

Osteogenic Differentiation of Hypertrophic Chondrocytes Involves Asymmetric Cell Divisions and Apoptosis

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Abstract. We have investigated the early cellular events that take place during the change in lineage commitment from hypertrophic chondrocytes to osteoblast-like cells. We have induced this osteogenic differentiation by cutting through the hypertrophic cartilage of embryonic chick femurs and culturing the explants. Immunocytochemical characterization, [³H]thymidine pulse-chase labeling, in situ nick translation or end labeling of DNA breaks were combined with ultrastructural studies to characterize the changing pattern of differentiation. The first responses to the cutting, seen after 2 d, were upregulation of alkaline phosphatase activity, synthesis of type I collagen and single-stranded

DNA breaks, probably indicating a metastable state. Associated with the change from chondrogenic to osteogenic commitment was an asymmetric cell division with diverging fates of the two daughter cells, where one daughter cell remained viable and the other one died. The available evidence suggests that the viable daughter cell then divided and generated osteogenic cells, while the other daughter cell died by apoptosis. The results suggest a new concept of how changes in lineage commitment of differentiated cells may occur. The concepts also reconcile previously opposing views of the fate of the hypertrophic chondrocyte.

ASYMMETRIC cell division refers to any cell division that produces two daughter cells with different fates (for a review see Horvitz and Herskowitz, 1992). These special types of divisions are repeatedly observed in the tissues of plants and invertebrates, where asymmetric cell divisions provide a mechanism for generating cell diversity during lineage commitment. In vertebrates, asymmetric cell divisions may be involved at branch points during lineage commitment, for example in human hematopoietic cells (Mayani et al., 1993), in the rat corneal epithelium (Lamprecht, 1990) and during differentiation of thymocytes in the chicken, guinea pig, and mouse (Sugimoto and Yasuda, 1983). Here we report that asymmetric cell divisions also occur during the phenotypic change from cartilage to bone-forming cells.

This osteogenic differentiation of hypertrophic chondrocytes takes place during endochondral ossification, i.e., the process whereby tissue that initially consists completely of cartilage is replaced bone. Although the majority of the bone-forming cells differentiate from marrow stromal cells (Wolbach and Hegsted, 1952; Howlett, 1980; Pechak et al., 1986; Roach and Shearer, 1989; Beresford, 1989), the notion that some hypertrophic chondrocytes

may also give rise bone-forming cells has been suggested repeatedly during the last three decades (Holtrop, 1966, 1972; Crelin and Koch, 1967; Lufti, 1971; Shimomura et al., 1975; Kahn and Simmons, 1977; Thesingh et al., 1986, 1991; Weiss et al., 1987; Yoshioka and Yagi, 1988; Moskalewski and Malejczyk, 1989; Galotto et al., 1994). The alternative view is that all hypertrophic chondrocytes are terminal cells which degenerate and die (Anderson and Parker, 1966; Bentley and Geer, 1970; Brighton et al., 1973; Hanaoka, 1976; Hunziker et al., 1984; Farnum and Wilsman, 1989; Farnum et al., 1990).

In recent years even more compelling evidence for the osteogenic differentiation of chondrocytes has been obtained from a variety of in vitro systems (Strauss et al., 1990; Closs et al., 1990; Roach, 1992a; Descalzi-Cancedda et al., 1992; Gentili et al., 1993). We have studied the process using an organ culture system of embryonic chick bones (Roach, 1990), where we had previously found that more chondrocytes could be induced to differentiate to bone-forming cells if the cartilage matrix was cut. This cut seemed to simulate the micro-mechanical changes that take place when cartilage matrix is resorbed in vivo. When cut explants of epiphyses and bone shafts were cultured for 12–15 d, bone matrix was present in many intact chondrocytic lacunae (Roach, 1992a). Here we follow the early cellular events during the phenotypic change from chondrocytes to bone-forming cells and show that one of the

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crucial events is an asymmetric cell division, where the fate of the daughter cells diverges: one cell remains viable and becomes osteogenic while the other is destined to die by apoptosis.

Materials and Methods

Dissection and Organ Culture Methods

Femurs were dissected from 14-d old chick embryos. At this stage the bones consist of approximately equal amount of epiphyseal cartilage and trabecular bone shaft with a central marrow cavity (Fig. 1). In avian bones the region of hypertrophic cartilage is fairly wide so that it is not difficult to cut through this region with a scalpel. The approximate site of the cut is indicated by a solid line in Fig. 1a. The femurs were either cultured whole (uncut controls) or cut into three pieces, i.e., two epiphyses and one bone shaft. The whole or cut femurs were maintained in organ culture as previously described (Roach, 1990, 1992b). Media were changed every 24 h when freshly prepared ascorbic acid (50 $\mu\text{g/ml}$) was also added (Roach et al., 1985). The bones or explants were harvested after 2, 3, 4, 6, 9, and 12 d.

Electron Microscopy

Thin slices of material near the cut edge were either fixed in 2% glutaraldehyde in neutral Na-cacodylate buffer or in 2.5% glutaraldehyde (in 0.1 M cacodylate buffer, pH 6.2) containing 1% Alcian blue. Alcian blue protected proteoglycans from solubilization and preserved chondrocyte and matrix morphology in a similar manner to the Ruthenium hexamine trichloride used by Hunziker et al. (1982), provided the tissue pieces were very thin. The sections were postfixed in 2% osmium tetroxide and 2% uranyl acetate, processed for standard TEM, and embedded in Spurr's resin. Semithin sections were stained with toluidine blue, the ultra-thin sections were contrasted with lead citrate or double contrasted with uranyl acetate and lead citrate.

Light Microscopy

The tissues were fixed either in 4% buffered paraformaldehyde or in 85% ethanol. The latter was a better fixative for alkaline phosphatase activity, proliferating cell nuclear antigen (PCNA)¹ antigenicity (Casasco et al., 1994) and [³H]thymidine autoradiography, whereas the aldehyde fixative was required for in situ detection of nuclear DNA fragmentation. Tissues were processed without decalcification through graded ethanols and chloroform into paraffin wax. Sequential sections were cut at 5–6 μm (or 10–20 μm for the confocal microscope) and stained by one of the following methods: Weigert's haematoxylin/alcian blue/sirius red (after Lison, 1954) was used as a nuclear stain and to distinguish bone matrix (red) from cartilage matrix (blue). Alkaline phosphatase was demonstrated with an azo dye coupling method (modified from Bancroft and Stevens, 1982; described in detail in Roach and Shearer, 1989).

Immunocytochemistry

The following polyclonal antibodies were used: the antibody for type I collagen (LF-67 from Professor L. Fisher) had been raised against the human COOH-terminal pro-peptide of the α -1 chain. This antibody detects pro- α -1, but also the fully processed α -1 chain (Fleischmajer et al., 1990). The osteopontin (details in McKee et al., 1990) and osteonectin (LF-8; details in Pacifici et al., 1990) antibodies were specific for chick and were donated by Dr. Yoze Gotoh (Boston) and Professor Larry W. Fisher (Bethesda), respectively. The osteocalcin antiserum (α bovine) was a gift from Dr. Simon Robins (Aberdeen), and the bone sialoprotein antiserum (α porcine, details in Chen et al., 1991) was kindly donated by Dr. Qi Zhang (Toronto). Although not specific for chick, the later two antisera cross-reacted with the corresponding chick proteins. 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. Control sections were incubated with rabbit serum (negative control, Sigma Chem. Co., St. Louis, MO), and then treated as above. No staining was found in controls. Proliferating cells were identi-

fied (a) by incubating with [³H]thymidine (2 $\mu\text{Ci/ml}$) for 12–24 h followed by autoradiography of the paraffin sections and (b) by using a monoclonal antibody against PCNA (PC-10, DAKO). [³H]Thymidine labels cells in the S-phase, whereas PCNA begins to accumulate during the G1 phase of the cell cycle, reaches maximal synthesis during the S-phase, and declines during the G2 phase (Kurki et al., 1988).

Pulse-chase Experiments

To determine the fate of those cells that had been in the S-phase during the early days of culture, [³H]thymidine was added to the culture medium between 24–48 h or 48–72 h. The cut or uncut bones were either harvested at the end of the labeling period or cultured for 6–9 d.

Autoradiography

Sections were dipped in photographic emulsion (K-5, Ilford, 1:1 dilution, 45°C), exposed in a dark box at 4°C for 2 wk, developed with Kodak Dektol (2 min), and fixed with 30% Na-thiosulphate (5 min). The sections were either counterstained with Van Gieson after development or the autoradiography was preceded by the alkaline phosphatase method, or immunocytochemistry using any of the above antibodies.

In Situ Hybridization

A specific cDNA probe of the 5' end of the α 1(I) cDNA (P1E1) was generated by PCR (Dietz et al., 1993). The construct was linearized with Eco RI and transcribed in vitro using T7 RNA-polymerase (Promega Southampton, England) to generate (α -³⁵S)UTP-labeled antisense transcripts. Not incorporated nucleotides were separated by alcohol precipitation. In situ hybridization was performed as described in detail elsewhere (Aigner et al., 1992). Briefly, paraformaldehyde-fixed wax sections were deparaffinized, digested with proteinase K (7 min, 20 $\mu\text{g/ml}$, RT), postfixed with 4% paraformaldehyde, and acetylated. The sections were hybridized for 12–16 h at 43°C with the riboprobe at a final activity of 4×10^7 cpm/ml. After washing, the sections were treated with RNases A (20 $\mu\text{g/ml}$) and T1 (50 U/ml), washed again for 2 h at 45°C, $2 \times \text{SSC}/50\%$ formamide/0.5% β -mercaptoethanol, and then three times in $0.1 \times \text{SSC}$ at RT, followed by dehydration. Autoradiography was performed (Kodak NTB-2 nuclear track emulsion) for 4 d and sections counterstained in 5% Giemsa dye.

In Situ Detection of DNA Breaks by Nick Translation or End Labeling

Two methods were used on paraformaldehyde-fixed sections: (1) the in situ nick translation method (ISNT) was based on that of Oberhammer et al. (1993), as modified by Edwards and Tolkovsky (1994) and (2) the end-labeling method of Gavrieli et al. (1992). DNA polymerases (holoenzyme and the Klenow fragment) and terminal transferase were purchased from Sigma Chem. Co. None of the enzymes had any endonuclease activity. Nicks in the DNA breaks can be "translated" by DNA polymerase I which, using the other strand as a template, will incorporate labeled dUTP. The holoenzyme has 3'- and 5'-exonuclease activity in addition to its polymerase activity. This means that single-stranded "nicks" can be widened by removal of existing nucleotides before repair. The Klenow enzyme lacks the 5'-exonuclease activity. It will label either "wide" single-stranded breaks or staggered double-stranded breaks. Terminal transferase does not require a template strand and will add labeled nucleotides to staggered or blunt-ended double-stranded DNA breaks.

Sections were dewaxed and hydrated, and then digested for 15 min at room temperature with proteinase K (5 $\mu\text{g/ml}$ in 0.1 M Tris/EDTA buffer, pH 8). The degree of digestion was crucial, higher concentrations of proteinase K (20 $\mu\text{g/ml}$) resulted in labeling of almost every cell, while in absence of proteinase K no cells were labeled, even in positive (DNase treated) controls. After washes, sections were refixed with 4% paraformaldehyde for 20 min at 4°C, washed, and then pretreated with the respective reaction buffer for 15 min (ISNT: 50 mM Tris/HCl, pH 7.4, 10 mM MgCl₂, 50 mg/ml BSA); (end-labeling: 30 mM Tris/HCl, pH 7.2, 1% BSA, 140 mM Na-cacodylate, 1 mM CoCl₂). Samples were then incubated for 1 h at 37°C in 25 μl of the respective reaction buffer containing 1 μl dNTPs (DIG-labeling mix, Boehringer Mannheim Corp. UK, Lewes, England) and the relevant enzyme (ISNT: 6 U of DNA polymerase, either holoenzyme or Klenow fragment); (end-labeling: 7.5 U terminal transferase). The reaction was stopped with 50 mM TRIS/HCl, 20 mM EDTA, pH 7.4 (for ISNT) or 0.1 M Tris/HCl containing 300 mM NaCl and 30 mM

1. *Abbreviations used in this paper:* ALP, alkaline phosphatase; ISNT, in situ nick translation; PCNA, proliferating cell nuclear antigen.

Na-citrate (for end labeling). After extensive washing, the incorporation of digoxigenin-labeled dUTP was detected using monoclonal anti-digoxigenin FAB fragments, linked directly to alkaline phosphatase (Boehringer Mannheim Corp.; 1:1,000 dilution; 1–2-h incubation), followed by color development with NBT/BICP as recommended by the manufacturer (40 min).

Controls. Positive controls were processed through proteinase K as described, and then treated with DNase (2 U/ml) for 1 h at RT (buffer = 30 mM Tris/HCl, pH 7.2, 140 mM Na cacodylate, 4 mM MgCl₂, 0.1 mM dithiothreitol). 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.

Results

Location of Chondrocytes Undergoing Osteogenic Differentiation

Unlike the mammalian growth plate where the hypertrophic zone consists of a few cell layers only, the avian growth plate is very wide and irregular (Fig. 1, and Roach and Shearer, 1989). Osteogenic differentiation was observed near the cut edges both in the epiphyses and the cartilage “plug” that remained within the cut bone shaft (* in Fig. 1 *a*, later referred to as “transition region”). The analyses of the osteogenic differentiation patterns were confined to those areas where any invasion of periosteal mesenchymal or bone marrow stromal cells could be excluded by (a) absence of marrow tunnels and (b) intactness of the chondrocytic lacunae. The latter was confirmed by serial sectioning and confocal microscopy (not shown). For electron microscopy, we were careful to select lacunae that were well away from any channels or marrow tunnels. All the cells shown in Figs. 2–4 were present inside intact lacunae and were not connected via any channels to the cut edge or marrow tunnels.

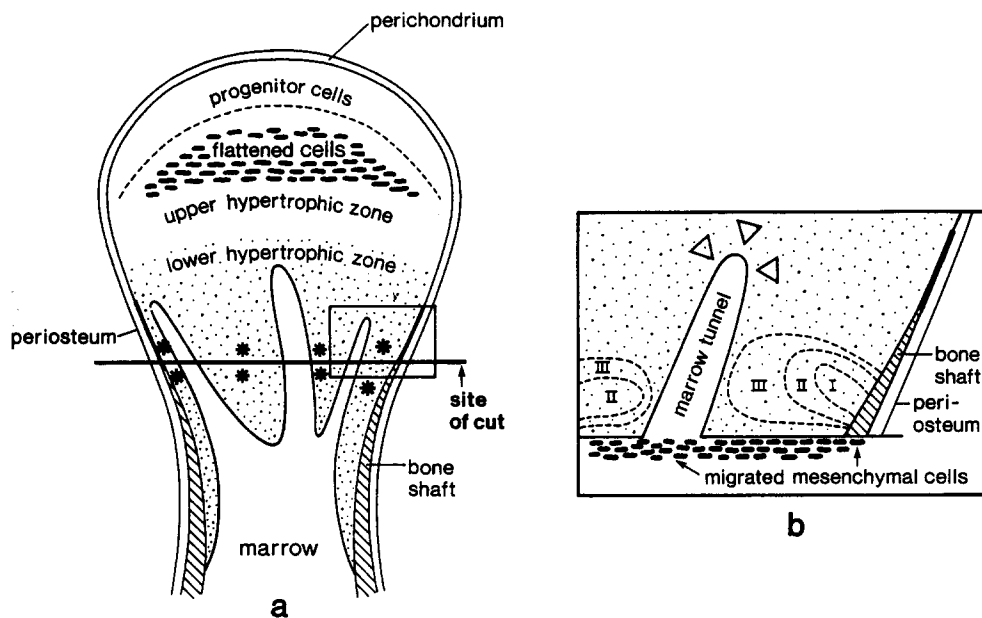


Figure 1. Diagram of the epiphysis of a femur from 14-d old chick embryos. The stages of chondrocyte differentiation are outlined and the approximate site of the cut indicated by the solid line (Fig. 1 *a*). Osteogenic differentiation was found in the separated epiphyses and shaft at the locations indicated by stars. Fig. 1 *b* illustrates the spatial progression of osteogenic differentiation with time in culture. Chondrocytes undergoing asymmetric cell division and the subsequent stages of osteogenic differentiation were first found near the bone shaft and a few cell layers away from the cut edge (region I in Fig. 1 *a*). With time in culture chondrocytes fur-

ther away from this area also responded (regions II and III). At the end of marrow tunnels (triangles) osteogenic differentiation also took place, but these regions were not studied in detail because the proximity of the lacunae to the marrow tunnel made interpretation of the results ambiguous.

Immediate Responses: Type I Collagen, Alkaline Phosphatase, and DNA Breaks

Within 48 h, changes were found in most chondrocytes in a region that was more extensive than the region where intra-lacunae bone matrix was present after 12 d. Around 10–30% of the lacunae contained cell doublets, but the responses described below were equally found in single chondrocytes. Type I collagen was present along the perimeter of many lacunae containing both single chondrocytes and cell doublets (Fig. 2 *a*); alkaline phosphatase activity was present in the cytoplasm (Fig. 2 *b*) and many nuclei contained single-stranded DNA strand breaks, as indicated with the ISNT method using the holo DNA polymerase I (Fig. 2 *c*). In the same sections differentiating cells, such as proliferating and aligned chondrocytes, and actively transcribing cells, such as osteoblasts, were also labeled by ISNT with the holoenzyme. On the other hand, no hypertrophic chondrocytes were labeled by the end-labeling method nor by ISNT when the Klenow enzyme was used, whereas in the same sections those cells which die during culture, such as some osteocytes and lots of marrow cells (Roach, 1990), were labeled. This suggested that the holoenzyme detected DNA nicks which were not related to apoptosis (Eastman and Barry, 1992; Ansari et al., 1993) but were perhaps due to the transient nicks which are produced by topoisomerases to relieve the torsional consequences of transcriptional elongation (Stewart et al., 1990). In studies where the holo DNA polymerase I was used for the detection of apoptotic cells (Gold et al., 1993; Oberhammer et al., 1993; Edwards and Tolkovsky, 1994), actively transcribing or differentiating cells might have been absent.

At this early stage, no positive reaction product was found in hypertrophic chondrocytes with immunocy-

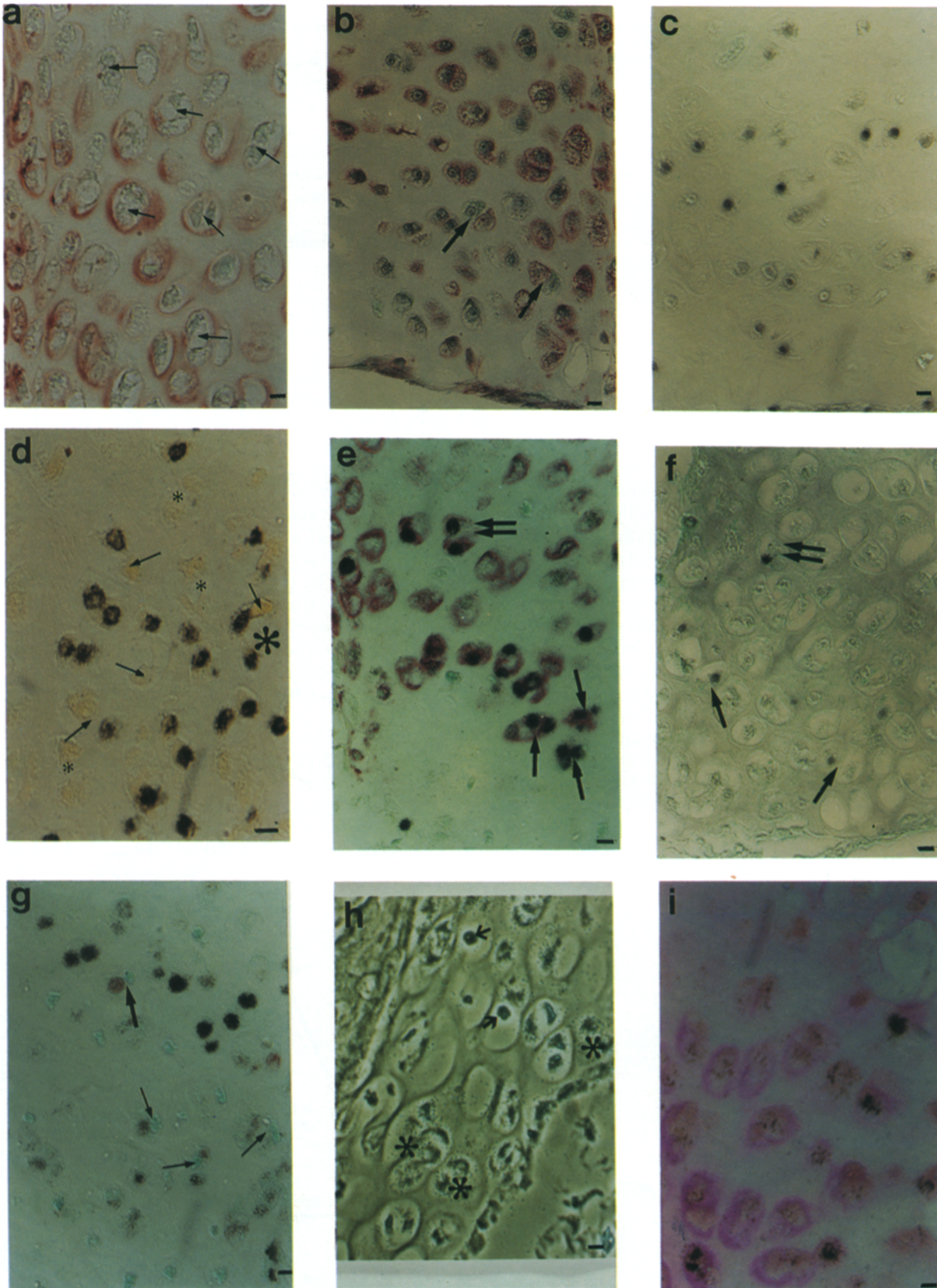


Figure 2. Cellular events during the osteogenic differentiation from chondrocytes. All examples are longitudinal sections from cut femurs after various times of culture. The cut edge is always at the bottom, but may be out of view. Bars, 10 μm . (a) Type I collagen around chondrocytes after 2 d. The bone shaft is just visible on the left. All lacunae contain a layer of type I collagen around the inner perimeter, sometimes spreading into the interterritorial matrix. Many lacunae contain cell doublets (*arrows*). (b) Alkaline phosphatase

Table I. Appearance of Typical Bone Proteins in Chondrocytic Lacunae in Cut and Uncut Cultured Femurs

	day 2		day 6		day 9	
	cut	uncut	cut	uncut	cut	uncut
Alkaline phosphatase	++	-	+++	±	++++	++
Type I collagen	++	-	++	-	+++	±
Osteonectin	-	-	++	±	+++	+
Osteopontin	-	-	+	-	+++	±
Bone sialoprotein	-	-	-	-	+++	±
Osteocalcin	-	-	-	-	+++	±

Femurs were cultured intact (uncut) or were cut through the hypertrophic cartilage and the separated epiphyses and bone shafts were cultured for the days indicated. The number of positive cells in the hypertrophic region was scored from absent (-) to present in many cells (++++). ± indicates presence on very few chondrocytes near the bone shaft.

tochemistry using antibodies to noncollagenous bone proteins (Table I).

At the ultrastructural level, hypertrophic chondrocytes were large, pale cells that filled their lacunae. Before culture, they were relatively quiescent with some signs of senescence: few mitochondria, rather short and narrow strands of RER and some fat globules (not shown). Culture alone seemed to activate metabolic activity, as indicated by the larger and more numerous mitochondria and more developed RER (Fig. 3 a). In cut femurs, cell doublets (Fig. 3 b) and single chondrocytes (not shown) were surrounded by collagen fibers that were thicker than the cartilage-specific type II collagen fibers and which showed the typical banding pattern of type I collagen (inset in Fig. 3 b), consistent with the light microscopic findings.

In uncut femurs, cultured for 2 d, chondrocytes were also activated, 1–2% of lacunae contained cell doublets, ~5% of chondrocytes were labeled with the ISNT (holoenzyme) method, but otherwise the chondrocytes showed none of the above responses.

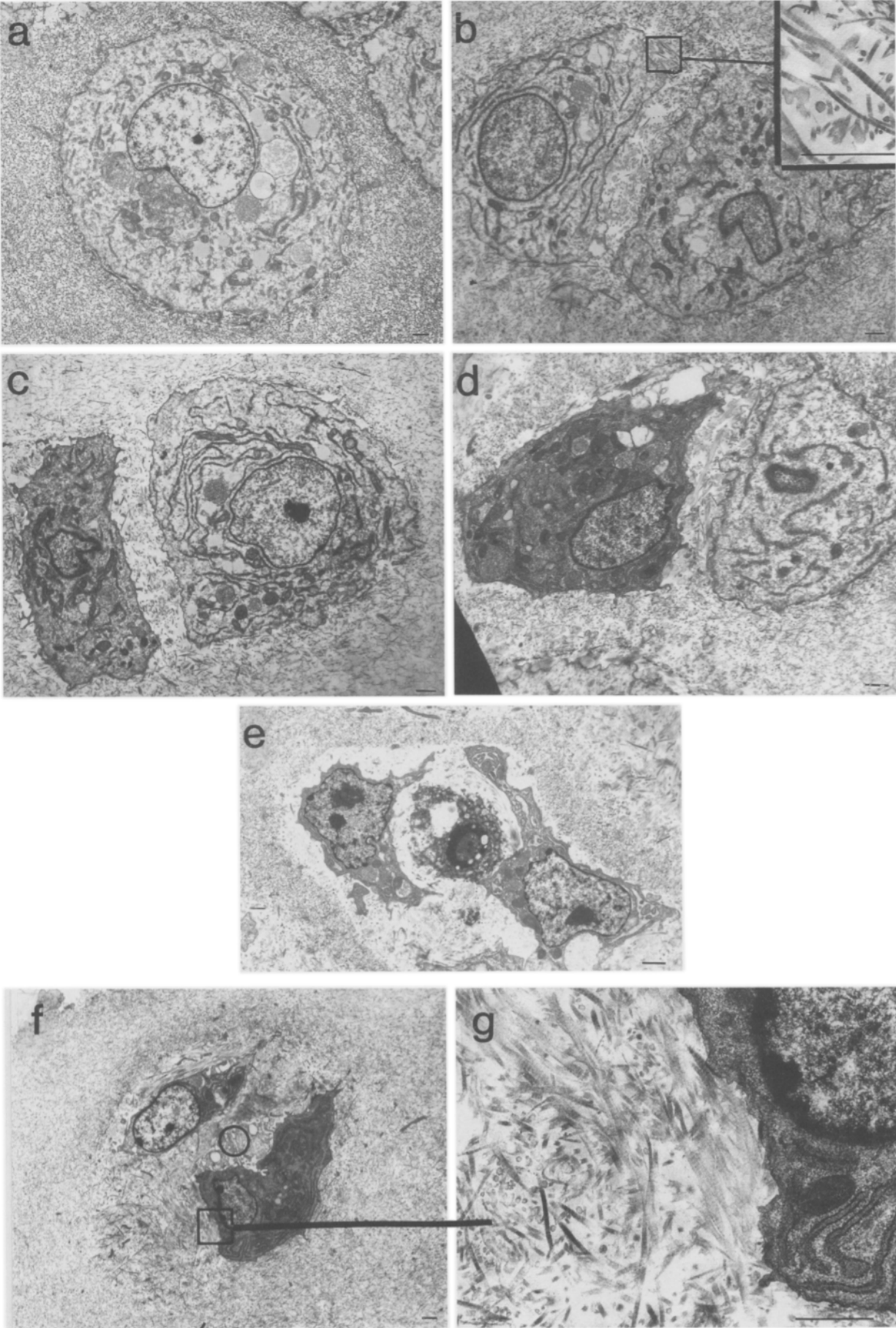
Two Different Cell Types within One Lacuna

Cell doublets where the two cells had different characteristics or morphology were present in ~5% of the lacunae in the transition region after 2–4 d. When incubating with [³H]thymidine between 24–48 or 48–72 h, labeling was sometimes present in only one cell of cell doublets (arrows in Fig. 2 d), suggesting that only one cell was in the S-phase during the labeling period. In cell triplets (*), two cells were heavily labeled whereas the third was not, consistent with the notion that only one cell had the capacity to continue proliferation.

After 4 d of culture, those cells that had gone through the cell cycle during day 2–3 were always positive for alkaline phosphatase (Fig. 2 e). Activity was still present in other chondrocytes, but tended to concentrate at the periphery of the lacunae. Some lacunae contained three cells in the plane of section (arrows in Fig. 2 e). The triplet where two of the nuclei were heavily radiolabeled and surrounded by alkaline phosphatase activity while the third one was not (double arrow in Fig. 2 e) suggested that the proliferating daughter cell maintained alkaline phosphatase activity while the other cell lost activity.

Care needed to be taken when interpreting the in situ techniques as evidence for apoptosis. The nuclei of embry-

activity in chondrocytes after 2 d. Most chondrocytes show enzyme activity in the cytoplasm. Again many cell doublets are present, in two such doublets enzyme activity is found in only one cell (arrows). In our view this ALP activity is not yet indicative of osteogenic differentiation. (c) Single-stranded DNA breaks after 2 d. Nuclei containing single-stranded nicks in the DNA were identified by in situ nick translation (ISNT) using the holoenzyme of DNA polymerase I. Most lacunae which contain a nuclear profile are labeled, in one instance both nuclei of a doublet are labeled. In parallel sections, using ISNT with the Klenow fragment or in situ end-labeling method with terminal transferase, no staining was found in the cells of this region. It is therefore unlikely that the labeling indicates apoptosis. (d) Burst of cell proliferation after 3 d. Many cells in the hypertrophic region have taken up [³H]thymidine between 48–72 h. Of the cell doublets present, some are unlabeled (small *), in others only one daughter cell is labeled, consistent with the notion that following asymmetric cell division, only one daughter cell is in the S-phase (arrows). In the lacuna marked by a large asterisk one daughter cell from the asymmetric cell division appears to have divided during the 24-h labeling period. (e) Alkaline phosphatase (ALP) activity and [³H]thymidine autoradiography after 4 d. [³H]Thymidine had been present in the medium on day 2–3. Most cells that had been in the S-phase during that time have gone through the M-phase. Four examples are shown, where the lacuna contains three nuclei (arrows). In one case the third nucleus is unlabeled (double arrows), consistent with the interpretation that the asymmetric division took place between day 1–2 and that the viable cell, which was in the S-phase during day 2–3, had now completed mitosis. In the other three cases the asymmetric division probably took place during the labeling period and the viable cell divided a second time during day 3–4. Note also that alkaline phosphatase activity is present in most cells, not just the radiolabeled ones. (f) In situ detection of apoptotic cells after 4 d. Apoptotic cells are characterized by double-stranded DNA breaks which were detected by end labeling with terminal transferase. Three lacunae contain doublets with one labeled nucleus (arrows). In one case (double arrows) two unlabeled cells share the lacuna with one labeled cell, similar to the example given shown in Fig. 2 e. Some single chondrocytes are also labeled. At the top left (*) a marrow tunnel is present and two cells are labeled which seem to be released into the marrow tunnel. (g) PCNA immunocytochemistry and [³H]thymidine autoradiography after 7 d. Labeling with [³H]thymidine took place during day 2–3. The approximate number of mitoses can be estimated from the dilution of the radiolabel. Some cells have only divided once (doublet with two intensely labeled cells), but most have divided 2–4 times. Note that no radiolabel is present over those cells with condensed (green) nucleus. Such a cell may share one lacuna with radiolabeled cells (arrows). One doublet can be seen where one daughter cell is positive for PCNA, while the other has a pyknotic nucleus and neither cell is radiolabeled (large arrow). This suggests that these cells were not in the S-phase during day 2–3, but asymmetric cell division has taken place since that time. (h) In situ hybridization for type I collagen mRNA after 6 d. The message is particularly strong in those lacunae that contain cell doublets (*), whereas single cells with condensed nuclei (arrows) only contain background grains. This is consistent with the view that the former cells were now osteoblast-like, while the latter cells were dying. (i) [³H]Thymidine autoradiography and osteoid formation after 9 d. Labeling with [³H]thymidine took place during day 2–3. Osteoid is identified by Acid fuchsin (red). All lacunae with osteoid contain diluted [³H]thymidine label, indicating that the bone-forming cells were derived via several mitoses from cells that were in the S-phase during day 2–3.



onic chick cells were much more sensitive to proteinase K digestion than rat ileum cells, since the usual regime of 20 µg/ml for 15 min at RT resulted in the labeling of almost every cell. However, when only 5 µg/ml of proteinase K were used, followed by ISNT with the Klenow enzyme or end labeling with terminal transferase, only those cells which are known to die in culture, such as osteocytes and marrow cells (Roach, 1990), were labeled together with ~5% of chondrocytes in the transition region. The similar distribution of cells labeled by terminal transferase and the Klenow enzyme suggested that the labeled DNA breaks were "staggered" double-stranded breaks. Such breaks would result from the cleavage of DNA into nucleosome-sized fragments by an endogenous endonuclease that is characteristic for apoptotic cells (Kerr et al., 1987; Bowen and Bowen, 1990; Wyllie, 1992; Parchment, 1993; Coates, 1994). This suggested that end tailing with terminal transferase or nick translation with the Klenow enzyme were selective for apoptotic cells.

When using the end-labeling method, doublets could be identified where only one cell was labeled (arrows in Fig. 2 f). In cell triplets, the cell between two unlabeled cells was heavily labeled (double arrows in Fig. 2 f). Comparing the triplets indicated by double arrows in Fig. 2, e and f suggested that the central cell (which had not proliferated and had lost alkaline phosphatase activity) was destined to die.

Ultrastructural studies (Fig. 3, c and d) also showed cells of different morphology in one lacuna. The cytoplasm of one cell remained pale (as in hypertrophic chondrocytes), while the other had darker, more basophilic cytoplasm which is characteristic for osteogenic cells. Some of the paler cell showed signs of disintegration (Fig. 3 d; note the diffuse RER), whereas the darker cell contained many mitochondria and an active REM, suggesting that it was a viable cell with active protein synthesis. In cell triplets, two smaller cells with basophilic cytoplasm, much RER and large nuclei surrounded a central cell with shrunken cytoplasm and condensed chromatin (Fig. 3 e). The compaction of the chromatin at the periphery of the nucleus was very characteristic for apoptotic cells, providing ultrastructural evidence for apoptosis of the central cell.

Subsequent Fates of the Apoptotic and Viable Cells

After 6–9 d, the cells which had been in the S-phase on day 2–3 had gone through 1–3 rounds of mitosis as judged by

the dilution of the [³H]thymidine label (Fig. 2, g and i) and by the number of smaller cells now present in the lacunae (Fig. 4, a–d). Frequently, the radiolabeled cells (Fig. 2 g) or BSP-positive cells (Fig. 4 d) shared a lacuna with one other cell which had little or no cytoplasm, but whose nucleus stained intensely with light green. Alkaline phosphatase activity, type I collagen, and the noncollagenous proteins were present in the smaller radiolabeled cells, but always absent from the condensed cell (not shown). No DNA breaks could be identified in the condensed cells, even in DNase-treated positive controls, when all other cells were labeled by ISNT or end labeling. This suggested that the nuclei were so tightly condensed that the DNA was inaccessible to the DNA polymerase or terminal transferase. It is possible that these nuclei were the remnants of apoptotic cells, presumably because their isolation inside lacunae prevented removal by phagocytosis. Immediately at the cut edge most lacunae contained single condensed cells after 6 d, whereas in the transition region up to 30% of lacunae contained such cells. With time in culture more and more lacunae were occupied by viable cells, possibly by migration of these cells from neighboring lacunae (Fig. 4, a and d).

The viable cells became positive for the markers of the osteogenic phenotype. After 6 d, in situ hybridization with a riboprobe for chick type I collagen mRNA confirmed that these cells expressed the message for type I collagen (Fig. 2 h). At the same time very strong alkaline phosphatase (ALP) activity was present inside the lacunae and the cells were positive for osteonectin and osteopontin (Fig. 4 c, Table I). After 9 d of culture, osteoid matrix was present along the inner perimeter of lacunae containing diffuse [³H]thymidine label (Fig. 2 i), confirming that the cells that had been in the S-phase during days 2–3 had synthesized the osteoid matrix. This matrix also stained with Sirius red (Fig. 4 a and Roach, 1992a) and contained the typical bone matrix components, such as collagen type I (Fig. 4 b) and osteopontin (Fig. 4 c). The cells were positive for osteocalcin and bone sialoprotein (Fig. 4 d and Table I), markers of the terminal osteogenic phenotype. The ultrastructural features of the cells were osteoblast-like: basophilic cytoplasm, large amounts of RER, and an eccentric nuclei (Fig. 3 f). Most of the space around and between them were filled by collagen fibers of random orientation with the diameter and typical striated appearance of type I collagen (Fig. 3 g).

Figure 3. Ultrastructural changes during osteogenic differentiation. (a) A hypertrophic chondrocyte from a cultured femur. It is a metabolically active cell characterized by a pale cytoplasm. Fixed with Alcian blue, hence proteoglycans are stained and the peri-lacunar cartilage matrix is clearly visible. (b) A cell doublet surrounded by noncartilage collagen. Chondrocytes appear to have divided by symmetric division. Noncartilage collagen fibers with the size and banding pattern of type I collagen (*inset*) surround the two chondrocytes. Such fibers are not present around chondrocytes in uncut femurs. (c and d) Asymmetric cell division: different cell types within one lacuna. One cell with darker, more basophilic cytoplasm shares a lacuna with another cell with paler cytoplasm. In Fig. 3 c the paler cell is still viable with the morphology of a chondrocyte, whereas in Fig. 3 d the paler cell shows signs of disintegration. Fibers, probably type I collagen, are present around and between the two cells. Fixed without Alcian blue, hence the the perimeter of the lacuna is not as distinct as in the other micrographs, but the cells are a clear distance away from neighboring cells, as confirmed at lower magnification. (e) Two smaller, viable cells surround an apoptotic cell. The two cells have dark cytoplasm and the high ratio of nucleus to cytoplasm that is typical for young cells. The cell in the center shows the typical ultrastructural features of an apoptotic cell: condensed chromatin which is compacted around the edge of the nucleus and shrunken, fragmented cytoplasm. Fixed with Alcian blue. (f and g) Osteoblast-like cells with osteoid matrix. The dark, basophilic cytoplasm, active RER, eccentric nucleus, and the high nucleus/cytoplasm ratio are all features typical for osteoblasts. A remnant of cellular debris (*open circle*) is present between the cells, but most of the lacunar space has been filled with needle-like collagen fibers. An enlargement of the fibers (Fig. 3 g) shows that the orientation of the fibers is random, some are seen in longitudinal section and others in cross-section. Fixed with Alcian blue. Bars, 1 µm.

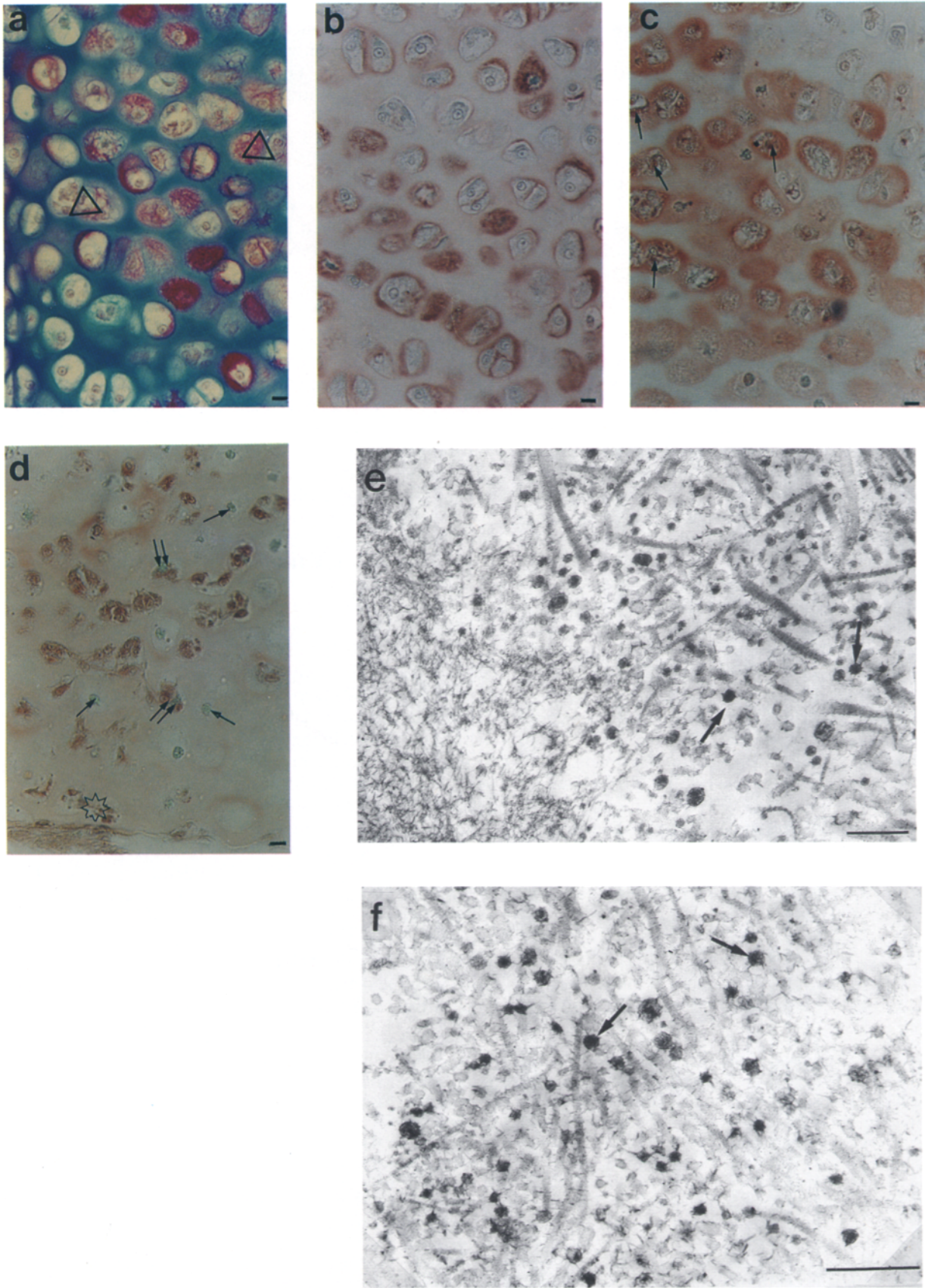


Figure 4. Evidence for the osteogenic phenotype after 6–12 d. (a) Osteoid matrix (red) is present inside chondrocytic lacunae after 6 d. Most lacunae are still intact and completely surrounded by cartilage matrix (blue), but two examples are shown where two lacunae have merged (triangles). These are not connected to a channel. (b) Type I collagen and (c) osteopontin inside or around chondrocytic lacu-

After 12 d of culture, the intra-lacunar osteoid matrix contained matrix vesicles (Fig. 4 e) and its ultrastructure was similar to that of de novo bone matrix produced by "definite" osteoblasts beneath the periosteum (Fig. 4 f). Previously, it had been shown with the Von Kossa stain that intra-lacunar matrix had mineralized after 12–15 d in culture (Roach, 1992a).

Discussion

An asymmetric cell division is any division where the daughter cells subsequently have different fates (Horvitz and Herskowitz, 1992). Theoretically such divisions would occur at every branch point during lineage commitment. Yet there are very few reports of asymmetric cell division in somatic cells in vertebrae (Sugimoto and Yasuda, 1983; Lamprecht, 1990; Mayani et al., 1993). This might be due to the difficulties of detecting the daughter cells of asymmetric cell divisions because (1) these are still morphologically identical; (2) no lineage markers are available to distinguish daughter cells, and (3) the daughter cells are interspersed among other cells. In the present experimental system these problems are overcome because (1) morphological differences are apparent in ultramicrographs; (2) several markers can distinguish the osteogenic from the chondrogenic phenotype, and (3) the starting cells, the hypertrophic chondrocytes, are fortuitously "locked" inside their lacunae and separated from neighboring cells by cartilaginous matrix. These three features made it possible not only to demonstrate asymmetric cell divisions, but also to follow the subsequent fates of the daughter cells.

The systematic analysis of the sequence of osteogenic differentiation depended on the ability to localize, by *in situ* techniques, those cells which had different characteristics within intact lacunae. In particular the possibility that other cell types had migrated into the lacunae from the cut edge or marrow tunnels needed to be eliminated. Lacunar integrity could always be confirmed by serial section analysis (or confocal microscopy) which established that channels were absent from the particular regions of hypertrophic cartilage under study.

Stages in the Switch from Chondrogenic to Osteogenic Expression

Metastable State. Two of the responses (type I collagen, alkaline phosphatase) observed within the first 48 h might be taken as the first indication of osteogenic differentiation, but we favor an alternative interpretation for the following reasons: (a) these responses were observed in cells that were morphologically still chondrocytes and (b) were

found in single chondrocytes as frequently as around cell doublets. (c) The region containing lacunae with chondrocytes with the above responses extended much further than the region whose lacunae contained osteogenic cells after 9–12 d. Although this evidence is still circumstantial, these immediate responses could reflect a dedifferentiation, a derepression of gene transcription, or activation of an inherent capacity. The single-stranded DNA breaks detectable with the holoenzyme of DNA polymerase I would be consistent with such a metastable state during which the regulation of gene transcription might be in a state of flux. Single-stranded DNA breaks play a role in gene transcription and/or differentiation (Johnstone and Williams, 1982; Dawson and Lough, 1988; Farzaneh et al., 1987; Coulton et al., 1992).

While it is not difficult to imagine an upregulation of alkaline phosphatase because hypertrophic chondrocytes produce the enzyme at a later stage, the presence of type I requires more explanation. Chondrocytes do not normally produce type I collagen, although the two type I collagen genes (α -1 and α -2) are transcribed in avian chondrocytes, but are not normally translated into proteins (Saxe et al., 1985). In the chicken, the genes for α -2(I) and α -1(II) overlap, with the cartilage-specific promoter lying within intron 2 of the α -2(I) gene (Bennett and Adams, 1990). It is therefore possible that the immediate secretion of type I collagen fibers might involve changes in the posttranscriptional regulation of α 1(I) synthesis or a switch in promoter utilization for the α 2(I) chain. Type I collagen expression was also found to be one of the earliest genes expressed during the osteogenic differentiation of hypertrophic chondrocytes in cultured mandibular condyles (Strauss et al., 1990; Closs et al., 1990).

Asymmetric Cell Divisions. Having ascertained the intactness of lacunae, the presence of two cells with different characteristics within one lacunae provided compelling evidence that an asymmetric cell division had occurred. The relatively low frequency (1–5%) of such "asymmetric" lacunae in any one section suggested that (a) not all chondrocytes underwent asymmetric division at the same time, (b) that the process was of short duration, and (c) that it did not happen in every chondrocyte.

The ultramicrographs show two cells of different morphologies within one lacuna. Their appearances were consistent with the notion that one cell was osteogenic (darker cytoplasm) while the other cell remained chondrocytic (pale cytoplasm). The former cell was viable and actively synthesizing proteins, as indicated by the many ribosomes and RER, while the later cell appeared to disintegrate. While morphology alone was insufficient evidence for dif-

nae. Parallel sections to Fig. 4 a. These confirm that the osteoid matrix contains type I collagen and osteopontin, the latter in preparation for mineralization. In some cells (arrows) osteopontin is still present in the cytoplasm, but mostly it is already present in the extracellular matrix. (d) Bone sialoprotein after 9 d. Lacunae now contain 2–4 cells which are positive for bone sialoprotein or single cells with a condensed nucleus (arrows). In two instances (double arrows) the BSP positive cells share a lacuna with a condensed cell, consistent with the notion that the condensed cell is the remnant of an apoptotic cell. Now more lacunae have merged, but none of these are connected to a channel. Near the cut edge (open star) migratory cells have invaded lacunae. Bars (a–d) 10 μ m. (e and f) Intra-lacunar osteoid contains matrix vesicles after 12 d. The ultrastructural appearance of intra-lacunar osteoid (Fig. 4 e) was compared with the matrix produced by definite osteoblasts beneath the periosteum (Fig. 4 f). Both matrices consist of collagen fibers of random orientation whose size and banding pattern are those of type I collagen. Matrix vesicles (arrows) are present in both. Bars, 1 μ m.

ferent phenotypes, it added weight to the light microscopic studies. These demonstrated that the viable cell immediately entered another S-phase, as shown by [³H]thymidine incorporation studies, while the nucleus of the other cell contained the double-stranded DNA breaks typical for an apoptotic cell. This suggested that, following the asymmetric cell division, one cell remained viable and divided again while the other cell was programmed to die by apoptosis.

Osteogenic Differentiation of the Viable Daughter Cell

The pulse-chase experiments with [³H]thymidine showed that 1–3 rounds of mitoses took place between days 3–9 in culture. This was consistent with the notion that the viable cell from the asymmetric cell division had completed the cell cycle and that further mitotic divisions of the progeny had occurred. This time all the daughter cells were committed to the osteogenic phenotype: within one lacuna all cells expressed type I collagen, alkaline phosphatase, osteonectin, osteopontin, bone sialoprotein, and osteocalcin. Although hypertrophic chondrocytes are also positive for the above markers (Mark et al., 1988; Pacifici et al., 1990; Chen et al., 1991; McKee et al., 1992; Lian et al., 1993), they only appear at a late stage. The temporal sequence of expression of these proteins in our experimental system was similar to that observed during osteogenic differentiation from mesenchymal cells in vivo (Robey et al., 1992) or in vitro (Ibaraki et al., 1992). Nevertheless, the best criterium for the osteogenic phenotype is the presence of a mineralized bone matrix because chondrocytes have never been shown to produce osteoid matrix. This criterium was fulfilled in the present system by those cells that had, within intact lacunae, secreted matrix that had the staining characteristics of bone matrix (including birefringence with polarized light, Roach, 1992a) consisted of type I collagen, contained osteopontin and matrix vesicles before mineralization, and was capable of mineralization. These findings indicate that in the present experimental system the hypertrophic chondrocytes had not only differentiated towards osteoblast-like cells, but also completed the differentiation to cells that were capable of producing a mineralized bone matrix.

Fate of the Apoptotic Cell. Apoptosis was identifiable in 5–10% of the cells in any one section during the earlier stages of culture, in support of the notion that double-stranded DNA breaks represent an early event in programmed cell death (Kerr et al., 1987; Bowen and Bowen, 1990; Wyllie, 1992), present for a limited time interval. In vivo apoptotic cells disappear within 1–2 h either by autolysis or phagocytosis of neighboring cells. In the present system the apoptotic cells remained isolated within the chondrocytic lacunae which probably prevented their rapid disappearance. However, we could not detect increased numbers of DIG-dUTP labeled cells with time in culture, whereas shrunk cells with a very condensed nucleus were always present, either alone or sharing a lacuna with osteogenic cells. Even when treated with DNase, no labeling could be detected in these cells, suggesting that their DNA was so condensed or fragmented that no nick translation or end labeling was possible. Eastman and Barry (1992) also found that the in situ methods could not detect highly fragmented DNA. It is feasible that these highly condensed cells were the remnants of those cells

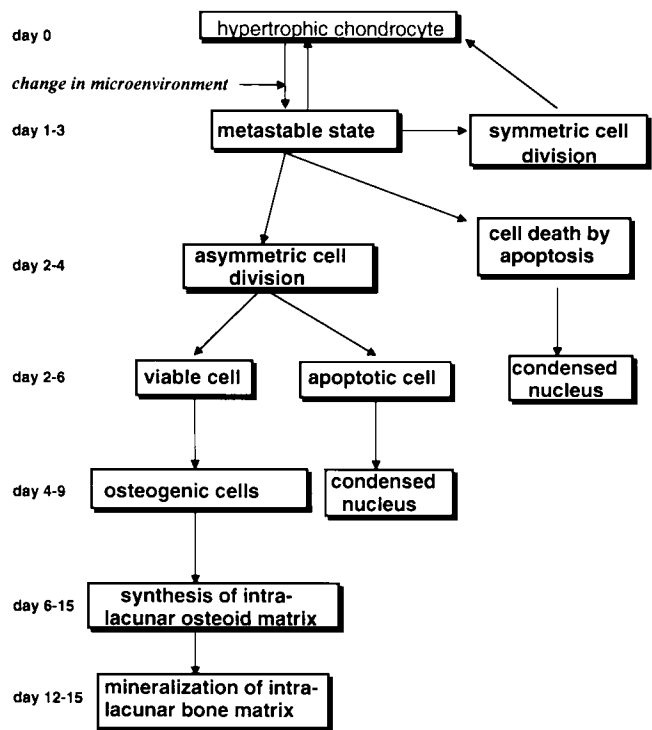


Figure 5. Possible sequence of cellular events during the phenotypic switch from chondrocytes to osteoblast-like cells. (See discussion for explanation.)

that contained double-stranded DNA breaks at an earlier stage.

Summary: Hypothesis of the Sequence of Cellular Events

Based on the data presented, we propose the following sequence of events during the phenotypic change from chondrocytes to osteoblast-like cells (Fig. 5). The initial stimulus is related to the change in the microenvironment resulting from cartilage resorption in vivo and simulated by the cutting through the hypertrophic region in vitro. This induces a metastable state (dedifferentiation? derepression?) during which the commitment of the cell might be redetermined. Depending on other, as yet unknown factors, cells will either remain chondrocytes (with or without symmetric division), become apoptotic or divide by asymmetric division. The latter is the crucial event in the switch from chondrogenic to osteogenic phenotype. Initially, this division results in one viable and one apoptotic cell. The viable cell enters the cell cycle and its progeny gradually differentiate to osteogenic cells. In the present system at least one further symmetric division seems to be required for the full redetermination of the phenotype as osteogenic. This contrasts instances in other tissues where a change in phenotype may occur without cell division (Beresford, 1990).

Apoptosis is known as a mechanism for the elimination of redundant cell subsets (Kerr et al., 1987; Bowen and Bowen, 1990; Wyllie, 1992; Parchment, 1993). In conjunction with asymmetric cell division apoptosis may provide the means of discarding redundant cellular material before

the viable cell becomes committed to the osteogenic lineage. It is of interest that the asymmetric divisions of chondrocytes, described by Sugimoto and Yasuda (1983), also gave rise to terminal transferase-positive cells together with functional T cells.

Whether the described osteogenic differentiation is a true "transdifferentiation", i.e., a sudden switch from the chondrogenic to the osteogenic phenotype, or a continuous "further" differentiation is still subject to debate. The osteogenic differentiation described in long term chondrocyte cultures (Descalzi-Cancedda et al., 1992; Gentili et al., 1993) and in certain chondrocytes near the bone shaft in vivo (Galotto et al., 1994) was interpreted as a gradual and continuous differentiation towards the osteogenic phenotype rather than a transdifferentiation. In the present system, the occurrence of asymmetric cell divisions plus the requirement of at least one further mitosis to redefine the phenotype as osteogenic are, in the authors' view, more consistent with a transdifferentiation than a gradual differentiation. However, further work is needed to support this concept, for example to establish whether there is a distinct break in phenotypic expression from that of a hypertrophic chondrocyte (expressing e.g., type X collagen) to that of an osteoblast-like cell which no longer expresses cartilage-specific markers.

If the concepts presented above are also applicable to what happens to hypertrophic chondrocytes as the cartilage is resorbed during endochondral ossification in vivo, the two previously opposing views of the fate of the hypertrophic chondrocyte may be reconciled: some chondrocytes die by apoptosis while others survive and become bone-forming cells. The phenotypic change from chondrocytes to osteoblast-like cells may also serve as a paradigm for other instances (for review see Okada, 1991) of phenotypic switches in differentiated cells as well as provide a good example of asymmetric cell division in differentiated cells.

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