Matrix changes during long-term cultivation of cartilage (organoid or high-density cultures)

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Summary. In high density (organoid or micromass) cultures of prechondrogenic mesenchymal cells from limb buds of 12-day-old mouse embryos typical cartilaginous tissue develops after 3 days. Immuno-morphological investigations have shown that it contains the typical components of the cartilaginous matrix, such as collagen type II and cartilage-specific proteoglycans. After a 2-week cultivation period hypertrophic cartilage cells develop to an increasing extent. Many of these cells as well as normal chondroblasts detach from the matrix from the 2nd week *in vitro* onwards to assume a fibroblast-like appearance. At the same time thick (25-65 nm) collagenous fibrils occur at the surface of these cells. These thick fibrils contain collagen type I, as shown by immuno-morphology. Hence, in these older cartilage cultures chondroblasts change their synthesis programme or direction of differentiation. Consequently, a model for the study of «dedifferentiation» of cartilage and possibly also transformation of cartilage cells to osteoblasts has become available.

Key words: Long-term organoid (high density) cartilage culture, Collagen type I, Dedifferentiation

Introduction

Cultivation of prechondrogenic mesenchymal cells from chicken or mouse limb buds at high density leads to the formation of cartilaginous tissue (Weiss and Moscona, 1958; Solursh and Meier, 1974; Ahrens et al., 1977; Merker et al., 1980a,b, 1981; Archer et al., 1985; Kistler et al., 1985; Rodger et al., 1989). Its matrix contains cartilage-specific components such as collagen type II as well as cartilage-specific proteoglycan (Schröter-Kermani et al., 1991). Collagen types I and III cannot be demonstrated; they only occur

in the perichondrium. After a cultivation period of 2 weeks or more hypertrophic cartilage develops and even mineralization of the matrix of cartilage and perichondrium is observed (Osdoby et al., 1979; Tacchetti et al., 1987; Flöttman, 1991; Gerstenfeld and Landis, 1991; Schröter-Kermani et al., 1991). The possible changes of the matrix and the cells during these long-term cultures (3 to 5 weeks) have not yet been fully elucidated. Preliminary electron microscopic and immunohistochemical investigations have shown the occurrence of thick collagen type I fibres at the surface of individual chondroblasts and a transformation into fibroblast-like cells in these old cultures. Confirmation of these findings is interesting for the following reasons: 1) this type of «dedifferentiation» would present an *in vitro* model for the study of pathological and age-dependent changes of cartilage (Abbott and Holtzer, 1966; Green, 1971; Levitt and Dorfman, 1984; Sokoloff, 1976; Müller et al., 1977; Oakes et al., 1977; von der Mark et al., 1976; Benya et al., 1978; Grundmann et al., 1980); and 2) these processes might play a role during osteogenesis. It is still controversial whether a newly immigrated cell population or the old hypertrophic cartilage cells together with an altered synthesis programme are responsible for the production of the new bone matrix (von der Mark et al., 1976; von der Mark and von der Mark, 1977). Therefore, we tried to demonstrate the occurrence of collagen type I in «old» organoid cartilage cultures (3-5 weeks) with immunomorphological means and to evaluate the accompanying processes morphologically.

Materials and methods

Limb bud mesenchymal cells were grown at high density at the medium/air interphase. Due to the occurrence of histotypic organoid structures formed by different cell types, this culture system is called «organoid culture» (Zimmermann, 1987; Zimmermann et al., 1988, 1990).

Cell culture

Upper and lower limb buds from day-12 mouse embryos (day 0 = day of conception) were dissected and incubated in 0.1% dispase (Boehringer, Mannheim, FRG) for 30 min at 37 °C by gentle agitation. Dissociation of the limb buds into single mesenchymal cells was performed by repeated pipetting in growth medium which was composed of nutrient mixture DMEM supplemented with 20% foetal calf serum, non-essential amino acids (BME-NEA), 50 µg/ml ascorbic acid, 50 µg/ml glucose, 100 µg/ml streptomycin, 100 IU/ml penicillin and 2.5 µg/ml amphotericin (Seromed, Berlin, FRG). The cells were washed twice in growth medium, filtered through a nylon mesh (pore size 20 µm) and sedimented by centrifugation at 600 rpm for 10 min. One or two 10 µl drops of the cell suspension (1.8 to 2.2 x 10^6 cells/10 µl) were placed onto a membrane filter (pore size 0.2 µm, SM 11307, Sartorius, Göttingen, FRG), which rested on top of a stainless steel grid at the medium air interphase. Complete medium changes were performed every three days. At certain time points (i.e. at 2, 7, 14, 21, 28, 35 days after plating) some of the cultures were processed.

Electron microscopy

The material was fixed in Karnovsky solution (3% glutaraldehyde plus 3% paraformaldehyde in 0.1M phospate buffer, pH 7.2) or in 2% glutaraldehyde plus 1% tannic acid in phosphate buffer or in 2.5% paraformaldehyde in 0.05M cacodylate buffer (pH 7.4) plus 0.7% ruthenium hexamine trichloride (= RHT, Aldrich, Steinheim, FRG). Postfixation used 1% OsO_4 in phosphate buffer. Dehydration was performed in ethanol, embedding in Epon. The sections were prepared using an Ultracut (Reichert), contrasted with uranyl acetate and lead citrate and inspected under a Zeiss EM10.

Immunofluorescence microscopy

The material was embedded in O.C.T. solution (Bayer, Munich, FRG) and frozen in liquid nitrogen. Ten µm-thick sections were preincubated with testicular hyaluronidase (1500 IU/mg, Serva, Heidelberg, FRG) for the immunolabelling of collagen type I and type II. FITC-coupled IgG-antibodies (Dianova, Hamburg, FRG) were used for the demonstration of specific binding.

Immunoelectron microscopy

Cultures were fixed with 3% paraformaldehyde, 0.25% glutaraldehyde in 0.1M phosphate buffer for 1 h. After dehydration in ethanol, the material was embedded in LR-white (Plano, Marburg, FRG). Ultrathin LR-white sections treated with testicular hyaluronidase (1500 IU/ml, Serva, Heidelberg, FRG) were immunolabelled with polyclonal anticollagen (1:2), and as second antibody goat antirabbit antibody (1:50) (Amersham, Braunschweig, FRG). Grids were examined on a Zeiss EM10 electron microscope.

Antibodies

Collagen type I was obtained from foetal mouse skin according to Trelstad et al. (1976), collagen type II from a transplantable chondrosarcoma of the mouse according to Schröter-Kermani et al. (1991). Fibronectin was isolated from rat serum according to Engvall and Ruoslahti (1977). The antibodies were grown in rabbits. Sera showing a high titer were immunoadsorbed in antigen-coated Sepharose columns. The specificity of the antibodies was verified using the ELISA technique (Gosslau and Barrach, 1979).

Results

Immunomorphology

Immunofluorescent microscopic experiments served to demonstrate the two typical components of the cartilaginous matrix, collagen type II and a cartilage-specific proteoglycan, in cartilaginous tissue at all stages. The perichondrium contained collagen type I. This collagen type also occurred in the pericellular region of single chondroblasts after a 2- to 3-week culture period (Fig. 1). The number of these cells steadily increased up to 5 weeks in culture, finally amounting to 100% of all chondroblasts.

Using antibodies against collagen type II and cartilage-specific proteoglycan, immuno-morphological investigations at electron microscopic level showed that gold particles were only located above the typical cartilage matrix (not shown here). Labelling with antibodies against collagen type I could not be demonstrated during the first two weeks in cartilaginous tissue *in vitro*. However, in the region of the thick fibrils at the surface of distinct chondroblasts the opposite was true: collagen type I could be demonstrated; however, not type II or PG (Fig. 2).

After cultivation of prechondrogenic mesenchymal cells from limb buds for 7 days at high density nodules and plates of typical cartilaginous tissue developed which were delimited by a perichondrium consisting of 2 to 5 layers of long, bipolar fibroblast-like cells. Loose mesenchyme of polymorphous cells was located between the cartilage nodules. The condroblasts were mainly round with small stubby microvilli at their surface (Fig. 3a). They were characterized by a pronounced rough endoplasmic reticulum, a large Golgi apparatus and the occurrence of other organelles or structures (mitochondria, small vacuoles, vesicles, granules and some components of the cytoskeleton). The cells flattened towards the perichondrium. The matrix in the cartilaginous tissue consisted of thin (18-22 nm) singly and irregularly running fibrils which attached to

the cell membrane (Fig. 2a). After tannic acid fixation, they came into contact or even fused in these areas with the surface coat of the cell membrane. The matrix of the perichondrium contained thicker fibrils (25-32 nm) which were mainly arranged in bundles parallel to the surface of the cartilaginous structures.

After a 2-week culture period, the situation had slightly changed. The centres of some cartilage nodules exhibited chondroblasts that contained organelle-free spaces occasionally showing glycogen structures. The relationship between cell membrane and matrix had not changed, neither had the composition of the matrix. In the perichondrium the number of single fibrils and fibrils per bundle had increased, as had their thickness (28-38 nm).

During the third week of cultivation the number and size of cells with organelle-free spaces had increased. In some cells the nucleus was floating in an organelle-free area. These cells corresponded morphologically to hypertrophic cartilage cells *in vivo*. The morphology of the cartilaginous matrix and the perichondrium had remained almost unchanged. However, some chondroblast-like cells were observed which were characterized by peculiarities that occurred singly or jointly: (1) The cell membrane detached from the matrix for distances of varying length (Fig. 3b), in other words, an optically empty space developed between cell membrane and matrix; (2) Cross-striated, mainly singly running fibrils rested on the surface of the cells. These fibrils were clearly thicker than those of the cartilaginous matrix (35-65 nm) (Fig. 4).

After a 4- to 5-week culture period the number of chondroblasts showing these two changes had steadily increased. In addition, the number of thick collagen fibrils in the pericellular area increased, which led to a greater thickness of this zone. The cell membrane detached from the matrix over longer distances. Single chondroblasts were occasionally located freely in the cavity. These cells stretched and formed plump processes, thus assuming a fibroblast-like appearance. The remaining cartilaginous matrix had not changed when compared with younger cultures.

Discussion

After a 2-week cultivation of prechondrogenic limb bud cells at high density (organoid or micromass culture) collagen type I occurs at an increasing extent at the surface of chondroblasts. This collagen type can be demonstrated with immunomorphological (light and electron microscopic) as well as morphological means (increased thickness of collagenous fibrils). *In vivo* and *in vitro*, too, embryonic type II fibrils are characterized by a low (18-22 nm), type I fibrils by a higher (35-65 nm) thickness (Merker et al., 1978). This situation is, of course, only true of embryonic or *in vitro*-formed cartilaginous tissue. The fibril thickness of mature tissue *in vivo* is different. The second finding observed in some chondroblasts - detachment of the cell membrane from the matrix, i.e. from the inside of the matrix cavity - is often followed by the occurrence of thick type I fibrils in the pericellular spaces. Before evaluating this finding, the problem of an artefact must be discussed. Hunziker et al. (1982, 1983) and Hunziker and Grabe (1986) were the first to emphasize the importance of the preparation technique for the detachment of proteoglycans and the ensuing disappearance of matrix - cell membrane connections; in other words, the occurrence of an optically empty gap in the pericellular space. However, in this case we are not dealing with this phenomenon, for
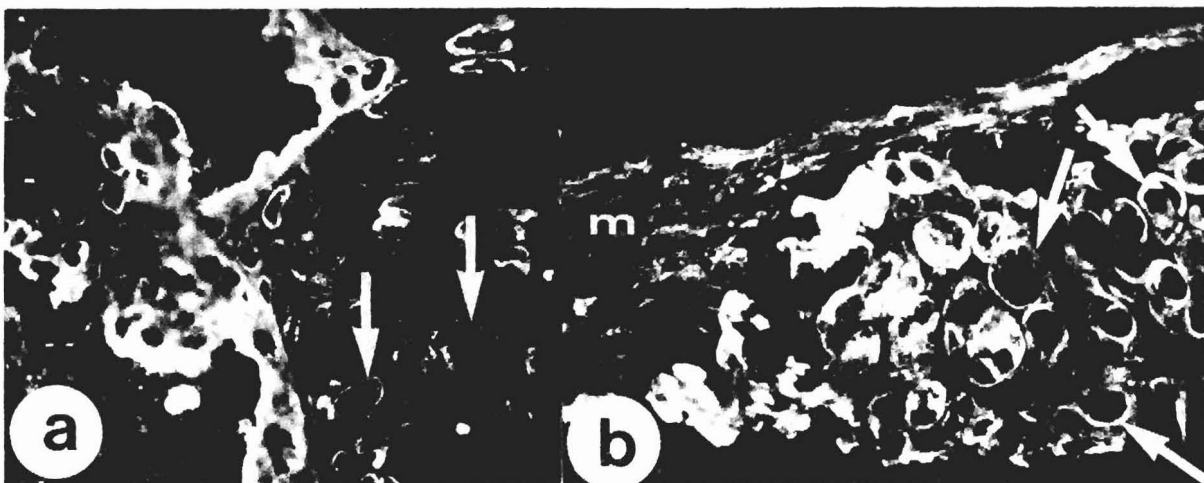

Fig. 1. Fluorescence microscopic demonstration of collagen types I and II and fibronectin in cartilaginous tissue of limb bud cells from 12-day-old mouse embryos in high density culture. **a.** Culture period: 16 days; demonstration of collagen type I. Onset of capsule formation around chondroblasts (arrow). x 160. **b.** Culture period: 21 days; demonstration of collagen type I. Capsule-like occurrence around chondroblasts (arrow). m - mesenchyme. x 160

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Fig. 2. Culture period: 21 days. Demonstration of collagen type I with gold-labelled antibodies. Gold particles seen only in the layer (arrow) around chondroblasts (C) with thick collagenous fibrils. This zone corresponds to the two stretched lines. Gold particles missing in the cartilaginous matrix (m). x 35,000
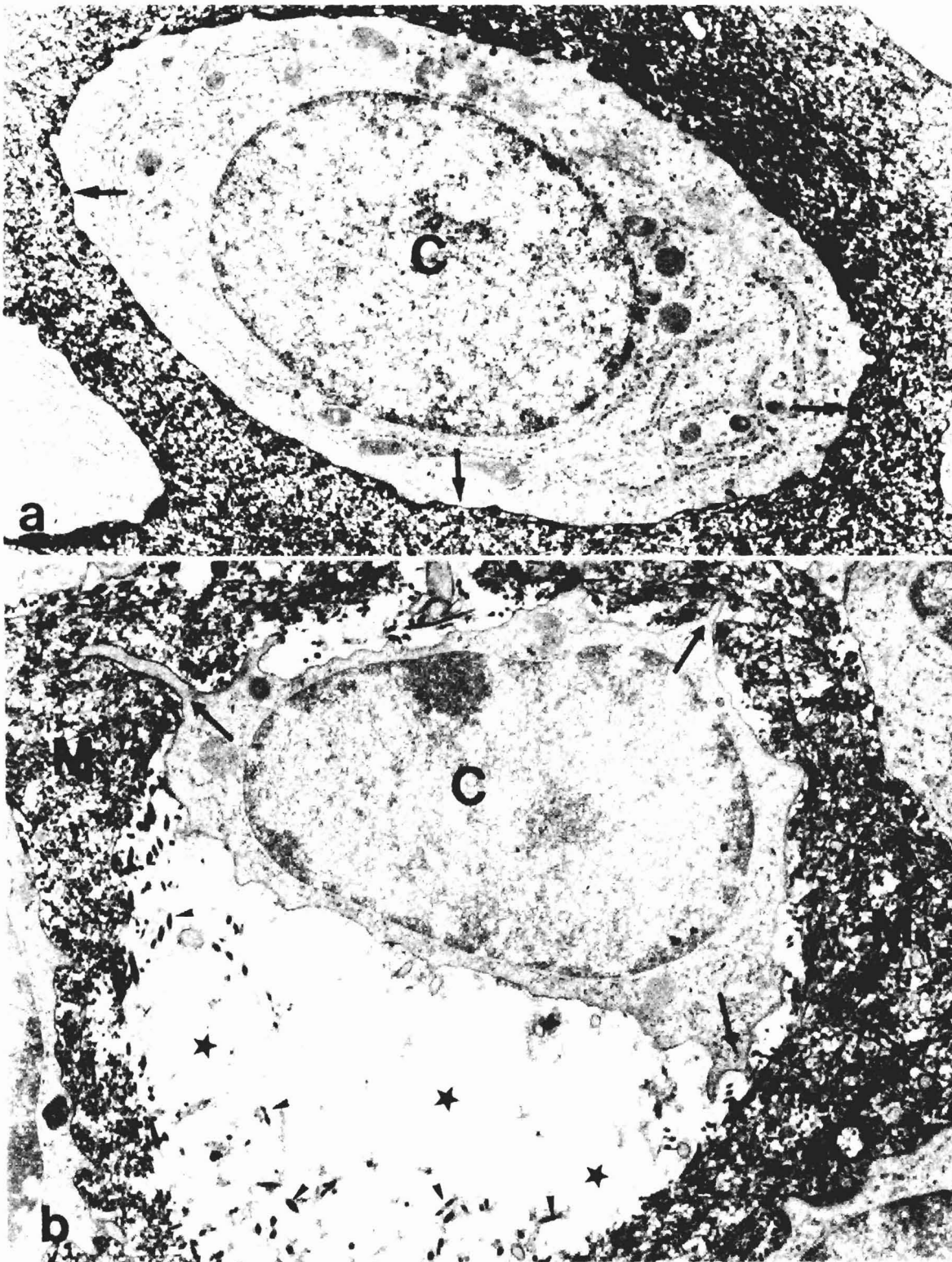
Fig. 3. Electron microscopic picture of cartilaginous tissue from high density cultures (limb bud cells of 12-day-old mouse embryos). **a.** Culture period: 7 days. Typical chondroblasts (C) with relatively smooth surface and numerous cavities of rough endoplasmic reticulum. Well developed matrix closely attached to the cell membrane (arrow). x 17,000. **b.** Culture period: 21 days. A chondroblast (C) has detached from the inner surface of the cartilage cell cavity. Optically empty spaces (*) occur. The cell shape has changed; cell processes (arrow) develop that occasionally extend into the matrix. Thicker collagenous fibrils (arrow head) occur in the surrounding of the cells. M = cartilaginous matrix. x 15,000
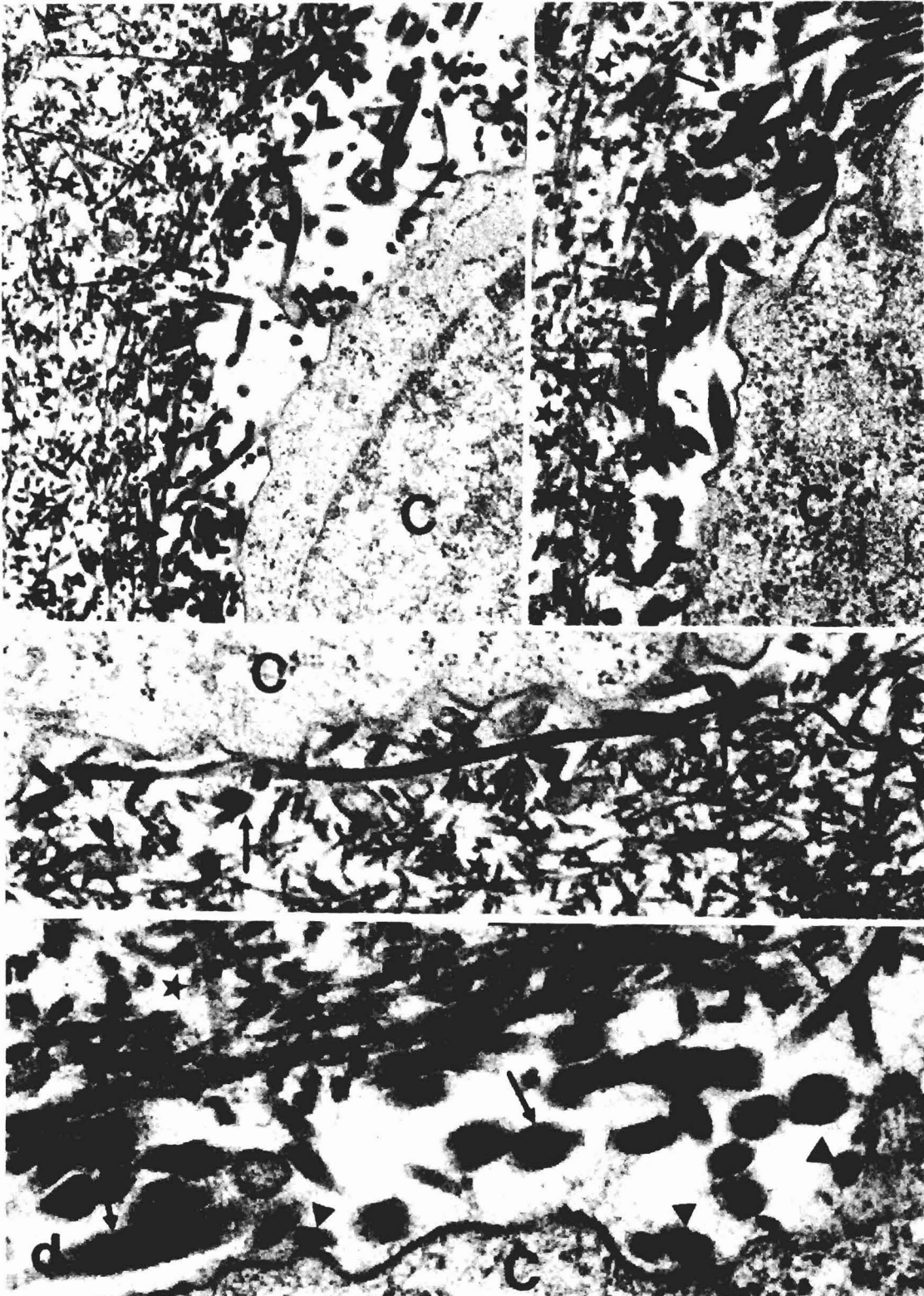
Fig. 4. Culture period: 21 days. Occurrence of thick collagenous fibrils (arrow) at the surface or in the surrounding of chondroblasts (C). Compare with the thin fibrils (18-22 nm) in the cartilaginous matrix (*). The thick fibrils are occasionally closely attached to the cell membrane (arrow head). **a.** x 33,000. **b.** x 60,000. **c.** x 60,000 and **d.** x 12,000

(1) with the used culture model the pericellular gap has never occurred, which is possibly due to the small size of the samples or the rapid fixation. (2) The described changes can also be observed in old cultures after Ruthenium Red fixation. (3) The shape of the cells changes during gap formation, i.e. they become fibroblast-like. Hence, an artefact cannot serve as an explanation for the loss of matrix-cell connections. The thick type I fibrils are often closely attached again to the cell membrane. These findings suggest that the matrix binding of the chondroblasts must change. First the binding disappears. This process may be due to an enzymatic detachment and/or loss of receptors for the cartilaginous matrix. The developing new cell type then forms other receptors which are capable of binding collagen type I or other associated matrix components, such as fibronectin. An altered direction of differentiation of the original chondroblasts must, therefore, be presupposed. Two goals of differentiation can be discussed in this connection: (1) the fibroblast-like cells. This assumption is corroborated by its morphology (stretching and process formation) and changes in the synthesis programme (synthesis of collagen type I). Thus, the behaviour of the chondroblasts in high-density cultures would be comparable with the changes of mature, differentiated chondroblasts in monolayer culture at low density. Under these conditions the cells alter their synthesis programme (no collagen type II, but types I and III) and their shape (stretching and process formation), detach from the matrix coat and become fibroblast-like (Grundmann et al., 1980; Merker et al., 1980a; for additional literature see «Discussion»). The occurrence of similar processes in the three-dimensional high-density culture would render this *in vitro* system suitable for the study of dedifferentiation and degenerative, pathological and age-dependent changes. The high density culture has a number of advantages over the monolayer culture (three-dimensional, more material, material is more easily obtainable, occurrence of many well-definable and reproducible morphogenetic processes) (Merker et al., 1980a,b,1981; Merker, 1991). (2) A second goal of differentiation might be discussed in this context: the osteoblast, although this appears to be less plausible. During chondral osteogenesis *in vivo* the stage of hypertrophic cartilage is followed by disintegration of cartilage and bone formation proper. The producers of the new osseous matrix are supposed to be the invading mesenchymal cells that have been transformed to osteoblasts. The original hypertrophic cartilage cells die during this process (Merker, 1968; Knese, 1979).

Other authors, however, believe that, after disintegration of their matrix, hypertrophic cartilage cells are able to change into osteoblasts (for literature see Knese, 1979). Since in high density cultures invasion of blood vessels and mesenchymal cells does not take place, the occurrence of collagen type I *in vitro* might be interpreted in terms of a transformation of hypertrophic cartilage cells to osteoblasts which also produce collagen type I.

Notwithstanding these problems of interpretation, long-term cultivation of prechondrogenic limb bud cells at high density appears to be a promising *in vitro* model for the study of maturation, disintegration and pathological changes of cartilaginous tissue.

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