Citation: BoneKEy Reports **4**, Article number: 663 (2015) | doi:10.1038/bonekey.2015.30

www.nature.com/bonekey

ORIGINAL ARTICLE

Structural differences in epiphyseal and physeal hypertrophic chondrocytes

Control of osmolarity and the use of ruthenium hexammine trichloride for histologic and ultrastructural study

Frederic Shapiro and Evelyn Flynn

Department of Orthopaedic Surgery, Orthopaedic Research Laboratory, Boston Children's Hospital, Boston, MA, USA.

We have observed that epiphyseal and physeal hypertrophic chondrocytes in BALB/c mice show considerable differences of light microscopic and ultrastructural appearance, even when the cells are at the same stage of differentiation. In addition, cell structure maintenance improved with tissue preparation controlled for osmolarity and for membrane stabilization using 0.5% ruthenium hexammine trichloride (RHT) for both light microscopy (LM) and electron microscopy (EM) or 0.5% lanthanum nitrate for LM. Physeal hypertrophic chondrocytes showed a gradual increase in size closer to the metaphysis and a change in shape as cells elongated along the long axis. The nucleus remained central, with uniformly dispersed chromatin, and the rough endoplasmic reticulum (RER) was randomly dispersed throughout cytoplasm with little to no presence against the cell membrane. Even the lowermost cells showed thin elongated or dilated cisternae of RER and intact cell membranes. Epiphyseal chondrocytes remained circular to oval with no elongation. Nucleus and RER were positioned as a complete transcellular central nucleocytoplasmic column or as an incomplete bud with RER of the column/bud always continuous with RER peripherally against the intact cell membrane. RER was densely packed with parallel cisternae with adjacent cytoplasm empty of organelles but often filled with circular deposits of moderately electron-dense material consistent with fat. Optimal technique for LM involved fixation using glutaraldehyde (GA) 1.3%, paraformaldehyde (PFA) 1% and RHT 0.5% (mOsm 606) embedded in JB-4 plastic and stained with 0.5% toluidine blue. Optimal technique for EM used fixation with GA 1.3%, PFA 1%, RHT 0.5% and cacodylate buffer 0.03 M (mOsm 511) and post-fixation including 1% osmium tetroxide. These observations lead to the possibility that the same basic cell, the hypertrophic chondrocyte, has differing functional mechanisms at different regions of the developing bone.

BoneKEy Reports **4**, Article number: 663 (2015) | doi:10.1038/bonekey.2015.30

Introduction

The hypertrophic chondrocyte has a major role in endochondral bone development.[1–4] The earliest site of chondrocyte hypertrophy is at the centre of the diaphysis of a developing long bone at the primary centre of ossification. As development proceeds toward both ends of the bone, the hypertrophic cell zone is positioned at the lower part of the physis (growth plate) between the proliferating cell layer that immediately precedes it in the developmental sequence and the metaphyseal bone. After the physis is established, the epiphyseal cartilage persists, and its central region between the articular cartilage and the physeal cartilage undergoes hypertrophic chondrocyte change at specific times as a precursor to formation of secondary ossification centre bone. The hypertrophic cells of the developing secondary centre are initially positioned circumferentially but as development proceeds are present in a hemispheric pattern adjacent to the articular cartilage and side walls of the epiphysis at the regions of outward expansion[3] (**Figure 1**).

The hypertrophic chondrocytes at each site are invaded at specific time intervals by blood vessels and osteoprogenitor cells, and bone tissue is formed on calcified cartilage cores. The central hypertrophic diaphyseal cells are invaded from the newly formed periosteal vessels of the primary ossification centre,[3] the physeal hypertrophic cells from the outward reaches

Correspondence: Dr F Shapiro, Department of Orthopaedic Surgery, Orthopaedic Research Laboratory, Boston Children's Hospital, 300 Longwood Avenue, Boston 02115, MA, USA.
E-mail: fdshapiro@gmail.com

Received 17 April 2014; accepted 19 January 2015; published online 29 April 2015

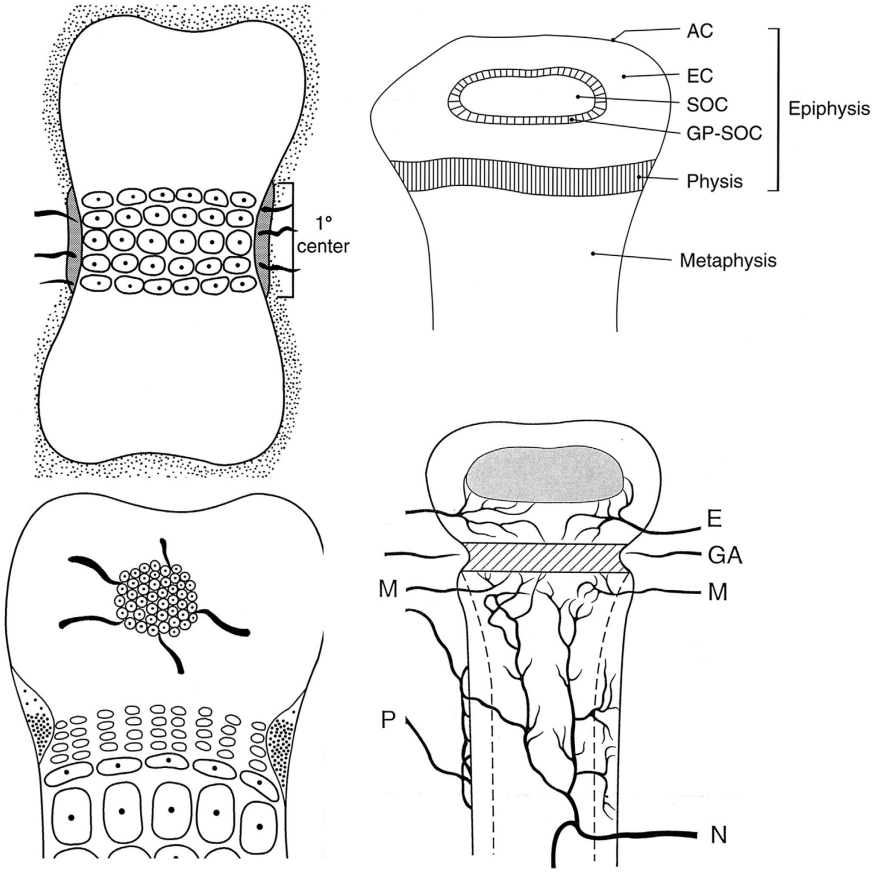

Figure 1 The first region of a developing bone to undergo chondrocyte hypertrophy is the central region of the bone (diaphysis) at the primary centre of ossification (upper left). Hypertrophic chondrocytes are seen next at the lower margin of the physis and then within the epiphyseal cartilage (secondary ossification centre) at the developing ends of the long bone (lower left). The regions of the developing end of a long bone are outlined at upper right. All the cartilage at the developing end is referred to as the epiphysis. This is subdivided into EC, epiphyseal cartilage; AC, articular cartilage; and physis, physeal cartilage. SOC, the secondary ossification centre, develops within the epiphyseal cartilage as central chondrocytes hypertrophy and then organize into a growth plate (physis), GP-SOC. Blood supply of the developing bone is seen at lower right. Epiphyseal vessels (E) supply the epiphyseal cartilage including the secondary ossification centre and the upper levels of the physis by diffusion. The blood vessels at the underside (metaphyseal side) of the physis enter from the periphery of the metaphysis (M) or are terminal branches of the intraosseous nutrient artery (N). The physis itself is avascular receiving its nutrition from a dual supply above and below. P, periosteal vessels and GA, perichondrial groove artery.

of the nutrient artery and metaphyseal vessels[5] and the epiphyseal hypertrophic cells from cartilage canals originating from the perichondrial vessels[6,7] (**Figure 1**). The best studied sequence of endochondral bone formation is the physis.[8–10] The lowermost 3–4 cells of the hypertrophic cell layer of the physis are invaded by metaphyseal vessels once their longitudinal cartilage trabeculae have calcified, and bone is then synthesized on the calcified cartilage cores. The hypertrophic chondrocytes are viable active cells[8,11–13] that synthesize type X collagen along with numerous other molecules, have a high level of vascular endothelial growth factor expression (which helps initiate vascular invasion from below) and eventually undergo apoptosis. Tissue preparation methods for microscopic examination can meaningfully alter the appearance of cells and either clarify or confuse any interpretation of their function. This is particularly true for the hypertrophic chondrocyte, an enlarged cell with increased fluid content whose organelles often appear to be sparse. Two major findings involving the importance of membrane stabilization[14–16] and the maintenance of intracellular osmolarity in a physiologic or near-physiologic range[17–19] during preparation of cartilage and especially of hypertrophic chondrocytes for histologic and ultrastructural examination have enhanced their structural stability. The hypertrophic chondrocytes at the developing diaphyseal, physeal and epiphyseal regions have always been considered to be the same by light microscopic criteria with no differentiating features described. This study, however, describes histologic and ultrastructural differences between epiphyseal and physeal hypertrophic chondrocytes in the BALB/c mice, which become more recognizable when specific structure maintaining steps in tissue preparation are taken. This leads to the possibility that the same basic cell, the hypertrophic chondrocyte, has different functional mechanisms at different positions in the bone.

Results

Light microscopy

Light microscopic (LM) sections indicated that the physes were well developed at distal metatarsal, proximal and distal femur, proximal tibia and proximal humerus and that the epiphyseal cartilage was undergoing central chondrocyte hypertrophy in this time frame. The region of epiphyseal central hypertrophy is relatively more extensive in the mouse than in larger vertebrates. Central chondrocyte hypertrophy was also occurring in some of

the tarsal bones. Vascular invasion and new bone formation of the secondary ossification centre were absent in some sections and underway in others. Both paraffin and plastic processed sections, in which the tissues had been fixed in 10% neutral buffered formalin, lacked sufficient cell detail to make the determinations described in this paper regarding the hypertrophic chondrocytes (method 1). Examination, however, of the 'thick' sections made as part of the tissue preparation process for ultrastructural studies began to demonstrate the findings (method 2). The findings were subsequently clearly defined by LM using method 3 in sections controlled for osmolarity, fixed with 1% PFA (paraformaldehyde) and 1.3% GA (glutaraldehyde) with either ruthenium or lanthanum fixation added, processed by the plastic embedding JB-4-Plus technique and stained with 0.5% toluidine blue (**Figures 2–4**). Using this preparation technique for LM, the cartilage cells remained intact including the hypertrophic cells of the physis and of the early developing secondary ossification centres within the epiphyseal cartilage. The nuclei remained round and the hypertrophic chondrocytes were for the most part filling each lacuna, which clearly implies outer membranes being intact and relating normally to the pericellular matrix. In cells undergoing condensation and early differentiation to chondrocytes, complete cell structure was maintained with the cell membranes immediately adjacent to the matrix (**Figure 2i**). The peripheral epiphyseal (**Figures 3i, ii**) and tarsal (**Figure 2ii**) cells maintained this appearance, but in the central epiphyseal regions the spectrum of characteristic appearing hypertrophic chondrocytes was seen (**Figures 2iii and 3i, ii**). Chondrocytes at the peripheral regions (**Figures 2iii and 3i, ii**) of the epiphyseal cartilage and in the physeal reserve, proliferating (palisading), and entire hypertrophic zones were well preserved filling each of their respective lacunae and showing intact nuclei and cytoplasm with diffusely dispersed structural content (**Figures 2iii and 3i, ii and iii**). The physeal hypertrophic chondrocyte structure was maintained with each cell filling its lacuna even at the lowermost region immediately adjacent to the advancing metaphyseal vessels and osteoprogenitor cells. The physeal hypertrophic chondrocytes had uniformly dispersed material (organelles) throughout the cytoplasm (**Figures 2i and iii and 3iii**), whereas the epiphyseal chondrocytes showed nucleocytoplasmic organization into either transcellular accumulations with clear appearing spaces on either side or buds from one part of the cell membrane into the cell interior (**Figures 2iii and 3ii**). This characteristic appearance was not seen in the physeal hypertrophic chondrocytes. Fixative solutions with osmolarity in the range of 600–730 (**Table 1**) and using RHT 0.5% preserved hypertrophic chondrocyte structure well. We also noted good preservation with lanthanum nitrate (LN) 0.5% as a fixative. **Figures 2 and 3** illustrate these findings with the 0.5% ruthenium fixation and **Figure 4** shows findings with 0.5% lanthanum, although very slight cell contraction was evident for some chondrocytes in the lanthanum group.

Transmission electron microscopy

Transmission electron microscopy assessed physeal and epiphyseal hypertrophic chondrocytes as they passed through their characteristic developmental cycles prior to undergoing vascular invasion as part of the endochondral sequence. Examination of sections that included hypertrophic chondrocytes of the epiphyseal cartilage and of the physis at the physeal–metaphyseal junction showed remarkable differences in hypertrophic chondrocyte ultrastructural appearance at the two sites. The physeal hypertrophic chondrocytes showed a gradual increase in size as development proceeded closer to the metaphysis along with a change in shape as the cell elongated along the longitudinal axis of the bone (**Figure 5i–iv**). The nucleus remained central, generally with uniformly dispersed chromatin, and the rough endoplasmic reticulum (RER) remained randomly dispersed throughout the cell cytoplasm as flattened cisternae or with some segments markedly dilated and containing a homogenous electron-dense substance. The nuclei were often irregular in shape in the tissue sections not fixed with ruthenium hexammine trichloride (RHT), but their roundness and nuclear membranes were preserved with RHT fixation (**Figures 5i, ii and iii**). Even the lowermost cells undergoing vessel invasion showed thin elongated and dilated cisternae of RER (**Figure 5iv**). The hypertrophic cell membrane at the cell periphery remained intact circumferentially and attached to the adjacent cartilage matrix in some of the preparations without RHT fixation, but in general cells were partially shrunken with the cell membrane pulled away from the adjacent matrix. Cell membrane continuity and positioning right at the matrix border and nuclear roundness was retained well with RHT (**Figures 5i–iv**). At the lowermost regions of the physis, blood vessels, red blood cells and undifferentiated mesenchymal cells originating from the metaphysis were seen entering and within the hypertrophic chondrocyte lacunae.

The epiphyseal hypertrophic chondrocytes also had intact cell membranes, which were immediately adjacent to the cartilage matrix (**Figures 6i–iv and 7i, ii**). Cell shape remained circular to oval, and the relative elongation of the physeal hypertrophic cell was not seen. The RER was arrayed in a different and a much more patterned manner. The nucleus and RER of the hypertrophic cell were positioned as a central complete transcellular nucleocytoplasmic column, or as an incomplete bud, but always with the (column/bud) RER continuous peripherally with the RER positioned against the intact inner cell membrane. The RER was densely packed with parallel cisternae in the regions it was present (inner cell membrane or central nucleocytoplasmic column/bud), whereas the cytoplasm, except for the column/spur, was empty of organelles (**Figure 6i–iv and 7ii**). This pattern was initially seen in tissues processed without RHT but was preserved and made clearer when RHT was added. The cytoplasmic spaces became increasingly prominent relative to the nucleus and RER as the epiphyseal chondrocytes hypertrophied. Although devoid of organelles they often contained moderately electron-dense globular material consistent with intracellular fat, seen most clearly in **Figures 6ii, iv and 7i and ii**. The nucleus almost always had patchy chromatin condensations. The nuclei were slightly to moderately irregular in shape in the tissue sections not fixed with RHT, but they retained their round shape in the RHT specimens (**Figures 6 and 7**). This was seen in sections both pre-vascular invasion and adjacent to early vascular invasion and bone formation. The hypertrophic chondrocytes were always immediately adjacent to the surrounding cartilage matrix with RHT fixation, whereas some without RHT partially retracted or collapsed within their lacunae away from the matrix at the periphery. At the cell periphery, the RER was clustered focally against the cell membrane (**Figures 6ii–iv and 7ii**) with a cytoplasmic collection passing continuously into the centre of the cell adjacent to the nucleus. The central and peripheral

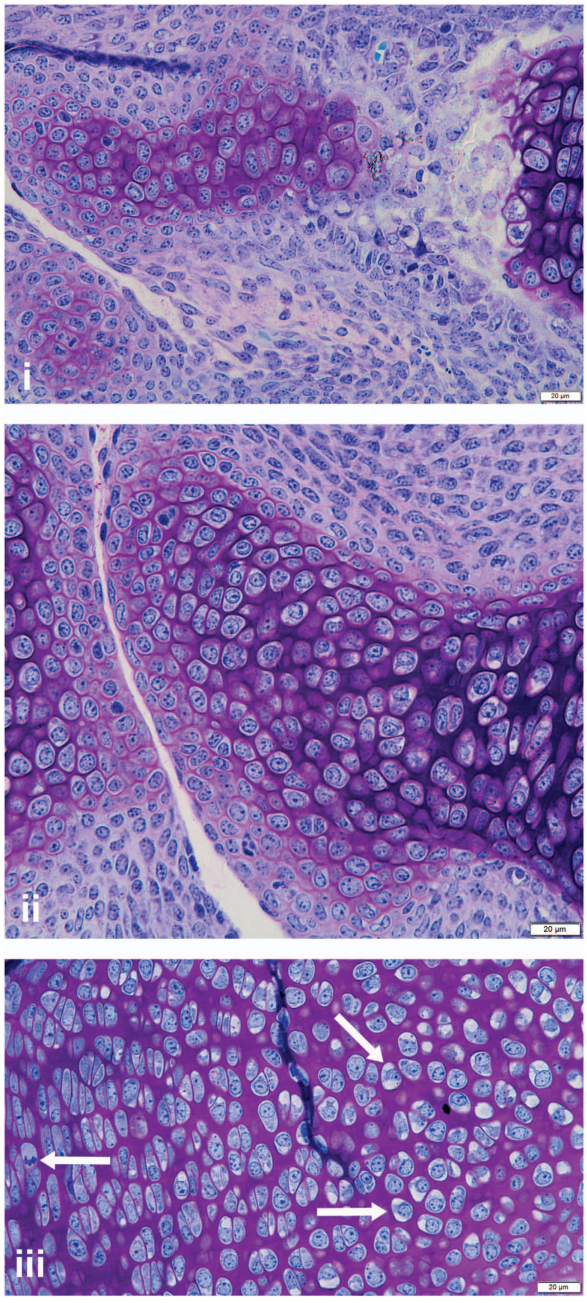

Figure 2 Light microscopy photomicrographs illustrate physeal and epiphyseal chondrocytes (newborn, lower extremity). Oblique section through a developing metatarsal shows (**i**) cartilage condensation (left), more differentiated cartilage centrally and physeal region (right). Chondrocytes are well preserved, including all physeal hypertrophic chondrocytes that fill each lacuna. (**ii**) Tarsal region. At left of joint cleft all chondrocytes are well fixed. To right, peripheral chondrocytes fill each lacuna, whereas toward the centre of the bone chondrocyte hypertrophy begins. A few cells show early characteristic changes (similar to those of epiphyseal cartilage) of central nucleocytoplasmic components and more peripheral lighter staining regions where cytoplasmic changes occur. (**iii**) Central region of a metatarsal bone showing part of physis at left (arrow pointing to a dividing proliferating layer chondrocyte). At right, the two arrows point to a characteristic early hypertrophic chondrocyte with central nucleocytoplasmic components and clearer staining spaces at periphery of cell in lacuna. Tissues were fixed in 1% PFA, 1.3% GA and 0.5% RHT (mOsm 606), embedded in JB-4 plastic and stained with 0.5% toluidine blue. Bars = 20 μm.

collections of RER were always continuous at least at one site per cell profile (bud) and often at two opposite sides of the cell (transcellular column; **Figures 6ii–iv and 7ii**). In early hypertrophic chondrocytes, the central RER usually formed a complete transcellular nucleocytoplasmic septum in continuity with the peripheral rim of RER adjacent to the intact cell membrane on both sides of the cell profile. At later stages, the nucleocytoplasmic collection was not completely transcellular but formed a bud-like accumulation, still always continuous with the RER against the inner cell membrane at one region of the cell profile.

Examination of the complete sequence of physeal hypertrophic cell ultrastructure from the proliferating layer of the physis to the end of the hypertrophic zone, in several hundred sections from several mice and the different epiphyses, always showed the RER randomly arrayed (**Figures 5ii–iv**) and specifically did not show the pattern of RER positioning as identified and described above in the epiphyseal hypertrophic cells. For most physeal cells, the cytoplasm was otherwise clear showing no electron-dense material or inclusions. Electron-dense material was seen in epiphyseal chondrocytes, however, to variable extents. The circular electron-dense material consistent with fat was never seen in the physeal hypertrophic chondrocytes. The ultrastructural assessments confirmed the findings described above whether assessing extremely small sections cut to involve the epiphyseal cartilage alone, the physeal–metaphyseal junction alone or narrow individual slices incorporating epiphyseal, physeal and metaphyseal regions in continuity on the same section. The ultrastructure of resting and proliferating chondrocytes appeared intact regarding cell membranes, nuclear shape and endoplasmic reticulum appearance in cells with and without RHT fixation. It was the hypertrophic chondrocytes fixed with RHT that maintained their structure best, whereas those without showed elements of shrinkage, withdrawal of the cell membranes from the adjacent matrix and irregular nuclear shapes.

Discussion

We report that the hypertrophic chondrocytes of the epiphyseal cartilage are structurally different from the hypertrophic chondrocytes of the physeal cartilage in the young post-natal BALB/c mice. These cells are at the same stage of structural differentiation based on light microscopic histology[3] and positive immunohistologic staining for vascular endothelial growth factor (not shown) at both sites, indicating imminent vascular invasion as part of the endochondral sequence.[20,21] Specific attention to controlling osmolarity toward a physiologic range, membrane stabilization with RHT and the use of plastic embedding for light microscopic sections allows these differences to be detected at both the light microscopic and ultrastructural levels. These are not occasional or isolated changes but characterize virtually all the hypertrophic physeal and epiphyseal chondrocytes in the BALB/c mice at this time frame (newborn to 1-week post-natal). In the physeal hypertrophic chondrocyte at the ultrastructural level, the RER is randomly arrayed and the nucleus central. The epiphyseal hypertrophic chondrocyte has a specific appearance markedly different from its physeal counterpart regarding the central nucleocytoplasmic column or bud and its continuity with peripheral RER collections. In the epiphyseal hypertrophic

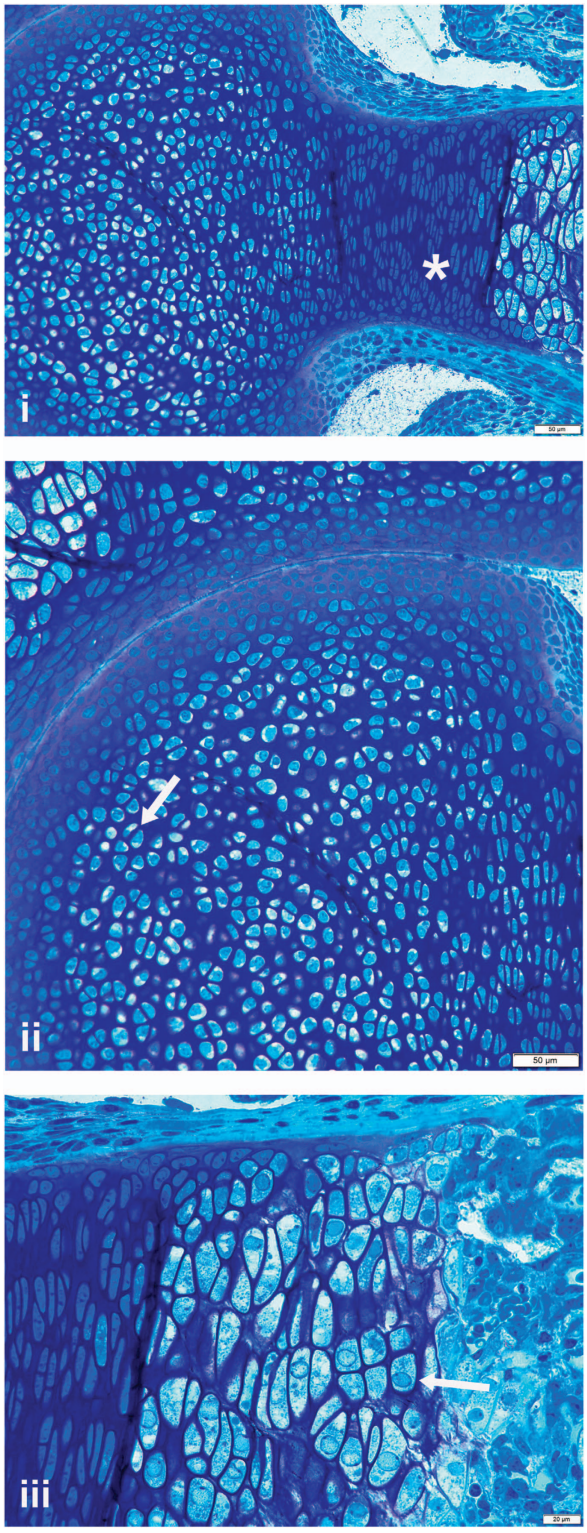

Figure 3 (**i**) Femoral head is present at left, physis (*) at centre and hypertrophic zone of physis at far right. All chondrocytes of physis are intact. Central cells within epiphysis (femoral head) show early hypertrophic changes. Bar = 50 μm. (**ii**) Higher power view of femoral head epiphysis shows characteristic epiphyseal hypertrophic cell changes, (arrow) and centrally. Bar = 50 μm. (**iii**) Higher power view of physis shows virtually all hypertrophic chondrocytes intact and filling their lacunae with preserved circular nuclei also seen. Even at the lowermost level, the hypertrophic cells remain intact (arrow). Bar = 20 μm. Tissues were fixed in 1% PFA, 1.3% GA and 0.5% RHT (mOsm 606), embedded in JB-4 plastic and stained with 0.5% toluidine blue.

chondrocyte, the RER is peripherally arrayed and continuous with a bud-like or complete transcellular central nucleocytoplasmic column composed of the nucleus and RER. Our findings appear to be the first to differentiate hypertrophic cell structural differences at the two regions. Maintenance of the same pattern of findings in specimens fixed with RHT and controlled for osmolarity on multiple assessments at different epiphyses further supports this contention. The physeal findings at the ultrastructural level have been well defined previously along with the improved membrane stabilization induced by the use of RHT in fixation.[9–16]

The hypertrophic cells of the physis had intact cell membranes, including those cells at the lowermost end immediately adjacent to sites of vascular invasion. Sections were seen where intact vessels containing red blood cells and undifferentiated mesenchymal cells entered the hypertrophic cell from the metaphysis. In both light microscopic and ultrastructural studies, the hypertrophic chondrocyte cell membranes were immediately adjacent to the cartilage matrix of the longitudinal columns and transverse septae. Epiphyseal hypertrophic chondrocytes also had intact cell membranes throughout along with being immediately adjacent to the cartilage matrix. The nuclei maintained their round shape. These findings are well accepted as ultrastructural indicators of appropriate cell fixation. It is thus realistic to interpret the differing RER and nucleocytoplasmic patterns of physeal and epiphyseal hypertrophic chondrocytes as being structurally site specific rather than due to fixation artefacts. At the light microscopic level (**Figures 2–4**), the differentiating epiphyseal and physeal changes were seen on single microscopic sections, which had been exposed to the same preparation technique. Most of the ultrastructural sections also included continuous cartilage tissues from epiphyseal hypertrophic chondrocytes to epiphyseal and physeal cartilage including physeal hypertrophic chondrocytes. Epiphyseal and physeal chondrocyte comparisons were made on tissues processed by the same methods at the same time and often on the same tissue sections, eliminating concern of differing preparation environments explaining the findings. The ultrastructural observations were made initially on cells processed without RHT but were seen in technically improved cells fixed and processed in an environment controlling for osmolarity and using RHT fixation.

Previous studies have been made almost exclusively from physeal hypertrophic chondrocytes and have not, with few exceptions, assessed hypertrophic epiphyseal chondrocytes as it has been assumed that cell changes in the epiphysis were the same as those in the physis. There have been a large number of structural studies on physeal growth cartilage where light microscopic,[10,22] light microscopic and ultrastructural,[12–16] and ultrastructural[8,9,11,23,24] photomicrographs define the appearance of the hypertrophic chondrocyte. With good fixation, the large majority of physeal hypertrophic chondrocytes are present as hydrated cells with central nuclei, cytoplasmic RER uniformly dispersed and intact cell membranes directly attached or immediately adjacent to the pericellular matrix. The physeal hypertrophic cells in our study (**Figure 6**) were similar to those described previously.

The epiphysis has been studied at the light microscopic level, and its pattern of development, including secondary ossification centre formation, is by the endochondral sequence.[1–4,25] Cartilage canals in the development of the

Table 1 Fixative solutions for light and electron microscopy

	N	*mOsm*	
Light microscopy			
GA 1.3%, PFA 1%, RHT 0.5%	6	606 (r: 594–616)	
GA 1.3%, PFA 1%, LN 0.5%	3	732	
GA 1.3%, PFA 1%, LN 1%	3	954	
Electron microscopy			pH
Solution A	6	450	6.8
GA 2%, RHT 0.7%, cacodylate buffer 0.05M		(r:347–516)	
Solution B	6	283	7.0
GA 1.3%, RHT 0.5%, cacodylate buffer 0.03M		(r:250–318)	
Solution C	6	511	7.2
GA 1.3%, RHT 0.5%, PFA 1%, cacodylate buffer 0.03 M		(r:427–653)	

Abbreviations: GA, glutaraldehyde; LA, lanthanum nitrate; N, number of osmolarity assessments; PFA, paraformaldehyde;r, range; RHT, ruthenium hexammine trichloride.

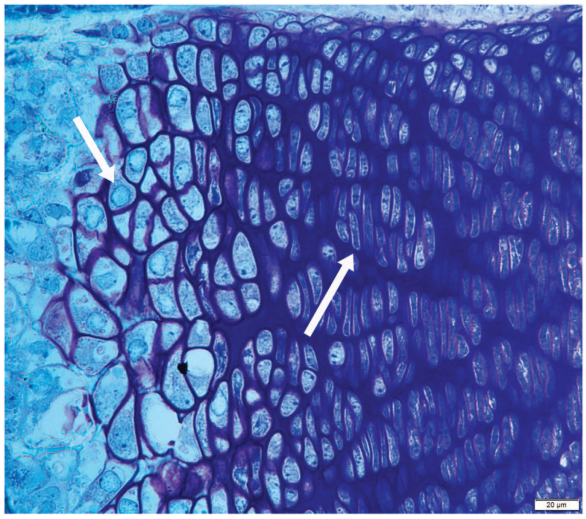

Figure 4 Cell membrane stability is also seen with lanthanum nitrate fixation in newborn metatarsal. Physis shows narrow circumferential clear space around each proliferating chondrocyte (right arrow) representing only minimal cell retraction. The hypertrophic zone chondrocytes are well stabilized (left arrow). Tissues were fixed in 1% PFA, 1.3% GA and 0.5% LN (mOsm 732), embedded in JB-4 plastic and stained with 0.5% toluidine blue. Bar = 20 μm.

secondary ossification centre have been well studied.[1,3,6,26] In the rabbit,[3,6] dog[27] and higher species including humans, the canals provide a source of nutrition to the enlarging epiphyseal cartilage and, several days to weeks later, vessels and cells for formation of the secondary ossification centre bone. In the mouse, the canals directly supply the secondary centre without a preceding nutritive role.[28] None of these studies, however, assessed specifics of individual epiphyseal hypertrophic cell structure.

Electron microscopic studies of epiphyseal tissue have been performed much less frequently. An early study assessed osteogenesis of the epiphysis but made no comments on the appearance of the hypertrophic chondrocytes.[29] Other studies included epiphyseal as well as physeal chondrocytes in the context of assessing their relationship to programmed (or physiologic) cell death and did not describe the differences we have noted.[30] It appears that the dissimilarity between physeal and epiphyseal chondrocytes identified in our study has not been appreciated or illustrated previously.

Once our observations were made ultrastructurally, we were able to see the differences between physeal and epiphyseal hypertrophic chondrocytes at the light microscopic level by examining the one micrometer 'thick' sections (from which the ultrastructural section had been cut). These tissues had undergone fixation and embedding in a far more specific way than is done normally in preparing tissues for LM by paraffin or plastic embedding. In the 'thick' sections stained with toluidine blue, we identify hypertrophic epiphyseal chondrocytes with central nuclei and adjacent cytoplasmic components continuous with the cell wall and either traversing the interior of a cell completely or bulging into the cell interior but not completely crossing to the opposite cell membrane. The resolution powers of LM cannot define the RER. When tissues are prepared for light microscopic examination using paraffin or plastic embedding after fixation with the commonly used 10% neutral buffered formalin the hypertrophic chondrocyte is not preserved well enough and the phenomenon is missed. It is now apparent that meaningful assessment of hypertrophic chondrocyte structure at the LM level requires tissue preparation techniques similar to those used for transmission EM.[10,15,17] We applied such techniques for plastic embedded sections controlling both for osmolarity and using the membrane stabilizing chemical RHT widely used for assessments of cartilage at the ultrastructural level. This approach allowed us to observe the same differentiating feature between epiphyseal and physeal hypertrophic chondrocytes at the light microscopic level.

We also studied LN as a fixation agent. Lanthanum has been used as an ultrastructural post-fixation agent to enhance visualization of gap junctions but also as a cell membrane stain owing to its affinity for membrane polysaccharides.[31–34] LN in 1% preparation showed osmolarity of 954 mOsm kg^{-1} but a 0.5% concentration lowered osmolarity to 732 mOsm kg^{-1}, and the 0.5% concentration only was used for LM fixation. There was a similar qualitative appearance of lanthanum and ruthenium processed sections in terms of improved hypertrophic chondrocyte structural preservation, but ruthenium was qualitatively slightly superior to lanthanum (see **Figures 2 and 3** for RHT fixation and **Figure 4** for LN fixation). There was slight cell contraction of all chondrocytes with lanthanum, although physeal hypertrophic cell preservation was still very good (**Figure 4**). Both are cationic dyes. Shaklai and Tavassoli,[31] assessing lanthanum as an ultrastructural stain, specifically noted its similarity of action with ruthenium.

Several techniques for tissue fixation and processing of growth cartilage, and particularly of hypertrophic chondrocytes, have been considered to have a role in maintaining normal cell and pericellular matrix structure and eliminating or at least minimizing artefactual change. Simple bisection of bone before fixation significantly reduces shrinkage artefact and fixation appears to be enhanced by limiting the time needed for the fixative to reach the cell by diffusion.[19] Cutting the tissue immediately upon removal into even smaller pieces as well as using newborn to 1-week-old tissues as in this study would be expected to further enhance fixation. Tissue preparation methods are a major consideration in interpreting structural findings at various levels of microscopic resolution. Recent studies on minimizing hypertrophic chondrocyte shrinkage by

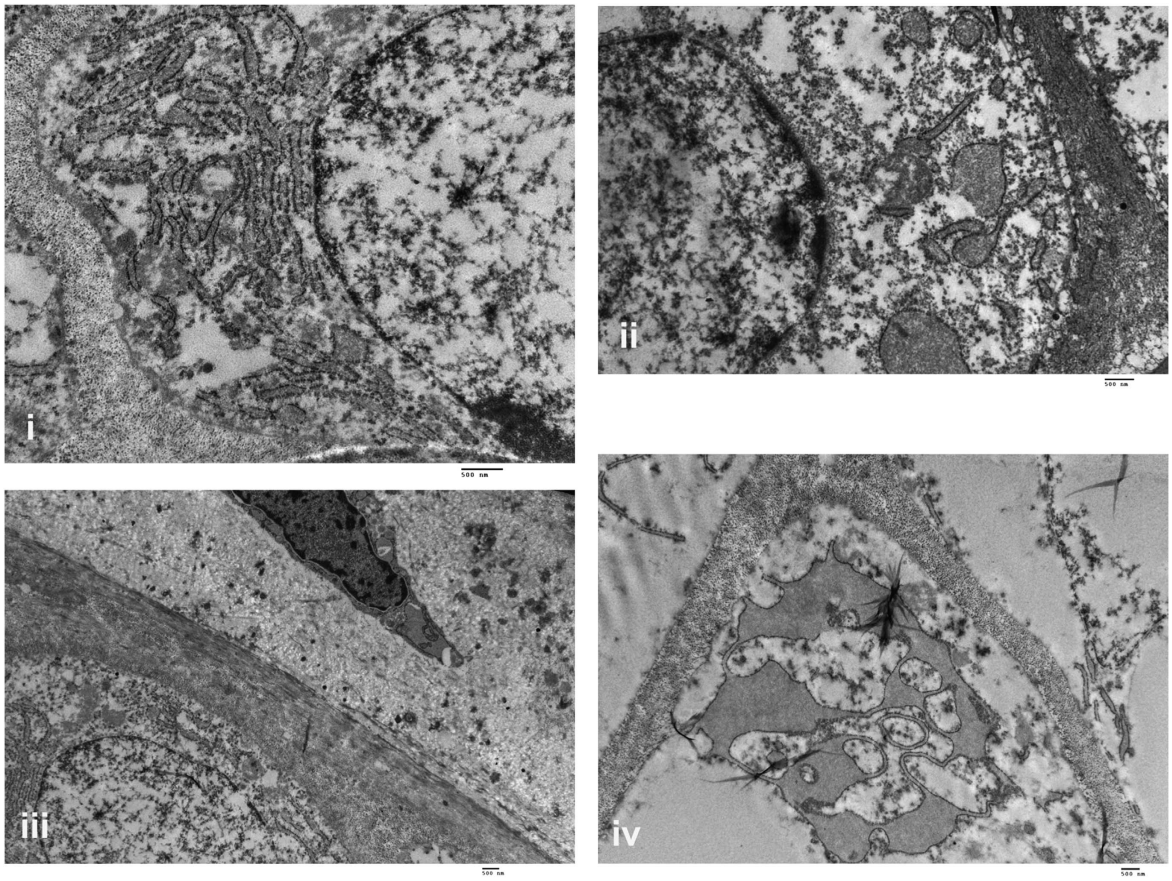

Figure 5 Electron micrographs illustrate physeal chondrocytes from upper hypertrophic zone passing to lowermost hypertrophic cells at the metaphyseal junction. Distal femur, newborn, solution B, mOsm 283. (**i**) Intact nucleus is seen at right, RER centrally and cartilage matrix at left. At this level and time in the sequence the RER is less densely packed. (**ii**) Further toward metaphysis the nucleus remains intact, cell membrane at right is intact adjacent to cartilage matrix and RER is diminished but present and active (dilated cisternae) with more space seen as cell hypertrophy increases. (**iii**) Intact hypertrophic chondrocyte, lower left, with circular nucleus, cell membrane intact and apposed to surrounding matrix, and some RER present. At upper right an osteoblast is synthesizing matrix onto the cartilage septum. (**iv**) Hypertrophic chondrocyte at lower level still shows active RER in the characteristic physeal conformation with linear and dilated cisternae randomly dispersed within the cytoplasm. The cytoplasm and cell membrane are immediately adjacent to the cartilage matrix implying good fixation. Bars = 500 nm.

adjusting fixative solution osmolarity to physiologic levels assessed cells by confocal microscopy and fluorescently labeled fixed and living chondrocytes.[19] This method displays the cell membrane well in relation to the lacuna wall but allows for little to no appreciation of nuclear and organellar structure. Different fixation and embedding are needed to control both for osmolarity, as well as pH and membrane, nuclear and organellar structure for LM and EM visualization. Hunziker and Schenk studied cartilage ultrastructurally using high pressure freezing, freeze substitution and low temperature embedding to demonstrate that all physeal hypertrophic chondrocytes remained morphologically intact without shrinkage or degeneration up to the point of blood vessel ingrowth.[12,16] Most of their hypertrophic chondrocyte studies, however, used the RHT chemical fixation technique[12,14–16,] which also was found to fully preserve cell shape and volume, although occasionally forming intracellular vacuoles. Our method of tissue processing has attempted to balance osmolarity, pH and fixation properties so as to maintain hypertrophic chondrocyte volume (prevent shrinkage), intact cell membrane relationships to the immediately adjacent matrix and both intracellular nuclear and cytoplasmic structure. These include adaptations of (i) the use of the cationic dye RHT,[14–16] (ii) rapid tissue fixation in a cold environment immediately at the time of euthanization,[23,24] (iii) sectioning of tissue into very small fragments to allow the fixative to diffuse quickly throughout the tissue,[13,14,19,23,24] (iv) maintenance of a physiologic osmolarity of the fixation solution[17–19,35,36] and (v) bathing the tissue in fixative as it is cut into small pieces. In this study, we adopted each of these mechanisms while attempting to strike a balance between osmolarity and the degree of fixation needed for both LM and ultrastructural observation. Excellent cell and cartilage matrix preservation for light microscopic and ultrastructural study have been achieved using fixation with 0.7% RHT[14,15,] and most studies now incorporate this method into studies of growth plate ultrastructure.[9,11,13,35] Although reasonably good ultrastructural preservation was obtained without the use of RHT, our second set of ultrastructural experiments with RHT markedly improved fixation, especially of the hypertrophic cells.

The intact cell membranes closely apposed to the pericellular matrix and the specifically patterned RER show that the fixation allowed for good hypertrophic cell preservation. The physeal and epiphyseal regions from each individual bone were dissected and prepared together and thus underwent the same processing. Many of the ultrastructural tissue sections spanned

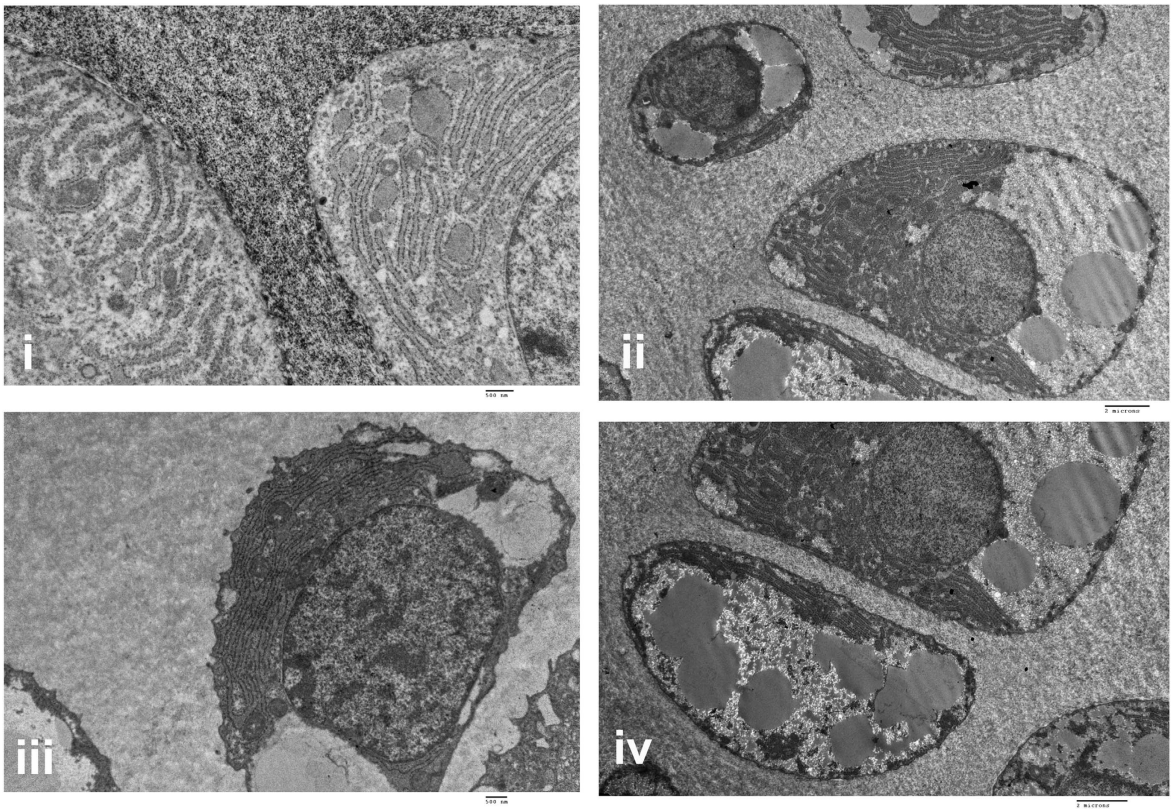

Figure 6 Electron micrographs illustrate epiphyseal hypertrophic chondrocytes. **i**, **ii** and **iv** illustrate metatarsal epiphysis, 1 week of age, solution C (including PFA 1%), mOsm 511; **iii** is from distal newborn femur, solution B, mOsm 283. (**i**) At peripheral region adjacent to central hypertrophic area parts of 2 active chondrocytes are seen. The RER is present but less densely packed than in the most peripheral regions (not shown), nuclear sections are round with membranes intact and cell membranes are immediately adjacent to cartilage matrices. Bar = 500 nm. (**ii**) Parts of four cells are all intact (cell membrane against matrix). In each hypertrophic cell, the RER is immediately adjacent to the cell membrane, a pattern which is not seen in the physeal hypertrophic cell. RER is not randomly dispersed within the epiphyseal cell cytoplasm but is either part of the nucleocytoplasmic mass or packed against the peripheral cell membrane and continuous with the nucleocytoplasmic mass. As the cytoplasm lacks RER presence, it often contains moderately electron-dense bodies consistent with intracellular fat. Bar = 2 μm. (**iii**) Electron micrograph illustrates an adjacent epiphyseal hypertrophic chondrocyte at a more advanced stage. Note nucleocytoplasmic continuity across central cell diameter. Peripheral regions of cytoplasm are empty of RER or other organelles. Bar = 500 nm. (**iv**) Metatarsal epiphysis, showing advanced stages of the epiphyseal hypertrophic chondrocytes continuous with image in ii with characteristic organellar patterns. There is RER packing against cell membrane at periphery and intracytoplasmic material consistent with fat. Bar = 2 μm.

the region continuously from the epiphyseal hypertrophic cells to the physeal hypertrophic cells and adjacent metaphysis. As the physeal hypertrophic cells were structurally intact, it appears reasonable to conclude that the epiphyseal hypertrophic chondrocytes represent an accurate depiction of differing structure compared with the physeal cells.

Cell shrinkage in growth plate chondrocytes has been demonstrated with commonly used fixative agents, including RHT, if the solutions are hyper-osmotic.[19] Osmolarity greater than 1000 mOsm leads to extensive cell shrinkage and can be associated with fixation using 0.7% RHT when the solution also contains PFA 2% and GA 2% along with 100 mM cacodylate buffer.[19] When osmolarity was adjusted in the fixatives to the osmotic pressure of normal extracellular fluid (~280 mOsm), the shrinkage artefact was minimized or prevented. The effects on cell shrinkage of several fixative combinations indicated that other factors than the use of 0.7% RHT affected the outcome.

Hypertrophic physeal chondrocytes have considerable biosynthesis capability *via* the endoplasmic reticulum and provide longitudinal growth by cell hypertrophy (generally considered to be swelling) and longitudinal axis orientation.[9,11–13] They are active cells synthesizing molecules even at the terminal cell levels where metaphyseal vessel invasion is occurring. Ultrastructural findings in both the physeal and epiphyseal chondrocytes are clearly supportive of these functions. The osmotic effect on hypertrophic cell volume has been appreciated and has a major role in assessing cell function for longitudinal physeal growth, as well as determining structural stability during fixation for microscopic studies.[17,18,36] The osmotic sensitivity of growth plate chondrocytes appears to be the same in the proliferating and hypertrophic cell zones even though cells in the latter zone are significantly larger. Recent understanding has the hypertrophic cells enlarging by the process of cell swelling due to fluid (water) accumulation. This would predict an increase in osmolarity at the hypertrophic zone cells as fluid accumulation occurs by osmosis with water drawn across the cell membrane into the cell by a higher concentration of organic and non-organic osmolytes.[17,18] The finding of no change in osmolarity, however, supports cell hypertrophy occurring by an increase in non-osmotically active cellular matter such as amino acids and sugars, which then synthesize intracellular proteins to increase cell volume. In the epiphyseal hypertrophic chondrocytes, large cytoplasmic regions, while devoid of organelles, contain moderately electron-dense, randomly dispersed globular material, the significance of which is not yet known.

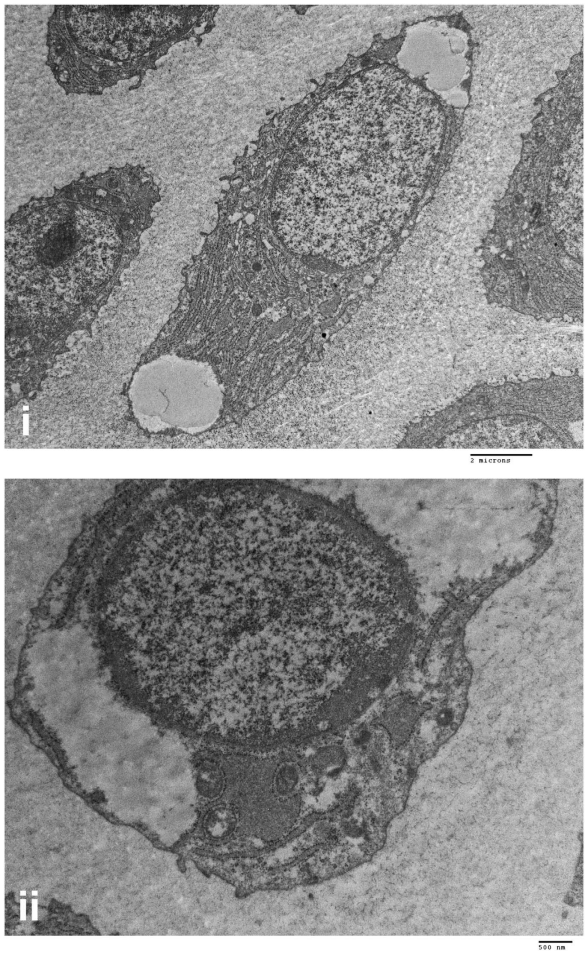

Figure 7 Electron micrographs are from newborn distal femur, solution B, mOsm 283. (**i**) Epiphyseal chondrocyte at periphery of hypertrophic region shows beginning hypertrophy with central nucleocytoplasmic region intact and peripheral clear intracytoplasmic spaces forming to either side with mildly electron-dense homogeneous material. Peripheral rim of RER persists around these clear areas. Bar = 2 μm. (**ii**) Closer toward the centre of the hypertrophic region the cell is intact and oval. The cell membrane is intact, nucleus round and the RER adjacent to the nucleus is active (dilated cisternae) and continuous with peripheral rim of RER. The cytoplasm to either side of the central nucleocytoplasmic column is clear and without organelles or material inclusions. Bar = 500 nm.

At the lower few cells of the physeal hypertrophic zone, the hypertrophic chondrocyte is generally considered to undergo apoptosis as part of its role in the terminal stages of the endochondral sequence leading to bone formation[20,] but the vast majority of hypertrophic chondrocytes do not develop the classic structural appearances described for apoptotic cells. In the mouse, with appropriate fixation and attention to osmolarity, the lowermost physeal hypertrophic cells show round intact nuclei, dispersed RER and intact cell membranes as they are invaded by the advancing mesenchymal cell/vascular collection from the metaphyseal side.

The differences in appearance of the physeal and epiphyseal hypertrophic chondrocytes led to a separate study using laser capture microdissection and GeneChip microarray analysis to assess hypertrophic chondrocytes at the same stage of histologic development in 7-day-old post-natal BALB/C mice.[37] Molecules upregulated in markedly higher levels in the epiphysis were gremlin (58-fold) and epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (25-fold), whereas molecules upregulated in higher levels in the physis were proline arginine-rich end leucine-rich repeat protein (PRELP; 15.6-fold), pyrophosphatase (inorganic) 1 (10-fold) and hedgehog-interacting protein (7.3-fold). The microarray study indicated an apparent site-specific role for hypertrophic chondrocytes in these two separate regions. Laser capture microdissection and microarray analysis appears valuable to correlate structure and molecular composition[38] in growth cartilage.

It is not known why the intracellular cytoplasm has mildly to moderately electron-dense lipid appearing collections in areas without organelles, whereas the physeal hypertrophic chondrocyte is clear with these materials never accumulating. Fat has been noted by LM in frozen sections of proliferating chondrocytes (fine lipid droplets with oil red staining)[14] and at the ultrastructural level in articular and costal cartilage.[39]

It remains to be demonstrated whether the structural differences outlined in this investigation are present at all ages in the developing BALB/c mice and in other vertebrate species. As a general rule, three-dimensional structure and intracellular nucleocytoplasmic organization of cells of the musculo-skeletal system have subsequently been found to have functional significance. This applies, for example, to the longitudinal growth of the physis conferred by the hypertrophic cell region itself and the continuing synthesis of site-specific molecules by the persisting RER of the hypertrophic cells. The concept of cellular self-organization involving dynamical cell shapes, intracellular organellar positioning and intranuclear compartmentalization is increasingly recognized as having functional implications.[40,41] It appears likely that intracellular water alone is not the sole mechanism for cell hypertrophy, which conceivably also involves the creation of a gel-like substance internally as well as molding by surrounding extracellular matrix pressures externally. The differences we describe might be related to the differing mechanical constraints on the cells at the two regions. Those in the physis are involved in well-oriented longitudinal growth, whereas the epiphyseal chondrocytes at this stage are involved in multiplanar circumferential epiphyseal expansion.[3] Extrinsic biophysical forces could be involved not only in shaping the configuration of the entire cell but also in affecting the internal nucleocytoplasmic structure. The orientation of the adjacent matrices in the two regions partially directs the shape of the chondrocytes; the restrictive circumferential tissues of the perichondrial ossification groove of Ranvier surround the physis, whereas the much less restrictive perichondrium surrounds most of the epiphysis. The study indicates (i) that preparation techniques maintaining osmolarity at or near-physiologic levels and enhancing membranes, nuclei and cytoplasmic organelles help maintain LM and EM hypertrophic chondrocyte structure; and (ii) the differing morphologies of physeal and epiphyseal hypertrophic chondrocytes possibly underlie site-specific structure–function relationships.

Materials and Methods

The study was performed in the BALB/c mice. Ages at study were newborn, 2 and 3 days and 1 week. Proximal and distal femoral, proximal tibial and proximal humeral epiphyseal–metaphyseal regions were studied along with distal metatarsal

and adjacent tarsus, as paraffin embedded toluidine blue stained sections studied by LM for other purposes had shown that at these time periods each of these physes was well established, whereas the chondrocytes of the central regions of the epiphyseal cartilage (and tarsal cartilage) were undergoing hypertrophic changes. Each mouse was killed either by decapitation or an intraperitoneal injection of sodium pentothal. The experimental studies were approved by the institutional review board.

Light microscopy

Tissues were prepared three ways for light microscopic assessment.

Method 1. For several years in our laboratory, the ends of the bones were quickly removed after euthanization, fixed in 10% neutral buffered formalin and decalcified in 25% formic acid for variable periods of time as needed prior to undergoing paraffin or plastic JB-4 embedding, sectioning at 5 micron thickness and staining with hematoxylin and eosin or 1% toluidine blue, respectively.

Method 2. Sections of 1–2 micrometer thickness of the tissues undergoing preparation for transmission electron microscopy were assessed by LM prior to trimming and thin sectioning. Sections were made from tissues prepared by each of the methods outlined in the transmission electron microscopy section below.

Method 3. Following rapid removal of the ends of bones, or of entire regions of bones in the newborn, the tissues were fixed in 1% PFA and 1.3% GA in 0.1 M sodium cacodylate buffer, pH 7.4, to which was added 0.5% RHT (Polysciences, Warrington, PA, USA) or 0.5% LN (Electron Microscopy Sciences, Hatfield, PA, USA). Osmolarities of the fixation solutions had been pre-determined (see below). After fixation for 3 h at room temperature, the specimens were washed three times in 0.1 M cacodylate buffer, pH 7.4, and decalcified where needed in 7.5% EDTA for 3 days. Dehydration was done with 70, 80 and 90% ethanol, twice each for 15 min per change. The specimens were infiltrated with JB-4 Plus (Electron Microscopy Sciences) and embedded in JB-4 Plus. The blocks were polymerized at 4 degrees. Tissues were cut with a tungsten carbide knife at 4 micrometer thickness and stained for 30 s with 0.5% toluidine blue. Photomicrographs were taken with an Olympus BX50 microscope (Olympus, Tokyo, Japan) with a CMOS digital camera.

Transmission electron microscopy

Tissues were prepared in two ways for electron microscopic assessment.

Method 1. Dissection was performed immediately after euthanization with tissues bathed in 2.5% GA in a cold environment to enhance rapid fixation. Each epiphyseal–metaphyseal region was surgically exposed with visualization under a mounted magnifying glass, removed quickly by cutting the joint capsule, ligaments and metaphyseal bone with scissors and scalpel, cut with scalpel into physeal, epiphyseal and epiphyseal–metaphyseal segments $1 \times 1 \times 0.2$ mm and fixed immediately in modified Karnovsky solution (1% PFA and 2.5% GA in 0.1 cacodylate buffer, pH 7.4) for 3 h at 4 °C. The tissue segments were washed twice for 10 min each time in 0.1 M cacodylate buffer, decalcified in 7.5% EDTA in 2.5% GA in 0.1 M cacodylate buffer for 3 days, post-fixed in 1% osmium tetroxide-sym-collidine buffer for 3 h, washed in 0.1 M cacodylate buffer, dehydrated in increasing concentrations of ethanol from 70 to 100% and infiltrated and embedded in Epon 812 resin (E.F. Fullam Inc., Latham, NY, USA). They were then sectioned at 1 μm thickness and stained with 1% toluidine blue for light microscopic assessment. Blocks chosen for ultrastructural assessment were trimmed, sectioned at 60 nm, stained with lead citrate and uranyl acetate and examined at 60 kV with a JEOL 100 C or JEOL 1200 EX transmission electron microscope.

In one set of studies, the distal metatarsal and distal femur were removed and prepared for transmission electron microscopy study. Four mice from the same litter were assessed using the distal third metatarsals and distal femurs from each mouse. Dissection of tissues into three groups was rapidly performed; in the first group epiphyseal cartilages alone were studied (five blocks: three metatarsal and two femoral), in the second group physeal–metaphyseal junctions were studied (five blocks: four metatarsal and one femoral) and in the third group continuous thin segments from articular surface to physeal–metaphyseal junction were studied (six blocks: three metatarsal and three femoral). Several sections were cut from each block and all were examined. In the next set of studies, four mice from the same litter were assessed using one distal femur and one proximal humeral epiphysis from each mouse with tissues also dissected into segments of epiphyseal cartilage alone, physeal–metaphyseal junctions alone and continuous thin segments incorporating epiphyseal-physeal–metaphyseal tissue. Selected sections were examined to confirm the findings from the first set of studies.

Method 2. In an effort to improve cartilage tissue and specifically hypertrophic chondrocyte fixation, close attention was paid to the osmolarity of the fixation solutions and to the use of 0.5 or 0.7% RHT. Three methods of preparing fixative with RHT were used in an effort to maximize and balance osmolarity, hypertrophic cell membrane preservation and organellar preservation. Solution A was composed of GA 2% and RHT 0.7%, solution B GA 1.3% and RHT 0.5% and solution C GA 1.3%, PFA 1% and RHT 0.5% (**Table 1**). The three solutions were based on the work of Loqman *et al.*[19] with modifications creating solution C by adding 1% PFA for improved ultrastructural fixation. The methods for dissection, immediate fixation, decalcification, post-fixation (including use of osmium tetroxide OsO_4), embedding, sectioning, staining and examination were the same as described above for method one (except that during dissection the tissues were bathed in the specific fixative to be used rather than 2.5% GA).

Osmolarity determinations

Osmolarity was determined using a Fiske Micro-Osmometer model 210 (Fiske Associates, Norwood, MA, USA) for the LM fixation solutions using ruthenium and lanthanum and for the ultrastructural studies using each of the three solutions listed below. Values were determined on 3 occasions several weeks apart with fresh fixative preparations. Each solution had 3 separate readings from which a mean value was calculated. Values were expressed as mOsm kg^{-1}.

Osmolarity readings were made for ultrastructural fixation solutions A, B and C (based on the Loqman *et al*[19] criteria plus our modification using PFA 1%). The 3 solutions were as follows: solution A GA 2%, RHT 0.7% and cacodylate buffer 0.05 M; solution B GA 1.3%, RHT 0.5% and cacodylate buffer 0.03 M; and solution C GA 1.3%, RHT 0.5%, PFA 1% and cacodylate buffer 0.03 M. Readings were made for fixatives for LM (method 3) study on two separate occasions. The fixatives chosen were based on a balance between osmolarity values and the need for good fixation for light microscopic and ultrastructural study. The osmolarity values are listed in **Table 1**.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We acknowledge the excellent work of James Koepfler, Medical Photographer in the Department of Orthopaedic Surgery, Boston Children's Hospital and Theresa Bui in preparation of the manuscript.

References

1. Retterer E. Evolution du cartilage transitoire. *J Anat Physiol* 1900; **36**: 467–565.
2. Stump CW. The histogenesis of bone. *J Anat* 1925; **59**: 136–154.
3. Rivas R, Shapiro F. Structural stages in the development of the long bones and epiphyses. A study in the New Zealand white rabbit. *J Bone Joint Surg Am* 2002; **84**: 85–100.
4. Dodds GS. Row formation and other types of arrangement of cartilage cells in endochondral ossification. *Anat Rec* 1930; **46**: 385–399.
5. Schenk RK, Wiener J, Spiro D. Fine structural aspects of vascular invasion of the tibial epiphyseal plate of growing rats. *Acta Anat* 1968; **69**: 1–17.
6. Shapiro F. Epiphyseal and physeal cartilage vascularization: a light microscopic and tritiated thymidine autoradiographic study of cartilage canals in newborn and young postnatal rabbit bone. *Anat Rec* 1998; **252**: 140–148.
7. Roach HI, Baker JE, Clarke NMP. Initiation of the bony epiphysis in long bones: chronology of interactions between the vascular system and the chondrocytes. *J Bone Miner Res* 1998; **13**: 950–961.
8. Brighton CT, Sugioka Y, Hunt RM. Cyoplasmic structures of epiphyseal plate chondrocytes: quantitative evaluation using electron micrographs of rat costochondral junctions with special reference to the fate of hypertrophic cells. *J Bone Joint Surg Am* 1973; **55**: 771–784.
9. Buckwalter JA, Mower D, Schafer J, Ungar R, Ginsberg B, Moore K. Growth-plate-chondrocyte profiles and their orientation. *J Bone Joint Surg Am* 1985; **67**: 942–955.
10. Farnum CE, Wilsman NJ. Cellular turnover at the chondro-osseous junction of growth plate cartilage: analysis by serial sections at the light microscopical level. *J Orthop Res* 1989; **7**: 654–666.
11. Buckwalter JA, Mower D, Ungar R, Schaeffer J, Ginsberg B. Morphometric analysis of chondrocyte hypertrophy. *J Bone Joint Surg Am* 1986; **68**: 243–255.
12. Hunziker EB, Schenk RK, Cruz-Orive LM. Quantitation of chondrocyte performance in growth-plate cartilage during longitudinal bone growth. *J Bone Joint Surg Am* 1987; **69**: 162–173.
13. Farnum CE, Wilsman NJ. Morphologic stages of the terminal hypertrophic chondrocyte of growth plate cartilage. *Anat Rec* 1987; **219**: 221–232.
14. Hunziker EB, Herrmann W, Schenk RK. Improved cartilage fixation by ruthenium hexammine trichloride (RHT). A prerequisite for morphometry in growth cartilage. *J Ultrastruc Res* 1982; **81**: 1–12.
15. Hunziker EB, Herrman W, Schenk RK. Ruthenium hexammine trichloride (RHT)-mediated interaction between plasmalemmal components and pericellular matrix proteoglycans is responsible for the preservation of chondrocytic plasma membranes in situ during cartilage fixation. *J Histochem Cytochem* 1983; **31**: 717–727.
16. Hunziker EB, Herrmann W. Ultrastructure of cartilage. In: Bonucci E, Motta PM (eds) Ultrastructure of Skeletal Tissues: Bone and Cartilage in Health and Disease (Electron Microscopy in Biology and Medicine). Kluwer Academic Publishers: Dordrecht, The Netherlands, Chapter 5, volume 7, 1990, pp 79–109, 300 pages.
17. Bush PG, Hall AC, Macnicol MF. New insights into function of the growth plate. Clinical observations, chondrocyte enlargement and a possible role for membrane transporters. *J Bone Joint Surg Br* 2008; **90**: 1541–1547.
18. Bush PG, Constantinos AP, Hall AC. The osmotic sensitivity of rat growth plate chondrocytes in situ; Clarifying the mechanisms of hypertrophy. *J Cell Physiol* 2008; **214**: 621–629.
19. Loqman MY, Bush PG, Farquharson C, Hall AC. A cell shrinkage artifact in growth plate chondrocytes with common fixative solutions: importance of fixative osmolarity for maintaining morphology. *Europ Cells Materials* 2010; **19**: 214–227.
20. Ballock RT, O'Keefe RJ. The biology of the growth plate. *J Bone Joint Surg Am* 2003; **85**: 715–726.
21. Maes C, Carmeliet G. Vascular and nonvascular roles of VEGF in bone development. In: VEGF in Development. Christiana Ruhrberg (ed.) Landes Bioscience and Springer Science + Business Media 2008, pp 79–90.
22. Gerstenfeld LC, Shapiro FD. Expression of bone-specific genes by hypertrophic chondrocytes: Implications of the complex functions of the hypertrophic chondrocyte during endochondral bone development. *J Cell Biochem* 1996; **62**: 1–9.
23. Holtrop ME. The ultrastructure of the epiphyseal plate I. The flattened chondrocyte.. *Calcif Tiss Res* 1972a; **9**: 131–139.
24. Holtrop ME. The ultrastructure of the epiphyseal plate. II. The hypertrophic chondrocyte. *Calcif Tissue Res* 1972; **9**: 140–151.
25. Haines RW. The evolution of epiphyses and of endochondral bone. *Biol Rev* 1942; **17**: 267–292.
26. Haines RW. Cartilage canals. *J Anat* 1933; **68**: 45–64.
27. Wilsman NJ, Van Sickle DC. The relationship of cartilage canals to the initial osteogenesis of secondary centers of ossification. *Anat Rec* 1970; **168**: 381–391.
28. Floyd WE, Zaleske DJ, Schiller AL, Trehan C, Mankin HJ. Vascular events associated with the appearance of the secondary center of ossification in the murine distal femoral epiphysis. *J Bone Joint Surg Am* 1987; **69**: 185–190.
29. Kalayjian DB, Cooper RR. Osteogenesis of the epiphysis. A light and electron microscopic study. *Clin Orthop Rel Res* 1972; **85**: 242–256.
30. Roach HI, Clarke NMP. 'Cell paralysis' as an intermediate stage in the programmed cell death of epiphyseal chondrocytes during development. *J Bone Miner Res* 1999; **14**: 1367–1378.
31. Shaklai M, Tavassoli M. Lanthanum as an electron microscopic stain. *J Histochem Cytochem* 1982; **30**: 1325–1330.
32. Shapiro F, Cahill C, Malatantis G, Nayak RC. Transmission electron microscopic demonstration of vimentin in rat osteoblast and osteocyte cell bodies and processes using the immunogold technique. *Anat Rec* 1995; **241**: 39–48.
33. Shapiro F. Variable conformation of gap junctions linking bone cells: A transmission electron microscopic study of linear, stacked linear, curvilinear, oval and annular junctions. *Calcif Tissue Int* 1997; **61**: 285–293.
34. Shapiro F. Cortical bone repair. The relationship of the lacunar-canalicular system and intercellular gap junctions to the repair process. *J Bone Joint Surg* 1998; **70A**: 1067–1081.
35. Farnum CE, Lee R, O'Hara K, Urban JPG. Volume increase in growth plate chondrocytes during hypertrophy: the contribution of organic osmolytes. *Bone* 2002; **30**: 574–581.
36. Studer D, Millan C, Öztürk E, Maniura-Weber K, Zenobi-Wong M. Molecular and biophysical mechanisms regulating hypertrophic differentiation in chondrocytes and mesenchymal stem cells. *Eur Cell Mater* 2012; **24**: 118–135.
37. Shapiro F, Flynn E, Calicchio ML. Molecular differentiation in epiphyseal and physeal cartilage. Prominent role for gremlin in maintaining hypertrophic chondrocytes in epiphyseal cartilage. *Biochem Biophys Res Comm* 2009; **390**: 570–576.
38. Belluoccio D, Bernardo BC, Rowley L, Bateman JF. A microarray approach for comparative expression profiling of the discrete maturation zones of mouse growth plate cartilage. *Biochim Biophys Acta* 2008; **1779**: 330–340.
39. Collins DH, Ghadially FN, Meachim G. Intracellular lipids of cartilage. *Ann Rheum Dis* 1965; **24**: 123–135.
40. Karsenti E. Self-organization in cell biology: a brief history. *Nature Rev Mol Cell Biol* 2008; **9**: 255–262.
41. Misteli T. The concept of self-organization in cellular architecture. *J Cell Biol* 2001; **155**: 181–185.