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Bone Formation in Cartilage Produced by Transplanted Epiphyseal Chondrocytes*

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Summary. Chondrocytes were isolated from rat epiphyseal cartilage, cultured in vitro, and exposed to exogenous tracers which accumulated in their lysosomes. The cells were then injected into the posterior tibial muscle of animals from the same outbred strain, where they reconstructed calcifying hyaline cartilage. The mineralization of the tissue was followed by ingrowth of blood capillaries from the host bed. Macrophage-like cells surrounding the vessels phagocytized degenerated chondrocytes and unmineralized matrix, whereas multinucleated chondroclasts removed some of the mineralized cartilage matrix. Mesenchyme-like cells accompanying the invading vessels attached to the remaining septa of calcified cartilage matrix and developed into osteoblasts depositing bone matrix on the surface of these septa. The apparent lack of inherent tracer labeling of the lysosomes in the different bone cells indicate that they were derived from the host. No signs of transformation of chondrocytes into bone cells were observed.

When isolated rat epiphyseal chondrocytes were injected into the wall of the hamster cheek pouch, calcifying cartilage was reconstructed without signs of subsequent ossification. Transplantation of cartilage reconstructed in the hamster into the dorsal muscles of rats was, however, followed by formation of bone by a sequence analogous to that described above. Such an osteogenetic response was also obtained when the cartilage had been devitalized before transplantation.

These experiments show that calcified cartilage, developing in or grafted into an intramuscular site, is able to induce and serve as a substrate for endochondral bone formation, similar to that occurring during normal

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development. They further indicate that bone induction by calcified cartilage does not require the presence of living chondrocytes.

Key words: Epiphyseal chondrocytes (rat) – Transplantation – Bone induction – Transmission electron microscopy.

Endochondral ossification represents a complex process including proliferation, hypertrophy, and calcification of cartilage followed by ingrowth of blood vessels surrounded by undifferentiated cells and the formation of bone on remnants of the calcified cartilage matrix (Bloom and Fawcett, 1975). Autoradiographic studies with tritiated thymidine have indicated that the bone cells are derived from mesenchymal elements accompanying the invading capillaries (Young, 1962; Scott, 1967). It is also believed that the chondrocytes of the calcifying cartilage degenerate and that the cell remnants are subsequently resorbed by phagocytic cells (Takuma, 1960; Anderson and Parker, 1966; Schenk et al., 1967; Brighton et al., 1973; Thyberg et al., 1975b). It is also possible, however, that hypertrophic chondrocytes may survive and transform into osteogenic cells (Holtrop, 1966; Shimomura et al., 1973).

A developmental sequence of the type described above can be observed not only during normal skeletal growth. Thus, a large number of methods exist for inducing cartilage and bone formation in laboratory animals (Urist et al., 1967; Ostrowski and Wlodarski, 1971; Anderson, 1976; Reddi and Anderson, 1976). Such systems have been used to study extracellular matrix influences on cartilage and bone cell differentiation. Nevertheless, a detailed understanding of these processes is still lacking. For example, little is known about whether the inducers evoke both chondro- and osteogenesis or whether the cartilaginous tissue, once established, is responsible for initiating bone formation. One way to approach this problem would be to use the chondrocyte transplantation system first described by Moskalewski and Kawiak (1965). We have previously used this model to show that isolated epiphyseal chondrocytes can reconstruct cartilage which both hypertrophies and calcifies (Thyberg et al., 1975a). Furthermore, the results of Shimomura et al. (1975) suggest that cartilage formed by isolated chondrocytes can initiate osteogenic differentiation.

In the present transmission electron microscopic study we describe the formation of bone in transplants formed after intramuscular injection of isolated rat epiphyseal chondrocytes. To trace the origin of the cells in the tissue found at the site of injection, the chondrocytes were first cultured in vitro and exposed to colloidal thorium dioxide or dextran. Such tracers are taken into the cells by endocytosis and accumulate in their lysosomes. The presence of labeled lysosomes should thus help to determine whether different cell types in the transplants represent a progeny of injected or host cells. To study further the ability of cartilage to induce bone information, isolated rat epiphyseal chondrocytes were injected into the hamster cheeck pouch, where they are known to reconstruct calcified cartilage without any signs of ossification (Kaminski et al., in press). This tissue was then removed and grafted back to an intramuscular site in rats, either directly or after devitalization. The observations indicate that calcified cartilage developing in or

grafted into an intramuscular site can induce and serve as a substrate for endochondral bone formation of a type similar to that occurring during normal development.

Materials and Methods

Isolation, Culture and Transplantation of Cells

Ham's medium F-12 (Gibco Bio-Cult) was used. It was supplemented with 10% fetal calf serum (Gibco Bio-Cult), 0.3% tryptose phosphate broth (Difco), 50 μg/ml of L-ascorbic acid, 150 units/ml of penicillin and 150 μg/ml of streptomycin. To stabilize the pH of the medium 10 mM each of the organic buffers HEPES and TES (Sigma) were also included (Eagle, 1971).

Cartilaginous epiphyses were dissected from 6-day-old rats of an outbred Sprague-Dawley strain and dissociated by digestion with 0.5% collagenase (type I; Sigma) and 0.05% DNase (Biomed, Warsaw, Poland) dissolved in full culture medium (Thyberg et al., 1975a). The isolated cells were seeded into 75 cm^2 Falcon plastic flasks (2×10^7 cells/flask) in 15 ml of medium and kept at 37°C in an atmosphere of 5% CO_2 in air. After 12–15 h culture medium was changed and the cells exposed to colloidal thorium dioxide for 16 h or to dextran for 48 h. Thorotrast (25% stabilized colloidal thorium dioxide by volume; Testagar) was used at a concentration of 0.06 ml per ml of medium. Dextran T40 and T150 (Pharmacia Fine Chemicals) were prepared in 40% (wt/vol) stock solutions in T-buffer (Ham and Sattler, 1968) and sterilized by autoclaving. After adjusting the pH to 7.3, the stock solutions were diluted with full culture medium to a final dextran concentration of 15% (wt/vol).

Following exposure to the tracers the cultures were rinsed 5 times with non-supplemented medium. To remove the extracellular matrix they were treated for 60 min with 0.25% collagenase dissolved in full culture medium and subsequently rinsed twice with non-supplemented medium. The cells were then harvested with 0.5% trypsin (Difco, 1 : 250) and rinsed twice with full culture medium. Control cells, not exposed to tracers, were treated identically with collagenase and trypsin. Samples of the harvested cells were then fixed for electron microscopy. For transplantation 7×10^6 cells were suspended in 0.2 ml of full culture medium and injected into the posterior tibial muscle of 250–300 g male rats of the Sprague-Dawley strain. The tissue formed by the injected cells was removed after 2, 3, 4 and 6 weeks, and a total of 30 transplants was studied.

Transplantation of Cartilage

Chondrocytes were isolated enzymatically from Sprague-Dawley rats as described above, rinsed three times with culture medium, and injected into the wall of the cheek pouch of Syrian hamsters (5×10^6 cells/pouch; Moskalewski and Kaminski, 1970). After four weeks the 1–2 mm diameter cartilage nodules formed by the injected cells were removed and using a dissecting microscope separated from the surrounding tissues. A few were fixed for electron microscopy. The rest were transplanted into the dorsal muscles of Sprague-Dawley rats either immediately or after being kept in an excess of sterile distilled water for 24 h at 4°C and constantly stirred. Six transplants were made in each group and left for four weeks. All transplants were recovered in the first group and three transplants in the second.

Light Microscopy

The transplants were fixed and decalcified as described below. They were then embedded in Paraplast, cut at 5–8 μm, and stained with hematoxylin-eosin or with aldehyde fuchsin followed by van Gieson's picro-fuchsin for differentiating cartilage and bone (Addison, 1973).

Electron Microscopy

Isolated cells were fixed in suspension in 0.1 M sodium cacodylate buffered 3% glutaraldehyde, pH 7.3, and then pelleted by centrifugation. Transplants were cleaned from surrounding tissues, cut into small pieces, and fixed by immersion in glutaraldehyde. About half of the pieces from each transplant were

decalcified with 0.1 M EDTA dissolved in glutaraldehyde fixative. After 2 h postfixation in 1% cacodylate- or Veronal acetate-buffered osmium tetroxide, the specimens were dehydrated in ethanol and embedded in Spurr's (1969) resin. Before dehydration most of them were treated at room temperature for 1 h with 0.5% uranyl acetate in Veronal acetate buffer, pH 5.0 (Farquhar and Palade, 1965).

For demonstration of dextrans the one-step fixation-staining procedure of Simionescu et al. (1972) was used. The fixative contains 1.5% formaldehyde, 2.5% glutaraldehyde, 0.66% osmium tetroxide and lead citrate (about 2–3 mg/100 ml) in 0.1 M phosphate buffer, pH 7.4. The specimens were fixed in icecold fixative for 2 h. They were then dehydrated in cold ethanol and embedded in Spurr's (1969) resin.

Thin sections were cut on an LKB Ultrotome I, either left unstained, stained with lead citrate (Reynolds, 1963) or double-stained with uranyl acetate followed by lead citrate, and examined in a Philips EM 300 electron microscope operated at 80 kV.

Results

Isolated Cells

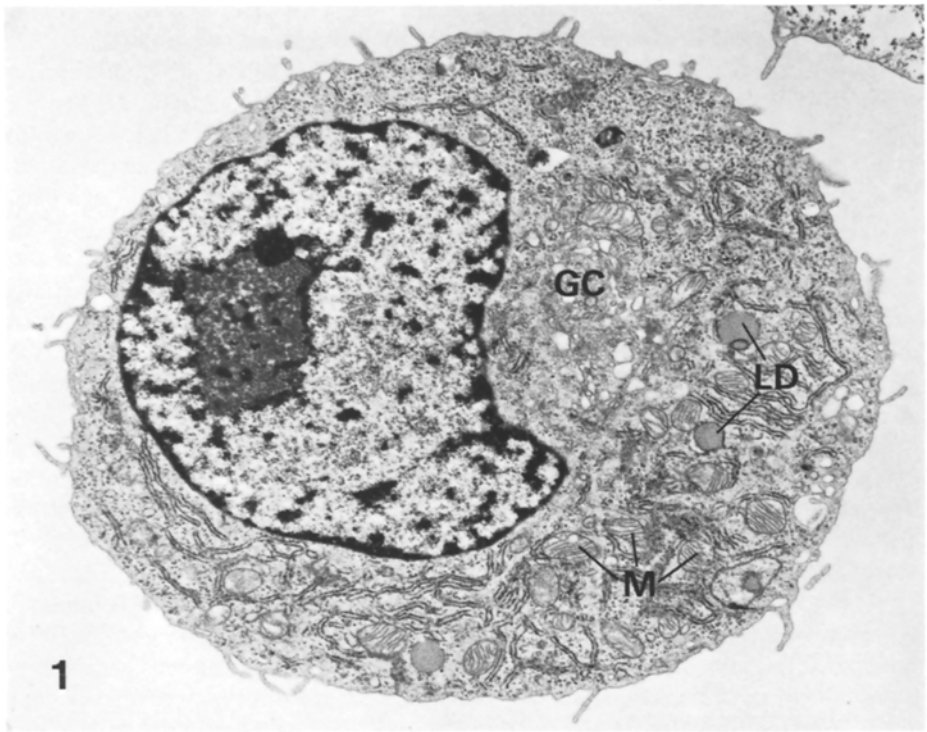
The cell population obtained by enzymatic isolation followed by short-time culture in vitro was homogeneous in structure with a characteristic chondrocyte appearance (Fig. 1). The extracellular matrix was normally completely removed by the collagenase digestion. Cells exposed to colloidal thorium dioxide (cf. Thyberg et al., 1975a) or dextrans showed accumulation of these exogenous tracers in endocytic vesicles and lysosomes (Fig. 2), but were otherwise identical to control cells. The number of labeled lysosomes was approximately the same with all tracers and there were no clear differences in the amounts of Dextran T40 and T150 ingested by the cells. After collagenase treatment the cell surfaces were essentially free of tracers. Thorium dioxide was, however, bound to fragments of degenerated cells and small numbers of these cells were usually present in the final cell preparations.

Bone Formation in Cartilage Produced by Chondrocytes Injected into Rats

The nodules of tissue formed after injection of cells into rats were mostly flattened in shape with dimensions of about 10–15 × 5–10 × 1–2 mm. After two weeks the transplants were composed of uncalcified cartilage with some hypertrophic cells. Subsequently hypertrophic cells were increased in number and regions with well advanced calcification were regularly observed. In 4- and 6-week-old transplants blood vessels with accompanying cells had grown into the cartilage and bone was

Fig. 1. A cultured chondrocyte of the type used for transplantation. The cell is rounded with a somewhat scalloped surface showing microvilli-like projections, an eccentrically located nucleus, an extensive rough endoplasmic reticulum, and a large Golgi complex (*GC*). *M* mitochondria, *LD* lipid droplets. No extracellular matrix can be detected. × 9000

Fig. 2. Chondrocyte from a culture exposed to Dextran T40 and fixed-stained with the one-step procedure. Five phagosomes/lysosomes (*L*) are shown. Two are heavily loaded and the rest moderately filled with dextran. Positively stained glycogen particles (*G*) in clusters or dispersed singly in the cytoplasm are also demonstrated. Part of the nucleus is also present. × 26,000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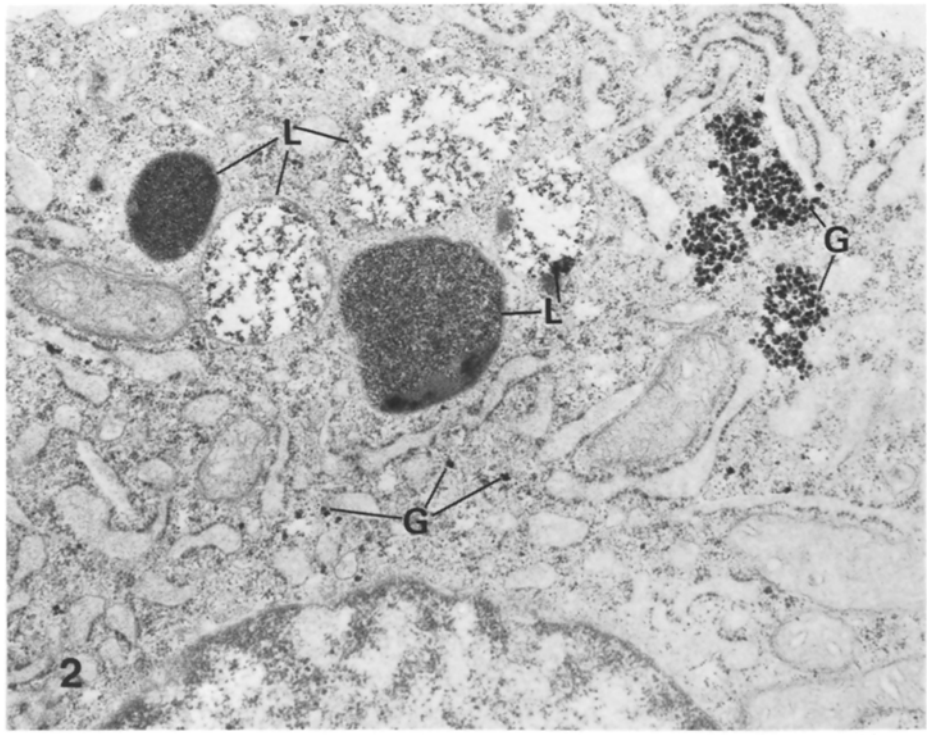
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laid down on remnants of mineralized cartilage matrix. No round cell infiltration or any other signs of immunologic rejection of the transplants were noted.

The formation and subsequent calcification of cartilage originating from transplanted chondrocytes has previously been described in some detail (Thyberg et al., 1975a). We will therefore concentrate on the processes of cartilage resorption and bone formation. Besides the presence of exogenous tracers in some cells, there was no difference between transplants formed by labeled and unlabeled chondrocytes.

2-Week-old-Transplants. The transplants were made up of hyaline cartilage surrounded by a perichondrium-like connective tissue. The chondrocytes were often grouped in cell nests, but were otherwise randomly distributed in the tissue. They had a rounded nucleus with prominent nucleoli, an extensive rough endoplasmic reticulum (RER), and a large Golgi complex (Fig. 3). Some degenerated cells were also noted. In transplants formed after injection of cells exposed to colloidal thorium dioxide or dextran most of the chondrocyte profiles showed labeled lysosomes (cf. Figs. 4 and 6). Moreover, one or a few fibroblast-like cells with labeled lysosomes were occasionally observed in the soft connective tissue surrounding the cartilage.

The cartilaginous matrix consisted of thin collagen fibrils and small rounded or polygonal granules believed to represent proteoglycans (Thyberg, 1977). The fibrils were partially aligned and thus formed septa between neighboring cells or cell nests. Strands with thicker and distinctly crossbanded collagen fibrils were also evident. They occurred most frequently around some of the degenerating chondrocytes. Early calcification of the intercellular substance was sometimes observed. The first mineral crystals occurred in clusters at midpoint between neighboring cells, i.e. within the above-mentioned septa, and often showed a close spatial relationship to matrix vesicles of varying appearance (cf. Thyberg et al., 1975a).

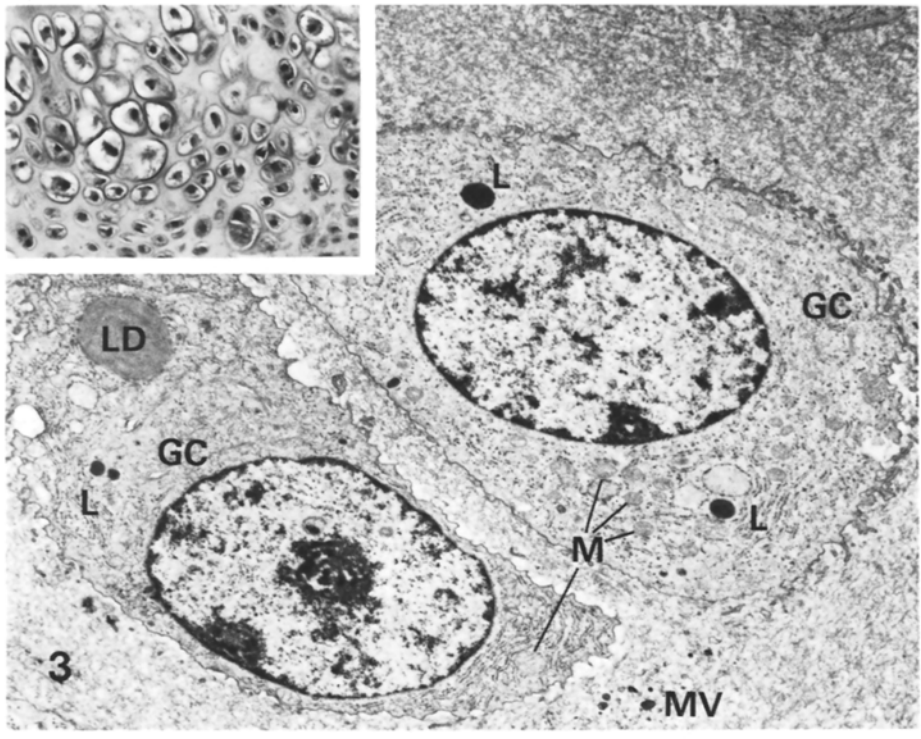
3-Week-old Transplants. Large parts of the cartilage were fully mineralized and early signs of endochondral bone formation could be observed. In decalcified specimens the borderline between calcified and uncalcified cartilage and calcified cartilage and bone was marked by a "lamina limitans" (Scherft, 1972). After decalcification, remnants of matrix vesicles were demonstrated within the calcified cartilage matrix and, less frequently, in the bone matrix.

Uncalcified cartilage was similar in appearance to that of 2-week-old

Fig. 3. Chondrocytes with surrounding matrix in a 2-week-old transplant formed after intramuscular injection of isolated cells. *GC* Golgi complex, *M* mitochondria, *L* lysosomes, *LD* lipid droplet, *MV* matrix vesicles. × 6000. The *inset* demonstrates the typical cartilage structure of transplants by light microscopy.

Fig. 4. Part of a chondrocyte with labeled lysosomes (*L*) in a 3-week-old transplant formed by cells exposed to Dextran T150. Tissue fixed-stained with the one-step procedure. × 64,000

Fig. 5. Labeled matrix vesicles in a 3-week-old transplant formed by cells loaded with Dextran T150. The vesicles are surrounded by a network of collagen fibrils. Tissue fixed-stained with the one-step procedure. × 80,000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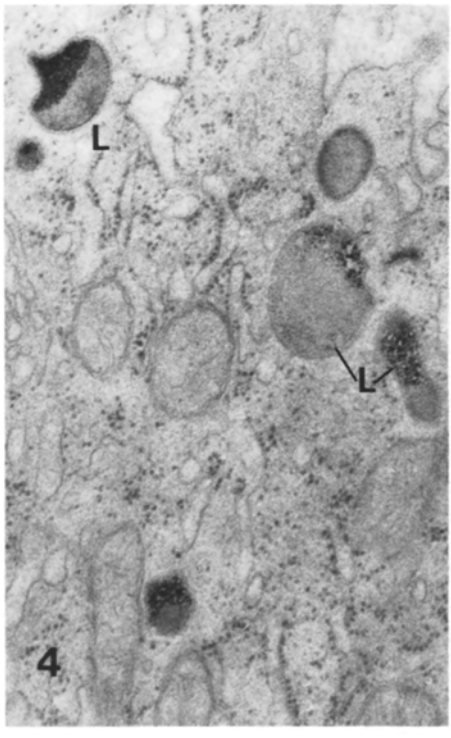
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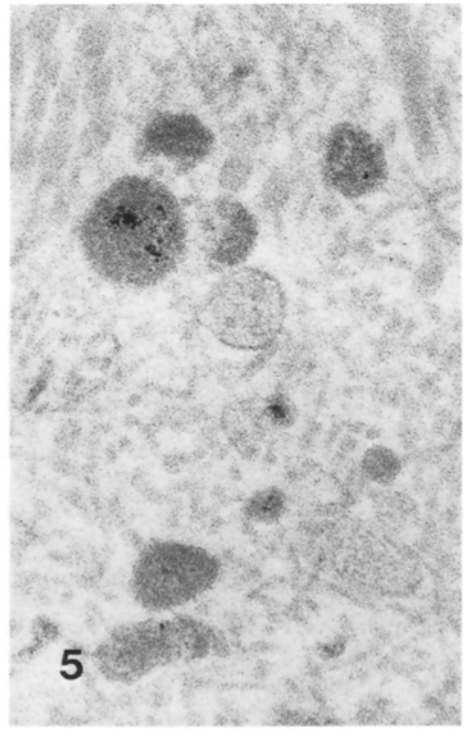
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transplants. The pattern of thorium dioxide and dextran labeling was also unchanged (Fig. 4). In connection with the disintegration of chondrocytes, lysosomes seemed to remain intact for some time because exogenous tracers were withheld. It was also possible to observe labeled matrix vesicles (Fig. 5), which confirmed previous reports on the existence of extracellular lysosomal material in calcifying cartilage (for a review see Thyberg and Friberg, 1978).

Chondrocytes surrounded by calcified septa were generally degenerate, whereas those located at the edge of the mineralized tissue often remained viable. A thin rim of non-mineralized matrix was present between the chondrocytes and the mineralized matrix, and small non-mineralized regions were also observed in the septa (cf. Fig. 7). The individual lacunae had diameters of about 25–40 μm.

Some transplants showed growth of blood vessels with accompanying cells into the degenerated cartilage and initial deposition of bone matrix on remnants of calcified cartilage matrix. A further description of these processes is given below.

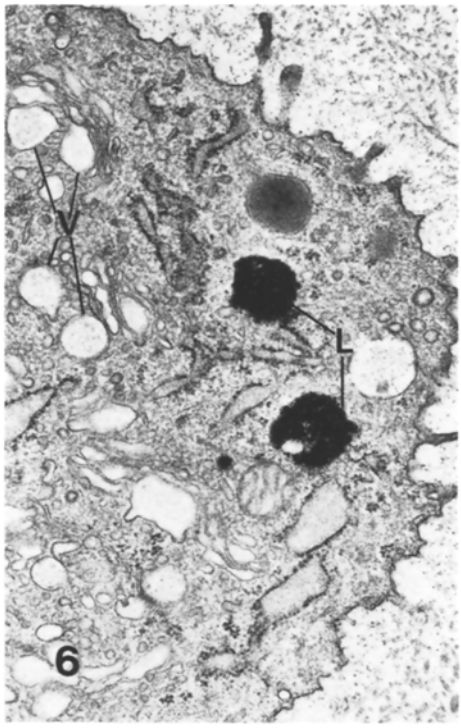
4- and 6-Week-old Transplants. Uncalcified cartilage was still evident and contained chondrocytes with a prominent secretory apparatus. There also remained exogenous tracers in the cells (Fig. 6). As in 3-week-old transplants, the cells of calcified cartilage were degenerate. Capillary sprouts were observed to penetrate the non-mineralized regions in the calcified septa and to grow into chondrocyte lacunae (Fig. 7). The capillaries were surrounded by cellular connective tissue which included many mesenchyme-like cells with poorly developed cytoplasmic organelles. Numerous macrophage-like cells and some chondroclasts were also found (Figs. 8–10). The former had a large Golgi complex, many phagosomes, and lysosomes containing cellular debris and other less clearly identifiable inclusions (Figs. 8 and 9). In transplants formed from chondrocytes exposed to exogenous tracers, the phagolysosomes of the macrophages were labeled (Fig. 9). Tracer molecules were also noted, but much less frequently, in phagolysosomes within chondroclasts. These large multinucleated cells occurred close to blood vessels and

Fig. 6. Part of a chondrocyte from an uncalcified region of a 4-week-old transplant, formed by cells loaded with colloidal thorium dioxide. Two heavily labeled lysosomes (*L*) are shown. Part of the Golgi complex with several large secretory vacuoles (*V*) is also seen. × 26,000

Figs. 7–12 demonstrate various aspects of vascularization and resorption of cartilage and the subsequent ossification of 4-week-old transplants formed after intramuscular injection of thorium dioxide-labeled cells. Demineralized specimens

Fig. 7. A capillary sprout (*CS*) in the process of penetrating an uncalcified region within a septum of calcified cartilage matrix (*CCM*). A macrophage-like cell precedes the tip of the invading vessel and has found its way into the former chondrocyte lacuna (*upper right*). *E* erythrocytes, *LL* lamina limitans delimiting the calcified cartilage matrix. × 5000

Fig. 8. A more advanced stage of vascularization with macrophage-like and small, mesenchyme-like (*Me*) cells in the space between two blood vessels, at some distance from the front of invasion of the cartilage. The macrophage-like cells have a prominent Golgi complex (*GC*) and a few large lysosomes (*L*) which appear to lack tracer particles. *Arrows* mark tight junctions connecting adjacent endothelial cells. × 8000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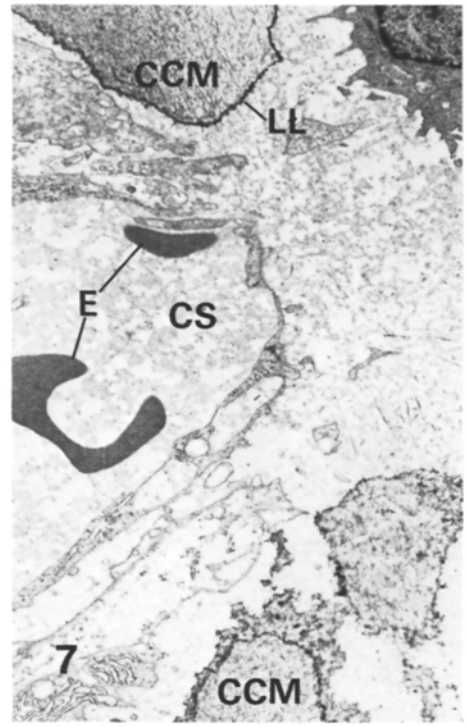
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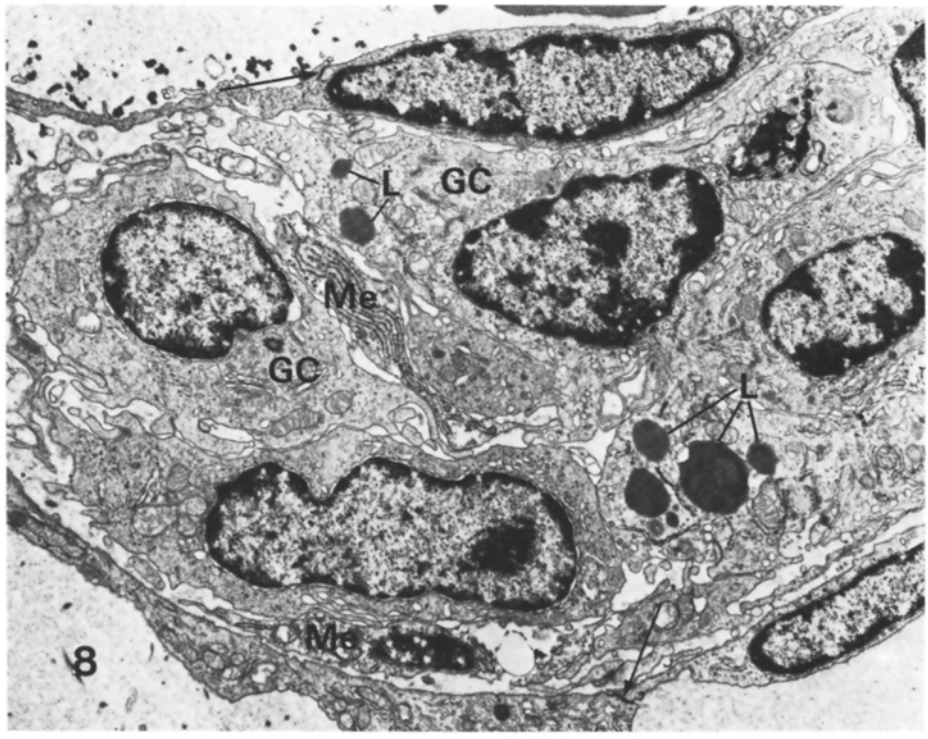
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were actively engaged in removing calcified cartilage. In the zone of resorption chondroclasts displayed a ruffled border with associated vesicles and vacuoles. Numerous lysosomes were widely distributed in the cells, and were mostly moderate in size with a dense homogenous matrix. The Golgi complex was prominent and consisted of stacks of cisternae located close to the nuclei. Mitochondria were abundant but RER sparse.

As a result of the capillary and cellular ingrowth described above, degenerated chondrocytes, the surrounding unmineralized and some mineralized matrix were all resorbed and the surface of the remaining calcified cartilage matrix exposed. Mesenchyme-like cells attached to this surface and developed into osteoblast-like cells (Figs. 10 and 11). The osteoblasts had a prominent RER and a large Golgi complex with many secretory vacuoles among which elongated structures with a collagen-like content were evident. The bone matrix consisted of cross-banded collagen fibrils, much wider than those of the cartilaginous matrix. Osteoblast processes and, possibly, pinched off fragments of such processes were also observed. Dense, lysosome-like vesicles of the type occurring in the cartilage (see Fig. 5) were, however, not seen. Although the bone matrix appeared to become rapidly mineralized, a zone of osteoid matrix was always found next to the osteoblasts (Fig. 11). Osteocytes were surrounded by mineralized bone matrix into which long cytoplasmic processes extended. Compared to the osteoblasts, the osteocytes had only small amounts of cytoplasm (Fig. 12). In transplants formed after injection of cells exposed to colloidal thorium dioxide or dextran, tracer molecules were only very occasionally present in lysosomes of osteoblasts and osteocytes.

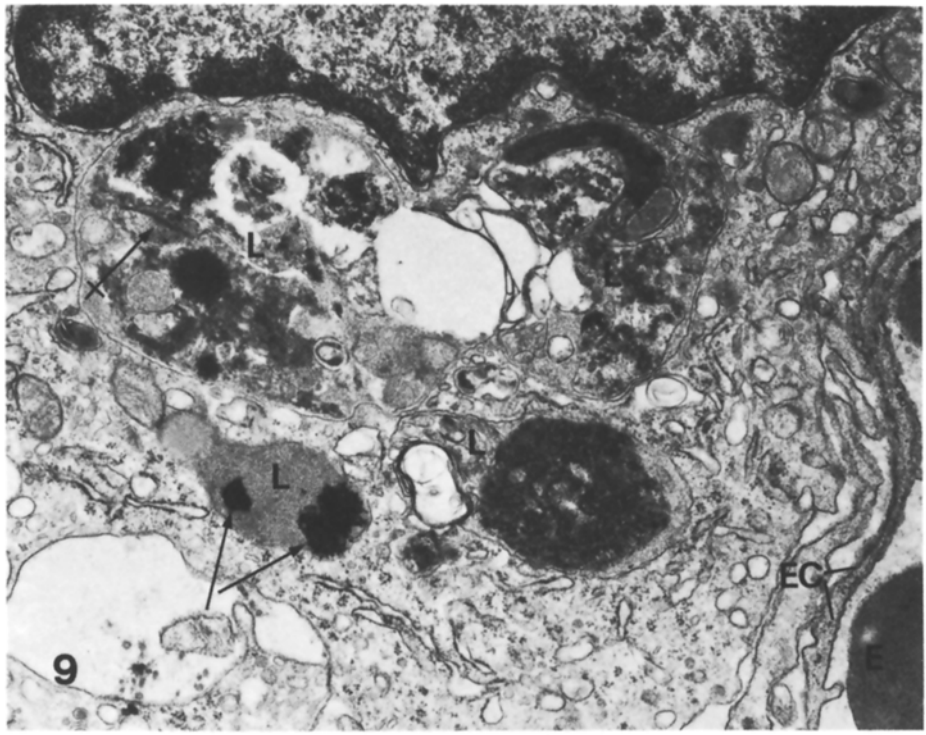
After the early stages of ossification described above, the primary bone trabeculae were resorbed by osteoclasts and replaced by new bone, in which bone marrow with erythropoietic and granulopoietic cells, megakaryocytes and fat cells appeared within the marrow cavities.

Bone Formation in Cartilage Produced in Hamster Cheek Pouch and Transplanted into Rats

After injection into the wall of the hamster cheek pouch, rat chondrocytes formed a calcified hyaline cartilage similar to that formed intramuscularly in the rat (see above). However, in accordance with previous observations (Kaminski et al., in press), no ossification was noted. Treatment of the cartilage with distilled water for 24 h resulted in the death of all chondrocytes and the dissolution of both nuclear

Fig. 9. Detail of a perivascular, macrophage-like cell showing large phagosomes/lysosomes (*L*) containing cellular debris including thorium dioxide particles (*arrows*). The latter occur in aggregates in the lysosomes of the macrophage (*arrow*) or within lysosome-like bodies (*crossed arrow*) evidently phagocytized by this cell. *E* erythrocyte, *EC* endothelial cell. × 23,000

Fig. 10. Perivascular, macrophage-like (*Ma*) and mesenchyme-like (*Me*) cells. The latter are attached to a septum of calcified cartilage matrix (*CCM*) and have started to lay down small amounts of osteoid-like matrix (*OM*) on its surface. *E* erythrocyte, *EC* endothelial cell, *L* thorium dioxide-labeled lysosome, *LL* lamina limitans. × 8000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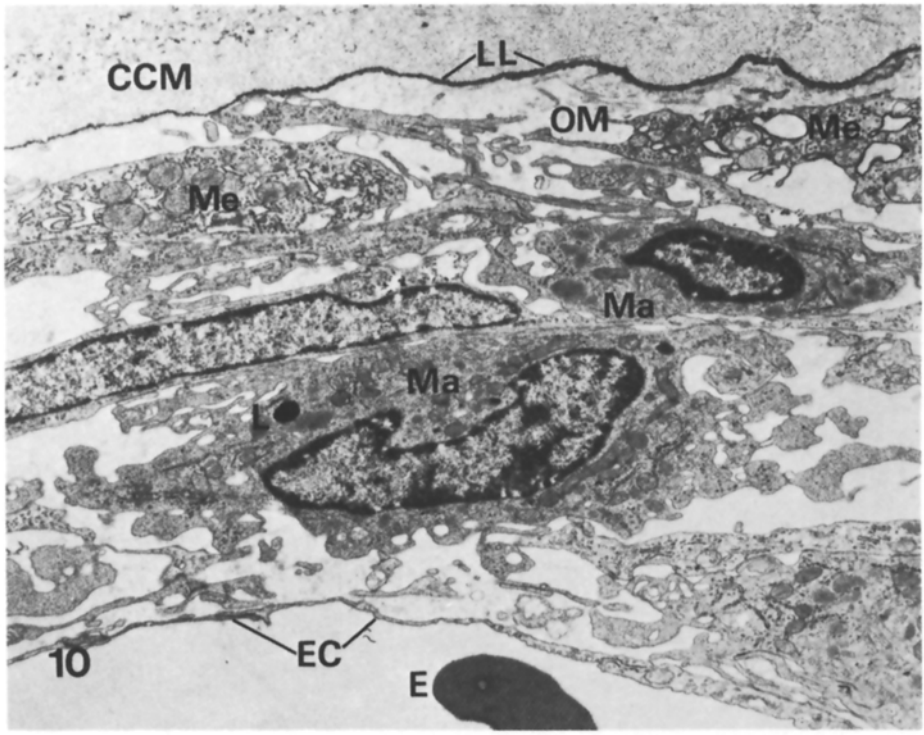
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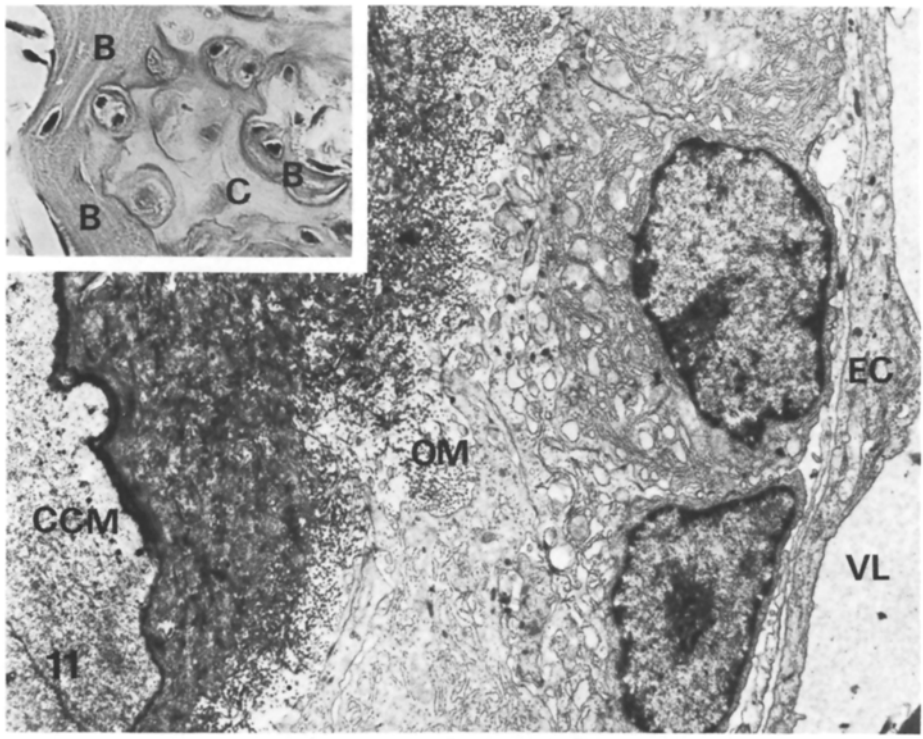
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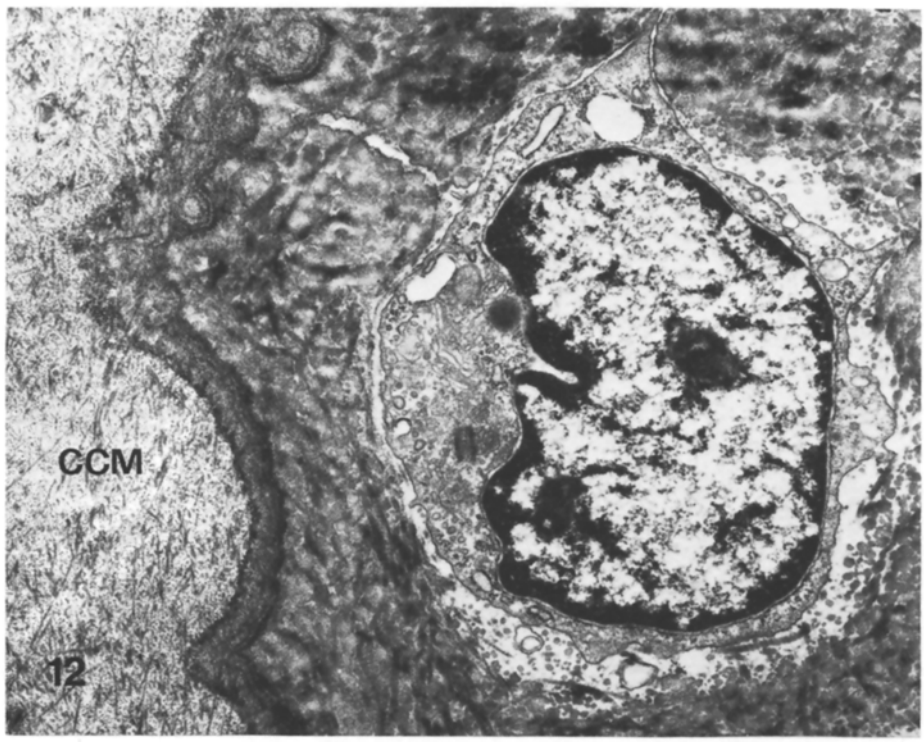
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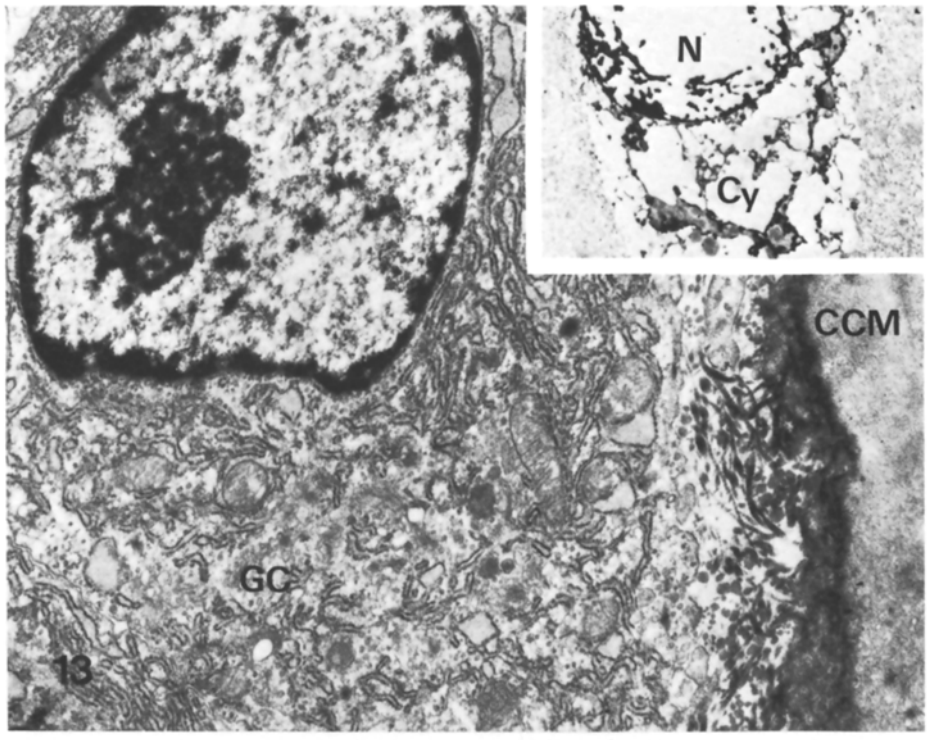

Fig. 13. Formation of bone in cartilage reconstructed in the hamster cheek pouch, devitalized, and then implanted intramuscularly in a rat. The micrograph shows part of an osteoblast with an extensive rough endoplasmic reticulum and a large Golgi complex (*GC*). Bone matrix is being deposited on the surface of a septum of calcified cartilage matrix (*CCM*). × 9000. The *inset* shows a chondrocyte and surrounding matrix in devitalized cartilage. Both the nucleus (*N*) and the cytoplasm (*Cy*) are lysed and partly extracted, whereas the matrix morphologically appears to be essentially unaffected. × 5000

and cytoplasmic structures. The extracellular matrix, on the other hand, remained largely unchanged morphologically (Fig. 13, inset).

Transplantation of cartilage formed in the hamster to an intramuscular site in rats was followed by deposition of bone. This appeared to take place in an identical way to that described under the previous heading, and did not depend on whether living or devitalized cartilage was used. Thus, in both cases, blood vessels with accompanying undifferentiated cells grew into the cartilage, chondrocyte lacunae

Fig. 11. Osteoblasts depositing bone matrix on the surface of a septum of calcified cartilage matrix (*CCM*). Close to the cells, a zone of loosely arranged collagenous stroma corresponds with the zone of unmineralized osteoid matrix (*OM*). *EC* endothelial cell, *VL* vascular lumen. No thorium dioxide labeling of the cells can be detected. × 7000. The *inset* is a light micrograph demonstrating bone tissue (*B*) deposited on cartilage (*C*).

Fig. 12. Osteocyte in bone deposited on the surface of calcified cartilage matrix (*CCM*). The collagen fibrils of the bone matrix are thicker and distinctly cross-banded when compared with those of the cartilage matrix. × 14,000

were broken up and cell remnants, both unmineralized and some mineralized matrices were resorbed by macrophage-like cells and chondroclasts. The bone matrix was finally laid down by osteoblasts on the surface of the remaining calcified cartilage matrix (Fig. 13), and size and thickness of the bone trabeculae so formed were similar in both groups.

Discussion

This study confirms previous observations on the ability of isolated epiphyseal chondrocytes to reconstruct calcifying cartilage in heterotopic sites (Thyberg et al., 1975a; Shimomura et al., 1975; Kaminski et al., in press). Moreover, the tissue formed by these isolated cells was capable of inducing and serving as a substrate for endochondral bone formation of a type similar to that occurring during normal development (see also Urist and Adams, 1968; Shimomura et al., 1975).

The cartilage produced in the first step showed a three-dimensional organization different from that in the epiphyseal plate. For example, no distinct cell columns were seen. Nevertheless, hypertrophy of cells and mineralization of extracellular matrix proceeded in an analogous way. Furthermore, in the final stages of mineralization, the cells were only partially surrounded by calcified matrix, making it possible to point out equivalents of mineralized longitudinal and unmineralized transverse septa in the epiphyseal plate (Thyberg, 1974).

When isolated rat chondrocytes were injected intramuscularly into animals from the same outbred strain (Sprague-Dawley), serving for practical purposes as an isogeneic system, the formation of calcified cartilage was followed by the ingrowth of blood vessels and deposition of bone. In contrast, and in agreement with previous observations (Kaminski et al., in press), no signs of ossification were detected after injection of rat chondrocytes into the wall of the hamster cheek pouch. Similarly, no ossification was noted after allogeneic transplantation of chondrocytes to intramuscular sites (Thyberg et al., 1975a; Kaminski et al., in press). It seems likely that this difference is due to immunological incompatibility between donor and host cells in the latter cases, which leads to round cell infiltration and a consequent disturbance of the osteogenetic process. This disturbance was not definite, however, and when cartilage formed by rat chondrocytes in the hamster was grafted back to rats, endochondral ossification followed. In the present study we used this latter system to demonstrate that bone induction by calcified cartilage does not require the presence of living cells. Bone was thus also produced when the cartilage had been devitalized before the grafting.

As already mentioned, the ossification process was analogous to that occurring in the normal epiphysis-metaphysis. It could be divided into three partly overlapping stages, namely:

(1) Invasion of the calcified cartilage by capillaries from the host bed.

(2) Resorption of remnants of degenerated chondrocytes and unmineralized matrix.

(3) Deposition of bone on the surface of the exposed septa of mineralized cartilage matrix.

Associated with the calcification of the cartilage was the degeneration of most chondrocytes. This was followed by growth of capillaries through unmineralized regions in the septa surrounding the cells and into the lacunae. Macrophage-like cells with phagosomes/lysosomes containing cellular debris and other inclusions appeared around the vessels close to their advancing tips. It is believed that these cells play a major role in the resorption of degenerated chondrocytes and unmineralized cartilage matrix (cf. Thyberg et al., 1975b; Thyberg, 1975; Thyberg and Friberg, 1978). Chondroclasts engaged in resorption of mineralized cartilage matrix were also present around the vessels. Structurally, they were indistinguishable from osteoclasts and together with them probably represent the same cell type. This suggestion is supported by the fact that, during reorganization of the bone, individual clast cells were seen to resorb mineralized bone and cartilage matrix simultaneously (cf. Thyberg and Friberg, 1978).

As a result of the activities of the macrophages and chondroclasts, cavities delimited by mineralized cartilage matrix were created. Undifferentiated, mesenchyme-like cells accompanying the invading capillaries attached to the walls of these cavities and developed an extensive rough endoplasmic reticulum and a large Golgi complex. The osteoblasts formed in this way then started to lay down osteoid matrix, using the aforementioned walls as a substrate. After being entrapped within the bone matrix, the osteoblasts turned into osteocytes. This transformation involved a marked decrease in cytoplasmic volume.

Because of the multiplicity of ways in which heterotopic bone formation can be induced (for a review, see Ostrowski and Wlodarski, 1971), it seems likely that there are several chemical and/or physical factors which can initiate this process. It may also be advisable to consider chondro- and osteogenesis separately. Several of the inductive agents described, for example decalcified bone matrix (Urist et al., 1967; Reddi and Anderson, 1976) and various epithelial cells (Anderson, 1976), give rise to bone by an endochondral sequence. It is possible that these agents merely bring about the formation of calcified cartilage and that this latter tissue then induces bone formation. The present study confirms that calcified cartilage has the power to do this.

Conceivably, the mineralization of the cartilage and the accompanying death of most of the chondrocytes plays an important role in initiating capillary ingrowth and the subsequent formation of bone. It has been shown that chondrocytes produce factors which inhibit angiogenesis (Brem et al., 1975; Kaminski et al., 1977; Kuettner and Pauli, 1978). The vascularization of calcified cartilage could therefore be the result of a decreased production of such factors due to cell death and/or the masking of inhibitors present in the extracellular matrix by the deposited minerals. The osteogenetic response following the vessel ingrowth could possibly be ascribed to the bone inductive properties of the exposed septa of calcified cartilage matrix (cf. Ham, 1932). The possibility that the interaction between the undifferentiated cells surrounding the blood vessels and the cartilaginous tissue is more complex and starts at an earlier stage cannot however be excluded. Nevertheless, our results indicate that living chondrocytes are not necessary for this interaction, although they may give a better yield of bone, for example by preparing more cartilage matrix for mineralization.

Alternative conclusions were reached by Urist and Adams (1968). In a series of

experiments they demonstrated that full-thickness transplants of articular cartilage from young inbred rats into the anterior eye chamber induced formation of bone in a way analogous to that described above. After devitalization of the cartilage by repeated freezing and thawing or devitalization and decalcification in hydrochloric acid before the transplantation, mainly fibrous tissue was formed. On the basis of these findings the authors suggested that interaction of viable chondrocytes in the transplant and connective tissue cells in the host induced differentiation of the latter into osteoblasts. The differences between these and the present results could be explained by the fact that neither the experimental systems nor the methods of devitalization of the cartilage were the same. There may also have been a difference in the amounts of calcified cartilage matrix present in the transplants.

Our studies with thorium dioxide- and dextran-labeled cells showed that the cartilage formed after intramuscular injection of chondrocytes was formed by the latter and not by differentiating host cells. Furthermore, the results indicated that the different types of bone cells originated from the host, i.e. from the cells surrounding the blood capillaries growing into the calcified cartilage. Admittedly, exogenous tracers were found in macrophage-like cells and, less frequently in chondroclasts, but only after these cells had become engaged in resorption of cartilage. It was thus obvious that the tracers found in the above-mentioned cells represented fragments of degenerated chondrocytes ingested by phagocytosis. Similarly, the small amounts of tracers which were found very occasionally in osteoblasts and osteocytes could represent material released from disintegrating chondrocytes and endocytized by osteoblasts or their precursor cells. It cannot be excluded, however, that a few cells with osteogenic potential may have been present among the injected chondrocytes or that a few chondrocytes were transformed into osteoblasts. It has previously been proposed that such a transformation may take place (e.g. Holtrop, 1966; Shimomura et al., 1973; Kahn and Simmons, 1977). Clear morphological signs of conversion of chondrocytes to osteoblasts were not, however, observed in the present study. Moreover, the fact that grafting of devitalized cartilage was followed by efficient bone formation suggests that such a conversion, if present, is the exception rather than the rule.

Previous fine structural studies of the epiphysis-metaphysis in rats (Scott, 1967) and guinea pigs (Thyberg et al., 1975b) and of fracture callus in rats (Göthlin, 1973) have pointed out that macrophage-like cells and chondroclasts/osteoclasts on one side, and osteoblasts, on the other, may originate from separate cell lines. In a study on the origin of cells in heterotopic bone formation induced by decalcified bone matrix, Buring (1975) reached the same conclusions. He used parabiosis in combination with autoradiography with tritiated thymidine to distinguish between cells of hematogenous origin and cells developing from the connective tissue at the site of implantation. His findings indicated that macrophage-like cells and chondroclasts/osteoclasts were derived from blood-borne monocytoid cells, the precursors of which were located in the bone marrow. Osteoblasts, on the other hand, developed from local perivascular cells. The present electron microscopic observations support the concept of two osteogenic cell lines, namely, one of phagocytic cells engaged in resorptive processes and one of cells producing bone matrix. The results of Jotereau and Dourin (1978), utilizing the quail-chick nuclear marker system, also point in this direction.

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