Epiphyseal Fusion in the Human Growth Plate Does not Involve Classical Apoptosis

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ABSTRACT: By the end of puberty, growth ceases and epiphyseal fusion occurs through mechanisms not yet completely understood. Human growth plate tissues were collected in various pubertal stages including a unique late pubertal growth plate, which was about to fuse. Apoptosis was studied by TUNEL staining, immunolocalization of pro- and antiapoptotic proteins, and electron microscopy (EM). Morphologic analyses of the fusing growth plate revealed disorganized, large chondrocytes surrounded by a border of dense, cortical-like bone. In the unfused growth plates, few chondrocytes were TUNEL positive. In contrast, the fusing growth plate contained no single TUNEL-positive cell. Antiapoptotic (Bcl-2 and Bcl-X_L) and proapoptotic (Bax, Bad, and cleaved caspase-3) proteins were detected in all growth plate zones without change in intensity during pubertal progression. Expression of antiapoptotic proteins was found in the fusing growth plate but of the proapoptotic proteins only Bad was detected. EM revealed no typical signs of apoptosis or autophagy in any of the growth plates. In contrast, morphological signs of hypoxia and necrosis were observed. We conclude that classical apoptosis is not likely to be involved in the process of human growth plate fusion. (*Pediatr Res* 66: 654–659, 2009)

Longitudinal growth occurs at the epiphyseal plate, a thin layer of cartilage entrapped between epiphyseal and metaphyseal bone, located at the distal ends of the long bones. The epiphyseal plate consists of three principal layers with immature cells lying toward the epiphysis, called the resting zone, more mature flat chondrocytes in the proliferating zone and the hypertrophic zone adjacent to this. Stem-like cells in the resting zone have a finite proliferative capacity that is gradually exhausted, which consequently results in fusion of the growth plate at the end of puberty (1). At the chondroosseous junction site of the growth plate, a nowadays generally accepted hypothesis is that terminally hypertrophic chondrocytes die by undergoing apoptosis leaving behind a scaffold of cartilage matrix for osteoblasts that invade and lay down bone resulting in growth plate fusion (1,2). Apoptotic cells in general show typical morphologic changes like cell

shrinkage, intact organelles and integrity of membranes, pyknotic nuclei by aggregation of chromatin, fragmented DNA, partitioning of the cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies), and absence of an inflammatory response (3,4). The Nomenclature Committee on Cell Death suggested to describe apoptosis at a biochemical level as a caspase-dependent process (5).

Apoptosis in growth plate chondrocytes has been reported in many *in vivo* and *in vitro* studies performed in different species applying mostly the TUNEL technique to label fragmented DNA (6–8). Additional studies in animals show that apoptosis-regulating proteins are predominantly expressed in the proliferative and hypertrophic zones with an increase of proapoptotic factors by age (7,9). Others have questioned apoptosis as the final mechanism through which chondrocytes die in the terminal hypertrophic zone. Ahmed *et al.* (10) hypothesized nonapoptotic mechanisms to be involved and described morphologic changes distinct from classical apoptosis. In addition, Roach and Erenpreisa (11) re-examined microscopic pictures of hypertrophic chondrocytes of *in vitro* cultured chick growth plates and hypothesized that terminal hypertrophic chondrocytes die not only through apoptosis but also through transdifferentiation to endochondral osteoblasts. In later studies, Roach and Clarke (12,13) studied rabbit growth plates and described chondrocytes with condensed chromatin, suggestive of apoptosis, but the “morphology of the cytosol” was unlike that of necrotic, apoptotic, or normal cells. In 2004, they came up with the name chondroptosis instead of apoptosis to describe the appearance of these cells (14). This study reported autophagic vacuoles in the chondroptotic cells, suggesting a role for autophagy in the process of cell death of the terminal hypertrophic cell. Autophagy and autophagosomes were also observed in avian hypertrophic chondrocytes and in chondrocytes of newborn mice (15,16).

Previous studies focusing on the fate of growth plate chondrocytes have been performed mostly in different animal models. It is uncertain whether the chick growth plate is comparable with the human, and studies in rodents are limited

Received June 10, 2009; accepted August 25, 2009.

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Supported by ZonMw (project 920-03-358) the Netherlands, the Swedish Research Council, and a visiting scholarship award from the European Society for Paediatric Endocrinology.

Abbreviations: TEM, transmission electron microscopy; TRACP, tartrate-resistant acid phosphatase

by the fact that growth plate fusion does not occur during sexual maturation in rats and mice. The only studies done in human tissues are not very extensive involving TUNEL analyses performed in spinal growth plates and slipped capital femoral epiphysis growth plates (6,17,18). To resolve this issue, we decided to jointly collect human growth plate tissue samples obtained during epiphyseal surgery performed in children at different stages of development. When analyzing all these samples of human growth plate tissues, we were lucky to discover that in one of the patients, a 17-y-old late pubertal female, the growth plate was captured just when it was in the process of undergoing epiphyseal fusion. This growth plate tissue can be considered as unique because epiphyseal fusion is a rapid process that is extremely difficult to capture. This specific human tissue specimen is therefore like a snapshot of epiphyseal fusion and could help us to better understand how growth plate fusion occurs and the underlying mechanisms involved. Using these tissues, we here aimed to describe the morphologic characteristics of a fusing human growth plate and possible mechanisms involved in the process of human epiphyseal fusion and discuss these in relation to previous published data obtained in different animal models.

METHODS

Patients and tissue preparation. Human proximal and distal femur growth plate tissues were collected from 14 girls and five boys at different pubertal stages who were undergoing surgery for different medical indications (Table 1). The study protocol was approved by the Local Medical Ethics Committees of The Leiden University Center, Leiden, the Netherlands, and The Karolinska University Hospital, Stockholm, Sweden. Informed consent was obtained from all patients and their parents. No patient used any continuous medication. Patient 13, the patient with a growth plate in the process of epiphyseal fusion, was mostly wheelchair bound and mentally retarded. Because of frequent painful hip luxations hip replacement surgery was performed. Figure 1 shows an x-ray of her right hip where a complete luxation can be observed. All tissue samples were processed in the same way. Specimens were immediately fixed in 10% formaldehyde overnight, decalcified in 10% EDTA for 5 d, and then embedded in paraffin.

Immunohistochemistry. Immunohistochemistry was performed as previously described (19) with the following modifications. After antigen retrieval by incubating with trypsin (Invitrogen) for 10 min at 37°C, the sections were additionally treated with 5 mg/mL hyaluronidase (Sigma Chemical Co.-Aldrich, Inc, Steinheim, Germany) for 30 min at 37°C. Anti-Collagen-X antibody was from Quartett (Berlin, Germany) and used in a 1:100 dilution. Anti-Bcl-2 antibody was from Upstate (#06-474 Upstate Biotechnology, Lake Placid, NY) and used in a 1:300 dilution. AntiBax antibody (P19) and antiBad antibody (C-20) were purchased from Santa Cruz Inc., (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and, respectively, used in a 1:300 and 1:200 dilution. Anti-Bcl-X_L antibody was purchased from Transduction Laboratories (Becton Dickinson AB, Stockholm, Sweden) and used in a 1:100 dilution. Anti-cleaved-caspase three primary antibody was purchased from Cell Signaling Technology (*In Vitro* Sweden AB, Stockholm, Sweden) and used in a 1:50 dilution. Secondary anti-rabbit biotinylated antibody (Jackson ImmunoResearch lab, West Grove, PA) or anti-mouse biotinylated antibody (DAKO, Glostrup, Denmark) were, respectively, used in a 1:1000 and 1:300 dilution, followed by incubation with avidin-biotin Vectastain ABC reagent according

Figure 1. x-ray of the right hip of patient 13 showing a complete hip luxation.

Table 1. IHC total growth plate staining

Patient	Diagnosis	Sex	(Distal/prox femur)	Age (y:mo)	Tanner	TUNEL	Bcl-2	Bcl-X _L	Bax	Bad	Casp3
1	Constitutional tall stature	F	Distal	11:6	B1	0	x	x	x	x	x
2	Leg length difference	F	Distal	9:6	B1	0	x	x	x	x	x
3	Leg length difference	F	Distal	9:6	B1-2	0	x	x	x	x	2
4	Hip luxations, femur head resection	F	Proximal	12:5	B2	0	1	2	1	1	2
5	Leg length difference	F	Distal	12:8	B2	1	x	3	x	2	2
6	Constitutional tall stature	F	Distal	13:7	B2	0	x	x	x	x	x
7	Constitutional tall stature	F	Distal	12:1	B2	0	2	x	x	x	x
8	Leg length difference	F	Distal	14:2	B2-3	0	x	2	1	2	2
9	Hip luxations, femur head resection	F	Proximal	13:5	B3	0	2	2	2	2	1
10	Leg length difference	F	Distal	13:8	B3	0	x	1	1	x	0
11	Cerebral palsy, femur head resection	F	Proximal	15:1	B4	0	2	2	1	2	1
12	Leg length difference	F	Distal	13:3	B4	0	x	x	x	x	x
13	Cerebral palsy, femur head resection	F	Proximal	17:0	B5	0	2	2	0	2	0
14	Leg length difference	F	Distal	12:1	B5	0	x	x	x	x	x
15	Marfan	M	Distal	12:3	G1	0	x	x	x	x	1
16	Leg length difference	M	Distal	12:6	G3	0	x	x	x	x	x
17	Leg length difference	M	Distal	15:4	G3-4	0	x	x	x	x	x
18	Leg length difference	M	Distal	14:8	G4	0	x	x	x	x	x
19	47XYY	M	Distal	14:0	G4-5	0	x	x	x	x	x

TUNEL and histology staining for Bcl-2, Bcl-X_L, Bax, Bad, and caspase-3 were scored from 0 to 3 for each patient. 0 indicating no staining in the growth plate and 3 indicating many positive chondrocytes throughout the growth plate. x indicates that no staining was performed. Age is given in years and months (y:mo).

to manufactures instructions (Vector laboratories, Burlingame, CA). Digital images were collected using a Nikon Eclipse E800 microscope equipped with an Olympus DP70 digital camera.

Apoptosis assay (TUNEL). Apoptotic cells were identified by TUNEL immunohistochemistry according to instructions for the TdT-FragEL DNA fragmentation kit (Oncogene Research, Boston, MA) with the following modifications. The sections were treated with 5 μ g/mL proteinase K for 15 min (20–22). Control experiments showed that when pretreating sections with distilled water instead of TdT, all cells were negative, whereas pretreatment with DNase enabled labeling of all cells.

Tartrate-resistant acid phosphatase (TRACP) staining. TRACP staining was performed using a combination of solutions that include naphthol-AS BI phosphate, dimethylformamide, tartaric acid, acetate buffer, vermoal buffer, sodium nitrite, and pararosaniline pH 5.2. Tissue sections were deparaffinized, rehydrated, and incubated with the reactive solution for 30 min as described in detail previously (23). After washing with distilled water, the tissue sections were counterstained with Mayer's hematoxylin.

Transmission electron microscopy. Small tissue pieces were cut out from blocks of paraffin embedded bone/cartilage tissue and the paraffin was dissolved in xylene at 60°C. The tissue was rehydrated in alcohol into distilled water and fixed in 2% glutaraldehyde + 0.5% paraformaldehyde in 0.1 M sodiumcacodylate buffer containing 0.1 M sucrose and 3 mM CaCl_2 , pH 7.4, 24 h at 4°C. Specimens were rinsed in 0.15 M sodiumcacodylate buffer containing 3 mM CaCl_2 , pH 7.4 postfixed in 2% osmium tetroxide in 0.07 M sodiumcacodylate buffer containing 1.5 mM CaCl_2 , pH 7.4 at 4°C for 2 h, dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, VT). Semithin sections were cut and stained with toluidine blue and used for light microscopic analysis. Ultrathin sections were cut and contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10 transmission electron microscope at 80 kV. Digital images were captured by a MegaView III digital camera (Soft Imaging System, GmbH, Münster, Germany).

RESULTS

Light microscopy. Figure 2A and C show representative pictures of an early pubertal female growth plate (patient 4) at 40 $\times$ (A) and 100 $\times$ (C) magnification. Figure 2B and D show pictures of the late pubertal patient (patient 13) with ongoing fusion of her growth plate stained with H&E at, respectively, 40 $\times$ and 100 $\times$ magnification. As expected, early pubertal growth plate chondrocytes were found to be organized in parallel columns maturing from resting to proliferative and then to large hypertrophic cells. In the late pubertal patient, growth plate remnants were found at the site where in less mature bones the epiphyseal plate is located. These remnants were small and surrounded by dense cortical-like bone. Chondrocytes within these growth plate remnants were disorganized and no columns could be found. In addition, the cells were very large with big lacunae and some of them were fused together. To study in more detail the interface of the fusing growth plate, immunohistochemistry for collagen type X and TRACP staining were performed. The unfused growth plates showed expectedly positive staining for collagen type X in the hypertrophic zone (Fig. 2E). However, surprisingly, the growth plate remnant with large hypertrophic-like cells did not show any positive staining (Fig. 2F). TRACP staining, a marker for osteoclasts, was seen at the chondro-osseous junction of prepubertal growth plates (Fig. 2G) and in early pubertal growth plates (data not shown). However, at the interface of the growth plate remnant, no TRACP staining could be detected (Fig. 2H).

Transmission electron microscopy. To allow comparative electron microscopic studies of cell death characteristics, small pieces were cut out from paraffin blocks containing growth plate tissues from the patient with ongoing epiphyseal fusion. For comparative analysis, three younger pubertal female growth plates (patients 4, 9, and 11) were processed in a similar way.

Figure 2. H&E staining of sectioned human growth plates. In early pubertal patients, growth plate chondrocytes were organized in parallel columns (patient 4; panel A 40 $\times$ and panel C 100 $\times$ magnification). In a late pubertal patient, the growth plate was diminished to a small remnant surrounded by dense cortical-like bone (patient 13; panel B 40 $\times$ and panel D 100 $\times$ magnification). All unfused growth plates stained positive for collagen type X in the hypertrophic zone (patient 2; panel E 100 $\times$ magnification), whereas the fusing growth plate did not show any staining (patient 13; panel F 100 $\times$ magnification). Panel G, shows positive TRACP staining in an early pubertal growth plate, indicated by arrows (patient 2, 100 $\times$ magnification), whereas the fusing growth plate stained negative (patient 13; panel H 100 $\times$ magnification). Bars in all figures indicate 200 μ m.

The fusing growth plate was found to be entirely surrounded by a border of very dense, cortical-like bone clearly isolating the growth plate from any vessels or osteoclasts (Fig. 3F). In contrast, in nonfusing growth plates, there was a small distinct border between loosely packed collagen type II in the cartilage matrix and collagen type I in the bone matrix (Fig. 3E). Collagen type I contains thick fibers compared with the very thin collagen type II fibers and therefore these two types of collagen could easily be distinguished. There were no signs of classical apoptosis in the fusing growth plate; e.g. no apoptotic bodies or patched chromatin and proteins could be found (Fig. 3B and D). In addition, no typical signs of inflammation were found although we did observe some signs

Figure 3. TEM images from the proximal femur epiphyseal growth plate in a mid-pubertal patient (patient 9; left panels, A, C, and E) and the late pubertal patient with a fusing growth plate (patient 13; right panels B, D, and F). In the midpubertal patient, hypertrophic zone chondrocytes displayed a normal morphology (A) and at a higher magnification, dense chromatin was found in the cell nucleus (C). In contrast, the fusing growth plate displayed a patchy chromatin pattern (B and D). At high-power magnifications of the interphase between cartilage and bone matrix, a distinct border between loosely packed cartilage matrix collagen type II (Coll-II) and bone matrix collagen type I (Coll-I) was found in the midpubertal patient (E), whereas in the fusing growth plate, the border was thicker and the bone matrix collagen type I more dense (F). Bars: A and B, 5 μ m; C and D, 2 μ m; E and F, 1 μ m.

of early necrosis like empty vacuoles (see arrows Fig. 3B). In addition, we observed signs of hypoxia, e.g. patchy distributed chromatin and defect plasma cell membranes. No double-membrane autophagosomes, like typically seen in cells undergoing autophagy, were found in the fusing growth plate.

In early-pubertal and mid-pubertal growth plates (Fig. 3A and C), we observed normal chondrocytes with nonpyknotic nuclei containing homogenous dense chromatin without signs of DNA fragmentation. We specifically focused on the terminal hypertrophic chondrocytes that were about to be incorporated into the newly formed bone. In these cells, some chromatin condensation was seen, but this was clearly different from what normally is seen in cells undergoing classical apoptosis. Cell membranes were intact and the cytoplasm

Figure 4. TUNEL and immunohistochemistry staining of prepubertal, pubertal, and fusing growth plates (100 $\times$ and inserts 200 $\times$ magnification). Panel A, TUNEL analysis patient 4, left insert showing positive cells in surrounding bone marrow. Panel B, TUNEL analysis fusing growth plate (patient 13). Panels C and D, Bcl-2 staining patient 4 and fusing growth plate. Panels E and F, Bcl-X_L staining patient 10 and fusing growth plate. Panels G and H, Bax staining patient 4 and fusing growth plate. Panels I and J, Bad staining patient 3 and fusing growth plate. Panels K and L, cleaved caspase-3 patient 3 and fusing growth plate. Bars indicate 100 μ m.

mostly empty. No autophagosomes could be found and no sign of necrosis was present.

TUNEL analysis. In all female (two prepubertal and 12 pubertal) and male (five pubertal) subjects, very few growth plate chondrocytes stained positive or showed an apoptotic-like morphology when applying the TUNEL method (Fig. 4A). The staining intensity was scored (0 to 3 points; 0 indicating no staining and 3 indicating many positive cells

throughout the growth plate), and the results are shown in Table 1, ranked according to pubertal stages. It is important to point out that when evaluated by experienced scientists in the field, most TUNEL-positive cells indeed were considered to have a viable appearance, as indicated by a complete absence of apoptosis-related morphologic changes, and should therefore be considered as nonspecifically stained. In the fusing growth plate, no single TUNEL-positive cell was detected (Fig. 4B). In contrast, the surrounding bone marrow was abundantly stained with the TUNEL technique and could therefore serve as an internal positive control as previously described (21,24). Negative controls showed no staining.

Antiapoptotic proteins. Bcl-2 staining was detected in cells throughout the whole growth plate in patients with nonfused growth plates ($n = 4$, Table 1) (Fig. 4C). When the relative staining intensity was scored (score 0 to 3), a possible tendency toward increased expression was found during maturation (Table 1). The fusing growth plate did show some staining for Bcl-2 (score 2; Fig. 4D).

Bcl-X_L, another antiapoptotic factor, was also localized in cells throughout the whole growth plate ($n = 6$, Table 1, Fig. 4E). No change in percentage positive cells or staining intensity was seen during maturation. In addition, in the fusing growth plate some cells stained positive for Bcl-X_L (score 2; Fig. 4F).

Proapoptotic proteins and caspase 3. Staining for Bax, a proapoptotic factor, was seen in chondrocytes throughout the whole growth plate ($n = 5$, Table 1, Fig. 4G). No change in staining intensity or percentage Bax positive cells was seen during pubertal development. Interestingly, in the fusing growth plate no staining for Bax could be detected (Fig. 4H).

Staining for Bad, another proapoptotic factor, was found throughout the whole growth plate ($n = 5$, Table 1, Fig. 4I). No change in staining intensity or percentage of positive cells was seen during development. Some staining for Bad proteins was observed in the fusing growth plate (score 2; Fig. 4J), unfortunately with abundant background staining.

Cleaved caspase 3, an effector caspase in the apoptotic cascade, was seen throughout the whole growth plate in all pubertal stages ($n = 8$, Table 1) with no change during maturation (Fig. 4K). In the fusing growth plate, no staining for cleaved caspase 3 was detected (Fig. 4L).

Negative controls showed no staining for all analyzed pro- and antiapoptotic proteins.

DISCUSSION

On the basis of well-established morphologic and histologic criteria of apoptosis, we could not detect any signs of classical apoptosis in human terminal hypertrophic growth plate chondrocytes. These findings were also confirmed by electron microscopy (EM). In a unique tissue specimen of a late pubertal fusing human growth plate, we found clear evidence that chondrocyte apoptosis is not likely to be involved in the end phase of growth plate fusion in humans. In contrast, signs of hypoxia and early necrosis were present in the fusing growth plate.

This is the first detailed study of apoptosis in the human pubertal growth plate that includes expression levels of pro- and antiapoptotic proteins together with morphologic analysis

based on light and EM. Our study includes the characterization of a unique tissue sample from a growth plate in the process of undergoing fusion where we made the novel observation that the chondrocytes were markedly enlarged, disorganized, and surrounded by a border of dense, cortical-like bone. No morphologic signs of classical apoptosis were found in this fusing growth plate. Our findings are in agreement with earlier studies describing terminal hypertrophic zone chondrocytes at the chondro-osseous junction to have a morphology, which is not typically seen in cells undergoing classical apoptosis (11,14,25,26). However, it is important to point out that all these previous studies were performed in animal growth plates; avians or rodents that do not fuse their growth plates by the end of sexual maturation.

The mechanism by which terminal hypertrophic chondrocytes disappear during endochondral ossification is believed to be related to the underlying cause of eventual growth plate fusion. However, these two events involving the epiphyseal growth plate might as well be two different and independent processes. In this article, we compared both. Interestingly, we found some signs of early apoptosis and also some signs of necrosis such as empty vacuoles in the fusing growth plate, suggesting that fusing growth plate chondrocytes appear in a sort of intermittent stage between apoptosis and necrosis. Importantly, no signs of inflammation were observed around the fusing growth plate. Erenpreisa and Roach (26) also reported signs of necrosis in dark chondrocytes of the embryonic chick growth plate, however, such dark chondrocytes were not found in our human growth plate tissue samples. To our knowledge, no other signs of necrosis have previously been reported in the growth plate. Interestingly, Shapiro *et al.* (16) has reported that the morphology of terminal hypertrophic chondrocytes suggest autophagy, a type II programmed cell death, to occur in the growth plate. However, we did not see any double membrane structures like autophagosomes suggestive for autophagy but only vesicles with a single membrane that we interpreted as vacuoles.

Apoptosis is the most widely accepted and described mode of cell death through which terminal hypertrophic chondrocytes disappear and are replaced by bone (27). Most studies rely on TUNEL technique only, which often overestimates the number of apoptotic nuclei, as it labels not only fragmented DNA but also DNA in the process replication and repair. In many studies, TUNEL positive chondrocytes were observed not only in the hypertrophic layer where cells are assumed to die before being incorporated into the newly formed bone, but also in all zones of the growth plate (6,17,18). When re-evaluating pictures of growth plate chondrocytes positive for TUNEL staining, a high diversity of results could be found. Cell morphology is not always complementing TUNEL positivity and when data are presented, cells often do not look like cells in the process of undergoing apoptosis, but as healthy viable cells without showing signs of cell shrinkage and pyknotic nuclei (6,17,18). Thus, the TUNEL method is very sensitive, requires precise temporal control of each step (28) and must be verified by other techniques like EM or molecular markers of apoptosis.

Molecular markers of apoptosis include cleavage of caspases and often regulation of pro- and antiapoptotic proteins of the Bcl-2 family (29). In this study, we are the first to investigate these proteins in the human postnatal growth plate and we found that they are indeed expressed throughout the whole growth plate. We did not observe any caspase-3 cleavage, the effector caspase of apoptosis in the fusing growth plate. Moreover, in the fusing growth plate, we observed clear expression of the antiapoptotic proteins Bcl-2 and Bcl-X_L and a possible down-regulation of the proapoptotic protein Bax. Thus, all three approaches including TUNEL technique, analysis of cell morphology by EM, and molecular markers by immunohistochemistry altogether suggest that there is no classical apoptosis occurring in the fusing human growth plate.

A novel and clear observation was that the fusing human growth plate is surrounded by a border of very dense thick bone, shelling the growth plate remnant. There are no vessels seen in or surrounding the fusing growth plate. Moreover, there were some signs of hypoxia in the fusing growth plate like patchy distributed chromatin. From these findings, one can hypothesize that this border of dense bone is functioning as a physical barrier for oxygen and nutrients to reach the fusing growth plate resulting in hypoxia and eventually cell death in a nonclassical apoptotic way through necrosis or a mixture of apoptosis and necrosis. White *et al.* (30) recently demonstrated by light microscopy bridging bone in the center of a distal human tibial growth plate obtained from a 12-y-old and 11-mo-old girl, which might be an early sign of this shelling process.

A limitation of this study is the relatively small number of growth plates that were analyzed and the fact that some were derived from pathologic conditions. To some extent, this is compensated by the fact that we were fortunate to obtain a tissue sample from a human growth plate just in the rapid process of undergoing epiphyseal fusion, which allowed us to investigate possible underlying mechanisms. In addition, the underlying mechanism of epiphyseal maturation and fusion will be the same for all growth plates regardless of the underlying disease of each patient because eventually longitudinal growth stopped in all patients by the end of puberty. When performing EM, the gold standard in cell death research, we found no clear signs of classical apoptosis in any of the human growth plates studied including the fusing one. It should be mentioned that fixation was not performed in the optimal way for EM. However, membranes were not disrupted in the way one would expect if tissue damage had occurred secondary to fixation problems. Furthermore, all tissues were processed in the same way, and there were clear differences in chromatin pattern and collagen fibers, which were nicely preserved.

In summary, a unique piece of fusing human growth plate tissue clearly demonstrate that classical apoptosis is not likely to be involved in the process of human growth plate fusion. We found signs suggestive for hypoxia and necrosis, but the exact mechanism through which chondrocytes disappear during epiphyseal fusion in humans still needs to be defined.

Acknowledgments. We thank the orthopaedic surgeons in the Leiden University Medical Center and at the Karolinska University Hospital in Stockholm for providing the growth plate samples.

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