An Ultrastructural Study of the Cells Involved in Endochondral Ossification in Humeral Growth Plates of Young Beagle Dogs

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Abstract: This study has attempted to clarify the processes of cartilage-to-bone replacement in endochondral bone formation of long bones. Three-week-old male beagle dogs were perfusion-fixed with buffered glutaraldehyde, and dissected distal humeri were examined by means of light and electron microscopy. Calcification of growth plate cartilage occurred in the central area of the longitudinal septa, but not in thin transverse septa. Chondrocytic lacunae were opened by the removal of transverse septa by perivascular mononuclear cells. These cells contained many RER cisterns, well-developed Golgi apparatuses, dense bodies, and pale vesicles, and they had developed membrane ruffling toward the transverse septa. Phagocytosis of cartilage fragments of longitudinal septa was also observed in mononuclear cells lacking membrane ruffling. A thin bone layer was deposited on the remaining longitudinal septa by invading osteoblasts. Osteoclasts in these lacunal canals lacked ruffled borders on the cartilage matrix, but formed these structures on the bone layer deposited on the longitudinal septa. Our results suggest sequential processes in the cartilage-to-bone replacement in growth plates: 1) removal of the transverse septa by perivascular RER-rich mononuclear cells, 2) formation of wider lacunal canals by phagocytosis of cartilage fragments in the longitudinal septa by mononuclear cells, 3) bone deposition over the remaining longitudinal septa, and 4) resorption of calcified cartilage and bone by differentiated osteoclasts.

Key words: growth plate cartilage, cartilage resorption, perivascular RER-rich mononuclear cells, osteoclasts.

Endochondral bone formation follows to terminal differentiation of chondrocytes in growth plate cartilage, in which calcification of longitudinal septa, erosion of transverse septa, and chondroblast death have been found to take place. Although the removal of uncalcified and calcified cartilage walls provides the pathways by which invading osteoblasts produce a bone layer over persisting longitudinal septa, the precise cellular mechanisms of cartilage removal in endochondral bone formation have yet to be fully elucidated. Osteoclasts, having been termed “chondroclasts,” have long been believed to be involved in calcified cartilage resorption during endochondral bone formation4). It is uncertain, however, whether chondroclasts are specialized cells in calcified cartilage resorption or are capable of resorbing both uncalcified and calcified matrices. Furthermore, osteoclasts are known to lack ruffled borders, the functional site of resorption, on uncalcified substratum9).
Cartilage resorbing cells have previously been recognized as being macrophages or fibroblasts in epiphyseal growth plates\textsuperscript{6-8}). Uncalcified cartilage has been reported to be resorbed by mononuclear phagocytic and/or fibroblast-like cells in rheumatoid joints\textsuperscript{9,10}). Moreover, Hunziker et al.\textsuperscript{11}) found monocytes associated with the ingrowth of vessels adjacent to uncalcified transverse septa and interpreted these monocytes as having initially penetrated an intact transverse septa and thus being responsible for the removal of chondrocytes and tissue debris. In fact, mononuclear phagocytes and macrophages are present at sites of calcified cartilage degradation in vivo and in vitro\textsuperscript{12-14}). Furthermore, in osteosclerotic (OC/OC) mice, despite a lack of ruffled borders in osteoclasts and completely disordered bone resorption\textsuperscript{15,16}), uncalcified and calcified cartilage can be removed and replaced by primary bone trabeculae. These observations indicate that uncalcified and calcified cartilage must be removed by cells other than the so-called chondroclasts.

In this regard, Lee et al.\textsuperscript{17}) have further suggested that the removal of uncalcified cartilage of transverse septa in epiphyseal growth plates is not due to chondroclasts, but it is a function of RER-rich mononuclear cells that secrete the proteolytic cysteine proteinase, cathepsin B. These cells were named “septoclasts,” specialized cells in the resorption of transverse septa. It is of great interest that septoclasts do not express the antigenicity common to monocytes, macrophages, and osteoclasts\textsuperscript{17}). Through ultrastructural observations of humeral growth plates in young beagles, we have attempted to clarify the ultrastructural features of cells involved in the resorption of uncalcified and calcified cartilage in the cartilage-to-bone replacement processes that occur during endochondral bone formation.

Materials and Methods

Three 3-week-old male beagle dogs were used in this study. The animal-use protocol was reviewed and all experiments were conducted according to the SOP code approved by the New Drug Development Research Center Committee, Inc. The animals were anesthesized with sodium pentobarbital and perfusion fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). The dissected distal humeri were further immersed in the same fixative for several hours at 4°C. Longitudinal slices (about 1 mm thick) of distal humeri were then postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, block stained with ethanolated 1% uranyl acetate, dehydrated through a graded ethanol series, and embedded in epoxy resin (Quetol 812, Nisshin EM, Tokyo). One-\textmu m and ultrathin sections were prepared with a diamond knife on a Reichert-Jung Ultracut Om U-4, stained with either toluidine blue (1-\textmu m sections) or tannic acid-uranyl acetate-lead citrate (ultrathin sections), and examined with an Olympus BHS light microscope or a Hitachi HU-12A electron microscope.

Results

1. Light Microscopic Observation

In the endochondral bone formation of distal humeri, the zones of chondrocyte proliferation, maturation, hypertrophy, and provisional ossification were clearly recognized. Most chondrocytes were distributed by the formation of regular longitudinal rows (Figure 1a). Thus cartilage matrix formed the longitudinal septa between adjacent chondrocytic rows and the transverse septa between chondrocytic lacunae within a row (Figure 1a). In the zone of provisional ossification, in which calcified cartilage was partially removed and bone deposition took place, the chondrocytic lacunae were opened by removal of the thin interlacunal partitious of cartilage matrix, the transverse septa (Figures 1a, b). This exposure of chondrocytic lacunae into the marrow cavity was also accompanied by the cell death of chondrocytes and the invasion of many mononuclear cells and capillary vessels into the opened lacunal canals (Figures 1a, b).

The longitudinal septa between adjacent rows of chondrocytes appeared to persist, forming the walls of opened lacunal canals. These remnants of longitudinal septa ultimately become enclosed in a thin bone layer, thus forming the primary bone trabeculae (Figures 1a, b). The longitudinal canals of opened chondrocytic lacunae were invaded by capillary vessels and many mononuclear cells (Figure 1b). These cells were in close contact with one another and with capillary endothelial cells, and the mononuclear cells generally exhibited an elongated or a cuboidal profile (Figure 1b).
chondroclasts were not observed over cartilage matrix in the zone of provisional ossification (Figures 1a, b). Differentiated osteoclasts were rarely observed over cartilage matrix in this zone, but were found along primary trabecular bones in the deeper zone of the metaphysis.

2. Electron Microscopic Observation

In transmission electron microscopic observations of the zone of provisional ossification in undecalcified ultrathin sections, clusters of electron-dense calcified granules were observed in the central portion of the longitudinal septa between the chondrocytic rows, but no calcified clusters were observed in the thin transverse septa between the lacunae within a row (Figure 2). Thus the cartilaginous walls of chondrocytic lacunae were not necessarily fully calcified.

Where the transverse septa had been removed, many perivascular mononuclear mesenchymal cells and capillary vessels with fenestrations were consistently observed (Figure 3). These mononuclear cells exhibited various cell configurations, but the usual profile was elongated or cuboidal (Figure 3). Mononuclear cells contained numerous polyribosomes, cisterns of rough-surfaced endoplasmic reticulum (RER), the Golgi apparatuses, mitochondria, dense bodies of various sizes and electron densities, and pale vesicles 50-60 nm in diameter (Figures 4a, b, c). The well-developed Golgi apparatuses consisted of 4-5 Golgi cisterns and pale Golgi vesicles of about 60 nm in diameter. The Golgi apparatuses formed an apparent Golgi area in the perinuclear cytoplasm, but secretory granules were not evident (Figure 4b). The nuclei were euchromatic with nucleoli and sometimes irregular in outline (Figures 3, 4a, b). Dense bodies and many pale vesicles were concentrated in the distal cytoplasm facing the transverse septa. Microtubules
were also distributed among these dense bodies and pale vesicles (Figure 4c).

These RER-rich mononuclear cells showed membrane ruffling only in apposition to uncalcified transverse septa (Figure 4d). Some cells extended long projections with membrane ruffling 0.2-1 μm deep toward transverse septa or open lacunae.

These areas of membrane ruffling lacked cell or-
ganelles and thus structurally resembled the osteoclastic ruffled border (Figure 4d). The formative rate of this membrane ruffling differed from cell to cell, with some cells having only very narrow ruffled cell surfaces. No clear zones were observed in them. In the area, from which transverse septa were being removed, these mononuclear cells always appeared in close association with capillary...
endothelium (Figures 3, 4a). Membranous fragments of cellular debris, perhaps derived from degenerated chondrocytes, were widely distributed in this region (data not shown).

Adjacent to these RER-rich mononuclear cells with membrane ruffling, RER-rich mononuclear
cells of cuboidal or round cell configurations contained membrane-bound cytoplasmic vacuoles, i.e., phagolysosomes, in which electron-dense round fragments of cartilage matrix were frequently observed (Figures 5a, b). These round fragments of cartilage matrix were frequently observed along the

Figure 8 An undifferentiated multinucleated osteoclast within a lacunal canal in the provisional ossification zone lacking the ruffled border-clear zone complex (a). A relatively differentiated multinucleated osteoclast with many cytoplasmic vesicles also lacks the ruffled border-clear zone complex facing the longitudinal septal cartilage matrix (b). a: ×3,000; b: ×5,000.

Figure 9 A fully differentiated osteoclast with a prominent ruffled border (RB) and clear zone (CZ) against the bone (Bo) and calcified cartilage matrices (CM) (a). A higher magnification view of the ruffled border and clear zones facing bone (Bo) and calcified cartilage matrices (CM) (b). a: ×4,000; b: ×12,000.
remaining longitudinal septa (Figures 2, 5a). The internalization of collagen fibers was not recognized in these mononuclear cells, which did not exhibit membrane ruffling toward the persisting septal cartilage (Figure 5a). Therefore these RER-rich mononuclear cells appeared to be involved in the formation of wider lacunal canals via phagocytosis of cartilage fragments in the longitudinal septa.

The osteoblasts were observed to form a single cell layer over the remaining longitudinal septa in opened lacunal canals in the ossification zone (Figure 6a). Concomitant to the removal of transverse septa, osteoblasts produced the first thin osteoid and bone layers over the remaining longitudinal septa (Figures 6, 7). In comparative observations of stained and unstained ultrathin sections, the first deposited bone matrix (0.3-0.7 μm wide) consisted of amorphous electron-dense materials, type-I collagen fibers, and mineral crystals, whereas the osteoid matrix consisted mainly of type-I collagen fibers (Figure 7).

Multinucleated osteoclasts were sometimes observed within these opened chondrocytic lacunae, but lacked structural polarity in cytoplasmic organization and also lacked ruffled borders in apposition to the exposed cartilage walls (Figures 8a, b). In the lower part of the provisional ossification zone, osteoclasts with prominent ruffled borders and clear zones were on the remaining septal cartilage covered with thin bone matrices (Figures 9a, b). In all cases examined, the induction of the osteoclastic ruffled borders and clear zones was closely associated with the presence of thin bone matrix over the remaining septal cartilage (Figure 9b).

**Discussion**

The present observations of humeral growth plate cartilage confirm that calcification of the cartilage matrix occurs in the longitudinal septa, whereas the thin transverse septa remain uncalcified. These uncalcified transverse septa appear to be degraded extracellularly and removed by perivascular RER-rich mononuclear cells, thereby creating chondrocytic canals. On the other hand, mononuclear cells without membrane ruffling incorporated cartilage fragments of longitudinal septa. Except for the absence of membrane ruffling, the structure of these mononuclear cells incorporating cartilage fragments resembled that of perivascular RER-rich mononuclear cells with membrane ruffling. It is uncertain, however, whether these mononuclear cells, those with and those without membrane ruffling, are an identical cell type. From the cartilage fragment incorporation, the RER-rich mononuclear cells are thought to be cartilage-resorbing cells essential for the resorption of uncalcified and calcified matrices needed to form wider lacunal canals.

Based on the observed structural features, these perivascular RER-rich mononuclear cells are thought to be identical to the “septoclasts” described by Lee et al.17 Because perivascular RER-rich mononuclear cells with membrane ruffling had no phagosomes containing cartilage fragments, they were assumed to secrete any proteolytic enzymes for cartilage degradation at an extracellular site. Cathepsin B in septoclasts is a possible candidate for the cartilage degrading enzyme17, but other proteolytic enzymes may also be involved in these processes. Cathepsin B is activated at an acidic pH18,19, but it is unlikely that the interface between transverse septa and the perivascular region is a sufficiently acidic microenvironment to activate secreted cathepsin B. Cole and Wezeman8 also characterized the perivascular cells in cartilage canals as RER-rich fibroblast-like cells with dense bodies, phagolysosomes, and cytoplasmic projections toward the cartilage matrix. They noted that such perivascular fibroblastic cells and the capillary endothelium were in contact with each other and with calcified cartilage8. Thus they speculated that fibroblastic cells secreted enzymes responsible for cartilage degradation. In this regard, fibroblastic cells20,21 and macrophages22,23, both present at the site of cartilage resorption, have been implicated in the secretion of chondrolytic enzymes. Although we have not yet characterized these perivascular mononuclear cartilage-resorbing cells, their structural similarity to fibroblasts and capillary endothelial cells is noteworthy because (1) capillary vessels appeared to have first invaded chondrocytic lacunae, and (2) this was always accompanied by the removal of transverse septa by cartilage-resorbing cells.

Takeuchi and Itakura24 found RER and lysosome-rich liberated chondrocytes in the calcifying zone and suggested that these cells might be respon-
sible for the digestion of uncalcified cartilage matrix. They also speculated that this cartilage digestion resulted in the induction of osteoclastic resorption of calcified cartilage. Although liberated chondrocytes were observed in this study, our cartilage-resorbing cells are structurally different from such liberated chondrocytes. It is also unlikely that liberated chondrocytes can resorb uncalcified cartilage because (1) liberated chondrocytes are not always present in significant numbers and (2) it remains unclear how such surviving chondrocytes, which are few, can resorb all transverse septa.

As the calcified cartilage of longitudinal septa is only superficially resorbed, the remaining calcified core is subsequently covered with a newly produced bone layer. This persisting cartilage core is thought to provide the site for primary trabecular bone deposition. The presence or exposure of calcified cartilage may not stimulate osteoclast recruitment. In fact, at sites where uncalcified and calcified cartilage are resorbed, no osteoblasts are found, and they are an essential coupling factor for terminal differentiation of osteoclasts\textsuperscript{25,26}. Soon after chondrocytic lacunae had been opened by cartilage resorbing cells, osteoblasts invaded the opened lacunal canals and immediately deposited a thin bone layer over the remaining longitudinal septa. This deposition may be an essential phenomenon for the induction of terminal differentiation of invading osteoclasts.

The multinucleated osteoclasts appearing in newly opened chondrocytic lacunal canals exhibited no cytoplasmic polarity and, most important, lacked ruffled borders, the site of bone resorption via secretion of protons\textsuperscript{27–29}, cathepsins\textsuperscript{30,31}, and other hydrolytic enzymes\textsuperscript{32–34}. It is therefore assumed that these ruffled borders lacking osteoclasts are unable to degrade or absorb uncalcified and calcified cartilage matrices. These cells developed prominent ruffled borders only in apposition to newly deposited bone matrix over persisting septal cartilage and appeared to degrade calcified cartilage and bone. We have already demonstrated that the presence of calcified matrix as bone and dentine is essential for the ruffled border induction in cultured osteoclasts\textsuperscript{33}. In the same culture system, we have also found that the coating of cocultured dentine slices with RGD peptides resulted in a disappearance of the osteoclast ruffled border and a prominent reduction in the resorption rate\textsuperscript{35}. This result suggests that any bone matrix constituents (possibly osteopontin including RGD sequences of amino acids) are necessary to induce the ruffled borders in osteoclasts. It can therefore be concluded that osteoclasts are not functioning as chondroclasts, which specifically resorb uncalcified and calcified cartilage matrix. Therefore so-called chondroclasts as osteoclasts\textsuperscript{1,3,8,14} are not thought to be present during cartilage resorption.

When all these results are taken together, the sequences of endochondral bone formation in humeral growth plate cartilage are defined as follows: (1) degradation and removal of the uncalcified transverse septa by perivascular RER-rich mononuclear cells concomitant with capillary vessel invasion into opened lacunal canals; (2) formation of wider lacunal canals by phagocytosis of cartilage fragments of longitudinal septa by RER-rich mononuclear cells; (3) the bone matrix production over persisting longitudinal septa by invading osteoblasts; and (4) resorption of both calcified cartilage and bone of longitudinal septa (primary bone trabeculae) by differentiated osteoclasts with ruffled borders and clear zones in the lower portion of the provisional ossification zone.

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Lucht U: Acid phosphatase of osteoclasts demonstrated by electron microscopic histochemistry.
