses or extracellular matrix events at a given site within bone. In this regard, it should be noted that Ritter et al. (1992) have demonstrated a strong interaction between osteopontin and osteocalcin, and Prince et al. (1991) have shown osteopontin to be a substrate for the protein cross-linking activity of transglutaminase. Surely, other similar, but as yet unidentified, interactions must exist among the molecules in bone, and it is clearly the nature of these interactions that allow osteogenic processes to proceed as they do.

## Acknowledgements

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## Discussion with Reviewers

**J.L. Matthews:** How does the morphology described enable bone cells to know where and when to resorb and form bone? Since various envelopes (periosteum, endosteum, haversian) respond differently to various signals and pathology, what interface differences might exist? How does the vascular supply limit or enhance the regional specificity of bone?

**Authors:** A lamina limitans is generally found at most cell-matrix interfaces in bone, except where osteoblasts are secreting new matrix (osteoid) and where osteoclasts are resorbing bone. Since practically all bone surfaces lined by bone-lining cells show this feature, it is unlikely that the organic components of this lamina limitans itself, such as osteopontin for example, act alone in mediating events related to site-specific bone formation and resorption. Clearly, regional and localized differences in tissue vascularity and in molecular interactions among a plethora of molecules, some produced locally and others systemically, define the pattern of bone modeling and remodeling.

**C.G. Groot:** It is possible that the small osteoclast vesicles you show in Figure 6b arise from cross-sections of finger-like invaginations of the plasma membrane with a microvillus somewhere near the bottom, like that seen for other acid-secreting cells (e.g. in the stomach). Could you give your opinion on that?

**Authors:** Although it is possible that a fortuitous plane of section may generate images from structures such as you have described, we feel that based on the regularity with which they are observed and on their presence in the cytoplasm sometimes far away from the ruffled border or from any other apparent site of membrane invagination for that matter, that they are distinct vesicular structures within the osteoclast. If they were indeed invaginated channels from the plasma membrane, then one would expect to see also some of these profiles mixed with those of the vesicles, whereas generally profiles of only one type are observed. However, to prove this definitively, imaging by serial-sectioning or by high-voltage stereo-electron microscopic reconstruction is necessary.

**C.G. Groot:** From our experience, we know that fixa-

tives do not penetrate in the mineralized areas of bone/or cartilage. This means that if these tissues are demineralized without the presence of a fixative, the proteins in the originally mineralized areas are not fixed and they are easily translocated, as you suggested yourself in your manuscript. A second point is that in these unfixed mineralized areas, the antigenicity of the proteins is not changed compared with the fixed unmineralized areas. This will undoubtedly lead to differences in detectability by antibodies, which might give an incomplete pattern of distribution over the whole tissue. I would like to hear your comments on that.

**Authors:** As stated in the text, we often add 0.1% glutaraldehyde to our decalcifying solutions. However, here it should be indicated that, although we have not directly quantified labeling intensity, immunolabeling patterns are generally the same whether or not glutaraldehyde has been added to the decalcifying solution.

**K. Väänänen:** Can the authors make any kind of estimation concerning what percentage of osteoblasts finally differentiate into osteocytes and whether osteocytes may be an important mediator of mechanical stress in bone?
**Authors:** Although we find it difficult from our own studies to estimate the numbers of cells that undergo the osteoblast-to-osteocyte transition, the reviewer and reader are referred to the excellent publications of Marotti and Palumbo and their colleagues for extensive descriptions of the formation of osteocytes and their potential roles.

**K. Väänänen:** The authors conclude that it is unlikely that osteoclasts secrete components of cement lines, although some researchers have shown that they express osteopontin. If that is the case, what is then the function of osteopontin expression in these cells?
**Authors:** At the moment, the significance of the expression and possible secretion of osteopontin by osteoclasts, as shown by others, is not clear. One possibility may be that, if secreted by the cell, osteopontin is not incorporated into bone matrix, but rather acts at the level of the osteoclast plasma membrane (or other cells for that matter) to influence cell dynamics or metabolism related to bone modeling or remodeling. On the other hand, although some osteopontin potentially secreted by the osteoclast may end up in the matrix, it may be a relatively small contribution as compared to that produced as a cement line or lamina limitans by cells of the osteoblastic lineage.

**J.E. Davies:** In Figures 11 a-c you illustrate both cement lines and laminae limitantes. These would seem to be of similar dimensions (I would estimate < 50 nm from the inset to Figure 11c) and mark matrix/matrix in-

terfaces and cell/matrix interfaces respectively, as you explain in the text. Nevertheless, cement lines were first described as linear structures which can be clearly seen in both decalcified and non-decalcified light microscopic sections, and are generally considered to be of approximately 500 nm in width, and thus an order of magnitude greater than the lines you demonstrate. Have you been able to image, using your TEM techniques, morphologically distinct structures of such proportions and are they labeled as you have illustrated herein?
**Authors:** Considering the similarity in the organic composition of cement lines and laminae limitantes that we have shown previously and reviewed here, our use of the two terms is at present an operational one: the former being used for the generally planar accumulation of organic material and mineral at matrix-matrix interfaces, and the latter for similar material at cell-matrix interfaces. Here, it should be noted that in this context, the interface between calcified cartilage and bone found in endochondral ossification should theoretically be classified as a cement line (or cement 'plane' for that matter). However, since the literature is replete with references to cement lines as separating older bone from newer bone, and Scherft [Scherft JP (1972) The lamina limitans of the organic matrix of calcified cartilage and bone. J. Ultrastruct Res. **38**, 318-331] originally used the term lamina limitans for the electron-dense periphery of calcified cartilage and bone, for the sake of clarity, we therefore, frequently use these traditional terms. It should be emphasized, however, that in many instances, direct continuity can be observed in sections of bone between the lamina limitans at the bone surface, the lamina limitans of osteocyte lacunae and their canaliculae, the lamina limitans (cement line) at the bone-calcified cartilage interface and cement lines at bone-bone interfaces, all of which contain high levels of osteopontin. Thus, in these cases, the use of distinct terminology for each of these site-specific structures may be questioned. The dimensions of laminae limitantes and cement lines may be similar and quite thin (ranging from 10-100 nm), or may appear as thicker structures as you have indicated. Indeed, we have observed substantial variation in the thickness, regularity and organization of organic components even within a single cement line. These differences most likely arise from the three-dimensional topography of the older, 'substrate' mineralized matrix upon which components of the cement line such as osteopontin are deposited by (pre)osteoblasts. Thus, differences in cement line appearance may arise from the physical state of the matrix left after resorption by osteoclasts, forming an often crenated *reversal line*, or, where prior resorption did not occur, a smoother, non-crenated *resting line* is formed at the site of new bone deposition.

**J.E. Davies**: Given the importance of the non-collagenous protein content of bone matrix which you discuss, and the belief expressed by some (Christoffersen and Landis, 1991) that the collagen compartment is an essential prerequisite for mineralization, would you speculate on whether collagen is necessary for calcium phosphate nucleation in bone or whether it serves to provide a framework for long-range order in the organization of the mineral component of this tissue?

**Authors**: As you are aware, substantial evidence exists to implicate practically every bone component in the mineralization process. Recent studies also suggest that specific interactions between individual constituents in bone are responsible for defining the parameters of mineral nucleation and crystal growth. Although early studies focused on collagen as being the primary mediator of mineral deposition, recent work has considered the role of non-collagenous proteins in this process. While substantial and exciting developments have resulted from the isolation and study of non-collagenous proteins, it would be premature at this time to ascribe definitive roles to them. Furthermore, it is entirely possible that the relative sequence of their secretion, and the nature of their spatial association with pre-existing 'substrate' molecules and/or mineral, determine the site- and tissue-specific mineralization pattern for hard tissues in general.

**Editor**: Could you please give some details of the paper in preparation by Nanci *et al?*

**Authors**: The paper by "Tissue response to titanium implants in a rat tibial model: Structural, immunocytochemical and lectin cytochemical characterization of the titanium-bone interface" Nanci *et al.* was presented at the last Cells and Materials meeting (May, 1993) at Los Angeles and will soon be submitted for publication to Cells and Materials.

In that implant study, our aim was to examine the tissue response to commercially pure titanium in a rat tibial model by 1) describing ultrastructural features of osteogenesis at, and near the implant surface, 2) characterizing the bone-implant interface using lectin gold cytochemistry for glycoconjugates and immunocytochemistry for non-collagenous bone and plasma proteins, and 3) comparing its composition with that of natural bone interfaces *in vivo* such as cement lines and laminae limitantes. The data show that bone formation occurs soon after implantation of a miniaturized titanium screw, first as spongy bone, which then remodels relatively quickly into more compact bone. The presence of either bone matrix or osteoblasts against the surface of the implant indicates that bone deposition can occur both toward and away from the implant. In this rat tibial system and under the experimental conditions used, the bone-implant interface was characterized by the presence of cells or of an intervening extracellular matrix layer between the bone matrix and the titanium surface. In the circumstance that bone deposition occurs away from the implant, osteoblasts may view the titanium surface as an interface between an older and a younger matrix and thereby secrete the equivalent of a cement line against the titanium surface. Our recent immunocytochemical data, in general, show that naturally-occurring cement lines are enriched in osteopontin and contain some bone sialoprotein, osteocalcin and fetuin. Likewise, in these implant studies, a layer rich in glycoconjugates (as shown by lectin-gold cytochemistry) and containing a high concentration of osteopontin was found at, or near, the surface of the titanium implant. Indeed, the host's response to create a bone-implant interface may be similar to the osteogenic processes required to form a naturally-occurring bone interface.