In Vitro Morphogenesis of Chick Embryo Hypertrophic Cartilage

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Abstract. Dedifferentiated chick embryo chondrocytes (Castagnola, P., G. Moro, F. Descalzi-Cancedda, and R. Cancedda, 1986, *J. Cell Biol.*, 102:2310–2317), when transferred to suspension culture on agarose-coated dishes in the presence of ascorbic acid, aggregate and remain clustered. With time in culture, clusters grow in size and adhere to each other, forming structures that may be several millimeters in dimension. These structures after 7 d of culture have the histologic appearance of mature hypertrophic cartilage partially surrounded by a layer of elongated cells resembling the perichondrium. Cells inside the aggregates have ultrastructural features of stage I (proliferating) or stage II (hypertrophic) chondrocytes depending on their location. Occurrence and distribution of type I, II, and X collagens in the in vitro-formed cartilage at different times of culture, show a temporal and spatial distribution of these antigens reminiscent of the maturation events occurring in the cartilage in vivo. A comparable histologic appearance is shown also by cell aggregates obtained starting with a population of cells derived from a single, cloned, dedifferentiated chondrocyte.

CHICK embryo hypertrophic cartilage develops through a series of differentiation events (18). Between stages 22 and 25 (9), in the center of limb buds, committed mesenchymal cells aggregate (5, 18, 19) and mature to proliferating chondrocytes. The chondrogenesis process is characterized by changes in the nature and amount of the extracellular matrix macromolecules that are synthesized. In the cartilage-specific matrix, chondrocytes undergo further maturation to fully differentiated hypertrophic cells. Matrix vesicles released by hypertrophic chondrocytes are the first site of calcium deposition (1). In the calcifying region cells degenerate, leaving empty lacunae which fuse to each other and generate large spaces subsequently invaded by incoming blood vessels. In this developmental pathway at least three different cell phenotypes can be identified: (*a*) committed mesenchymal cells, found first in the limb bud and then in the perichondrium—these cells produce type I collagen and fibronectin and possibly basal level of type II collagen (14); (*b*) stage I (proliferating) chondrocytes—these cells produce large amounts of type II collagen and cartilage-specific proteoglycans (10, 14, 20); and (*c*) stage II (hypertrophic) chondrocytes localized in the hypertrophic calcifying cartilage—these cells are characterized by the synthesis of type X in addition to type II collagen (3, 13, 17).

We have recently shown that chondrocytes isolated from tibial cartilage of early chick embryos, when cultured on plastic dishes, assume a fibroblast-like morphology and shift from the synthesis of type II to the synthesis of type I collagen. These dedifferentiated cells, when transferred to suspension culture, resume the chondrocytic phenotype and continue their maturation to single, isolated hypertrophic chondrocytes. The process begins with a transient cell aggregation that starts within few hours after seeding (4).

In the present study, we have cultured in suspension dedifferentiated cells in the presence of ascorbic acid, a cofactor of collagen hydroxilases which is an absolute requirement for the correct tridimensional assembly of collagen fibrils. In these conditions the aggregated dedifferentiated cells do not evolve to isolated hypertrophic chondrocytes but develop into a tissue strongly resembling the hypertrophic cartilage in vivo.

Materials and Methods

Primary Culture

Culture medium was Coon's Modified Ham F-12 (2) additioned with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland), penicillin 50 IU/ml, and streptomycin 50 μg/ml (Gibco Ltd., Paisley, Scotland). Cell cultures were performed with slight modifications of the already described procedure (4). Briefly stage 28–30 chick embryo tibiae were removed, cleaned, washed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), and digested for 15 min at 37°C with 2 mg/ml collagenase I (Cooper Biomedical, Inc., Malvern, PA) and 0.25% trypsin (Gibco Ltd.). After sedimentation the supernatant, containing tissue debris and perichondrium, was discarded and the pellet was digested for 45–60 min with the same dissociation buffer supplemented with 1,000 U/ml of collagenase II (Cooper Biomedical, Inc.). Dissociated cells were seeded in 10-cm plastic culture dishes at a concentration of 5–6 × 10^6.

Suspension Culture

After 2–3 wk of anchorage-dependent growth, cells were harvested by tryp-

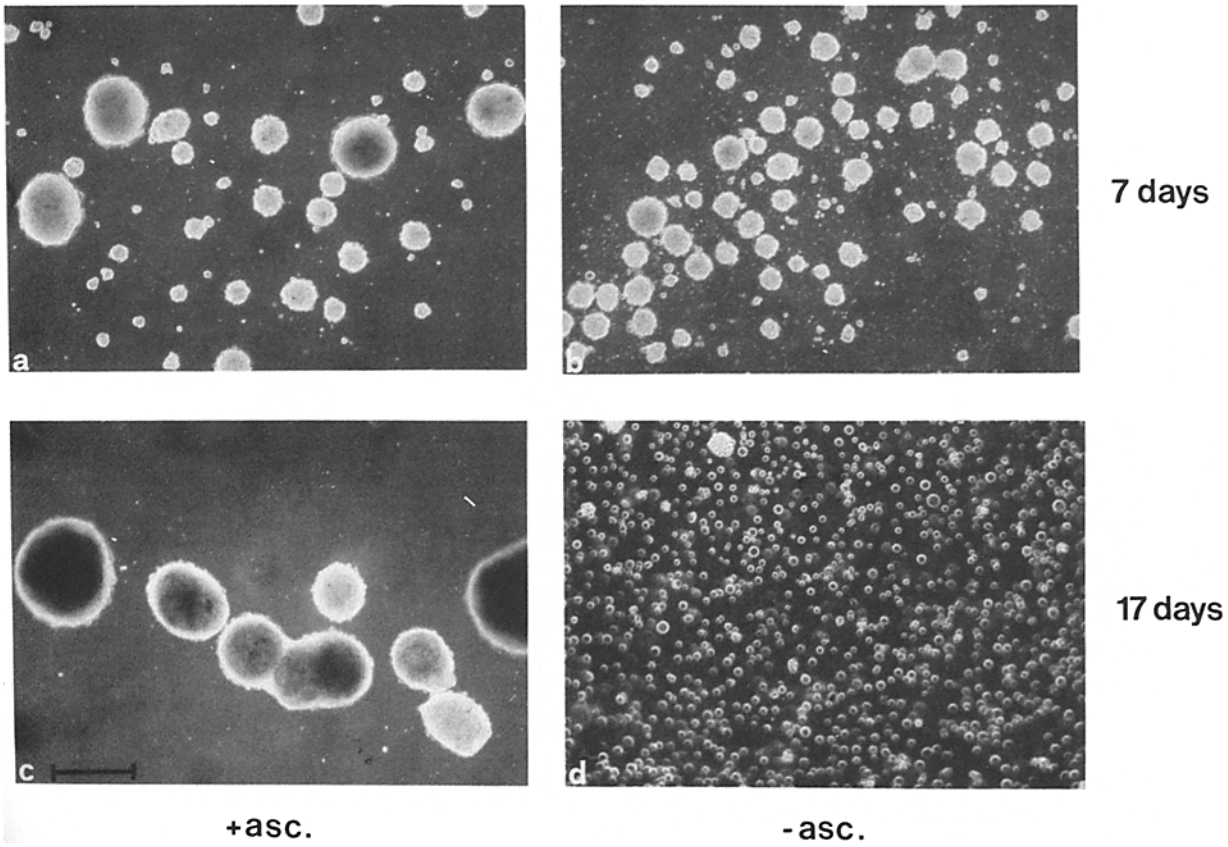

Figure 1. Cultures performed in the presence (*a* and *c*) or in the absence (*b* and *d*) of ascorbic acid (*asc.*) 7 (*a* and *b*) and 17 d (*c* and *d*) after transfer to agarose-coated dishes. Note the increased size of the cell aggregates in *c* compared with *a*. In *d* the complete dissociation of cell aggregates into single hypertrophic chondrocytes is evident. Bar, 250 μm.

sinization and transferred to tissue culture dishes previously coated with a thin layer of 1% agarose (type II, Sigma Chemical Co., St. Louis, MO). Where indicated 50 μg of ascorbic acid (Sigma Chemical Co.) per ml of medium were added daily. Culture medium was changed every third day.

Cell Cloning

Freshly dissociated cells as described above, were seeded in 96-well tissue culture plates at a 0.3 cell/well ratio. Cells were grown in dedifferentiated cell-conditioned medium passed through a 0.45-μm filter and diluted 1:1 with complete fresh culture medium. Clones showing fibroblast-like morphology were selected and expanded for 3 wk before transferring to suspension culture.

Light and Electron Microscopy

At several times after transferring to suspension culture, cells were collected and washed in PBS. Samples were fixed for 20 min in 2.5% glutaraldehyde (Polysciences, Inc., Warrington, PA) in 0.1 M cacodylate buffer, pH 7.3, postfixed for 20 min in 1% osmium tetraoxide (Polysciences, Inc.) in the same buffer, en bloc stained with uranyl acetate (Polysciences, Inc.), and embedded in Poly-bed 812 (Polysciences, Inc.). 1-μm-thick sections were stained with toluidine blue. Gray-silver thin sections were stained with uranyl acetate and lead cytrate (Polysciences, Inc.) and observed in a Philips 400 T electron microscope.

Immunofluorescence

1-μm-thick sections of cell aggregates fixed and embedded as described above were cleared of the resin by 10-min treatment with sodium ethoxyde/ethanol (1:1) followed by three washes of 5 min each with absolute ethanol and one wash with 50% ethanol. After rinsing for 30 min in PBS, sections were digested for 15 min with trypsin and for 90 min with 150 U/ml hyaluronidase (Sigma Chemical Co.). Sections were exposed for 1 h at room temperature to rabbit antisera against type I, II, and X chicken collagens, and to FITC-labeled goat anti-rabbit IgG (Jackson Immunochemical Research Laboratories, Inc., Avondale, PA). To reduce background all solutions of antibodies contained 4 mg/ml of goat gamma globulins. Pictures were taken with an Olympus Vanox-T fluorescence microscope; to make pictures directly comparable, sections stained with the same antibodies were photographed with the same exposure time and pictures were printed all the while maintaining constant exposure and developing times. The rabbit antisera to type I collagen used have been previously characterized (15). Antisera to type II and X collagens were raised in rabbits using electrophoretically pure proteins and tested for specificity by radioimmunoassay, immunoprecipitation, and immunofluorescence as will be described elsewhere (unpublished data).

Results

In the Presence of Ascorbic Acid Aggregates of Dedifferentiated Cells Do Not Separate into Single Isolated Chondrocytes

Ascorbic acid was added to the culture medium at the time dedifferentiated cells were transferred from the adherent to the suspension culture. Within a few hours the cells formed aggregates indistinguishable from aggregates obtained in the absence of the vitamin. These structures enlarged and by day 7 they were clearly bigger than the ones maintained in the absence of ascorbic acid (Fig. 1, *a* and *b*). By day 17 aggregates had further increased in size and often fused to one another (Fig. 1 *c*) to form structures which might be several millimeters in size. By that time control cultures were composed of single, isolated hypertrophic cells (Fig. 1 *d*).

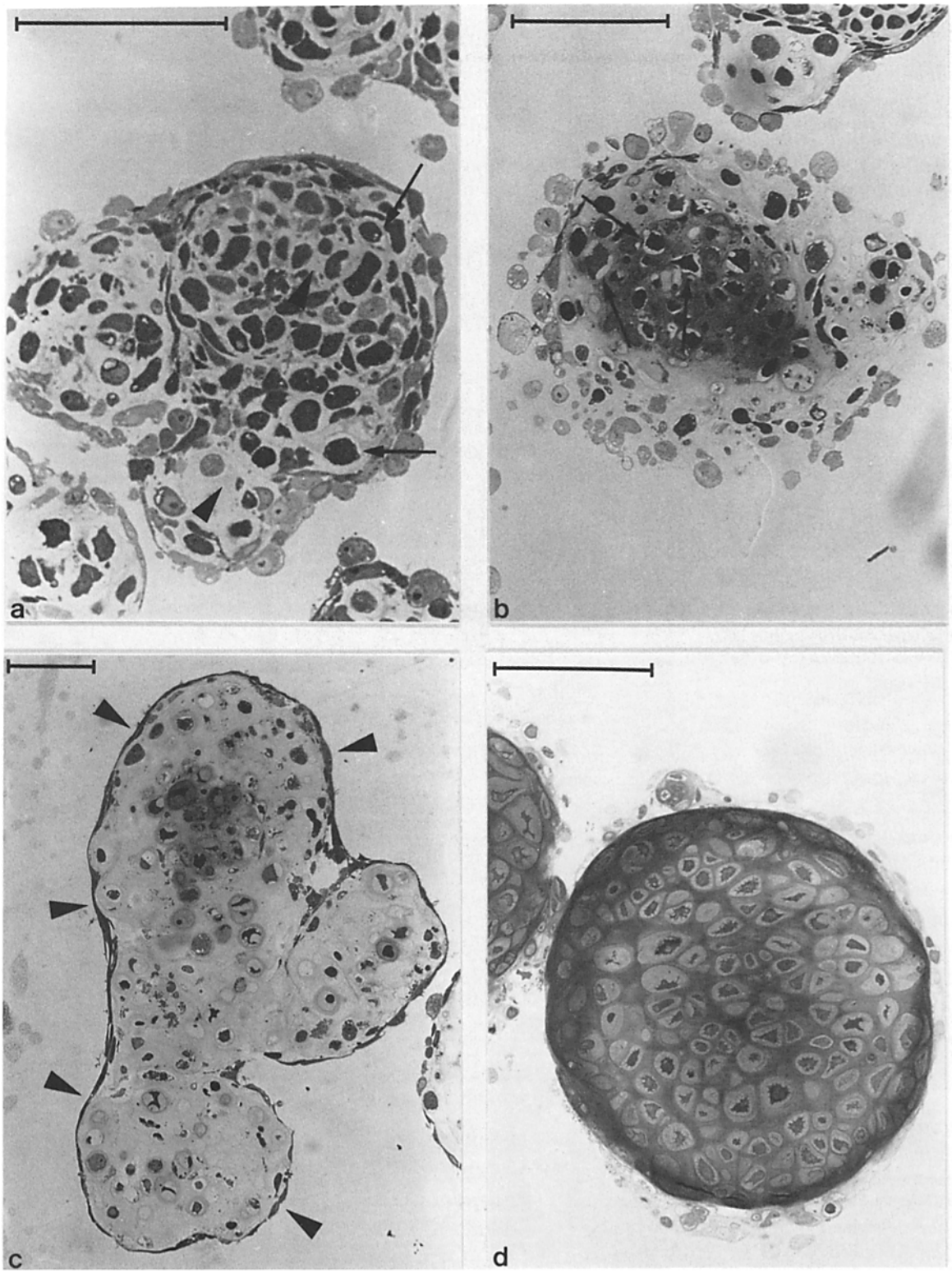

Figure 2. (*a–c*) Sections of aggregates from dedifferentiated cells cultured in the presence of ascorbic acid. (*a*) At day 3 of culture cells have a round or elongated shape; note the presence of a pale matrix between them (*arrowhead*). Only few cells are surrounded by a lacuna (*arrow*). (*b*) At day 10 of culture, note the dark central area. Most of the cells are contained in lacunae (*arrow*). (*c*) 17 d of culture. The aggregate is surrounded by a layer of elongated cells (*arrowhead*). Cells inside the aggregate are all contained in lacunae separated by an abundant extracellular matrix. A darkly stained area is present in the upper portion of the aggregate. (*d*) Cell aggregate obtained after 10 d of culture in the presence of ascorbic acid of a cloned population of dedifferentiated cell. Cells show the histological appearance of hypertrophic chondrocytes. Noteworthy is the absence of the layer of elongated cells at the periphery at variance with *c*. Bars, 100 μm.

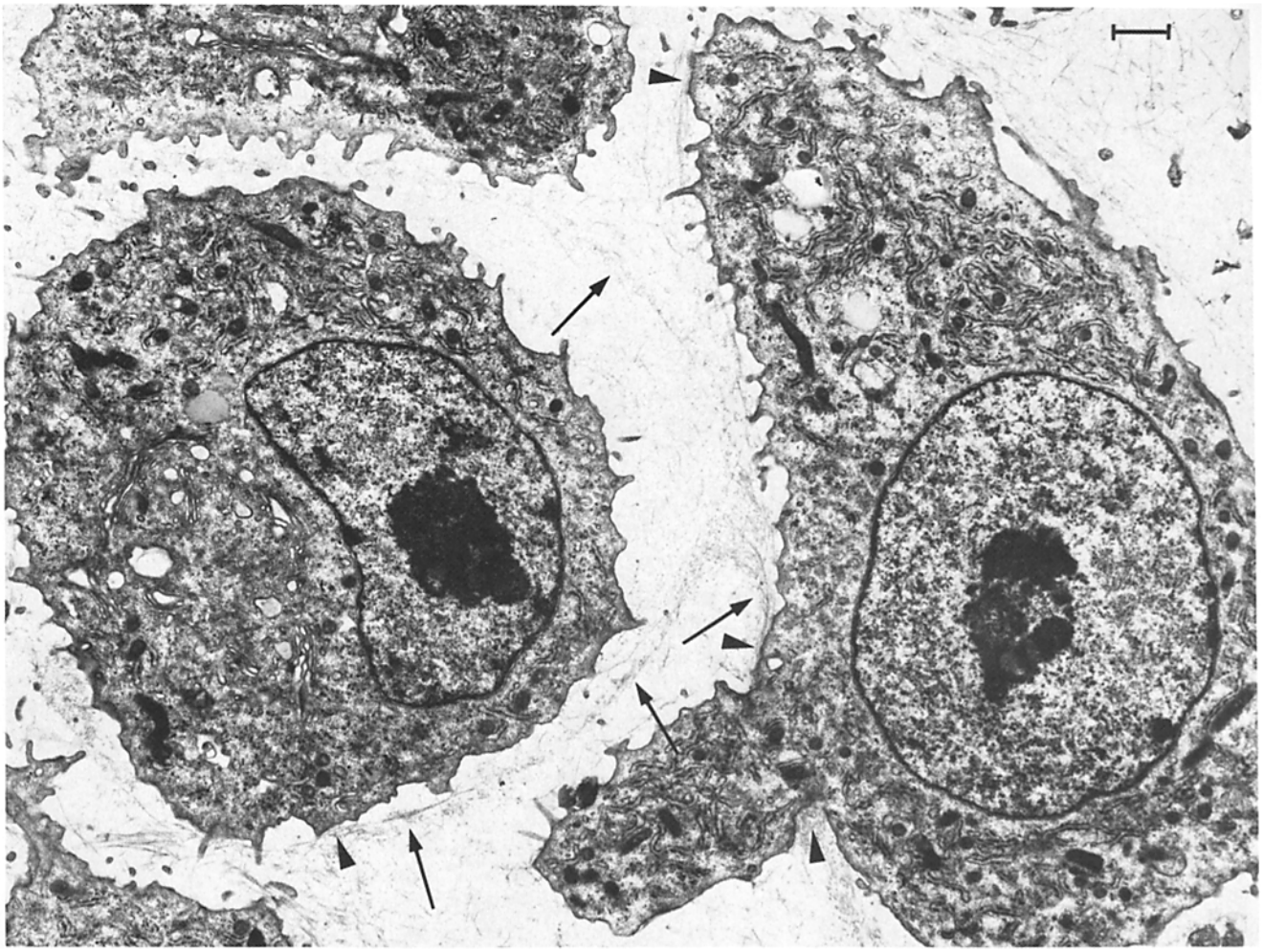

Figure 3. Central portion of a 3-d culture cell aggregate formed in the presence of ascorbic acid. Cells are separated by a fibrillar matrix (*arrows*) closely adjacent to the cell membrane (*arrowheads*). These cells produce the same collagens as chondroblasts (see text) and, like these cells, lack a surrounding lacuna (compare with Figs. 5 and 6). Bar, 1 μm.

Maturation of Cell Aggregates into Hypertrophic Cartilage

By 3 d of culture, thick sections of cell aggregates, formed in the presence of ascorbic acid, showed a mixed population of cuboidal and elongated cells embedded in a pale matrix (Fig. 2 *a*). At the ultrastructural level, cuboidal cells presented the characteristic morphology of chondroblasts, i.e., immature cartilage cells not yet surrounded by lacunae and embedded in a matrix made of thin fibrils (Fig. 3). By day 7 the central area in the aggregates stained darker with toluidine blue. Only some of the cells of the area were contained in lacunae. The heavily stained region was more pronounced after 10 d (Fig. 2 *b*). After 17 d most of the aggregates showed a gradient of cartilage maturation from the periphery to the center with cells enclosed in progressively larger lacunae. In the center some degenerated cells were observed. In most cases the edge of the aggregates was surrounded by a layer of elongated cells resembling a perichondrium (Fig. 2 *c*). Analysis on the electron microscope revealed that all lacunae contained a granular matrix with the already described characteristics of a proteoglycan network (11, 12). Differences were observed in the territorial matrices (i.e., matrices forming the edge of lacunae) which were much more defined and organized in the central area than at the periphery of the aggregates (Figs. 4 and 5). Cells with the ultrastructural morphology of hypertrophic chondrocytes were observed in the central areas already after 7 d of culture (Fig. 5). By this time matrix vesicles could be detected in the proximity of the territorial matrix (*inset*, Fig. 5).

Occurrence and Distribution of Collagens in Cell Aggregates

To evaluate whether the morphologic resemblance of aggregates to hypertrophic cartilage was associated with the same temporal and spatial distribution of the most represented collagens in the cartilage during development, we have localized by indirect immunofluorescence type I, II, and X collagens in sections of aggregates at different times after transfer to suspension culture. Collagen type I was evenly localized in the aggregates at day 3 and highly reduced at later times when it was mostly detectable at the periphery (Fig. 6, *a*, *d*, and *g*). Antibodies to type II collagen gave only a pale staining at day 3 but at later times strongly decorated the central region of the aggregates, clearly defining the territorial ma-

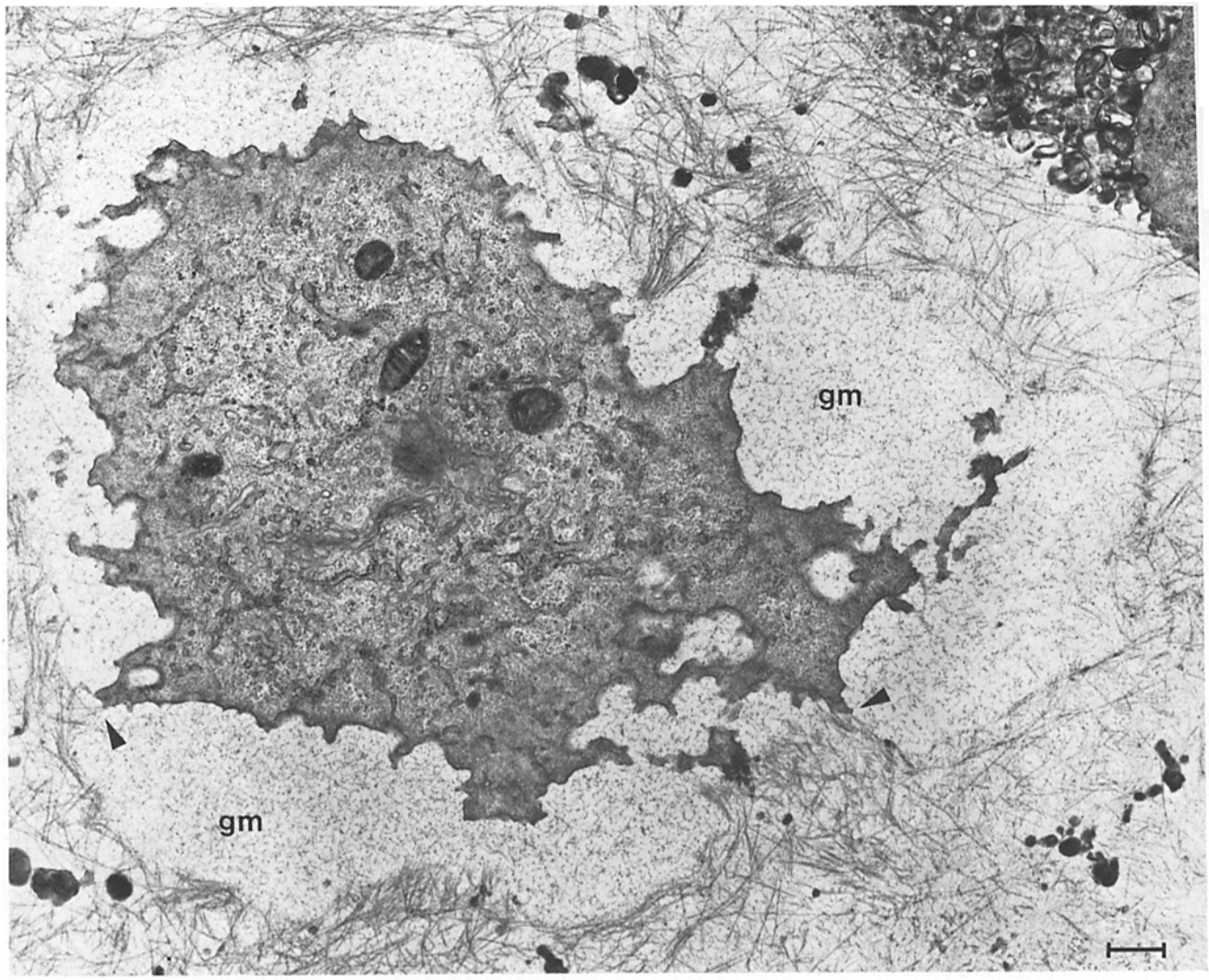

Figure 4. Chondrocyte in the pale, stained peripheral area of a 10-d aggregate. The cell is embedded in a lacuna containing granular matrix (*gm*). Collagen fibrils from the periphery of the lacuna make occasional contact with cell cytoplasmic processes (*arrowheads*). A defined territorial matrix is not clearly detectable (see Fig. 6 for comparison). At the upper right corner of the micrograph, there is a degenerated cell. Bar, 0.5 μm.

trix of lacunae. (Fig. 6, *b*, *e*, and *h*). Type X collagen was detectable only in the 7- and the 17-d aggregates and localized in the central area, staining both territorial and interterritorial matrices (Fig. 6, *c*, *f*, and *e*). A good overlap between the staining of type II and X collagen and the areas that stained darker with toluidine blue was always observed. Analogous results were obtained with paraffin-embedded material (data not shown).

Cartilage Morphogenesis from Cloned Dedifferentiated Cell Populations

To rule out the possibility that some of the phenomena we observed were due to selection of a cell subpopulation and not to the in vitro maturation of committed cells, we looked at the cartilage obtained in vitro from a cloned population derived from a single dedifferentiated cell. The tissue showed the same morphologic characteristics of the central area of aggregates obtained from primary cultures (Fig. 3 *d*).

Discussion

In a previous report (4) we have described how dedifferentiated cells cultured in suspension in the absence of ascorbic acid can mature into single hypertrophic chondrocytes. Furthermore, we showed that the differentiation process goes through at least one intermediate stage of maturation (stage I chondrocytes). These data are confirmed by our recent studies on the kinetics of the mRNA coding for type I, II, and X collagen (Castagnola et al., manuscript submitted for publication) and studies on the kinetics of the cell cycles of these dedifferentiated cells after different times from transferring in suspension culture (Giaretti et al., manuscript submitted for publication).

In this article we have shown that dedifferentiated cells, when grown in suspension culture in the presence of ascorbic acid, can mature to form a tissue strongly resembling hypertrophic cartilage. Ascorbic acid is necessary for the full

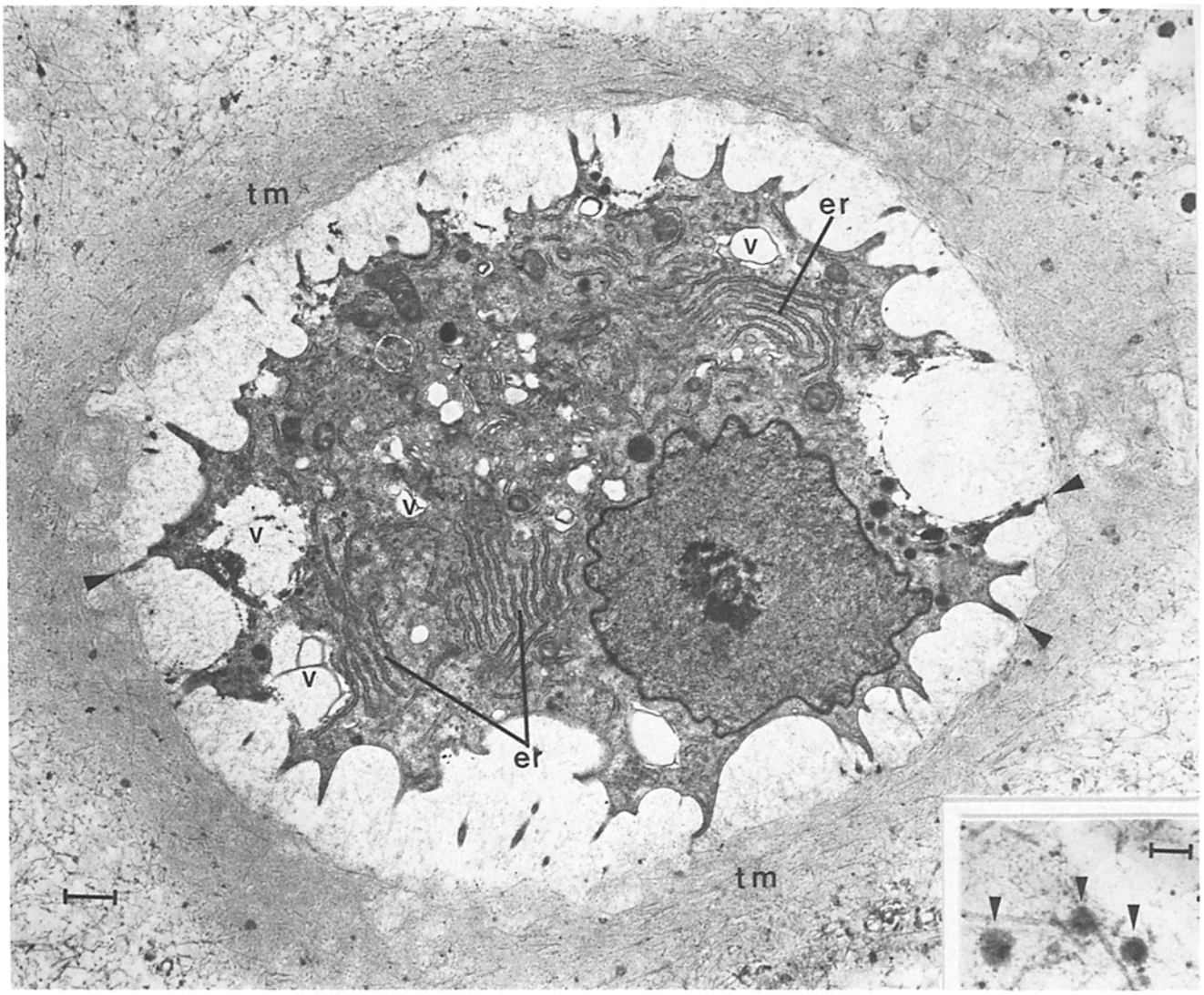

Figure 5. 7 d of culture. A hypertrophic chondrocyte from the central darkly stained area of an aggregate formed in the presence of ascorbic acid. Note the well-represented rough endoplasmic reticulum (*er*) and the characteristic vacuoles (*v*). The irregular cell membrane makes occasional contact with the edge of the lacuna (*arrowheads*). The territorial matrix (*tm*) is well defined. Bar, 1 μm. (*Inset*) Matrix vesicles (*arrowheads*) are present in the territorial matrix surrounding hypertrophic chondrocytes already at this time of culture. Bar, 0.1 μm.

maturation of collagen molecules and the organization of a tridimensional matrix. We suggest that dedifferentiated cells transferred in suspension culture in the presence of the vitamin remain embedded in this tridimensionally organized, newly secreted matrix and mature in this environment giving rise to the cartilage tissue. The initial aggregation of the dedifferentiated cells, observed also in the absence of ascorbic acid, might be mediated by (an) extracellular molecule(s) or by a direct cell–cell interaction. In the absence of the vitamin, cells separate from each other probably because of the interposition of secreted macromolecules such as proteoglycans unable to be organized by a collagen network. Histologic and ultrastructural data support this possibility because in the presence of ascorbic acid cells are surrounded by extracellular matrix fibrils. A gradient of cartilage maturation can be observed in cell aggregates cultured in the presence of ascorbic acid, going from chondroblasts secreting type I and low amounts of type II collagen to hypertrophic chondrocytes (stage II) synthesizing type II and X collagens. Thus the sequence of events of maturation in vitro is analogous to the sequence observed in vivo. Most of the aggregates are surrounded by a layer of elongated cells resembling the perichondrium. No evidence of these peripheral cells can be observed in the cartilage obtained from the cloned cell population. This observation raises the question of whether the peripheral cells are inhibited in proceeding through the maturation process, possibly because of their position in the aggregates, or whether a mechanism of sorting out the cells, not committed to the chondrogenic lineage and present in the original heterogeneous population, is active at this time. Further analysis of several clones that we have isolated will probably help to answer this question. Oakes et al. (16), using high-density cultures of differentiated chondrocytes from 13-d-old chick embryo tibiae and femora, obtained similar results. In this respect, it is noteworthy that in our hands, when the vitamin is added to hypertrophic chondrocytes obtained by culture in the absence of ascorbic acid, cells aggregate to form structures analogous to the cartilage obtained

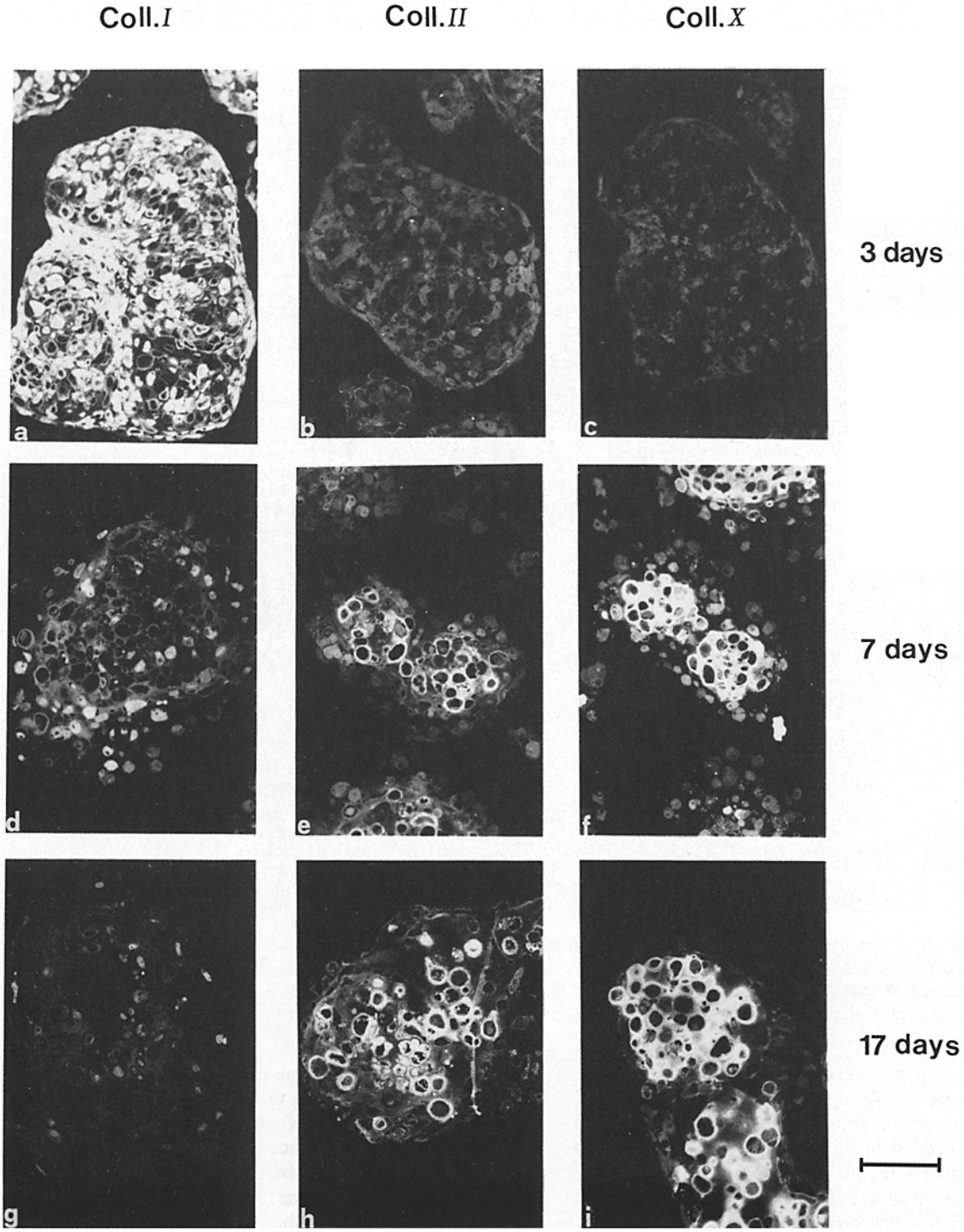

Figure 6. Immunofluorescence localization of type I (*a*, *d*, and *g*), type II (*b*, *e*, and *h*), and type X (*c*, *f*, and *i*) collagens in cell aggregates 3, 7, and 17 d after transfer of dedifferentiated cells in suspension culture. Whereas by day 3 the staining for type I collagen is brightly and evenly distributed on the entire section of the aggregate (*a*), type II gives only a very pale staining (*b*) and type X no staining at all (*c*). *a*, *b*, and *c* show sections of the same aggregate. Type X collagen can be seen as a staining of the territorial and interterritorial matrix in aggregates at 7 d (*f*); at this time the distribution of type II collagen is more in the territorial matrix with a brighter staining than the one at 3 d (*e*), and at this time type I collagen starts to disappear (*d*). *e* and *f* show different sections of the same aggregate. By 17 d the major change is at the level of type I collagen which is almost undetectable (*g*), whereas the distribution of type II (*h*) and type X (*i*) collagens is almost unchanged. Bar, 100 μm.

from primary cultures but with very few peripheral mesenchymal cells (Tacchetti et al., unpublished data). The importance of ascorbic acid in the extracellular matrix accumulation in chick embryo chondrocytes cultures has been shown also by other authors (8). In this article we also describe the distribution pattern of type I, II, and X collagens in the in vitro-formed cartilage and show that it is analogous to the pattern described in vivo (17).

The occurrence of matrix vesicles and the presence of type X collagen, which has been suggested to play a role in the endochondral calcification (3, 6, 7, 13, 17), suggest that calcification could occur in these aggregates. The presence of degenerating chondrocytes, especially in the central area of the aggregates is another sign of cartilage maturation in that degeneration of the chondrocytes occurs in the area of calcification. Indeed, we have preliminary evidence that calcium is deposited in the extracellular matrix organized in the presence of ascorbic acid (Tacchetti et al., manuscript in preparation). Therefore in vitro obtained endochondral cartilage is able to perform its main function, which is to prepare the field for the following ossification. We are presently using this model to investigate the regulation of the calcification process and ossification and the accompanying phenomenon of angiogenesis.

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