Contribution of matrix vesicles and alkaline phosphatase to ectopic bone formation

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Abstract

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Endochondral calcification involves the participation of matrix vesicles (MVs), but it remains unclear whether calcification ectopically induced by implants of demineralized bone matrix also proceeds via MVs. Ectopic bone formation was induced by implanting rat demineralized diaphyseal bone matrix into the dorsal subcutaneous tissue of Wistar rats and was examined histologically and biochemically. Budding of MVs from chondrocytes was observed to serve as nucleation sites for mineralization during induced ectopic osteogenesis, presenting a diameter with Gaussian distribution with a median of 306 ± 103 nm. While the role of tissue-nonspecific alkaline phosphatase (TNAP) during mineralization involves hydrolysis of inorganic pyrophosphate (PP_i), it is unclear how the microenvironment of MV may affect the ability of TNAP to hydrolyze the variety of substrates present at sites of mineralization. We show that the implants contain high levels of TNAP capable of hydrolyzing p-nitrophenylphosphate (pNPP), ATP and PP_i. The catalytic properties of glycosyl phosphatidylinositol-anchored, polidocanol-solubilized and phosphatidylinositol-specific phospholipase C-released TNAP were compared using pNPP, ATP and PP_i as substrates. While the enzymatic efficiency (k_{cat}/K_m) remained comparable between polidocanol-solubilized and membrane-bound TNAP for all three substrates, the k_{cat}/K_m for the phosphatidylinositol-specific phospholipase C-solubilized enzyme increased approximately 108-, 56-, and 556-fold for pNPP, ATP and PP_i, respectively, compared to the membrane-bound enzyme. Our data are consistent with the involvement of MVs during ectopic calcification and also suggest that the location of TNAP on the membrane of MVs may play a role in determining substrate selectivity in this micro-compartment.

Introduction

Biological calcification is a tightly regulated process in which different types of tissues, cells, organelles, and biomolecules participate in the coordination and regulation of metabolic events involved in accumulating calcium phosphate (1-7).

Bone formation can be reproduced experimentally by the subcutaneous implantation of demineralized bone matrix, a procedure that leads to the sequential development of cartilage and bone (8,9). The ectopic implantation of bone morphogenetic proteins (10) also leads to osteogenesis but by a mechanism that is unclear. Elucidating the molecular mechanisms controlling ectopic bone formation is of paramount interest because understanding these mechanisms will enable the partial or *de novo* reconstruction of bone tissue (11) and contribute to the establishment of therapeutic strategies for bone diseases.

We have studied ectopic bone formation, including some of the enzymes (9,12-14) and lipids present in membranes of cells and/or organelles associated with this process (15,16). There is good evidence that endochondral calcification involves the participations of matrix vesicles (MVs) (7,17, 18), but it is not clear whether calcification of ectopically implanted demineralized bone matrix also proceeds via MVs. According to several investigators, these extracellular organelles capable of mineral accumulation bud by exocytosis from the plasma membrane and serve as the site of primary mineralization in the matrix of bone, cartilage and dentin (4,7,17-22).

Electron microscope studies have indicated that biological calcification occurs in the MVs in two steps, the first related to the initial deposition of hydroxyapatite within the lumen of the MVs and the second to the propagation of mineral outside the vesicles (2-4,18). Tissue-nonspecific alkaline phosphatase (TNAP) has been shown to be highly enriched in MVs (17,18). TNAP's function in bone mainly relates to its ability to hydrolyze inorganic pyrophosphate (PP_i), a potent mineralization inhibitor, while simultaneously producing P_i, which as a byproduct is available for deposition as hydroxyapatite (23,24). Studies on MVs isolated from hypophosphatasia patients (25) or from knockout mice deficient in TNAP (26) have indicated that the first steps of mineralization remain intact in the MVs, while it is the propagation step that is severely affected in the absence of TNAP.

In the present study, we demonstrate the presence of functional MVs in the initial stages of ectopic mineralization caused by subcutaneous implantation of decalcified bone matrix. We also show that the kinetic properties of TNAP vary greatly in the presence or absence of diacylglycerol or other membrane components, suggesting that its location on MVs during mineralization may be a factor that contributes to establishing substrate specificity.

Material and Methods

Material

All aqueous solutions were made using Millipore (Bedford, MA, USA) MilliQ ultra-pure apyrogenic water. Bovine serum albumin, Tris hydroxymethyl amino methane (Tris), 2-amino-2-methyl-propan-1-ol (AMPOL), trichloroacetic acid, N-(2-hydroxyethyl) piperazine-N'-ethenesulfonic acid, adenosine 5'-triphosphate disodium salt (ATP), sodium dodecylsulfate, p-nitrophenylphosphate (pNPP), and polyoxyethylene 9 lauryl ether ($C_{12}E_9$ - polidocanol) were from Sigma (St. Louis, MO, USA). Analytical grade reagents were used without further purification.

Preparation of rat osseous plate

Adult Wistar rats of both sexes, weighing

350-400 g, were killed by decapitation and the muscle-free thigh bones were removed and demineralized according to Reddi and Huggins (8). Briefly, diaphyseal bones were dehydrated and grounded in 0.125 to 0.175 mm fragments, treated with 0.5 M HCl in a proportion of 25 mM HCl/g bone, with constant stirring and 100 mA of alternating current for 3 h at 25°C. Finally, the remaining bone matrix was exhaustively washed using distilled water and newly dehydrated. Ten to twenty milligrams of rat demineralized diaphyseal bone matrix was introduced through a small incision in the dorsal subcutaneous tissue of anesthetized young male Wistar rats (50-60 g). Fourteen days after implantation (the period required for the development of maximal alkaline phosphatase activity), the newly formed plate was removed, rinsed in ice-cold 0.9% (w/v) NaCl, and used for microscopical examination and for alkaline phosphatase extraction (15).

Electron microscopy

The growth plate obtained after 14 days of induction was sectioned (1 μm) and incubated in 0.1 M phosphate buffer, pH 7.3, containing 3% (w/v) glutaraldehyde for 1 h, followed by incubation with 1% (w/v) osmium solution prepared in the same buffer. After 1 h, the sample was washed in 0.1 M sodium phosphate buffer, pH 7.3, centrifuged and dehydrated with acetone/water mixtures of increasing acetone concentration and the final dehydration was accomplished by 3 washes with 100% acetone for 10 min. Finally, the pellet was embedded in (1:1) araldite resin at 37ºC for 48 h and in pure araldite for 72 h at 60ºC. Thin sections were cut using a microtome and stained with 1% (w/v) Toluidine blue and ultrafine sections (0.5 μm) were mounted on copper gilder grids (200 mesh). Finally, they were stained with 4% (w/v) uranyl acetate, pH 12, for 10 min followed by 0.3% (w/v) lead citrate, pH 12, for 10 min. The sample was examined using a Philips (Model 208; Prague, Czecho-slovakia) transmission electron microscope.

Preparation of rat osseous plate alkaline phosphatase

An osseous plate membrane fraction rich in alkaline phosphatase, polidocanol-solubilized and phosphatidylinositol-specific phospholipase C (PIPLC)-released alkaline phosphatase was prepared as described by Camolezi et al. (15). Briefly, 14 days after implantation, the growth plate formed was removed, rinsed in ice-cold 0.9% (w/v) NaCl and homogenized with 10 mM Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl (3 mL buffer/g osseous plate) in a high-speed shearing homogenizer, for 2 min. The homogenate (crude extract enzyme) was centrifuged at 15,000 g for 20 min and the supernatant was dialysed overnight, at 4ºC, against 5 mM Tris/HCl buffer, pH 7.5, containing 2 mM $MgCl_2$ and 0.15 M NaCl. The dialysed homogenate was centrifuged for 1 h at 160,000 g. The pellet, corresponding to the membrane-bound enzyme was resuspended in 5 mM Tris/HCl buffer, pH 7.5, containing 2 mM $MgCl_2$. Samples of membrane fraction (0.2 mg/mL) were treated with 1% polidocanol for 1 h with constant stirring at 25°C or aliquots (2 mg/mL) were incubated with 0.1 U PIPLC from *B. thuringiensis* for 1 h under constant stirring at 25°C. Both samples were centrifuged at 100,000 g for 1 h, at 4ºC. The supernatants were the source of solubilized or enzymatically released alkaline phosphatase, respectively.

Enzymatic activity measurements

p-Nitrophenylphosphatase (pNPPase), adenosine-5'-triphosphatase (ATPase) and pyrophosphatase (PP$_i$ase) activities were assayed as described by Camolezi et al. (15). Briefly, pNPPase activity was assayed discontinuously at 37ºC in a Spectronic (Genesys 2) spectrophotometer by following the liberation of p-nitrophenolate ion (1 M ε, pH

9.4, 17,600 M^{-1} cm^{-1}) at 410 nm. Standard conditions were 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 1 mM pNPP in a final volume of 1.0 mL. The reaction was initiated by the addition of the enzyme and stopped with 1.0 mL of 1.0 M NaOH at appropriate time intervals. For ATPase, the activity was also assayed discontinuously by measuring the amount of inorganic phosphate liberated, adjusting the assay medium to a final volume of 1.0 mL. The reaction was initiated by the addition of the enzyme, stopped with 0.5 mL of cold 30% trichloroacetic acid at appropriate time intervals. The reaction mixtures were centrifuged at 4,000 *g* prior to phosphate determi-

nation. Standard assay conditions were 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 2 mM ATP. For PP$_i$ase activity assays, standard conditions were 50 mM Tris/HCl buffer, pH 8.0, containing 2 mM $MgCl_2$ and 2 mM sodium pyrophosphate, and processes performed as described for ATPase activity.

All determinations were carried out in triplicate and the initial velocities were constant for at least 90 min provided that less than 5% of the substrate was hydrolyzed. Controls without added enzyme were included in each experiment to account for the non-enzymatic hydrolysis of substrate.

Determination of kinetic parameters

Maximum velocity, catalytic constant (k_{cat}), apparent dissociation constant ($K_{0.5}$), and Hill coefficient obtained from substrate hydrolysis were calculated using the software described by Leone et al. (27). Data are reported as the mean of triplicate measurements of three different enzyme preparations, which was considered to be statistically significant at P ≤ 0.05.

Statistical analysis

Kinetic data were submitted to analysis of variance in a completely randomized design and differences between means were verified by the Tukey test (P < 0.05).

Results and Discussion

Ectopic bone formation can be reproduced experimentally by implantation of demineralized bone matrix (8) or by implantation of morphogenic proteins at ectopic sites (10). This process permits clear visualization of a sequence of mineral nucleating events in the osteoid, in the absence of an established mineralizing front (28). In particular, implantation of decalcified bone matrix triggers the cascade leading to endo-
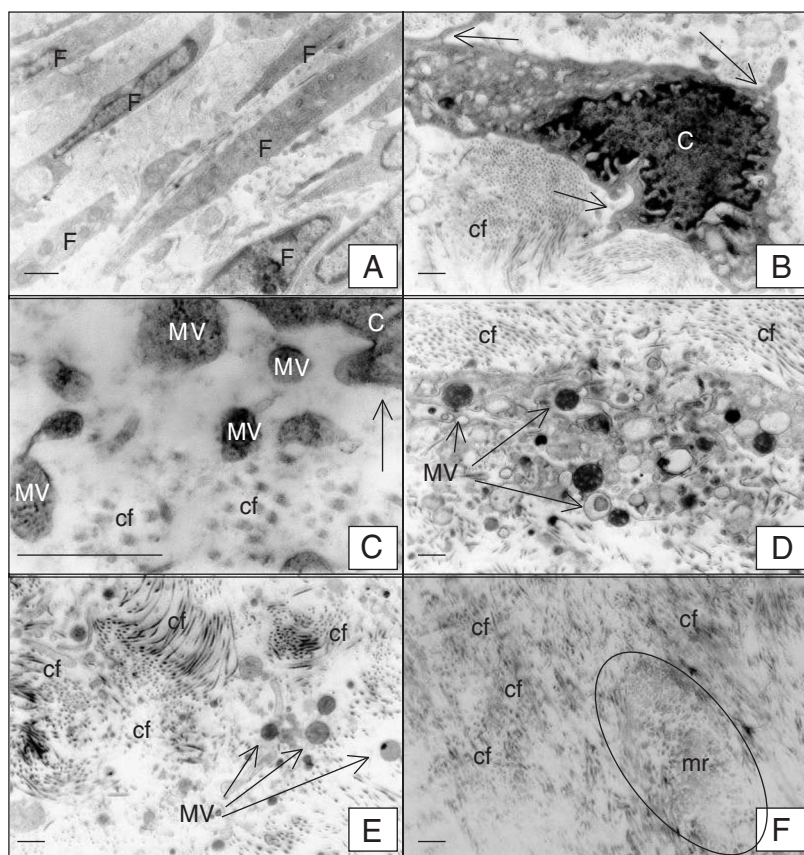

Figure 1. Mineralization was induced by implanting demineralized rat diaphyseal bone matrix into the dorsal subcutaneous tissue of young male Wistar rats. Fourteen days post-implantation the tissue was removed and processed for electron microscopy as described in Material and Methods. *A*, Fibroblast "F"; *B*, chondrocyte "C"; *B* and *C* details of matrix vesicles (MV) budding from chondrocyte membrane (arrows); *D* and *E*, extracellular MV and collagen fibers "cf"; *F*, stage of advanced mineralization ("mr" indicates mineralization region previously occupied by hypertrophic chondrocytes). Bars correspond to 500 nm.

chondral bone formation. The histological profile of the tissue 14 days after implantation of demineralized bone indicates the presence of fibroblasts and chondrocytes. The 14-day endpoint was chosen because TNAP activity of the implant was maximal at this stage (9). By means of electron microscopy, fibroblasts (Figure 1A), hypertrophic chondrocytes (Figure 1B), osteoblasts can be identified in the specimens in agreement with previous studies (8,9,11,28,29). The presence of fibroblasts has been considered a characteristic of the initial stages of the calcification process while chondrocytes are characteristic of later stages (8,9,14). The histological profile of the implanted demineralized bone matrix 14 days after implantation of bone matrix shows typical features of the later stage of endochondral osteogenesis, namely bone and cartilage (Figure 1F).

We found MVs budding from chondrocytes (see Figure 1C and E) and the presence of collagen fibers in the extracellular matrix (Figure 1E). Many chondrocyte-derived MVs appeared to serve as nucleation sites for mineralization during induced ectopic osteogenesis as they contained needle-like crystals consistent with hydroxyapatite (Figure 1D and E). These vesicles had a Gaussian size distribution of 150 to 600 nm with a median of 306 ± 103 nm (Figure 2). This median diameter is similar to that reported by us and others during mineralization of epiphyseal cartilage as well as in cultured

chondrocytes (7,30,31). This wide size distribution may indicate that more than one type of MV exist. However, it is as yet unknown whether a single cell produces multiple sub-classes of MVs at a specific stage of differentiation or if each cell produces only one class of MV (30,31).

The implants contained high levels of TNAP capable of hydrolyzing pNPP, ATP and PP$_i$ (Table 1). Since TNAP is not the only enzyme present in the membrane (4,13, 14,19,32-37) the enzymatic studies were carried out using isolated membrane-bound, polidocanol-solubilized and also PIPLC-released TNAP. There were significant differences (P < 0.05) in terms of substrate utilization by the different purified TNAP preparations. The PIPLC-solubilized enzyme displays approximately a 700-fold increase in
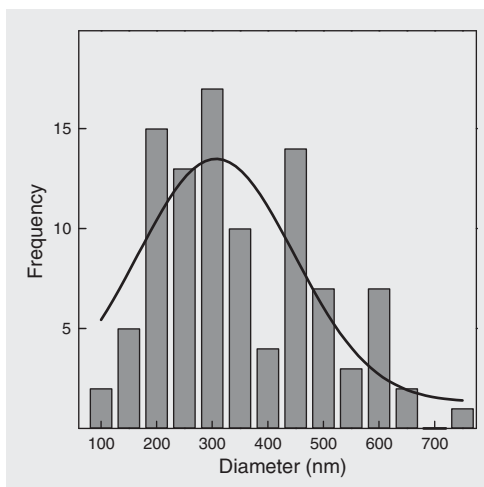

Figure 2. Gaussian distribution of the diameter of 100 matrix vesicles determined from electron micrographs of 14-day-old mineralized implants.

Table 1. Kinetic parameters of tissue-nonspecific alkaline phosphatase present in the matrix vesicles acting on different substrates.

Substrate	pH	Membrane fraction		k_{cat}/K_m	Polidocanol-solubilized		k_{cat}/K_m	PIPLC-solubilized		k_{cat}/K_m
		k_{cat} (s^{-1})	K_m (M)	(M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (M)	(M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (M)	(M^{-1} s^{-1})
pNPP	9.4	0.79	1.0×10^{-4}	7.3×10^4	2.21	4.1×10^{-5}	5.5×10^4	550.2*	7.0×10^{-5}	7.9×10^6
ATP	9.4	1.23	1.5×10^{-4}	8.2×10^3	0.65	0.9×10^{-5}	7.2×10^3	59.2*	1.3×10^{-4}	4.6×10^5
PP$_i$	8.0	0.23	1.4×10^{-3}	1.6×10^2	0.52	1.1×10^{-3}	4.7×10^2	87.5*	9.8×10^{-4}	8.9×10^4

Preparations were buffered with 50 mM 2-amino-2-methyl-propan-1-ol containing different concentrations of substrates as described in Material and Methods. Data are reported as the mean of triplicate measurement of three different enzyme preparations. PIPLC = phosphatidylinositol-specific phospholipase C; k_{cat} = catalytic constant; K_m = dissociation constant; pNPP = p-nitrophenylphosphate; ATP = adenosine-5'-triphosphate; PP$_i$ = pyrophosphate.
*P < 0.05 compared to membrane fraction (Tukey test).

k_{cat} for pNPP, 48-fold for ATP and 380-fold for PP$_i$ with respect to the membrane-bound enzyme. However, comparing the polidocanol-solubilized enzyme to the membrane-bound enzyme the values for k_{cat} barely changed and even decreased as in the case of ATP. Furthermore, while the enzymatic efficiency (k_{cat}/K_m) remained comparable between the polidocanol-solubilized and the membrane-bound enzyme for all three substrates, the value for k_{cat}/K_m for the PIPLC-solubilized enzyme (P < 0.05) increased approximately 108-, 56-, and 556-fold for pNPP, ATP and PP$_i$, respectively, compared to the membrane-bound enzyme. Since TNAP is anchored to the membrane by a phosphatidylinositol moiety (13,33), this suggests that the catalytic efficiency of TNAP is strongly affected by diacylglycerol or by molecules binding to it.

These data are relevant to interpreting studies aimed at understanding the functional consequences of mutations in TNAP known to cause hypophosphatasia (36,38, 39). We recently reported that several hypophosphatasia mutations in mice displayed selectivity towards PP$_i$ versus pyridoxal-5'-phosphate, another natural substrate of the enzyme, and that this selectivity could explain in part the variable expressivity of epileptic seizures in patients harboring those mutations (36). We have also recently obtained experimental evidence to support the previous contention that the primary role of TNAP in bone is to hydrolyze PP$_i$, a potent inhibitor of hydroxyapatite deposition, thus helping to restrict the pool of extracellular PP$_i$ and ensure proper mineralization (24,40). The main source of PP$_i$ at the level of MVs is the pyrophosphohydrolase activity of NPP1 that converts extracellular ATP into AMP and PP$_i$. However, TNAP, which co-localizes with NPP1 on the MVs, hydrolyzes ATP to produce ADP and P$_i$ while being affected allosterically by ATP (33) and being subjected to competitive inhibition by the product of this reaction, i.e., P$_i$. In turn, both ADP and AMP can also serve as substrates of TNAP. Thus, the relative concentrations of ATP, ADP, AMP, PP$_i$, and P$_i$ will have an influence on TNAP function. Our data suggest that the location of TNAP on the membrane of MVs may play a role in determining substrate selectivity in this micro-compartment. These data also suggest that assays of TNAP mutants bound to MVs or to liposome-based systems may be more biologically relevant than assays done on solubilized enzyme preparations.

We have shown here that the ectopic mineralization process induced by ectopic implantation of demineralized bone matrix occurs via an MV-mediated mechanism analogous to that of endochondral ossification. The MVs contain high levels of TNAP able to function as ATPase and pyrophosphatase, although the kinetics towards the substrates is greatly affected by the presence of diacylglycerol moieties and other cell membrane components. It is still unclear if the size and localization of the MVs may further affect the kinetic properties of TNAP but studies in this regard should further our understanding of the biological role of this enzyme during bone mineralization and better explain the abnormalities caused by different hypophosphatasia mutations.

Acknowledgments

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