Tissue-nonspecific alkaline phosphatase (TNSALP) and Ca-ATPase are known to play roles in bone mineralization, but how these enzymes contribute to appositional mineralization has been illusive. Here we examined the active sites of these enzymes in appositional mineralization using the bones of young rats being administered with 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) for 5 days. The doses of HEBP totally abolished mineralization of newly formed bone matrix except in matrix vesicles (MVs), and hence allowed precise localization of MVs and phosphatase reactions within non-mineralized extracellular matrix. Intense TNSALP and ATPase reactions were confirmed along the limited portions of osteoblast membranes where intimate cell-cell contacts were maintained. Diffuse reactions of these enzymes were throughout the osteoid implicating efflux of TNSALP and ATPase molecules into extracellular matrix from the osteoblast membranes. Phosphatase reactions associated with MVs varied both in intensity and location among the individual vesicles; newly formed MVs were almost free of reactions but appeared to gain those activities later in the osteoid. These data suggest that TNSALP and ATPase are released from the osteoblast membrane and later integrated into MVs within the osteoid. The osteoblasts may thus regulate appositional mineralization of bone from a distance at least in part by providing phosphatases via MVs.

Key words: bone, TNSALP, ATPase, Matrix vesicles, HEBP

Introduction

Mineralization of bone matrix is a biphasic process; matrix vesicles (MVs)-mediated initial mineralization and subsequent process of appositional mineralization \(^{1}\). MVs are osteoblast-derived extracellular lipid bilayer-enclosed microstructures ranging from 30 to 300 nm in diameter. These extracellular vesicular structures are known to serve as a site for Ca\(^{2+}\) and inorganic phosphate (Pi) accumulation and create a specific micro environment whereby the initial deposition of amorphous calcium phosphate and its conversion to needle-like crystals of hydroxyapatite (HA) are induced\(^{2-6}\). The second phase of mineralization starts with a release of elongated HA crystals through the ruptured vesicle membranes. These crystals serve as a template for the formation of crystalline arrays in the surrounding matrix (osteoid), thus leading to formation and growth of calcified nodules that eventually coalesce and form a calcification front of bone\(^{7,8}\). In contrast to abundance of MVs at the site of initial mineral deposition in the rapidly forming embryonic bones and the mineralization front of growth plate cartilage, MVs had been scarcely found to be associated with appositional mineralization of bone. Therefore, MVs have been thought to contribute exclusively to the initial phase of mineral induction and that subsequent appositional mineralization of bone proceeds independent of MVs.
However, electron microscopic studies of bones of hypophosphatasia patients as well as those of tissue-nonspecific alkaline phosphatase (TNSALP) deficient mice, all reported existence of MVs containing HA crystals within the bone matrix, which failed to properly mineralize due to insufficient inorganic phosphate (Pi) and/or elevated pyrophosphate (PPI) concentrations. In the animals experimentally made rachitic by systemic administration of 1-hydroxyethylidene-1-, 1-bisphosphonate (HEBP), a type of bisphosphonates, we also confirmed a large population of MVs scattered throughout the non-mineralized matrix of bone, many of which were containing electron-dense crystalline figures. All these data suggest constitutive involvement of MVs in the process of appositional mineralization. Taken together, although detailed mechanisms whereby MVs regulate bone mineralization is yet to be fully explained, it is safe to state that MVs play pivotal roles in both the initial- and appositional phases of bone mineralization.

The onset and regulated progression of bone mineralization depend on a delicate balance of local concentrations of Ca\(^{2+}\), Pi and PPI, and other extracellular matrix components such as matrix gla protein (MGP), osteopontin, and proteoglycans, all known to have inhibitory effects on mineral deposition and crystal growth. Biochemical analyses of isolated MVs indicate abundance of enzymes and other functional molecules relevant to regulation of Ca\(^{2+}\) and/or Pi levels in and around the MVs, such as TNSALP, adenosine triphosphatase (ATPase), trypsin-like phosphophosphohydrolase (NTPP or NPP1, PC-1), annexins, phospholipids of high calcium-binding affinity, and other calcium binding proteins. Among these, TNSALP is thought to contribute to elevate local Pi concentrations by hydrolyzing natural substrates including ATP and PPI, the latter being a potent inhibitor of bone mineralization. Ca-ATPase is also suggested to be involved in the regulation of Ca\(^{2+}\) and Pi levels and mineralization in MVs.

Thus, although TNSALP and Ca-ATPase associated with MVs have been thought to play important roles during bone mineralization, the exact sites and mode of actions of these enzymes in bone have been largely speculative, particularly with respect to those in appositional mineralization. Under normal conditions, once mineralized matrix layer is established in bone, it becomes extremely difficult to identify MVs and MVs-associated enzyme activities in the mineralization front because of the high electron-dense background of mineralized matrix, when viewed in the transmission electron microscope.

In this study, therefore, we sought to localize active sites of TNSALP and Ca-ATPase associated with the bones undergoing appositional mineralization, with special references to the relation of these enzymes with MVs. For this purpose, we used an experimental animal model in which whole population of MVs were made visible by preventing mineral deposition in the bone matrix by long-term administration of HEBP, a synthetic analogue of PPI, to the animals at early stages of postnatal development.

**Materials and Methods**

Protocols for animal experiments had been approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University. All experiments were properly carried out under the Guidelines for Animal Experimentation at the Tokyo Medical and Dental University.

**HEBP injection**

Five-day-old and 16-day-old rats of Wistar strain (n=6 each, all female) were used for the experiment. Each group of rats received subcutaneous injections of HEBP (8mgP/kg per day) (Sumitomo Pharmaceutical Co., Osaka, Japan) in sterile normal saline once a day for 5 to 7 days in the dorsal regions. In each group, two rats were subcutaneously injected with the same volume of normal saline under the same schedule, and served as controls. A younger group of rats were breast fed with milk during the experimental period.

**Fixation and tissue preparation**

One day after the final injection of HEBP or normal saline, the rats were deeply anesthetized with an intraperitoneal injection of chloral hydrate solution (400 mg/ Kg body weight) and fixed by perfusion via the left ventricle either with a mixture of 1% glutaraldehyde (GA) and 1% paraformaldehyde (PFA) or with 4% PFA in 0.1 M sodium cacodylate buffer (pH 7.4). After completion of fixation, the animals were subjected to soft X-ray imaging using Sofron SRO-M50 (Sofron Ltd, Tokyo, Japan), and then the long bones were carefully excised and further fixed by immersion in the identical fixatives for additional 2 h at 4°C. Thereafter, some of the long bones were decalcified in neutralized 10% ethylenediaminetetraacetic acid (EDTA) solution at 4°C for 7 days.
After thorough rinse in 0.1M cacodylate buffer at 4°C, some of the PFA-fixed bones (decalcified or non-decalcified) were dehydrated through graded series of ethanol at 4°C and embedded in water miscible resin, Technovit 7100 (Heraeus Kulzer GmbH, Werrhein, Germany). To prevent heat inactivation of the enzymes, polymerization was carried out at 0°C for 7 h. Some of the GA-PFA fixed bones were further fixed with 1% osmium tetroxide solution for 2 h at 4°C, dehydrated through ethanol series and acetone, and embedded in epoxy resin (Epon812) (TAAB, Aldermastor, UK) for ultrastructural observations. Other specimens were soaked in 30% sucrose solution overnight at 4°C and subjected to cryosectioning with a freezing microtome equipped with CO2-freezing stage (FX-801) (Yamato Ko-ki, Asaka, Japan). Fifty-µm-thick frozen sections were collected as free-floating sections, and stored in cacodylate buffer solution at 4°C until use.

Histochemical localization of active sites of phosphatases

1. Histochemical staining of TNSALP on Technovit sections

Two-µm-thick sections of Technovit 7100-embedded specimens were collected on glass slides and incubated for histochemical localization of active sites of TNSALP by the NBT/BCIP method using the NBT tablet (Boehringer Mannheim, GmbH, Germany) according to the manufacturer’s instruction. Some adjacent sections similarly prepared as above were not incubated for enzyme histochemistry but processed for von Kossa staining modified for plastic sections to distinguish between the mineralized and non-mineralized matrices within the HEBP-affected bone.

2. Histochemistry of TNSALP and ATPase by the lead-salt method

For histochemical localization of TNSALP active sites, 50-µm-thick nondecalcified frozen sections of the HEBP-affected bones were incubated in a medium containing 33 mM Na-β-glycerophosphate (substrate), 8% dextrose and 2.28 mM lead citrate in 32 mM tris-HCl buffer at pH 9.2 for 10 to 15 minutes at 4°C. The sections were briefly rinsed in distilled water to terminate enzymatic reactions and then soaked in 2% ammonium sulfide solution to convert the colorless reaction products (lead phosphate) to visible brown precipitates of lead sulfide.

To detect ATPase active sites, some of 50-µm-thick frozen sections were incubated in the precipitate-free lead citrate medium for ATPase at alkaline pH after slight modifications. The medium consisted of 80 mM Tris-Maleate buffer (pH 8.7), 8% glucose, 3 mM lead citrate, 1 mM sodium ATP, 3 mM CaCl2 and 3 mM MgCl2. To improve the specificity of ATPase reactions, 0.25mM ouabain and 1 mM levamisole were added to the incubation medium to eliminate the influence of Na-K ATPase and TNSALP, respectively. After 15 min of incubation at 37°C, the specimens were treated as described for those incubated for TNSALP reactions. The specimens incubated in the medium without substrate were used as controls in all cases.

All the sections thus incubated for TNSALP or ATPase were fixed by 1% osmium tetroxide solution for 2 h at 4°C, dehydrated through a series of graded ethanol and acetone, and flat-embedded in Epon 812. Ultrathin sections were cut with a diamond knife, routinely stained with uranyl acetate and lead citrate solutions, and examined in the Hitachi 7100 transmission electron microscope (TEM) (Hitachi Co., Tokyo, Japan).

Results

HEBP-affected bones and bone forming cells

Daily injections of HEBP (8 mg P/kg/day) for 5 or 7 consecutive days to the rats did not cause notable changes in structural features and the matrix forming activity of the affected osteoblasts, whereas it almost completely abolished the mineral deposition in the newly formed matrix, resulting in extensively thickened osteoid layers in both trabeculae and cortical bones (Figs. 1, 2). Soft X-ray imaging of the HEBP-affected bones of young rats indicated lack of the secondary ossification center and overall decrease in X-ray opacity relative to that of the normal bone (Fig. 1A). The osteoblasts particularly those on the periosteal surfaces were enriched with cytoplasm and contained well-developed Golgi apparatus and rough endoplasmic reticulum, indicating highly active matrix synthesis by these cells (Fig. 1C). Lack of electron-dense mineral deposits in the newly formed matrix made it easy to identify MVs, particularly those containing electron-dense crystals (Fig. 1D-G).

TNSALP in HEBP-affected bones

A comparison of TNSALP reactions in bones of HEBP-affected rats and of normal controls indicated a significant decrease in the enzymatic activity in the HEBP-affected bones (data not shown), but the localization patterns of reactive products of this enzyme
were identical between the two groups.

In semi-thin Technovit sections incubated for TNSALP by the NBT/BCIP method, brown-colored reaction products were located along the entire perimeter of the osteoblasts as well as the adjacent preosteoblast-like cells (Fig. 2A-C). Weak enzymatic reaction was also depicted in the Golgi apparatus and lysosomal structures in the cytoplasm of osteoblasts (Fig. 2C). Under the TEM, distinct reaction products of TNSALP (lead phosphate), were shown to be deposited along the basolateral aspects of the osteoblast membrane. The osteoblast membrane facing the osteoid matrix did
Fig. 2. TNSALP reactions in HEBP-affected bone

A. Serial cross-cut sections of humerus of HEBP-affected rat, stained for histochemical demonstration of mineralized matrix (left) and for localization of TNSALP reactions (right).

B. C. Higher magnification of a portion of (A, right) showing intense reactions of TNSALP in the osteoblast layer (arrows). Arrows in (C) indicate diffuse extracellular TNSALP reactions in the osteoid.

D. Transmission electron micrograph of osteoblasts and osteoid of the HEBP-affected femur showing electron-dense deposits of TNSALP reactions. In osteoblasts (OB), distinct reactions of ALPase are limited to the portions of the membrane in close contact with the adjacent cells (arrows). Note deposition of diffuse reaction products in the osteoid.

E-I. Matrix vesicles in the osteoid of HEBP-affected bone. TNSALP reactions associated with matrix vesicles are restricted to the outer surfaces and shown to vary considerably in intensity among the individual vesicles.
not show notable reactions, whereas the surface layers of the osteoid contained diffuse reaction products of TNSALP (Fig. 2D). Among the MVs scattered in the osteoid matrix, some did not show TNSALP reactions, but most of MVs displayed TNSALP reactions at variable intensity associated primarily along the outer surface of the vesicle membrane (Fig. 2E-I). In the MVs containing elongated crystals and hence have had the membrane ruptured, TNSALP reactions were shown to be associated with the crystals (data not shown).

**ATPase in HEBP-affected bones**

In the HEBP-affected bones, distinct ATPase reactions were located along the basolateral membranes of osteoblasts, but those along the osteoidal surface of the osteoblasts differed considerably among the cells and also between the planes of sections within a single cell. A careful comparison between the serial sections of the osteoblasts incubated for ATPase revealed that the enzyme reaction along the osteoidal aspect of the osteoblast was weak or negligible in a section which had been cut through the cell center, whereas it was strongly positive in the sections cut through the periphery of the individual cells (cf. Figs. 3A and 3B). The same was true in the ultrathin sections viewed under the TEM, but the position-related differences in the intensity of enzymatic reactions along the osteoidal aspect of the osteoblasts were not as distinct as shown in semi-thin sections (Fig. 3C, D). It was verified in our observations that strong ATPase reactions were restricted to the portions of the osteoblast- or pre-osteoblast membranes where an intimate cell-cell contact was maintained. The portions of plasma membranes exposed to the tissue fluid or the osteoid only showed weak to barely detectable reactions (Fig. 3C-E). As anticipated, the reaction products of ATPase (lead phosphate) were scattered through the osteoid matrix with a decreasing concentration gradient from the surface to the deeper layers (Fig. 3D). The reaction products of ATPase were identified in the Golgi complex and the related organelles of the osteoblasts indicating synthesis of this enzyme by these cells (Fig. 3E).

Most of MVs in the osteoid displayed ATPase reactions at different intensity and hence could be identified under TEM even at low magnifications (Fig. 4A). The active sites of ATPase associated with MVs were primarily along the outer surface but, unlike in the case of TNSALP, strong reactions were also localized within the vesicles in many cases (Fig. 4B-F).

**Discussion**

Current observations have provided first histocytochemical evidence indicating possible release and translocation of the two types of ectoenzymes of osteoblasts (TNSALP and ATPase) to the MVs scattered in the osteoid. Since MVs are known to contribute to progressive mineralization along the mineralization front, we propose a hypothesis that the dynamics of these two phosphatases are one of the mechanisms whereby the osteoblasts located at some distance from the mineralization front regulate appositional mineralization of bone.

**Validity of HEBP-affected bones as experimental model**

In this experiment, we examined the bones of HEBP-affected rats, in which mineralization of newly deposited bone matrix had been arrested and hence allowed direct observations of the entire population of MVs in the non-mineralized osteoid matrix. The HEBP-affected bones further allowed us to localize enzymatic reactions of TNSALP and ATPase in the individual MVs, in addition to those in the extracellular matrix and the osteoblast layer.

Bisphosphonates are divided into two groups, simple bisphosphonate and nitrogen-containing bisphosphonate. HEBP belongs to the group of simple bisphosphonate, has a similar chemical structure to that of inorganic pyrophosphate (PPI), and can prevent ectopic mineralization as well as normal mineralization of calcified tissues through direct impairment of the calcification process by acting as poisons of hydroxypatite crystal. Besides well known detrimental effects on osteoclasts, bisphosphonates have also been shown to affect differentiation and function of osteoblasts in a divergent manner in vivo. In vitro analysis of the effect of HEBP on osteoblasts indicated that HEBP accelerates collagen synthesis of osteoblasts in early culture period but makes no difference in the total amounts of matrix being deposited by these cells, and thus explained one of the reasons for the inconsistency of previous reports. Bisphosphonates are also reported to increase ALPase activity in osteoblast cell cultures as a consequence of accelerated differentiation at low doses (Fleisch, 1998). However, high doses of HEBP have been shown to inhibit ALPase by making a complex with Zn in the active center of the enzyme.

In our current observation of the long bones of HEBP-affected rats, osteoblasts generally maintained...
Fig. 3. ATPase reactions in HEBP-affected bones
A,B. Light micrographs showing ATPase reactions in the osteoblast layer viewed in the adjacent sections. The intensity of ATPase reactions along the osteoidal domain of osteoblast membrane differs within the same cell in the adjacent sections (compare arrows in A and B), whereas it is consistent in basolateral domains. 
C. Transmission electron micrograph showing ATPase reactions in osteoblasts (OB). In contrast to intense enzymatic reactions along the basolateral membranes, the osteoidal domain of osteoblasts only shows weak reactions except in the cell boundary regions (thick arrows). The areas of basolateral membranes not in contact with the adjacent cells also display only weak reactions (thin arrows). Asterisk, artificial separation between osteoid and osteoblasts. 
D. ATPase reactions in the osteoblasts (OB) in contact with the osteoid. Note diffuse enzyme reactions throughout the osteoid. 
E. In the cytoplasm of osteoblast, enzyme reaction is located in the Golgi apparatus (G) (arrows) and lysosomal structures (Ly). Black arrow indicates intense ATPase reactions along the plasma membrane in contact with the adjacent cell. Plasma membrane not in contact with the opposing membrane only shows weak reactions (white arrow).
rich cytoplasm and continued to form extracellular matrix, whereas osteoclasts had been mostly diminished from the bone surface. This condition resulted in accumulation of thick layers of non-mineralized matrix in the cortical and trabecular regions leaving only narrow spaces of bone marrow as shown in Figs. 2 and 3. Despite altered internal structures of bone and the lack of mineral deposition in the newly formed extracellular matrix, the ultrastructural features of osteoblasts and enzymatic reactions of TNSALP and ATPase associated with these cells were indistinguishable from those of the osteoblasts in normal animals. We therefore regard the HEBP-affected bones as useful study model of bone biology allowing for close examination of the internal structures of bone matrix and its spatial and temporal relations with the osteoblasts and MVs at the ultrastructural level, which would have been totally disrupted and/or concealed by the dense precipitation of growing crystals under physiological conditions. Similarity of ultrastructural features and distribution patterns of the mineral-loaded MVs in the non-mineralized matrix of HEBP-affected bones with those in the bones of human hypophosphatasia patients and TNSALP deficient mice provides a strong support for the use of the HEBP-affected model of osteogenesis for the study of pathophysiology of bone mineralization. It is also noteworthy that the osteoblasts in TNSALP deficient mice maintain all the functional parameters compa-

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**Fig. 4.** ATPase reactions in osteoblasts (OB) and non-mineralized matrix of HEBP-affected bone

A. Numerous matrix vesicle-like electron-dense structures are scattered throughout the non-mineralized matrix. Inset: Closer view of a cluster of ATPase-positive matrix vesicle-like structures. Osteocytes (OC) do not show ATPase reaction. B-F. High magnification of matrix vesicles showing ATPase reactions. ATPase reactions are located in both outer and inner aspects of the limiting membrane and vary in intensity among the individual vesicles.
rable to those in normal animals except TNSALP. TNSALP and ATPase in osteoblast layer and its translocation to osteoid

Akisaka et al. (1988) reported that, in the osteoblasts of chick bones, Ca-ATPase is located along the basolateral membrane domains but not in the osteoidal surface of the osteoblasts. Watson et al. (1989) examined TNSALP and Ca-ATPase reactions in 3-day-old rat calvarium and gained the same results for both enzymes; intense reactions in basolateral domains and modest or negligible ones in the osteoidal domain of osteoblast membrane. The active sites of TNSALP and ATPase depicted in the bones of HEBP-affected rats in our current observations are essentially identical to those reported in previous studies, although histochemical reaction products tended to appear more diffuse in the HEBP-affected bones, particularly in the case of TNSALP.

It is our understanding that the uneven distribution of enzymatic reactions of these phosphatases along the osteoblast membranes is not necessarily related to the enzymatic characteristics of the three major membrane domains, (basal, lateral, and osteoidal domains) but rather, to the spatial relation of the osteoblast membranes to the surrounding cells or the matrix. It may appear obvious from these previous and current observations that the intense reactions for TNSALP and ATPase in the osteoblasts are restricted to the portions of the plasma membranes being in contact with the adjacent cells in both normal and HEBP-affected bones. In other words, with respect to basolateral domains of osteoblast plasma membranes, it is along the portions of the membranes open for the tissue fluid where the enzymatic reactions are weak or negligible. The diffuse enzymatic reactions in the intercellular spaces of osteoblasts and preosteoblasts appear to indicate that a large proportion of TNSALP and ATPase molecules associated with the osteoblast plasma membrane are released and diffuse into the surrounding milieu unless the membrane is intimately in contact with the adjacent cells. Presence of strong ATPase reactions restricted to the portions of the osteoid near the intercellular regions of overlying osteoblasts (Fig. 3A, 3B, 3D) appears to be the consequence of extracellular diffusion of ATPase enzymes, liberated from the osteoblast membrane, and translocated into the osteoid matrix through the paracellular channels. It is not determined from our current observations alone as to whether or not the putative release of certain membrane-associated enzyme(s) is also occurring in the osteoidal domain of osteoblast membrane.

MVs in the osteoid derive from the osteoidal domain of the osteoblast plasma membrane, which does not show distinct phosphatase reactions by conventional histochemical methods. It is therefore interesting to note that many of the MVs in the osteoid of HEBP-affected bones display strong enzymatic reactions for both TNSALP and ATPase as shown in Fig. 2E-I and Fig. 4B-F. We assume that while the enzymes released from the osteoblast membrane and diffused into tissue fluid are quickly washed away, those migrated in the osteoid should stay in the matrix and eventually be adsorb to MVs, and may further integrated into the vesicle membrane. Similar phenomenon (release of membrane ATPase into extracellular matrix and its subsequent adsorption to MVs) is known to occur also in the early stage of dentin matrix formation and mineralization in rat incisors. Figure 5 is a diagrammatic summary of the active sites of TNSALP and ATPase in the osteoblast layer, illustrating putative translocation of these enzymes into the osteoid and its subsequent adsorption to and possible integration into the vesicle membrane. How extracellular enzyme is integrated into vesicle membrane and further internalized in the vesicles is an intriguing question to be explored. It is reported that the MVs in chicken growth plate cartilage contain phospholipase C, which digests membrane phospholipids (phosphatidyl choline / ethanolamine), and that the concentration of this enzyme increases toward the mineralizing areas. Accordingly, it may be possible that the lipid bilayer of MVs membrane in the osteoid is partially disintegrated by phospholipase C, and allows for integration and possible internalization of the enzymes attached to the membrane surface.

Regulation of the initial mineral induction and subsequent processing of mineralization of bone depends largely on a delicate balance of local concentrations of Pi and PPI. In this regard, current data appear to suggest that hydrolysis of PPI by the osteoblast-derived extracellular phosphatases is not sufficient to induce mineral deposition in the newly formed osteoid matrix and, consequently, indicate the importance of mineral induction by MVs. The extracellular phosphatases in the osteoid and those secondarily associated with MVs in the osteoid may facilitate propagation of MVs-derived crystals in the osteoid as well as the steady mineral deposition and crystal growth at the mineralization front of the bone. Figure 6 illustrates hypothetical summary of our interpretation of involvement of osteoblast-derived phosphohydrolases...
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(TASALP and ATPase) in appositional mineralization of bone, where MVs are thought to contribute to it throughout the process.

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