New Aspects of Endochondral Ossification in the Chick: Chondrocyte Apoptosis, Bone Formation by Former Chondrocytes, and Acid Phosphatase Activity in the Endochondral Bone Matrix

HELMTRUD I. ROACH

ABSTRACT
A detailed histological study of the growth plates from 9- to 20-day-old embryonic chick long bones was carried out with the aim of clarifying the long-debated question of the fate of the hypertrophic chondrocytes. Since resorption in chick bones does not occur synchronously across the plate as it does in mammals, specialized regions develop and the fate of the chondrocyte depends on its location within the growth plate. Where resorption took place, as at the sites of primary vascular invasion or at the main cartilage/marrow interface, chondrocytes underwent apoptosis before the lacunae were opened. In addition, spontaneous apoptosis of chondrocytes occurred at apparently random sites throughout all stages of chondrocyte differentiation. In older chick bones, a thick layer of endochondral bone matrix covered the cartilage edge. This consisted of type I collagen and the typical noncollagenous bone proteins but, in addition, contained tartrate-resistant acid phosphatase in the mineralized matrix. Where such matrix temporarily protected the subjacent cartilage from resorption, chondrocytes differentiated to bone-forming cells and deposited bone matrix inside their lacunae. At sites of first endochondral bone formation, some chondrocytes underwent an asymmetric cell division resulting in one daughter cell which underwent apoptosis, while the other cell remained viable and re-entered the cell cycle. This provided further support for the notion that chondrocytes as well as marrow stromal cells give rise to endochondral osteoblasts. (J Bone Miner Res 1997;12:795–805)

INTRODUCTION
ENDOCHONDRAL OSSIFICATION takes place whenever pre-formed cartilage is replaced by bony tissue, for example, at the growth plate, the secondary center of ossification, and during fracture repair. The overall cellular strategies for the cartilage-to-bone transition are well known: parts of the cartilage matrix are resorbed and replaced by vascular/marrow elements and new bone is then laid down onto the scaffold of unresorbed cartilage spicules. One important unresolved question is the precise origin of the bone-forming cells. Although it is generally assumed that they differentiate from marrow-derived osteogenic stem cells (reviewed by Marks and Popoff and Beresford), the evidence rests on the osteogenic potential of marrow stromal cells rather than direct proof that this actually occurs during endochondral ossification in vivo. An alternative view is that chondrocytes themselves become bone-forming cells. Observations over many years have been consistent with a chondrocyte-to-osteoblast transition, but unambiguous proof that this occurs in vivo has not yet been obtained, and the notion has remained controversial.

The currently accepted view is that the terminal chondrocytes die and that marrow-derived osteoprogenitor cells migrate to the cartilage edge, differentiate into osteoblasts, and lay down the new bone matrix. Since developmental cell death usually takes the form of apoptosis, the expectation has been that terminal chondrocytes are eliminated by this process. However, verification of this and in situ quantification of apoptosis in chondrocytes has proven difficult.
The morphological characteristics, such as apoptotic bodies, are very small and thus easy to miss. In addition, the number of cells with apoptotic morphology is low at any given time point—even if many cells undergo programmed cell death with time. Using end-labeling of DNA strand breaks (TUNEL method of Gavrieli et al.), a relatively large percentage of hypertrophic chondrocytes were labeled: 20–30% in chick sterna and 30–40% in adult chick tibia. However, using flow cytometry, the latter authors estimated that only 8% of chondrocytes were apoptotic at any one time, which suggested that the TUNEL method sometimes overestimates the number of apoptotic chondrocytes. Bronckers et al. found far fewer chondrocytes with positive TUNEL staining in the growth plate of rodents.

The problem in ascertaining whether chondrocytes undergo apoptosis or become bone-forming cells in vivo is that, once released from their lacunae, any cartilage-derived cells would become interspersed among the marrow cells and hence impossible to trace. Previous work had, therefore, concentrated on in vitro situations where resorption was reduced so that lacunae that would have opened in vivo remained intact in vitro. In organ cultures of cut embryonic chick bones, the chondrocytes became bone-forming cells which produced mineralized osteoid matrix within intact lacunae after 9–12 days. More recently, we established that one of the crucial early events in the chondrocyte-osteoblast transition was an asymmetric cell division, where the fate of the daughter cells diverged: one cell remained viable, while its sister was programmed to die. These in vitro experiments provided convincing data on changes in phenotypic expression, but they did not directly address the question of the fate of the terminal hypertrophic chondrocyte in vivo. In the present study, many growth plates from 9- to 19-day-old chick embryos were examined in great detail. Particular attention was paid to the characteristics of the cells at, or just behind, the cartilage/marrow edge.

**MATERIALS AND METHODS**

**Tissue processing and light microscopy**

Femurs and tibiae were dissected from 9- to 20-day-old chick embryos and either fixed immediately in 4% phosphate buffered paraformaldehyde (pH 7.4) or the whole bones were cultured for 18 h as previously described. It is known that hypertrophic chondrocytes are extremely fragile cells which shrink with these conventional fixation methods. However, fixation with gluteraldehyde and ruthenium hexamine trichloride, which preserves cartilage morphology, led to impaired enzyme- and immunoreactivity. The shrinkage of the late hypertrophic chondrocytes was therefore accepted as inevitable. The bones were processed without decalcification for 9- to 17-day femurs, whereas the bones from 17- to 20-day-old chick embryos were decalcified in 5% EDTA (in 0.1 M Tris/HCl buffer, pH 7.4) for 2–3 days. The bones were processed through graded ethanol and chloroform into paraffin wax.

Twenty to eighty sequential longitudinal sections were cut off each bone at 5–6 μm, with the number depending on the age and hence thickness of each bone. The sections were stained by one of the following methods: Weigert’s hematoxylin/alcan blue/sirius red (after Lison) was used as a nuclear stain and to distinguish bone matrix (red) from cartilage matrix (blue). Acid phosphatase activity was demonstrated with an azo dye coupling method (modified from Bancroft and Stevens) described in detail in Roach and Shearer. To test for tartrate or fluoride inhibition, 50 mM Na tartrate or 0.1–10 mM NaF was present in the reaction mixture. Sections were counterstained with 0.2% light green (1 minute) and 1% alcian blue (1 minute).

**Immunocytochemistry**

The following polyclonal antibodies were used. The antibody for type I collagen (LF-67 from Prof. L. Fisher) had been raised against the human C-terminal propeptide of the α1 chain. This antibody detects pro-α1, but also the fully processed α1 chain. The antibodies to the noncollagenous proteins were all specific for chick. The osteopontin (details in McKee et al.) antiserum was a gift from Prof. L. Gerstenfeld (Boston, MA, U.S.A.). The osteonectin (LF-8; details in Pacifici et al.) antiserum was donated by Prof. Larry W. Fisher (Bethesda, MD, U.S.A.). The primary antibodies were visualized using the avidin/biotin method with peroxidase and 3-amino-9-ethylcarbazole (AEC). The sections were counterstained with 0.2% light green and 1% alcian blue. Control sections were incubated with rabbit serum (negative control, Sigma Chemical Co., Poole, Dorset, U.K.), then treated as above. No staining was found in controls.

**Autoradiography**

Proliferating cells were identified by incubating the whole femurs with 3H-thymidine (2 μCi/ml) for 18 h followed by autoradiography of the paraffin sections. Sections were pre-stained with light green/alcan blue, dipped in photographic emulsion (K-5, 1:1 dilution, 45°C, Ilford, Mobberley, Cheshire, U.K.), exposed in a dark box at 4°C for 3 weeks, developed with Kodak Dektol (2 minutes), and fixed with 30% Na-thiosulphate (5 minutes).

**Confocal microscopy**

Forty-micrometer-thick paraffin sections were cut. Type I collagen was visualized with anti-rabbit IgG-FITC as the second antibody, and nuclei were stained with propidium iodide. Dual series of 36 confocal optical slices were obtained by two-channel scanning, using a Leica TCS confocal microscope with a krypton/argon laser light source (Leica UK Ltd., Milton Keynes, U.K.).

**In situ detection of apoptotic cells**

Localizing DNA breaks by end-labeling (TUNEL): The end-labeling method of Gavrieli et al. was used, except that and no predigestion with proteinase K or Triton X-100
was carried out after preliminary experiments had shown that it led to overstaining. The terminal transferase and dUTP-DIG labeling mix were purchased from Boehringer Mannheim (Mannheim, Germany). The incorporation of digoxigenin-labeled dUTP was detected using monoclonal antidigoxigenin Fab-fragments, linked directly to either horseradish peroxidase or alkaline phosphatase (Boeringer Mannheim; 1:1000 dilution; 1–2 h incubation), followed by color development with AEC and H₂O₂ (15 minutes) or 4-Nitroblue tetrazolium chloride/5-bromo-4-cloro-3-indolyl-phosphate (NBT/BICP) (40 minutes). For some sections, the TUNEL method was followed by the reaction for acid phosphatase activity.

Negative controls were processed without any enzyme or without the anti-DIG antibody. Some negative controls were pretreated with DNase as above. No reaction was observed in negative controls, even when DNA breaks had been created with DNase.

**Morphological assessment: Condensed nuclei and apoptotic bodies:** Since the TUNEL method is selective rather than specific for apoptosis and also detects DNA strand breaks in other physiological and pathological states, and since nuclear morphology is still the most reliable method for quantifying apoptosis, those sections routinely stained with hematoxylin/sirius red/alcian blue were examined for the presence of pycnotic nuclei and apoptotic bodies.

**RESULTS**

**The growth plates of embryonic chick femurs**

Although the cellular events of endochondral ossification are similar in birds and mammals, there are several crucial differences in the strategies used to achieve longitudinal growth. In the mammalian growth plate, vascular invasion and resorption occurs in synchrony at a similar horizontal “plane” throughout the disc-like growth plate. Expansion by proliferation is more or less matched by resorption so that the thickness of the growth plate stays reasonably constant. This is in marked contrast to the avian growth region where the overall thickness of the growth plate increases considerably. For example, the thickness of the hypertrophic zone increases from around 0.5 mm (9 days in ovo, Fig. 1A) to 1.8 mm (14 days, Fig. 1B) and nearly 3 mm (19 days, Fig. 1C), whereas the thickness of the corresponding zone in a fast-growing rat tibia is just 0.37 mm. In addition, there is no secondary center of ossification until after hatching, although some vascular canals (vc, Fig. 1B) are present in the epiphyses of embryonic bones.

Primary resorption takes place at the tip marrow “tunnels” (mt), followed by secondary resorption at the “walls” of the tunnels. Since these tunnels push up to the region of flattened cells, it is not only hypertrophic chondrocytes that are in proximity to the vascular/marrow elements. At the lateral walls of the marrow tunnels, resorbing surfaces alternate with bone-forming surfaces where new bone matrix is deposited onto the tunnel walls analogous to the deposition of bone onto cartilage spicules below the mammalian growth plate. Specialized regions, not present in the mammalian growth plate, can be distinguished within the hypertrophic zone of the avian growth plate. Where endochondral bone covers the cartilage, the subjacent region is temporarily protected from further resorption (protected regions). Marrow tunnels tend to be positioned around the periphery near the bone shaft (Fig. 1D), and regions between two marrow tunnels appear to “anchor” the bulk of the epiphysis to the bone shaft (anchor regions, * in Fig. 1C). Resorption at the main cartilage/marrow interface is carried out by both mono- and multinuclear osteoclasts, depending on whether the cartilage matrix has calcified. Protected anchor regions frequently survive as cartilage remnants in the main marrow space for some time.

**Identification of apoptotic cells with the TUNEL method and morphological criteria**

Preliminary experiments had shown that when the tissues were predigested with proteinase K or treated with Triton, staining was extremely variable and inconsistent. Even without such treatments, the number of cells containing TUNEL-labeled nuclei was often higher than the number of cells with “apoptotic morphology” (pycnotic nuclei or apoptotic bodies). When sections were stained with hematoxylin and获利oxulin/sirius red, normal chondrocytes appeared as pale cells with light, scarcely visible nuclei (Fig. 2A). The weak nuclear staining with hematoxylin was due to partial removal of the stain by the subsequent alcin blue/sirius red counterstain, but reflected the diffuse chromatin of interphase chondrocytes. Other cells, such as chick erythrocytes or osteoblasts, retained a more intensely stained nucleus. Interspersed among these pale chondrocytes were cells with an intensely stained, pycnotic nucleus (arrows). At low magnification, such cells were easy to miss, but at high magnification their intense nuclear staining clearly distinguished them from their pale neighbors. Very occasionally, smaller fragmented bodies were found that contained DNA strand breaks, as indicated by the TUNEL reaction (* in Fig. 2B).

**Incidence and localization of apoptotic chondrocytes**

In the proliferative region, 0–2% of chondrocytes contained TUNEL-labeled nuclei, and a similar number had an apoptotic morphology, as assessed by hematoxylin staining. In the hypertrophic region away from marrow tunnels, 0–5% of cells were labeled with the TUNEL method, roughly corresponding to the number of hypertrophic chondrocytes with pycnotic nuclei. These apoptotic chondrocytes were found at apparently random locations throughout the epiphyses and were present in femurs from 9-day-old embryos as frequently as in 20-day-old embryos. It is noteworthy that in some sections no apoptotic cells were present, whereas in others several apoptotic cells were found in clusters. In addition to single apoptotic cells, cell doublets were occasionally present where only one nucleus labeled with the TUNEL reaction (arrow in Fig. 2B).

The incidence of apoptosis was increased near sites of resorption. At the “top” of marrow tunnels, 5–15% of...
chondrocytes contained condensed nuclei (Fig. 2A). Such cells were found up to 10 cell diameters around the distal tip of the marrow tunnel. At the “resorbing” lateral walls of marrow tunnels, apoptotic bodies were occasionally found in opened lacunae (not shown). At the main cartilage/marrow interface, up to 5% of cells contained a condensed nucleus (not shown) or DNA breaks (Fig. 2C). In the chick, the interface between the marrow space (M) and the cartilage (C) sometimes contained intermediate regions (I) in which the “ground substance” of the cartilage matrix had disappeared, but some structure still remained. Very condensed TUNEL-labeled cells were frequently found within this structure, as if temporarily “trapped.” It is likely that these cells were apoptotic chondrocytes that would otherwise have dispersed within the marrow space. The staining in the cells in the area marked by the open circle is an artifact.

**Endochondral bone formation**

To obtain clues as to the origin of the bone-forming cells, the lateral “walls” of marrow tunnels were examined in great detail. In the bones from younger chick embryos, the majority of edges appeared to be “quiescent,” i.e., there was no evidence for either bone formation or resorption. “Late” bone-forming regions, i.e., regions where the walls of marrow tunnels were already covered with bone matrix, were readily identified, particularly in bones from 18- to 20-day-old embryos. It was, however, extremely difficult to find “early” bone-forming regions, i.e., where a resorbing sur-

**FIG. 1.** Overall structure of the growth plates in femurs from 9- to 19-day-old chick embryos. All four sections are of the same magnification (×15, bars = 100 μm). (A, B, and C) Longitudinal sections of chick femurs, stained with sirius red and alcian blue. (A) Nine-day embryo; (B) 14-day embryo; (C) 19-day embryo; (D) transverse section through the distal epiphysis of a femur from a 17-day-old embryo, Von Kossa stain. The areas marked by squares show the approximate locations of regions shown in higher magnification in later figures, the numbers corresponding to the figure numbers. “Anchor” regions between two marrow tunnels are indicated by stars in (C). bs, bone shaft; pz, proliferative zone; fz, zone of flattened cells; mz, maturing zone; hz, hypertrophic zone; mt, marrow tunnel; vc, vascular canals.
face was just changing to a bone-forming surface. Such regions were identified by the presence of a thin layer of type I collagen (not shown). If chondrocytes became bone-forming cells and if this process involved an asymmetric cell division,\(^{(16)}\) then one would expect to find “asymmetric” cell doublets, i.e., where only one cell showed evidence of apoptosis, or only one cell entered the cell cycle. However, if chondrocytes underwent apoptosis, one would expect single apoptotic chondrocytes in lacunae near the edge. The presence of one apoptotic and one osteogenic cell inside opened lacunae near the edge would be consistent with either possibility.

At bone-forming surfaces, cell doublets were frequently present, whereas away from the edge chondrocytic lacunae only contained one cell. Incubation was with \(^{3}H\)-thymidine–labeled cells that had entered the S-phase, for example, most cells in the proliferative zone and in the osteogenic layer of the peristeam (not shown). Some cells in the marrow tunnels were also labeled (small arrows in Fig. 2D) and an “asymmetric” lacuna was present, still separated from the marrow space by a thin layer of matrix, where only one cell had taken up the label (large arrow). The cells in the marrow tunnel could either have been released from the cartilage or migrated to the edge. The “asymmetric” doublet, however, provides evidence that an asymmetric division had occurred and that one daughter cell had re-entered the cell cycle. Cell doublets were not present in the hypertrophic region away from marrow tunnels.

At a slightly later stage, identified by a thicker layer of type I collagen along the edge (Fig. 2E), osteoblasts (ob) now lined the edge and had covered the surface with bone matrix. Some intact lacunae “behind” this edge also contained type I collagen and usually two or more cells (arrows). Since this matrix had been formed inside lacunae, it will be termed intralacunar bone matrix. As the endochondral bone matrix thickened, the edge no longer had a scalloped appearance but became smooth and contained osteocytes (Figs. 2F–2K). This temporarily protected the subjacent cartilage from further resorption. In those areas, cell doublets were found within intact lacunae, in which one cell contained DNA strand breaks while the other one did not (lacuna 4, large arrow in Fig. 2K).

**TRAP activity in the new endochondral bone matrix**

The endochondral bone matrix contained the typical noncollagenous proteins of bone, such as osteopontin, osteonectin, bone sialoprotein, and osteocalcin (not shown). In addition, the matrix contained TRAP activity (Figs. 2G and 2I), which colocalized with type I collagen in lacunae 1, 2, and 3. The activity was inhibited by 2 mM NaF, whereas osteoclast-associated TRAP activity required 10 mM NaF for inhibition. TRAP activity was absent from a thin line of unmineralized osteoid immediately beneath the cuboidal osteoblasts (small triangles in Figs. 2G and 2I). In the same sections, no TRAP activity was found in the mineralized matrix of the intramembranous bone shaft (bs in Figs. 2F and 2G), except sometimes at “reversal lines” (arrows in Fig. 3A) where it indicated previous osteoclast activity.\(^{(36,39)}\)

**Intralacunar bone formation in anchor regions**

In the bones from 17- to 20-day chick embryos, small pieces of former anchor regions were sometimes found within the main marrow cavity. Since the \(\alpha\) type I collagen antibody had been raised against the extension peptide of the \(\alpha 1(I)\) chain,\(^{(30)}\) it always stained the recently synthesized collagen more strongly than the mature, processed collagen. In Fig. 2F, the bone matrix of the bone shaft (bs, on right) and the mineralized part of the endochondral bone matrix stained weakly, indicating that this was mature collagen. However, the intralacunar bone matrix and the osteoid of the endochondral bone matrix showed very strong immunoreactivity. This indicated that the latter two were formed more recently than the first types of bone matrix.

In anchor regions, the occasional cell doublet was found in which only one cell contained DNA strand breaks (Figs. 3A and 3B). More frequently, lacunae were filled with type I collagen matrix and contained several cells. Such lacunae were usually close to the marrow tunnel, but not always connected to it, as shown by a series of confocal images (Figs. 3C–3F), confirming that the type I collagen had been formed inside intact lacunae.

Figure 3G shows the last remnant of an anchor region, where the endochondral bone matrix had already been resorbed by giant osteoclasts (oc) which will proceed to resorb the whole anchor region. Here the intralacunar bone is positive for acid phosphatase, whereas the bone of the shaft (bs, lower right) does not contain acid phosphatase activity. It is likely that this intralacunar bone had acquired tartrate-resistant acid phosphatase (TRAP) activity with mineralization, just as the endochondral bone had.

**DISCUSSION**

Since the requirement for rapid longitudinal expansion in birds is much greater than in mammals, they have adopted different strategies to achieve longitudinal growth. In mammals, the majority of longitudinal growth results from advancing the whole “plate,” i.e., cell proliferation and matrix synthesis at the top of the plate is more or less matched by resorption at the bottom of the plate. In birds, the thickness of the growth plate increases in addition to distal expansion. Resorption does not occur synchronously across the plate, rather primary vascular invasion takes place at the tip of “marrow tunnels,” which are regularly spaced around the periphery, leaving the bulk of the cartilage initially intact. Resorption of the remaining cartilage takes place at a later time, at the lateral walls of the marrow tunnels and, even later, at the main interface of cartilage with the marrow cavity. There are some consequences of this different strategy: (1) most chondrocytes have a longer life span than in mammals, particularly in the hypertrophic stage; (2) the stages of resorption and endochondral bone formation are temporally and spatially separated to a much greater extent than in mammals, which facilitates study of the sequence of events.
FIG. 2. Apoptosis in chondrocytes and endochondral bone formation. (A) LS of the hypertrophic region around a marrow tunnel in a 19-day femur, alcian blue/sirius red/hematoxylin. Many chondrocytes with morphological signs of apoptosis (arrows) are present around the “tip” of the marrow tunnels. (B) TUNEL of the proliferative region of a 14-day femur (see B for location). Apoptotic bodies are seen in one lacuna (+), while another lacuna (open arrow) contains an “asymmetric doublet” where only one cell shows positive TUNEL labeling. (C) TUNEL-labeled cells and TRAP-positive mononuclear resorbing cells (red) at the main cartilage (C)/marrow (M) interface (19-day femur; see C for location). Note that some cells with TUNEL-labeled nuclei seemed to be “trapped” in an intermediate region (I), which is sometimes found in the chick, where some “structure” remains, although the “ground substance” of the cartilage matrix has been resorbed. Some artifactual staining is present in the cells marked by the open circle, possibly due to endogenous alkaline phosphatase activity. (D) Autoradiography of an early bone-forming region in a femur which had been incubated with 3H-thymidine for 18 h. Isotope label overlies several cells in the marrow tunnel (small arrows) and also one cell of an “asymmetric doublet” whose lacuna is still separated from the marrow by a thin cartilage bridge (large arrow). (E) Type I collagen immunocytochemistry showing a slightly later stage in endochondral bone formation. A continuous layer of type I collagen (brown) has been deposited onto the wall and is also present intracellularly in the osteoblasts (ob) that have accumulated along the edge. “Behind” the edge, type I collagen is present on the inner perimeter of two lacunae (arrows) which contain two smaller cells each. (F and G) Serial sections of a former anchor region, (F) showing Type I collagen and (G) and TRAP activity. TRAP activity co-localizes with the more mature collagen, but is absent from the newly-synthesized endochondral and the intra-lacunar bone. (H, I, and K) Serial sections of a “protected” edge showing the localization of (H) Type I collagen; (I) tartrate-resistant acid phosphatase activity and (K) TUNEL-labeled nuclei. Lacunae that are recognizable in more than one section have been numbered. Resorption has taken place at the lower part of the picture, and lacuna 1 has been opened. TRAP activity colocalizes with type I collagen in lacunae 2 and 3 and in the endochondral bone matrix, except for a thin line which represents nonmineralized osteoid (small triangles). Lacuna 4 contains an “asymmetric” doublet where only one nucleus contains DNA strand breaks. Lacuna 1 contains three nuclei, of which one is labeled by the TUNEL method. The nonspecific blue reaction product in the osteoblasts lining the edge is due to endogenous alkaline phosphatase activity. Bars, 10 μm.

Programmed cell death in chondrocytes

At the bottom of the growth plate, cartilage tissue and cells need to be eliminated to make way for vascular canals or bone marrow. In mammals, hypertrophy precedes resorption, and the accepted view is that chondrocytes undergo apoptosis at the terminal stage of hypertrophy. The corresponding areas in the chick would be the main cartilage/marrow interface or resorbing regions at the lateral walls of marrow tunnels. The present study confirmed that hypertrophic chondrocytes underwent apoptosis at these sites. In addition, primary resorption at the “tip” of marrow tunnels occurred in the regions of resting or maturing chondrocytes. The chondrocytes just ahead of the advancing “tip” underwent apoptosis prior to vascular invasion, suggesting that hypertrophy was not a prerequisite for apoptosis but that chondrocytes at any stage of differentiation may be programmed to die.

This notion was strengthened by the findings that a small number of apoptotic chondrocytes were present at apparently random sites throughout the epiphysial cartilage. This suggested that sporadic apoptosis occurred continuously throughout chondrocyte differentiation. Although at any one time the apoptotic index was low (0–2%), with time a significant proportion of chondrocytes might be affected, since the characteristic morphological structures of apoptosis remain visible histologically for only a few hours. In a variety of experimental tumors, the apoptotic index was 0.3–2.2%, which nevertheless accounted for an overall cell loss of up to 90%. Even in thymocytes, the “classic” cells in which apoptosis has been extensively studied, only a small number of cells show an apoptotic morphology at any one time. Nevertheless, this seems to be sufficient for the elimination of autoreactive T-cell clones during the development of cellular immune self-tolerance. Apoptosis of chondrocytes during differentiation suggests that, like thymocytes, chondrocytes are generated in excess of eventual requirement. It is possible that apoptosis serves to correct “mistakes” during differentiation by eliminating faulty or inappropriate cell types.

Origin of the osteoblasts in endochondral bone formation

This has been the subject of considerable debate and controversy for at least 30 years. On the one hand, the evidence for differentiation from marrow-stromal cells rests on the osteogenic potential of the marrow stroma, as indicated by marrow transplantation or diffusion chamber experiments. On the other hand, autoradiographic studies, chick-quail chimaeric studies, organ culture studies of mouse metatarsalia, or cut embryonic chick femurs have provided convincing evidence of the osteogenic potential of chondrocytes. Since it is not possible to follow the fate of individual marrow stromal cells or chondrocytes in situ, the crucial question of whether these osteogenic potentials are realized in vivo has remained unanswered, although it is generally assumed that marrow stromal cells give rise to the endochondral osteoblasts.

Our previous study identified asymmetric cell divisions as early events during the chondrocyte-to-osteoblast transition in vitro. This concept provided a new approach for in situ investigations of the fates of chondrocytes. In vitro, the osteogenic differentiation of chondrocytes was induced by a cut through the hypertrophic region, which resulted in (as yet unknown) changes in the extracellular microenvironment of the chondrocytes. Since the dif-
FIG. 3. Intralacunar bone formation in an anchor region. (A) Overview of an anchor region bordered by marrow tunnels and the bone shaft. TUNEL method combined with TRAP histochemistry. In the bone shaft (left), TRAP activity is only present along reversal lines (small arrows). The strong activity in the bone matrix in the upper part (star) suggests endochondral origin of that matrix. (B) An enlargement of the central area shows one lacuna containing an “asymmetric doublet” with one TUNEL-labeled cell. (C, D, E, and F) Four confocal images of an anchor region where the bone shaft is on the left, the marrow tunnel at the bottom. Five lacunae near the edge contain type I collagen (green, FITC) inside the lacunae and up to four nuclei each (orange, stained with propidium iodide). The lacunae more distal to the tunnel are intact. (G) TRAP histochemistry of a late remnant of an anchor region undergoing resorption by giant osteoclasts (oc). The activity within the chondrocyte lacunae is present in intralacunar bone matrix, as seen in a parallel section stained for Type I collagen. Note that many lacunae contain more than one cell. Bars, 10 μm.
ferentiation state of chondrocytes is partly regulated by their extracellular environment, it is likely that cartilage resorption in vivo also causes changes in the environment of those chondrocyte near the resorptive front. These changes might alter the differentiation state of the chondrocytes and provide the stimulus for a change in cell type. The present study showed that “asymmetric doublets” were located near regions where the cartilage matrix had been resorbed, supporting the above concept. However, commitment to change phenotype was confined to chondrocytes near bone-forming surfaces, i.e., at any one time the vast majority of hypertrophic chondrocytes were not destined to change phenotype. This would explain why isolated chondrocytes rarely become bone-forming cells unless additional conditions are fulfilled, e.g., intrarenal transplantation, coculture with brain tissue or the addition of retinoic acid, although mouse Meckel’s cartilage chondrocytes seem to have the capacity to transform to osteocyte-like cells in vitro without any additional factors.

Even at bone-forming surfaces, the incidence of “asymmetric doublets” was low, suggesting either that only a few chondrocytes transdifferentiated to bone-forming cells at any one time and/or that an asymmetric cell division was a very short-lived event. The occurrence of an asymmetric cell division in itself did not prove that the viable cell of that division would become osteogenic. This required further evidence, such as the expression of typical bone proteins or, at a later stage, the deposition of bone matrix. This evidence was readily obtained where the surface of the cartilage had been covered with endochondral bone matrix, as in the “protected” and “anchor” regions of the chick growth plate. Here the lacunae remained intact, and unambiguous proof that chondrocytes had become bone-forming cells was obtained. Similarly, a direct chondrocyte-osteoblast transition had been demonstrated in the cartilaginous center of the so-called “mixed” spicules during fracture repair. In view of this, the possibility that the viable cell of an asymmetric cell division became osteogenic after release into the marrow space cannot be discounted.

**TRAP activity in the endochondral bone matrix**

TRAP is generally recognized as a histochemical marker for skeletal resorbing cells, such as osteoclasts and their precursor cells. Hence, it was surprising to find TRAP activity in the mineralized matrix of endochondral bone, although in all other aspects the endochondral bone matrix was identical to intramembranous bone matrix. The presence of TRAP activity thus provided a way to distinguish the two types of bone matrix. Similar results were obtained for the medullary bone which is formed in egg-laying hens. No enzyme activity was detected in osteoblasts or the osteoid of endochondral bone, just as the osteoblasts or osteoid of medullary bone did not have any TRAP activity. Either an inactive form of the enzyme was secreted by osteoblasts, which became activated with calcification of the matrix or acid phosphatase was present in the osteoblasts, but activity was below the level of detection. There have been reports that chick and rat osteoblasts contain fluoride-sensitive TRAP activity, albeit much less than osteoclasts. Hence, the osteoblasts that lay down the endochondral bone matrix are in at least one respect different from the periosteal osteoblasts which lay down intramembranous bone matrix.

The presence of TRAP also raises the question as to the function of the enzyme in endochondral and medullary bone. Both are temporary bone structures, destined for resorption once they have fulfilled their function. Nevertheless, during an interim time period, the presence of endochondral bone is crucial in protecting selected regions of cartilage from resorption. TRAP dephosphorylates osteopontin and bone sialoprotein and thereby detaches osteoclasts, so the presence of TRAP activity throughout the matrix of endochondral bone might temporarily prevent remodeling.

**The fate of the hypertrophic chondrocytes at the avian growth plate**

Apart from the sporadic apoptosis that occurred in a small number of chondrocytes at all stages of differentiation, the fate of avian chondrocytes depended on their location within the growth plate. Where resorption was the prime requirement, as at the top of marrow tunnels or at the main cartilage/marrow interface, the available evidence suggested that chondrocytes underwent apoptosis. However, where selected areas of cartilage required “strengthening,” as in anchor regions, bone formation took place within lacunae by former chondrocytes. In bone-forming regions at the walls of marrow tunnels, chondrocytes underwent asymmetric cell divisions, consistent with a chondrocyte-to-osteoblast transition. This strengthens the evidence that chondrocytes as well as marrow stromal cells give rise to endochondral osteoblasts. The novel finding that the avian endochondral bone matrix contains TRAP activity indicates that endochondral osteoblasts may be different from periosteal osteoblasts.

**Do chondrocytes become bone-forming cells at the mammalian growth plate?**

There have been many excellent and detailed histological studies of the terminal chondrocytes in the mammalian growth plate, but evidence of an osteogenic differentiation was never found in that region. This is not surprising, since the site of vascular invasion in mammals would correspond to the top of marrow tunnels in birds, where no evidence of osteogenic differentiation was found either. In mammals, most of the cartilage spicules that provide the scaffolds for endochondral bone formation do not contain cells, and hence the conditions for a chondrocyte-osteoblast transition are not present. However, sometimes chondrocytes do persist within these spicules and become trapped by the deposition of endochondral bone, analogous to the “mixed spicules” of the fracture callus. It is in those areas, which are found at the side or below the actual growth plate, where one might expect bone-forming cells within intact chondrocytic lacunae.
ACKNOWLEDGMENTS

The author gratefully acknowledges the technical assistance of Mr. Adrian Wilkins, the provision of antiserum by Prof. Larry Fisher (Bethesda) and Prof. Louis Gerstenfeld (Boston), and the financial support of the Wishbone Trust.

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Address reprint requests to:
Dr. H.I. Roach
Academic Orthopaedic Unit
CF 86, MP 817
Southampton General Hospital
Tremona Road
Southampton, SO16 6YD, U.K.

Received in original form September 9, 1996; in revised form December 6, 1996; accepted January 8, 1997.