Vitamin D Metabolites Regulate Matrix Vesicle Metalloproteinase Content in a Cell Maturation-Dependent Manner

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Abstract. Matrix vesicles are extracellular organelles produced by cells that mineralize their matrix. They contain enzymes that are associated with calcification and are regulated by vitamin D metabolites in a cell maturation-dependent manner. Matrix vesicles also contain metalloproteinases that degrade proteoglycans, macromolecules known to inhibit calcification in vitro, as well as plasminogen activator, a proteinase postulated to play a role in activation of latent TGF-β. In the present study, we examined whether matrix vesicle metalloproteinase and plasminogen activator are regulated by 1,25(OH)2D3 and 24,25(OH)2D3. Matrix vesicles and plasma membranes were isolated from fourth passage cultures of resting zone chondrocytes that had been incubated with 10-10-10-7 M 1,25(OH)2D3 or growth zone chondrocytes incubated with 10-11-10-8 M 1,25(OH)2D3, and their alkaline phosphatase, active and total neutral metalloproteinase, and plasminogen activator activities determined. 24,25(OH)2D3 increased alkaline phosphatase by 35-60%, decreased active and total metalloproteinase by 75%, and increased plasminogen activator by fivefold in matrix vesicles from resting zone chondrocyte cultures. No effect of vitamin D treatment was observed in plasma membranes isolated from these cultures. In contrast, 1,25(OH)2D3 increased alkaline phosphatase by 35-60%, but increased active and total metalloproteinase three- to fivefold and decreased plasminogen activator by as much as 75% in matrix vesicles isolated from growth zone chondrocyte cultures. Vitamin D treatment had no effect on plasma membrane alkaline phosphatase or metalloproteinase, but decreased plasminogen activator activity. The results demonstrate that neutral metalloproteinase and plasminogen activator activity in matrix vesicles are regulated by vitamin D metabolites in a cell maturation-specific manner. In addition, they support the hypothesis that 1,25(OH)2D3 regulation of matrix vesicle function facilitates calcification by increasing alkaline phosphatase and phospholipase A2 specific activities as well as metalloproteinases which degrade proteoglycans.

Key words: Metalloproteinases — Matrix vesicles — Chondrocytes — Calcification — 1,25(OH)2D3 — 24,25(OH)2D3.

Matrix vesicles are extracellular organelles produced by cells that mineralize their matrix [1-3]. It has been hypothesized that these organelles are either the initial sites of calcification in vivo or are intimately associated with these sites [4-6]. They have been found to be enriched in proteolipids and calcium-phospholipid-phosphate complexes, macromolecules believed to be responsible for organizing the first mineral phase on the surface of the membrane [7-10]. In the electron microscope, hydroxyapatite crystals have been observed on the inner leaflet of the matrix vesicle membrane [6]. Matrix vesicles also contain enzymes, such as alkaline phosphatase, phospholipase A2, and NTP-pyrophosphohydrolase, which are increased in regions of active mineralization, such as is found in the growth plate [11-19].

Factors that regulate mineralization also regulate matrix vesicle structure and function, including matrix vesicle enzyme activity [15, 16, 20]. The effect of vitamin D3 metabolites on alkaline phosphatase and phospholipase A2 activity in chondrocyte and osteoblast cultures is targeted to matrix vesicles [17, 18]. Moreover, it has recently been found that vitamin D3 metabolites directly affect matrix vesicle enzyme activity via nongenomic mechanisms [20, 21].

Although a function of matrix vesicles in mineralization is to provide sites for hydroxyapatite crystal formation, it is likely that matrix vesicles function in maturation of the extracellular matrix as well. It has been recognized that proteoglycan aggregates are inhibitors of mineralization [22, 23] and that they must be removed by aggregate breakdown, proteolysis of the core protein, or glycosaminoglycan chain degradation for mineralization to occur [24-29]. Hirschman et al. [30] found neutral proteases in matrix vesicles and suggested that they may participate in matrix maturation. Katsura and Yamada [31] have made similar observations using matrix vesicles from chick growth plates, and Einhorn et al. [32] have postulated that matrix vesicle-derived proteases may also be involved in fracture callus remodeling.

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Matrix vesicles are selectively enriched in metalloproteinases which degrade proteoglycan [33]. The highest levels of metalloproteinase activity are found in matrix vesicles produced by growth zone chondrocytes, suggesting a mechanism whereby the more mature cell modulates the matrix for calcification [33]. Moreover, osteoblast-like cells also produce matrix vesicles enriched in proteoglycan-degrading metalloproteinases, and enzyme specific activity is higher in matrix vesicles isolated from cultures growing under conditions favorable for mineralization [34].

Recently, we found that matrix vesicles isolated from growth zone chondrocyte cultures could activate latent transforming growth factor-beta 1 (TGF-β1), a process that has been postulated to be mediated by matrix vesicle enzymes [35]. The activation of latent TGF-β1 by matrix vesicles was regulated by 1,25(OH)₂D₃ via direct, nongenomic action on the isolated organelle. This effect was specific to 1,25(OH)₂D₃ and limited to matrix vesicles produced by growth zone chondrocytes; 24,25(OH)₂D₃ did not elicit the effect, nor did 1,25(OH)₂D₃ stimulate matrix vesicles isolated from resting zone chondrocyte cultures to activate the latent growth factor.

With these observations in mind, we hypothesized that extracellular matrix processing enzymes might also be regulated by vitamin D₃ metabolites and that the stage of cell maturation might also affect the quantity and/or profile of enzymes present. To examine this hypothesis, we used a well-characterized chondrocyte model in which cells are derived from the growth zone and resting zone of costochondral cartilage. These chondrocytes are at two different stages of cell maturation and retain their phenotypic differences, including differential responsiveness to vitamin D metabolites, through four passages in culture [15–17, 20, 34–43]. Cells were grown to fourth passage, treated with varying levels of 1,25(OH)₂D₃ or 24,25(OH)₂D₃ for 24 hours, and the matrix vesicles and plasma membranes were isolated for assay of alkaline phosphatase activity, metalloproteinase activity on proteoglycans, and phospholipase A₂ activity. The data confirm that metalloproteinase activity in matrix vesicles and plasma membranes is differentially regulated by vitamin D₃ metabolites.

Materials and Methods

Cell Culture

Chondrocytes were isolated from the costochondral cartilage of 125 g Sprague-Dawley rats (Charles River Breeding Labs, Wilmington, MA), as described by Boyan et al. [36]. Rib cages were removed by sharp dissection and placed in Dulbecco’s modified Eagle’s medium (DMEM). The costochondral resting zone and adjacent growth zone cartilage were carefully separated to limit cross-contamination of cell zones. This technique takes advantage of the linear architecture of the growth plate in that the intervening cartilage zone (proliferative zone) and calcified cartilage are discarded to limit contamination with cells at other stages of differentiation. Cartilage was then sequentially digested with 1% trypsin (Gibco, Gaithersburg, MD) for 1 hour, and 0.02% collagenase (type II, Worthington, Freehold, NJ) for 3 hours. Both enzymes were dissolved in Hank’s balanced salt solution (HBSS) (Gibco). Cells were separated from tissue debris by filtration through 40-mesh nylon, followed by centrifugation through 40-mesh nylon, followed by centrifugation of the filtrate at 500 × g for 5 minutes. Cell viability was 95%, as demonstrated by Trypan blue exclusion.

Cells were then plated in 25-mm culture dishes at initial densities of 10,000 cells/cm² for resting zone chondrocytes and 25,000 cells/cm² for growth zone chondrocytes. Incubation was conducted in DMEM containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 50 µg/ml sodium ascorbate in an atmosphere of 5% CO₂ at 37°C and 100% humidity for 24 hours. At this time, culture media were replaced at 72-hour intervals. At confluence, cells were subcultured to T-75 flasks using the same plating densities as before and allowed to return to confluence. All cell populations took 5–8 days (7-day average) to reach confluence in primary culture and in each subculture. Fourth passage cultures were used for these experiments because a number of studies have demonstrated that these cells retain their differentiation phenotype, including response to 1,25(OH)₂D₃ and 24,25(OH)₂D₃, up to this number of passages in culture [15–18, 20, 34–43]. In addition, prior studies have shown that enzyme activity in costochondral chondrocytes is regulated by vitamin D metabolites in a cell maturation-specific manner. For example, it has been demonstrated that only 1,25(OH)₂D₃ regulates alkaline phosphatase [15, 17], phospholipase A₂ [16], and protein kinase C [49] in growth zone chondrocytes, whereas 24,25(OH)₂D₃ regulates these enzymes in resting zone chondrocytes. For this reason, we only investigated the effects of the metabolites on their target cells.

Each vitamin D metabolite was added to cultures of its target cells for 24 hours at both physiological and pharmacological concentrations. Resting zone chondrocytes were incubated with 10⁻¹⁰–10⁻⁷ M 24,25(OH)₂D₃, and growth zone chondrocytes were incubated with 10⁻¹⁰–10⁻⁸ M 1,25(OH)₂D₃. Ethanol was used as the diluent for both metabolites, since this solvent was used in our earlier work as well as that of other laboratories, and found to have no effect on the cells at the dilutions used. For the current study, hormone stock solutions were diluted by at least 1:5000 in DMEM before addition to the cells to minimize any toxic effects of the ethanol. As an internal control, ethanol at the same concentration found in the least diluted sample was added to selected cultures. Vitamin D metabolites were a gift from Dr. Milan Usokovic (Hoffmann-LaRoche, Nutley, NJ).

Isolation of Plasma Membrane and Matrix Vesicle Fractions

At harvest, the culture medium was removed and replaced by 1% trypsin in HBSS. Cells were separated from the trypsin digest by centrifugation for 10 minutes at 500 × g; pelleted cells were resuspended in HBSS and counted using a cell counter. Chondrocytes were then homogenized in a Ten Broeck homogenizer and plasma membranes were prepared according to the method of Fitzpatrick et al. [44]. Following assay for protein content [45] and alkaline phosphatase [46], the resultant plasma membranes were suspended in 0.9% NaCl and frozen at −70°C.

The supernatant of the 500 × g centrifugation prepared above was centrifuged again at 21,000 × g for 10 minutes to pellet cell debris, including mitochondria and endoplasmic reticulum. The resulting supernatant was centrifuged at 100,000 × g for 1 hour to pellet matrix vesicles. This protocol exceeds the effective g-forces recommended for clearance of matrix vesicles from epiphyseal cartilage digest supernatants [4]. Following assay for protein content [45] and alkaline phosphatase activity [46], matrix vesicles were suspended in 0.9% NaCl and frozen at −70°C.

Matrix vesicles isolated in this manner [47] typically exhibit greater than twofold enrichment of alkaline phosphatase specific activity when compared with the plasma membranes [15–17, 36] and have a transmission electron microscopic appearance consistent with matrix vesicles in vivo [15]. The purity of the preparations has been established using the ratio of specific activity of S1 nucleotidase to that of alkaline phosphatase as a marker [15].

Release of Enzymes from Matrix Vesicle and Plasma Membrane Fractions

Proteolytic enzymes were released from membrane fractions using the method previously described by our laboratory and found to optimally release all enzyme activity from the membranes and preserve enzyme activity as well [33, 34]. Matrix vesicle or plasma membrane suspensions in 1 ml 0.9% NaCl derived from one T-150