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Matrix Vesicles Mediate Mineralization of Human Thyroid Cartilage

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Abstract. Mineralization and ossification of human thyroid cartilage first starts after the end of adolescence when the previously cartilaginous human skeleton has become ossified and the epiphyseal discs are in the process of closing. However, the mechanisms involved in mineralization and ossification of human thyroid cartilage are not well understood. Ultrastructural analysis of human thyroid cartilage revealed that mineralization started close to cartilage canals in a matrix containing gigantic collagen fibers (asbestoid fibers). Matrix vesicles were detected in mineralized areas and were often associated with needle-like crystals. For the first time we were able to isolate matrix vesicles from human thyroid cartilage by mild enzymatic digestions and ultracentrifugation. These particles were oval and varied in size; some were heavily calcified. They were enriched in alkaline phosphatase, calcium, and inorganic phosphate, suggesting that the particles contain Ca^{2+}-P_i complexes. Immunoblot analysis of these vesicles revealed the presence of annexins II, V, and VI, membrane-associated, channel-forming proteins, which allow influx of Ca^{2+} into the vesicles and intralumenal crystal growth. In addition, the vesicles were associated with types II and X collagen, suggesting that this association not only anchors the vesicles to the extracellular matrix, but, as shown previously, also stimulates Ca^{2+} influx into these particles. In conclusion, matrix vesicles isolated from human thyroid cartilage contain all the components, enabling them to initiate and mediate the mineralization process in human thyroid cartilage.

Key words: Thyroid cartilage — Mineralization — Matrix vesicles — Annexin — Collagen.

Terminal differentiation and mineralization of human thyroid cartilage occurs after the end of adolescence. At that time, most of the previously cartilaginous human skeletal elements have become ossified, and the epiphyseal discs are in the process of closing. Ossification starts in both sexes at the posterior border, the lower margin, and inferior horn of thyroid cartilage. The male thyroid cartilage is ossified in most of its parts around the age of 70 but the female cartilage never ossifies completely, leaving the ventral half cartilaginous [1]. Other permanent cartilage, including human first rib cartilage and bronchial cartilage, also start to mineralize and ossify after the end of adolescence [2, 3].Endochondral ossification is a multistep process during which resting immature chondrocytes first undergo rapid cell proliferation, then become postmitotic, enlarge into hypertrophic chondrocytes, undergo mineralization and cell death, and are finally replaced by bone cells [4, 5]. This whole process leads to the longitudinal growth of bones. In contrast, mineralization and bone formation in human thyroid cartilage occur after the larynx has reached its final size and proceed slowly until advanced ages [1, 2]. Many studies have been reported on the mechanisms involved in mineralization of growth plate cartilage during endochondral ossification, however, very little information is available about the mineralization process in human thyroid cartilage. It is now well established that matrix vesicles, small membrane-enclosed particles released from the plasma membrane of hypertrophic chondrocytes, are the nucleation sites for mineralization of growth plate cartilage (for review see [6]). They contain key components, such as alkaline phosphatase, annexins II, V, and VI, metalloproteinases, phospholipases, surface-attached types II and X collagen, and Ca^{2+}-P_i-phospholipid complexes, which enable these particles to initiate and mediate the mineralization process (for review see [6]). Thus, mineralization is a highly regulated process and only mineralization-competent hypertrophic chondrocytes release these vesicles [7]. In addition, mineralization of growth plate cartilage occurs in a matrix that is enriched in type X collagen, a protein specifically produced by hypertrophic chondrocytes [8, 9]. This protein is thought to play a role in the mineralization process. It is associated with matrix vesicles and stimulates Ca^{2+} influx into these particles [10, 11], and it binds Ca^{2+} with high affinity [12].

The few histological findings published on the mineralization process in human thyroid cartilage have suggested that mineralization and ossification are based on degenerative processes. However, we have previously shown that mineralization of human thyroid cartilage is preceded by type X collagen synthesis [13], raising the following questions: (1) Is the mechanism of mineralization in these so-called permanent cartilages, such as thyroid, first rib, and bronchial cartilage, similar to those in transitional cartilages such as limb and sternal cartilage or are they controlled by different mechanisms? (2) Is the mineralization process in human thyroid cartilage also mediated by matrix vesicles? To address these questions, we investigated the ultrastructural organization of mineralized areas of human thyroid cartilage, and isolated and characterized matrix vesicles from human thyroid cartilage.

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Materials and Methods

Tissue

The larynges were obtained from autopsies and laryngectomies. Larynges from three male adults (22, 26, and 33 years) were used to isolate matrix vesicles. Larynges from two female adults (26 and 57 years) and one male adult (50 years) were used for electron microscopy.

Electron Microscopy

To study cell morphology, tissue was fixed in 2% glutaraldehyde in 0.05 M sodium cacodylate (pH 7.4) containing 0.7% ruthenium hexammine trichloride (osmolarity adjusted with NaCl to 330 mosmol/liter) and postfixed in 1% osmium tetroxide as described previously [14]. Using this fixation technique, large cartilage proteoglycans are made visible as matrix granules [15]. To study the morphology of collagen fibrils and membrane-enclosed particles (cell organelles, matrix vesicles), tissue was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) containing 2% tannin (osmolarity adjusted with NaCl to 280 mosmol/liter) and postfixed in 1% osmium tetroxide [16]. To study the morphology of collagen fibrils and proteoglycans, tissue was fixed in 2% glutaraldehyde in 0.05 M sodium cacodylate (pH 7.4) containing 0.2% ruthenium red (osmolarity adjusted with NaCl to 340–420 mosmol/liter). The tissues were dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were cut on an Ultratome (Reichert & Jung, Germany), picked up on formvar-coated grids, and contrasted with uranyl acetate and lead citrate. Specimens were evaluated in a transmission electron microscope (Zeiss EM 900; Germany) operated at 80 kV.

Isolation and Characterization of Matrix Vesicles

Human thyroid cartilage was cut into small pieces which were incubated in Hank's buffered saline solution (HBSS) (pH 7.4) containing 0.1% trypsin (type III) (Sigma Chemical Co., St. Louis, MO) at 37°C for 30 minutes. After washing twice with HBSS, cartilage pieces were incubated with HBSS containing crude collagenase (200 U/g tissue; type IA) (Sigma) at 37°C for 3 hours. Matrix vesicles were harvested by differential ultracentrifugation as described previously [11]. Briefly, pieces of undigested cartilage and cells were removed by a slow speed centrifugation step at 1,500 × *g* for 10 minutes. Subcellular particles were removed by ultracentrifugation at 30,000 × *g* for 20 minutes. Matrix vesicles were precipitated by ultracentrifugation at 100,000 × *g* for 1 hour. Protein content of isolated vesicles was analyzed by the assay from BioRad (BioRad Laboratories, Hercules, CA). Alkaline phosphatase (ALP) activity was determined using p-nitrophenyl phosphate as substrate [17]. To measure calcium and phosphate, matrix vesicles (100 μg of total protein) were sedimented by centrifugation and resuspended in 50 μl of 0.1 N HCl. Aliquots of the suspensions were analyzed for calcium using the microcolorimetric method of Baginsky et al. [18], and inorganic phosphate was determined by a modified method of Ames [19].

Antibodies

The preparation and specificity of polyclonal rabbit antibodies against human type X collagen are described elsewhere [20]. The monoclonal antibody CIID3 was an IgG2a, kappa isotype antibody cloned after immunization of a DBA/1 mouse with native chicken type II collagen [21]. It reacts with type II collagen from chick, mouse, rat, bovine and human cartilage [21]. The preparation and specificity of polyclonal antibodies against annexin II, V, and VI are described elsewhere [22].

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting

Samples were dissolved in 3% SDS sample buffer with dithiothreitol, denatured at 100°C for 5 minutes, and analyzed by electrophoresis in 8, 10 or 12% (wt/vol) polyacrylamide gels. Gels were stained with Coomassie blue, or proteins were electroblotted onto nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH). After blocking with low fat milk protein, blotted proteins were immunostained with the appropriate antibodies using peroxidase-conjugated secondary antibodies and α-chloronaphthol as a color substrate.

Results

Electron Microscopic Findings

Electron microscopic analysis of sections from human thyroid cartilage revealed that the formation of asbestoid fibers preceded mineralization (Fig. 1A). These asbestoid fibers were characterized as gigantic collagen fibers (approximately 0.4–0.8 μm in width). The spaces among these collagen fibers were filled with smaller collagen fibers and matrix granules representing fixed large aggregating proteoglycans (Fig. 1A). Asbestoid fibers were only visible in the territorial and interterritorial matrix; no fibers were found in the pericellular matrix (Fig. 1B). In areas of asbestoid fibers, the amount of matrix granules was less than in a matrix containing no asbestoid fibers (Fig. 1B,C). In addition, the pericellular matrix contained more matrix granules (proteoglycans) than the territorial matrix (Fig. 1B). Cartilage canals invaded areas of asbestoid fibers leading to vascularization and initiation of cartilage mineralization followed by deposition of bony tissue upon mineralized cartilage (Fig. 1C). Cartilage mineralization often started in close proximity to these cartilage canals. Chondrocytes in these areas were similar in size and shape to hypertrophic chondrocytes in growth plate cartilage (Fig. 1D). These cells had round nuclei, only a few mitochondria, a poorly developed endoplasmic reticulum, and many glycogen particles (Fig. 1D). Matrix vesicles with various size and shape were visible along the mineralization front; needle-like crystals were associated with these vesicles (Fig. 1E). Mineralized areas not only contained viable hypertrophic-like chondrocytes, but also dead cells, as seen in Figure 1F. Needle-like crystals (Fig. 1G) surrounded remnants of cell organelles including fragments of the cell nucleus. Once the mineralization process progressed, osteoid was deposited on mineralized cartilage. Matrix vesicles associated with crystals were also found in the mineralizing osteoid matrix (Fig. 1H).

Isolation and Characterization of Matrix Vesicles

We next isolated matrix vesicles from human thyroid cartilage by mild trypsin and collagenase digestion followed by ultracentrifugation. Matrix vesicles containing approximately 13 mg of total protein were isolated from 20 g of human thyroid cartilage. The matrix vesicle fraction was enriched in ALP activity and contained significant amounts of calcium and inorganic phosphate (Table 1). Ultrastructural analysis demonstrated that matrix vesicles isolated from human thyroid cartilage varied in size, shape, and content. In general, they were round to oval, with diameters ranging from 30 to 150 nm. Some vesicles were associated with needle-like crystallites (Fig. 2A) or were heavily calcified (Fig. 2B).

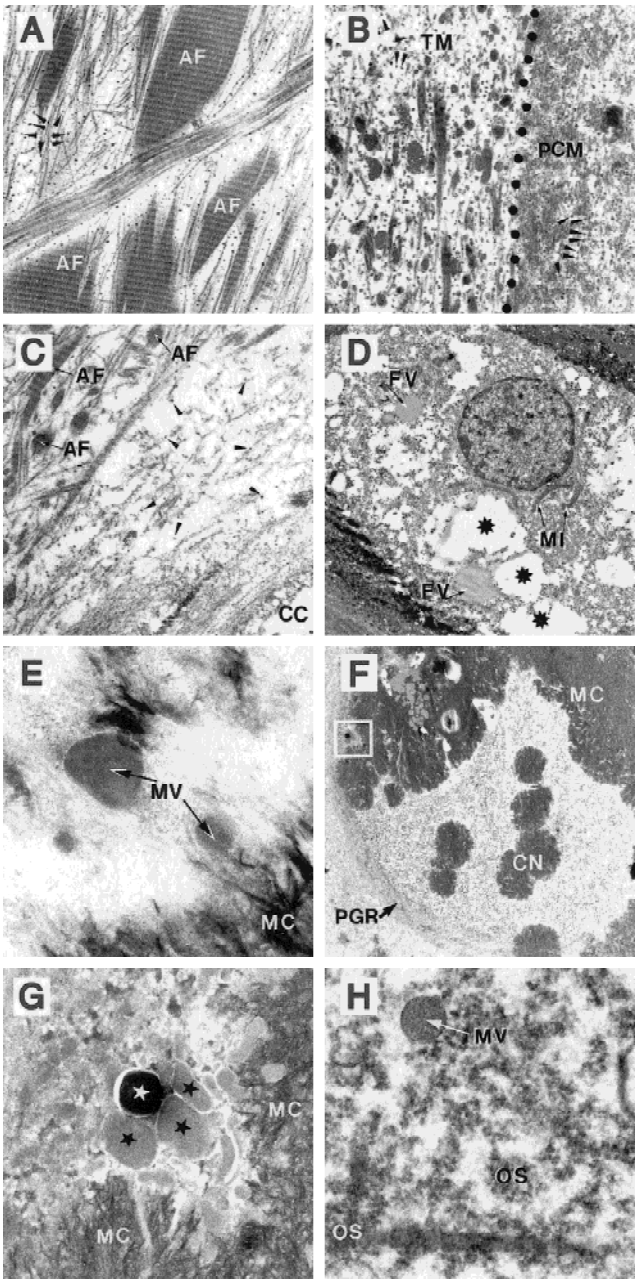

Fig. 1. Transmission electron photomicrographs of mineralizing and ossifying human thyroid cartilage. **(A–C)**: Thyroid cartilage from a 50-year-old male adult. **(A)** Matrix with gigantic collagen fibers (asbestoid fibers, AF) contains less matrix granules (resembling fixed large aggregating proteoglycans, arrowheads) than non-asbestoid fiber containing matrix in **(B)**. **(B)** Matrix granules (arrowheads) in pericellular (PCM) and territorial matrix (TM). Note that matrix granules are more abundant in the pericellular matrix than in the territorial and interterritorial (not shown) matrix. The dotted line indicates the border between pericellular and territorial matrix. **(C)** Extracellular matrix close to a cartilage canal (CC) contains asbestoid fibers (AF, arrows) and a few matrix granules (arrowheads). **(D–G)** Thyroid cartilage from a 57-year-old female adult. **(D)** Chondrocyte adjacent to the calcified area, containing fat vacuoles (FV) and few mitochondria (MI); the asterisks mark areas of washed out glycogen. **(E)** Matrix vesicles (MV) close to the mineralization front (MC). Note that needle-like crystals are associated with these vesicles. **(F)** Chondrocyte likely to have been destroyed by apoptosis close to the mineralization front (MC). Calcification nodules (CN) have developed on this destroyed chondrocyte. PGR; rim of proteoglycans. **(G)** Higher magnification of inset: cell organelles and fragments of the cell nucleus (*) close to the mineralization front (MC). **(H)** Thyroid cartilage from a 26-year-old female adult. Matrix vesicles (MV) are also present in the osteoid (OS) close to ossifying areas of thyroid cartilage. **(A–C)** ruthenium red (×20,000); **(D,F)** ruthenium hexammine trichloride (×2,800); **(E)** tannin (×46,000); **(G)** ruthenium hexammine trichloride (×42,000); **(H)** tannin (×51,000).

Table 1. Characterization of matrix vesicles isolated from human thyroid cartilage

	Matrix vesicle fraction
Alkaline phosphatase activity (nmol/min/mg protein)[a]	17,263
Calcium content (μmol/mg protein)	0.106
Phosphate content (μmol/mg protein)	0.102

[a] Alkaline phosphatase activity is expressed as nmol of p-nitrophenyl phosphate hydrolyzed per min/mg protein.

Previously it has been shown that annexins II, V, and VI are major components of matrix vesicles isolated from chicken growth plate cartilage [23]. Annexin V mediates Ca^{2+} influx into the vesicles and binds to types II and X collagen thereby anchoring the vesicles to the extracellular matrix [10, 11]. In addition, binding of types II and X collagen to the vesicle surface stimulates Ca^{2+} uptake by these particles [11]. Thus, we asked whether matrix vesicles isolated from human thyroid cartilage contained these components enabling them to initiate the mineralization process. Vesicle samples were subjected to SDS-PAGE and immunoblotting analyses. Figure 3B shows the protein profile of vesicles isolated from human thyroid cartilage. Immunoblot analyses using antibodies against annexin II, V, or VI revealed that matrix vesicles isolated from human thyroid cartilage contained these three annexins (Fig. 3C AII, 3D AV, 3E AVI). In addition, staining with antibodies against types II and X collagen showed that the vesicle fractions also contained significant amounts of types II and X collagen (Fig. 3F II, 3G X). Besides the major bands of types II and X collagen at approximately Mr of 110 kDA (Fig. 3F II) and 55 kDA (Fig. 3G X), respectively, some minor bands were visible which probably represent degradation products of these molecules caused by the isolation of vesicles by mild collagenase digestion.

Discussion

In contrast to transitional cartilage such as growth plate and sternal cartilage, permanent cartilage such as articular cartilage, thyroid cartilage, first rib cartilage, and bronchial cartilage never calcify and ossify or do so late in development or under pathological conditions [24]. Though a great deal of information is available on the differentiation, mineralization, and ossification of these transitional cartilages, very little is known about these processes in permanent

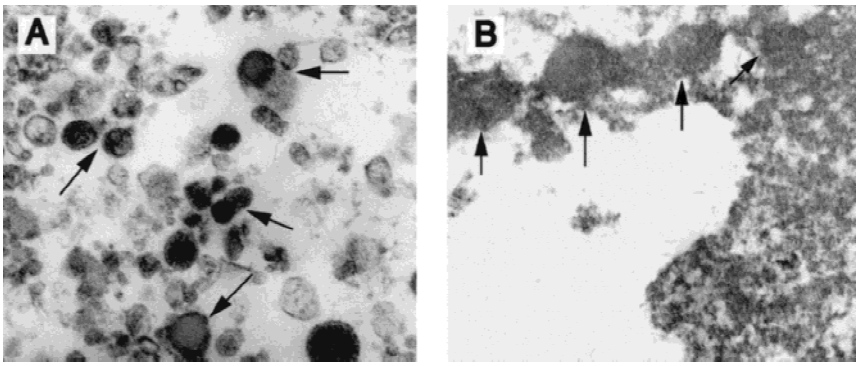

Fig. 2. Transmission electron micrographs of matrix vesicles isolated from human thyroid cartilage. **(A)** Matrix vesicles isolated from human thyroid cartilage vary in size and shape. Note that some matrix vesicles are associated with needle-like crystals (arrows) (×47,500). **(B)** Heavily calcified matrix vesicles (arrows) isolated from human thyroid cartilage (×47,500).

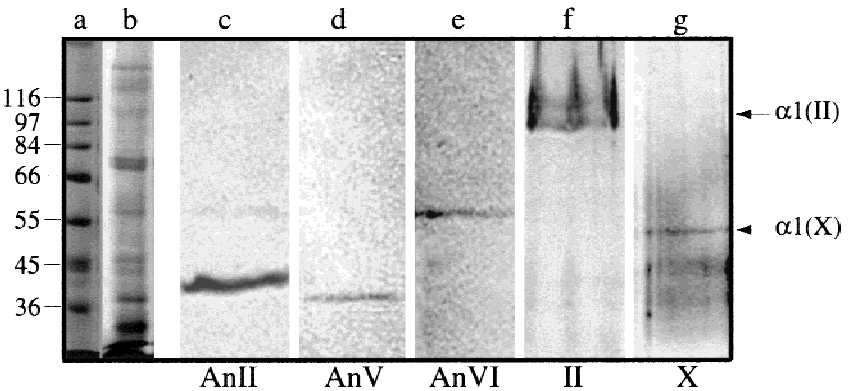

Fig. 3. Immunoblot analyses of matrix vesicles isolated from human thyroid cartilage. Proteins (50 μg) from vesicles isolated from human thyroid cartilage were separated on SDS-polyacrylamide gels. Lanes a and b were stained with Coomassie blue; lanes c–g were electroblotted onto nitrocellulose membranes and immunostained with antibodies against annexin II (lane c, AnII), annexin V (lane d, AnV), annexin VI (lane e, AnVI), type II (lane f, II), and type X collagen (lane g, X). Lane a: Molecular weight marker; lane b: matrix vesicle protein profile.

cartilages. Knowledge of the mechanisms involved in these processes in permanent cartilages will also be important for the prevention of uncontrolled mineralization during pathological conditions. In this study, we demonstrate that mineralization and ossification of human thyroid cartilage is preceded by the formation of asbestoid fibers, which resemble gigantic collagen fibers. At the mineralization front, matrix vesicles are associated with needle-like crystals. In addition, for the first time we were able to isolate and characterize matrix vesicles from mineralizing human thyroid cartilage. These particles are enriched in ALP and contain annexins II, V, and VI and significant amounts of calcium and inorganic phosphate. In addition, they are associated with types II and X collagen.

Matrix vesicles isolated from human thyroid cartilage contain all the components required for the induction of calcification. First, by being enriched in ALP activity, the vesicles have the capacity to generate inorganic phosphate ions from a variety of organic phosphate compounds [25–27]. Second, the vesicles contain annexins II, V, and VI which we and others have shown to be Ca^{2+} channel-forming proteins that mediate Ca^{2+} influx into phosphatidylserine-rich liposomes and matrix vesicles [28–30]. Third, the vesicles are associated with types II and X collagen. Binding of these collagens to matrix vesicles is mediated by annexin V and stimulates Ca^{2+} influx into these particles [11]. Fourth, matrix vesicles isolated from human thyroid cartilage contain high amounts of Ca^{2+} and P_i, suggesting that these vesicles contain a nucleational core complex. As shown previously, this nucleational core complex and the annexins keep the concentration of free Ca^{2+} and P_i ions inside the vesicles low, allowing continuous influx of mineral ions into the vesicles [31–33]. In addition, the nucleational core serves as the nucleational site for the first crystal phase to form and grow [33]. Thus, matrix vesicles isolated from human thyroid cartilage have the structural composition and functional properties to initiate the mineralization process.

The matrix remodeling process has been shown to play a crucial role during cartilage-bone metamorphosis and enables special functions of the tissues. For example, some studies have indicated that matrix remodeling during chondrocyte hypertrophy is necessary for initiation of cartilage mineralization [9, 34, 35]. In this study we provide evidence that matrix remodeling in thyroid cartilage seems to be important also for the initiation of mineralization in human thyroid cartilage. Mineralization starts in a matrix containing gigantic collagen fibers (asbestoid fibers) in close proximity to cartilage canals, perichondrial invaginations of blood vessels, and connective tissue [36, 37]. In addition, this matrix contains less large aggregating proteoglycans than the non-calcifying thyroid cartilage matrix.

The role of proteoglycans in the calcification process is controversial. Studies have suggested that focal accumulations of the large aggregating proteoglycans in hypertrophic cartilage may be the nucleational site for calcification. Because of their high negative charge density, these proteoglycans would bind large amounts of Ca^{2+}-ions; the presence of free inorganic phosphate ions would then lead to salt precipitation and mineral deposition (for review see [38, 39]). Other studies, however, have demonstrated that large aggregating proteoglycans are inhibitors of calcification [40]. Our electron microscopic studies revealed that the calcifying, asbestoid fibers-containing extracellular matrix of human thyroid cartilage contains less large aggregating proteoglycans than the pericellular, noncalcifying matrix, sug-

gesting that a loss of proteoglycans may favor mineralization, and that these molecules are not involved in the initiation of mineralization. Mineralization also occurs in surface areas of osteoarthritic cartilage. These mineralized areas correspond to the zone of loss of proteoglycans [41, 42]. These and our observations are in agreement with previous findings showing that mineralization-competent matrix vesicles and vesicles that do not mineralize contain similar amounts of proteoglycans, and that the removal of proteoglycans from matrix vesicles isolated from hypertrophic growth plate cartilage does not change their ability to take up Ca^{2+} [7].

In conclusion, the results presented here show that matrix vesicles initiate mineralization of human thyroid cartilage. These vesicles contain ALP, annexins II, V, and VI, surface-attached types II and X collagen, and significant amounts of calcium and phosphate. Thus, they contain the same major components as matrix vesicles isolated from mineralizing growth plate cartilage [7, 43], suggesting that the mechanisms involved in the initiation and propagation of human thyroid cartilage mineralization are similar to those in mineralization of growth plate cartilage during endochondral ossification. Ca^{2+} influx during the initial phase of mineralization is mediated by the annexins, enabling the first crystal phase to grow from the preexisting nucleational core complex inside the vesicles [32]. In addition, annexin V mediates the binding of types II and X collagen to the matrix vesicle surface thereby anchoring the vesicles to the extracellular matrix; binding of these collagen also stimulates Ca^{2+} influx into the vesicles [11]. Once the intralumenal crystals have reached a certain size, they rupture the vesicle membrane and grow out into the matrix [6]. Since mineralization and ossification of human thyroid cartilage are much slower than during endochondral ossification, the thyroid cartilage provides an ideal system to study these processes.

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