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Behaviour of Epiphyseal Mouse Chondrocyte Populations in Monolayer Culture

Morphological and Immunohistochemical Studies

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Summary. Growth and dedifferentiation of a heterogeneous mouse chondrocyte population, prepared from epiphyses of mouse embryos (day 17 of gestation), were studied in primary monolayer culture. At different times of culture, light and electron microscopic investigations were carried out and the change of collagen types was shown by immunofluorescence microscopy. During the first four days in culture, chondrocytes express their typical phenotype. Round or polygonal cells are embedded in a metachromatically staining matrix and produce type II collagen. After four to eight days in vitro most of the chondrocytes lose their matrix capsule and alter to fibroblast-like cells. Simultaneously, a switch of collagen synthesis to type III and type I collagen occurs, whereas the type II collagen synthesis is stopped. Altered cells and transitional stages have intracellular glycogen like typical chondrocytes, but show phagocytosis and indications of cell migration like fibroblasts. It is proposed that these cells, originating from a subpopulation of epiphyseal cartilage, are able to differentiate *and* dedifferentiate in vitro.

Key words: Chondrocytes – Monolayer culture – Dedifferentiation – Collagen.

Introduction

Numerous studies have been concerned with the dedifferentiation of chondrocytes in monolayer culture (Holtzer et al. 1960; Oakes et al. 1977; Sokoloff 1976; for review see also Levitt and Dorfman 1974). All authors agree in describing this phenomenon as a change of phenotypic behaviour: a) the round or polygonal chondrocytes are transformed into bipolar, fibroblast-like cells (FLC) (Abbott and Holtzer 1966; Chacko et al. 1969; Green 1971; Horwitz and Dorfman 1970); b) a switch of collagen synthesis from typical cartilage type II to type I and III occurs (Benya et al. 1977; Benya et al. 1978; Layman et al.

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1972; Müller et al. 1975; Müller et al. 1977; von der Mark et al. 1977) accompanied by c) a change and diminution of proteoglycan production (Abbott and Holtzer 1966; Kim and Conrad 1977; Lavietes 1971).

The appearance of dedifferentiation of chondrocytes in vitro has also been considered to be a result of experimental procedures as, for instance, in the overgrowth of contaminating fibroblasts (Bryan 1968; Hough and Sokoloff 1975; Kuroda 1964a, b; Norby et al. 1977). Other reports, however, have referred to an alteration of chondrocytes themselves (Chacko et al. 1969; Müller et al. 1975, 1977; Schlitz et al. 1973; Sokoloff 1976). Overgrowth by immature chondroblasts which are present in the heterogeneous cell population of embryonic cartilage has also been considered (Layman et al. 1972; Sokoloff 1976).

This paper describes the behaviour of epiphyseal mouse chondrocytes in monolayer culture. We have used light and electron microscopy and the immunofluorescent staining method to investigate the time dependence of the alteration of chondrocytes in vitro. It is demonstrated that, under our culture conditions, a distinct cell population grows as typical chondrocytes, which are shown to differentiate and dedifferentiate in a characteristic manner after some time in culture. Certainly, a small portion of cells cannot be considered to be "chondrocytes", but are immature chondroblasts and/or perichondral FLC.

Methods

1. Chondrocyte Cell Culture

Chondrocytes were obtained from the long bone epiphyses of fore and hind limbs of mouse embryos. Embryos were removed from the uteri at day 17 of gestation (day 0 = day of fertilization) and collected in Hanks balanced salt solution (Hanks BSS). The limbs were dissected from the body and freed of skin and hand. Muscle and connective tissue were removed from the long bones by two treatments with 0.2% trypsin (EG 3.4.21.4., Sigma, München) dissolved in Ca – Mg-free saline (Wiepjes and Prop 1970), pH 7.4 for 30 min at 37° C by vigorous stirring. Trypsin was then inactivated by fetal calf serum (FCS) and the bones were washed several times with Hanks BSS.

The epiphyseal cartilage was dissected from the long bones and incubated with 0.2% collagenase (from Clostridium histolyticum, 0.15 U/mg, Boehringer, Mannheim) in Hanks BSS for 60 min at 37° C. Isolated chondrocytes were suspended by pipetting several times and filtered through a gauze filter to remove undigested cartilage fragments. After centrifugation the cells were washed twice with complete Ham F12 nutrient mixture containing 15% FCS, 75 µg/ml ascorbic acid, 50 µg/ml streptomycin, 50 U/ml penicillin, and 2.5 µg/ml amphotericin B (Seromed, München).

Washed chondrocytes were resuspended in complete Ham F12 nutrient mixture. The initial cell density was adjusted to 1.5×10^6 cells/ml ($\triangleq 3 \times 10^5$ cells/cm^2). Cells were cultured as monolayers on cover slips in 35 mm plastic petri dishes. The cultures were maintained at 37° C in 95% air/5% CO_2. At different time intervals samples were taken for histological and immunohistochemical staining.

2. Preparation of Collagen Antibodies for Immunofluorescent Staining

a) Isolation and Characterization of Different Collagen Types. Type I collagen and procollagen type III were isolated from the skin of young mice (up to three weeks old). Type I collagen was prepared by neutral salt extraction with 1 M NaCl (Bornstein and Piez 1966, modified) and

purified by sequential salt precipitation (Trelstad et al. 1976) and diethylaminoethyl (DEAE) cellulose chromatography (Miller 1971). Procollagen type III was extracted with 0.15 M NaCl at neutral pH according to the method of Smith et al. (1977).

Type II collagen and procollagen type II were isolated from Swarm rat chondrosarcoma (Smith and Martin 1975). For preparing type II collagen, rats were placed on a diet containing β-aminopropionitrile (βAPN, 3 g/kg food) during growth of tumors. This pretreatment was not necessary when preparing procollagen type II. Type II collagen was isolated from chondrosarcoma of 3–4 cm diameter by neutral salt extraction (Smith and Martin 1975) and DEAE cellulose chromatography. Further purification was achieved by precipitation with 2.5 M NaCl, pH 7.5 and dialysis of the supernatant against 0.01 M Na_2HPO_4. Procollagen type II was prepared by the method of Byers et al. (1974).

After DEAE cellulose chromatography procollagen type II fractions were purified from remaining type II collagen using activated thiol-sepharose 4B for covalent chromatography (Angermann and Barrach 1979).

The purity of collagen and procollagen samples was tested by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Maurer 1971) and carboxymethyl cellulose (CMC) chromatography (Piez et al. 1963). Further characterization was carried out by amino acid analysis (Miller and Piez 1966) and precipitation of segment long spacing (SLS) aggregates (Lilja, in preparation).

b) Preparation and Purification of Specific Antibodies. 0.5 mg lyophilized collagen or 0.25 mg procollagen were suspended in 0.9% NaCl mixed with an equal volume of complete Freund's adjuvant (Behringwerke, Marburg). This suspension was injected into the popliteal lymph node of the hind leg of rabbits. The first booster injection containing no Freund's adjuvant was given intravenously after four weeks and the second booster was injected after another four weeks into the lymph node of the same hind leg of the rabbit.

Anticollagen-antibodies in rabbit sera were detected by the *e*nzyme-*l*inked *i*mmuno*s*orbent *a*ssay (ELISA; Engvall and Perlman 1972; Gosslau and Barrach 1979). Those sera which showed the highest titer and least cross-reactions with other collagen types were purified by passing through different collagen-bound Sepharose 4B columns (von der Mark et al. 1976a). Type specific antibodies were obtained by immunoadsorption to the homologous collagen type.

Anti-fibronectin antibodies were a generous gift of Dr. W. Dessau (MPI für Biochemie, Martinsried). For immunofluorescent staining experiments with these antibodies, chondrocytes were grown in complete Ham F12 nutrient mixture with fetal calf serum, which was previously purified from fibronectin by rinsing over a collagen-bound Sepharose 4B column.

3. Staining Methods for Light Microscopy

a) Giemsa Staining. Chondrocytes were fixed in 10% formalin with 0.2% cetrimide (Sigma, München) for 30 min, washed with aqua dest. and stained with Giemsa solution overnight (Merck, Darmstadt; one drop per ml aqua dest.). After destaining with 70% 2-propanol, dehydration and immersion in xylol the cover slips were embedded in Eukitt. Typical staining showed light blue cytoplasm and nuclei, dark blue nucleoli and chromosomes and a bright red metachromasia of matrix.

The relative number of chondrocytes was determined with the metachromatic index (MI) according to Lavietes (1970). Measurements were made optically with a microscope ocular, which contains a screen with twenty-five dots on the field. The number of dots falling on metachromatic or non-metachromatic cells was recorded at twenty randomly placed areas of the culture. The MI results from the ratio of the total number of dots falling on chondrocytes and those falling on FLC. Consequently, a MI of 1.0 means 50% chondrocytes and 50% FLC without consideration of cell density.

b) Indirect Immunofluorescent Staining. Chondrocytes on glass cover slips were washed with phosphate buffered saline and air dried. Cells which were to be stained with anti-type II collagen antibodies were first incubated with 1% hyaluronidase (from bovine testes, 3,000 U/mg, Serva, Heidelberg) for 15 min. The following staining procedure was carried out according to the method of von der Mark et al. (1976a, b) using several antibody fractions and fluorescein (FITC)-conjugated

anti-rabbit γ-globulins (Miles, Frankfurt). Fluorescence photographs were taken with a Zeiss photomicroscope III, equipped with overhead light from a HBO 50 W lamp, KP 490/500 filter and 560 blocking filter for FITC fluorescence. Agfachrome professional 50 S films were used.

4. Electron Microscopy

Chondrocytes grown on Thermanox cover slips (Seromed, München) were fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 and 1% tannic acid at room temperature for 1 h. Fixation with tannic acid leads to a good contrast of collagen and proteoglycans. After washing twice with cacodylate buffer post-fixation was carried out for 1 h in 1% OsO_4 in the same buffer. The cover slips were washed, dehydrated in acetone, topped with a gelatine capsule filled with Mikropal (Ferak, Berlin) and polymerized. When separating the capsule from the cover slips, the chondrocyte monolayer is embedded in Mikropal.

Thin sections were prepared with a LKB Ultrotom in horizontal direction. They were stained with uranyl acetate and lead citrate and photographed with a Siemens EM 101 electron microscope.

Results

1. Light Microscopic Studies

At day one of culture chondrocytes grow as single cells. After four days a nearly confluent monolayer has been formed. The chondrocytes express their typical round or polygonal shape and a metachromatically staining matrix. The relative number of chondrocytes has been determined by the metachromatic index (MI; Lavietes 1970; see Methods). We can show in Fig. 1 that the MI

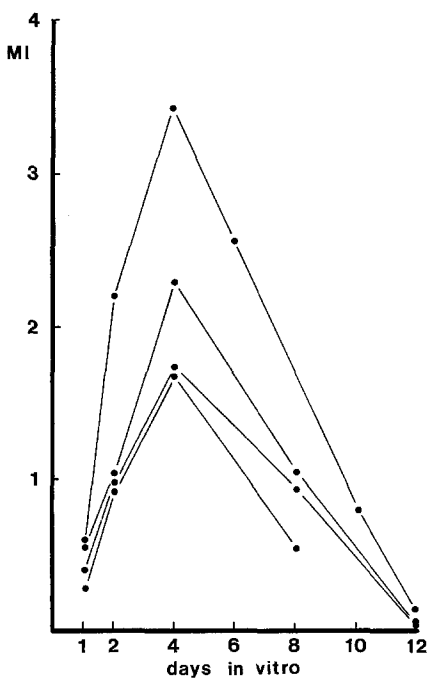


Fig. 1. Metachromatic index (*MI*) of mouse chondrocyte monolayer culture over a period of twelve days. The ratio of metachromatic (chondrocytes) to non-metachromatic cells (FLC) is shown in the ordinate (*MI*). The greatest number of chondrocytes was counted at day four of culture. Results of four different experiments are shown

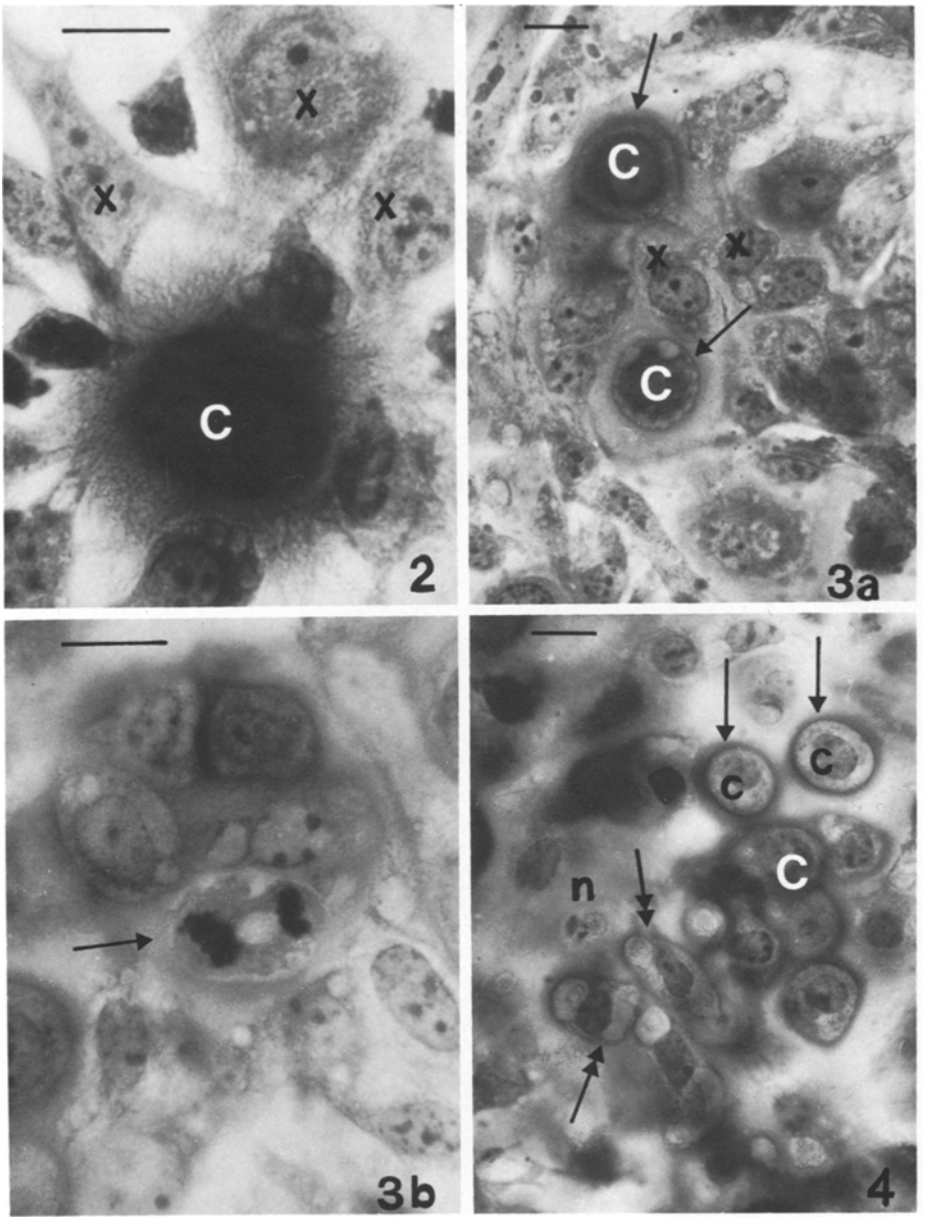


Fig. 2. Chondrocyte monolayer after one day in vitro (Giemsa staining). Besides polygonal cells (*x*) single chondrocytes (*c*) with filamentous matrix halo are observed. ×1,400, bar = 10 μm

Fig. 3a and b. Chondrocyte monolayer after two days in vitro (Giemsa staining). **a** A homogeneous matrix area with polygonal cells (*x*) and chondrocytes (*c*), which have just begun to produce a matrix capsule (→). ×850, bar = 10 μm. **b** In some areas, chondrocytes with metachromatically staining matrix are in mitosis (→). ×1,400, bar = 10 μm

Fig. 4. Chondrocyte monolayer after four days in vitro (Giemsa staining). Metachromatically staining matrix areas with typical chondrocytes (*c*) surrounded by matrix capsules (→). Besides necrotic cells (*n*) vacuolized chondrocytes (→→) usually found in hypertrophic cartilage are seen. ×850, bar = 10 μm

increases from 0.3–0.5 at day one of culture to 1.0 at day two. Maximal values are reached at day four corresponding to 70–80% chondrocytes. At day eight, the MI decreases to about 1.0 and at day twelve, only 2% chondrocytes can be detected (MI→0).

After one day in culture we primarily find polygonal cells, without matrix and single chondrocytes with a filamentous matrix halo (Fig. 2) besides some FLC. Furthermore, some necrotic cells can be found (see Electron Microscopic Results). One day later, round and polygonal chondrocytes form matrix plaques. Round chondrocytes in particular show a matrix capsule (Fig. 3a) and in these areas mitoses can sometimes be seen (Fig. 3b). The largest quantity of chondrocytes is present at day four of culture. Round chondrocytes with strongly marked matrix have large vacuoles as hypertrophic chondrocytes in vivo (Fig. 4).

After eight days, the matrix seems to be reduced. In diminished and enlarged matrix halos the cells show pseudopodia and the cytoplasm appears granulated with vacuoles (Fig. 5a). Some cells become necrotic; others look like fibroblasts and seem to migrate (Fig. 5b). During further cultivation (day twelve), these cells are freed of matrix and can be detected near almost dissolved matrix plaques (Fig. 6a). The main part of the cells, however, are bipolar fibroblasts and partly show parallel arrangement (Fig. 6b).

2. Electron Microscopic Studies

Directly after chondrocyte isolation we find round cells with a smooth cell membrane; the cytoplasm contains normal quantities of cell organelles. Figure 7 shows a freshly isolated chondrocyte with a prominent Golgi apparatus and flat cavities of rough ER near the nucleus. Seen from the morphological point of view, this cell can be considered to be a young chondrocyte.

After one day in culture we can distinguish two cell types as described in Light Microscopic Results. We find round chondrocytes with a Golgi apparatus rich in vesicles and enlarged rough ER (Fig. 8a). A characteristic matrix halo encloses the cells completely. It is evident that this halo has been formed in vitro because we cannot find any matrix components on freshly isolated chondrocytes. The matrix is composed of collagenous fibres with a thickness of 25 nm, but without clear cross striation pattern, and of proteoglycan granules with a diameter of 15–20 nm and fine filaments (Fig. 8b). Furthermore, several necrotic cells can be detected, which are surrounded by a small matrix border (Fig. 8c). These cells are mature chondrocytes, which are able to produce some matrix in vitro before they die.

The other cell type found in early culture stages are polygonal cells which grow close to each other and do not produce any extracellular matrix. Intracellularly we can demonstrate minimally enlarged vesicles of the rough ER, electron dense lysosomes and a small Golgi apparatus (Fig. 8d).

After four days large areas of the culture are covered with matrix, in which the cells are embedded. In the electron microscope the matrix is seen to be composed of a dense irregular network of collagen, whereas proteoglycan granules are diminished when compared with the day-one culture. The number

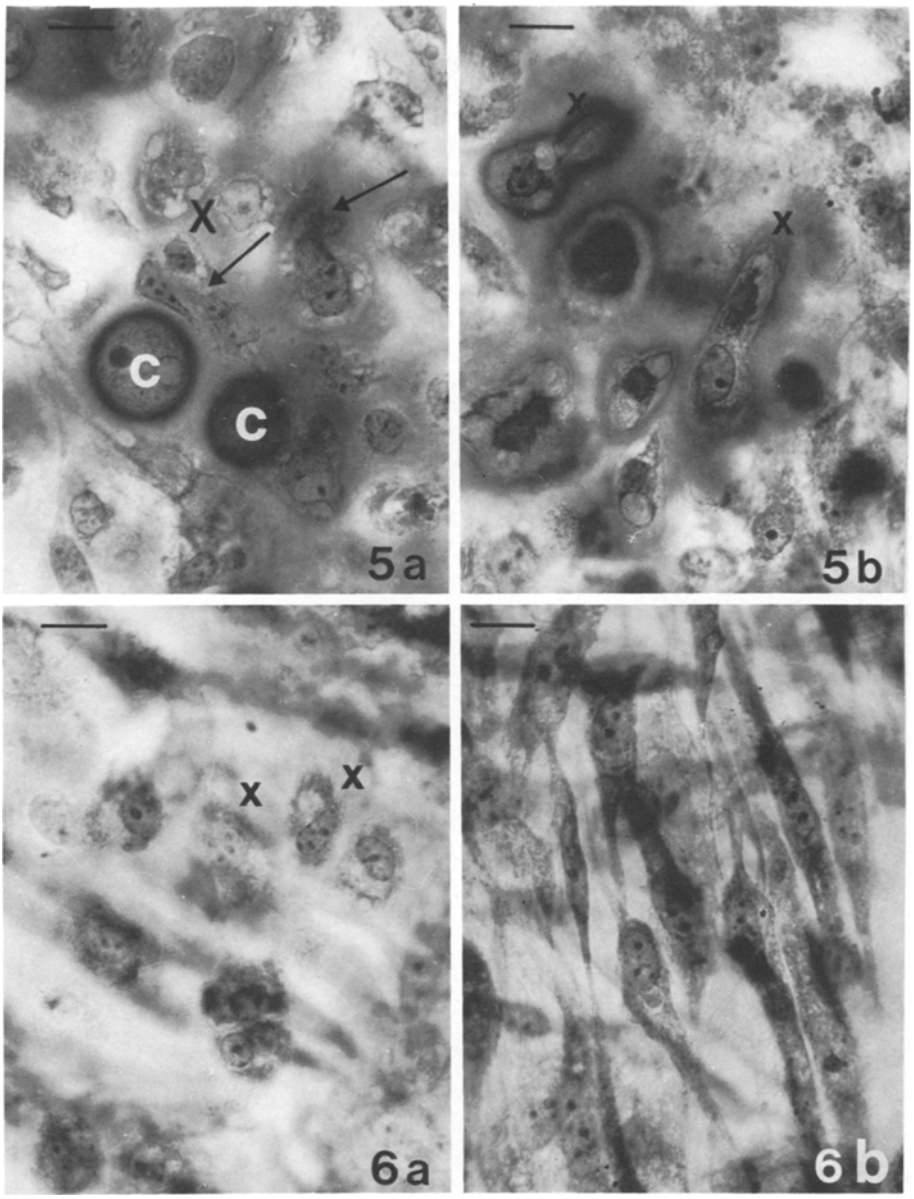
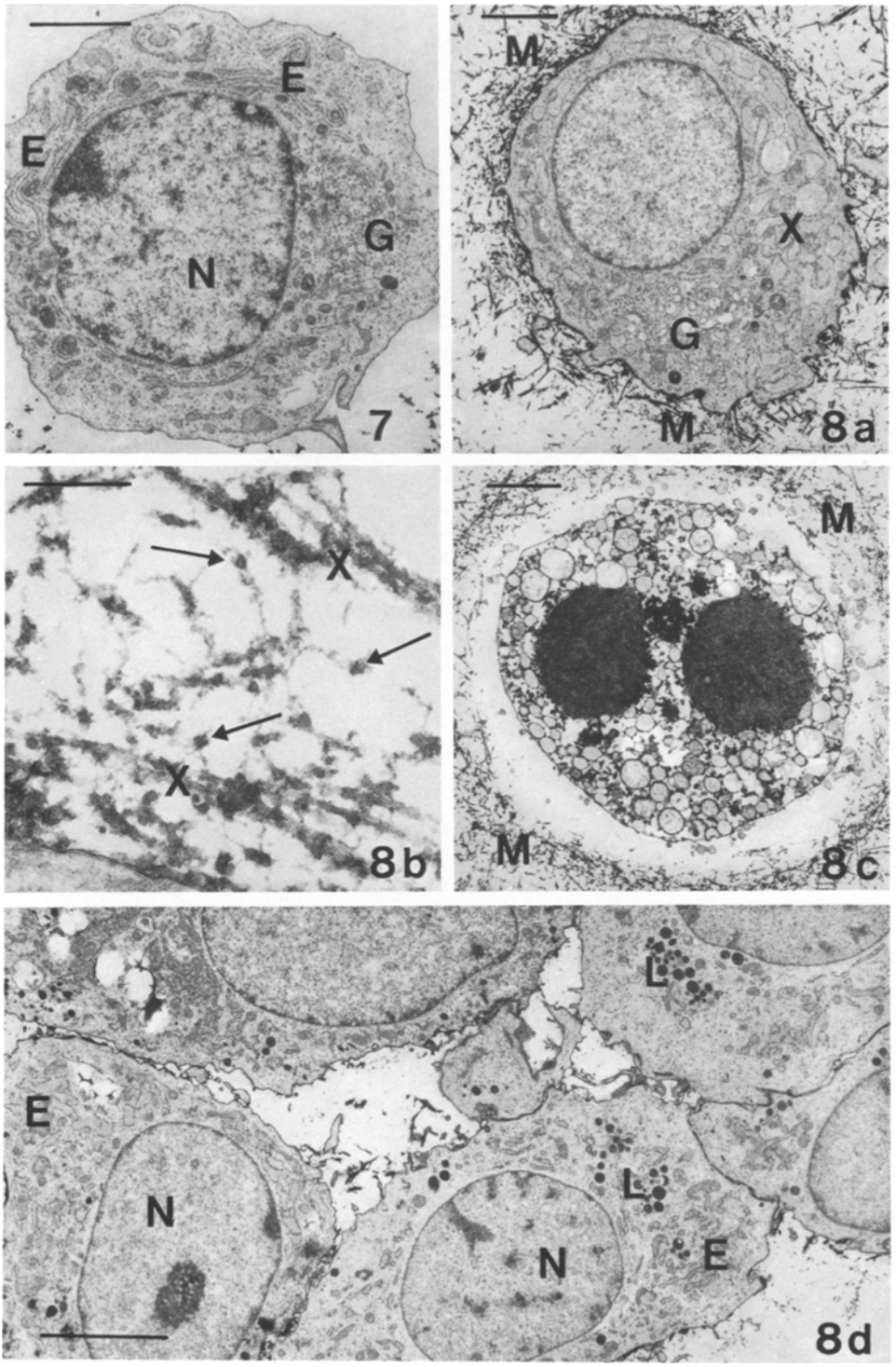


Fig. 5a and b. Chondrocyte monolayer after eight days in vitro (Giemsa staining). **a** Metachromatic matrix is clearly reduced. Besides some typical chondrocytes (*c*) vacuolized cells (*x*) with pseudopodia (→) are present. ×850. **b** Some cells show distinct indications of cell migration and elongation (*x*) within their reduced matrix. ×850, bar = 10 μm

Fig. 6a and b. Chondrocyte monolayer after twelve days in vitro (Giemsa staining). **a** Cartilage matrix areas are almost completely dissolved. Transitional stages of those cells shown in Fig. 5, and FLC are visible. They have granules and vacuoles in the cytoplasm and a bipolar shape (*x*). ×850, bar = 10 μm. **b** In other areas bipolar fibroblasts are arranged in parallel to each other. ×850, bar = 10 μm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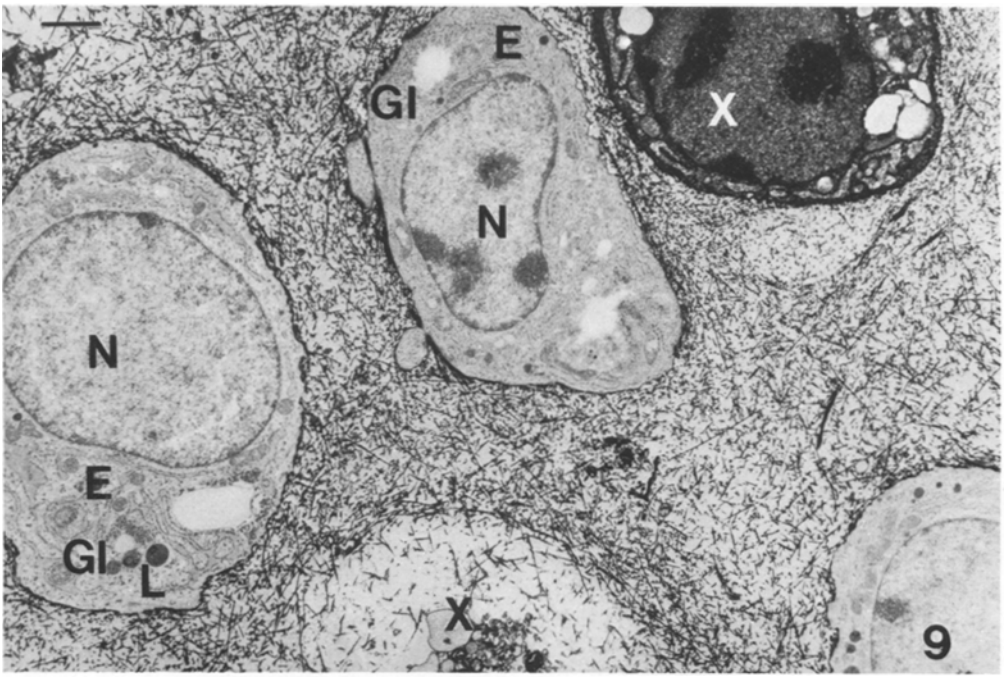

Fig. 9. Chondrocyte monolayer after four days in vitro. Typical chondrocytes are embedded in a dense filamentous matrix besides some necrotic cells (*x*). *N* nucleus, *E* rough ER, *GL* glycogen, *L* lysosomes. ×4,300, bar = 2 μm

of chondrocytes is clearly increased; intracellular glycogen can be found regularly. Furthermore, darkly stained cells with condensed cytoplasm and large vacuoles can be considered as beginning necroses (Fig. 9).

After eight days in culture, another cell type predominates. These cells have dark lysosomes, enlarged cisternae of the rough ER and conspicious Golgi apparatus. As in typical chondrocytes glycogen is detectable (Fig. 10a) and the cells phagocytize extracellular material (Fig. 10b). They are longitudinal in shape and show prominent cell extensions. Large pseudopodia filled with mitochondria, glycogen particles and rough ER give the impression of cell migration (Fig. 10c). In the matrix collagen fibres with a thickness of 25–30 nm are arranged parallel to each other; proteoglycan granules are now absent

Fig. 7. Electron microscopic picture of a newly isolated chondrocyte. *N* nucleus, *G* Golgi zone, *E* rough ER. The chondrocyte is completely freed of matrix. ×7,000. bar = 2 μm

Fig. 8a–d. Electron microscopic picture of a chondrocyte after one day in vitro. **a** Chondrocyte with enlarged rough ER (*x*) and Golgi vesicles (*G*), embedded in a filamentous matrix (*M*). ×5,100, bar = 2 μm. **b** At higher magnification collagenous fibres with a thickness of 25 nm (*x*) and proteoglycan granules with a diameter of 15–20 nm (→) can be identified. ×60,000. bar = 250 μm. **c** Necrotic cell, surrounded by a small matrix border (*M*). ×5,300, bar = 2 μm. **d** Polygonal cells grow close to each other without extracellular matrix production. *N* nucleus, *E* rough ER, *L* lysosomes. ×3,800, bar = 5 μm

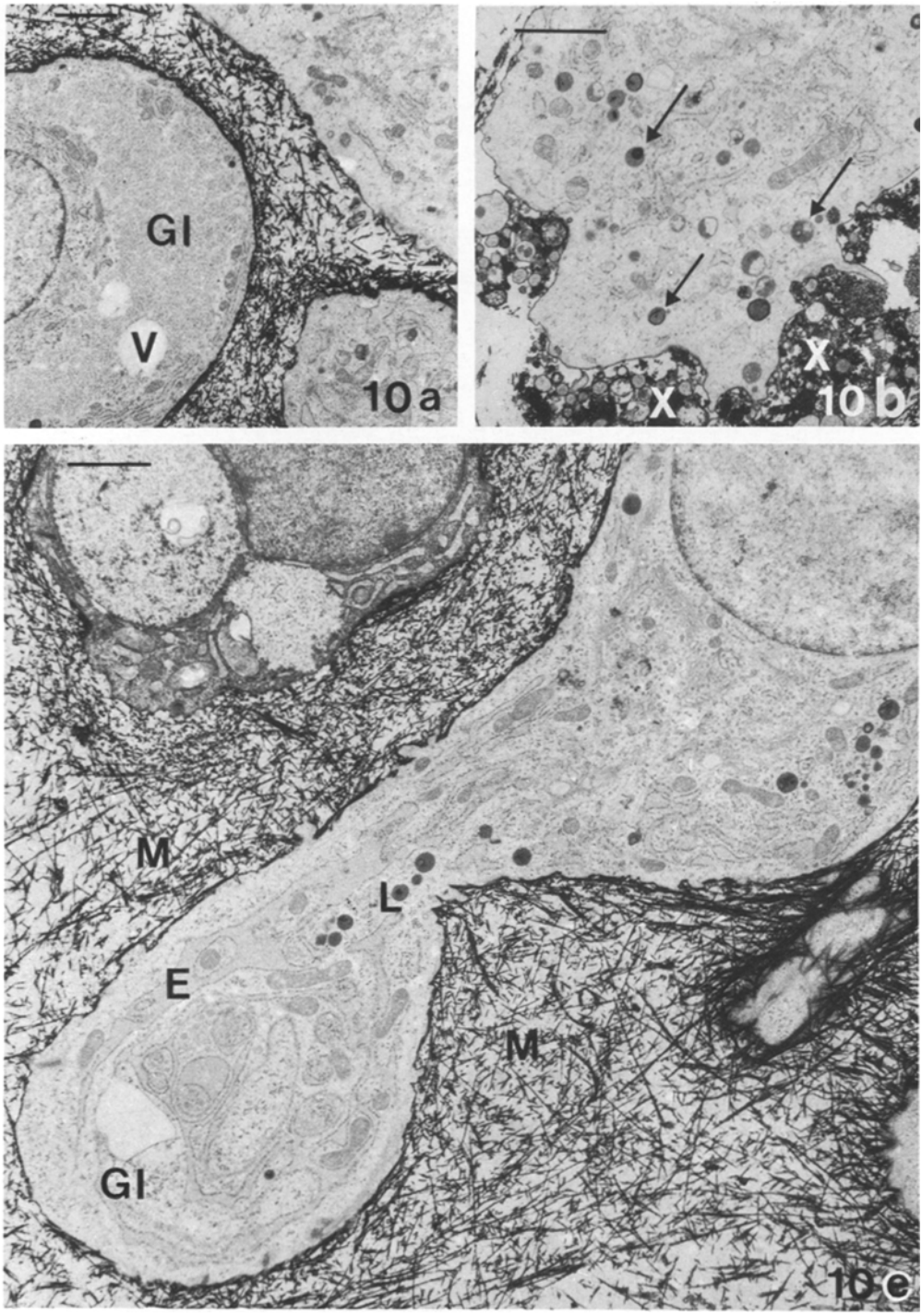

Fig. 10a–e. Chondrocytes after eight days in vitro. **a** The cell shows many glycogen particles (*GL*) and some vacuoles (*V*). ×4,800. bar = 2 μm. **b** Some cells phagocytize cell debris of necrotic cells (*x*) and have numerous phagolysosomes (→). ×6,800, bar = 2 μm. **c** The cell shows the formation of pseudopodia within the filamentous matrix (*M*) as well as enlarged rough ER (*E*), lysosomes (*L*) and glycogen (*GL*). ×6,600, bar = 2 μm. **d** Extracellular collagenous fibres in parallel formation without attached proteoglycan granules are seen. ×60,000, bar = 250 μm. **e** Collagenous filaments in parallel bundles are in direct contact with the cell membrane. The membrane is cut tangentially (*x*). ×60,000, bar = 250 μm

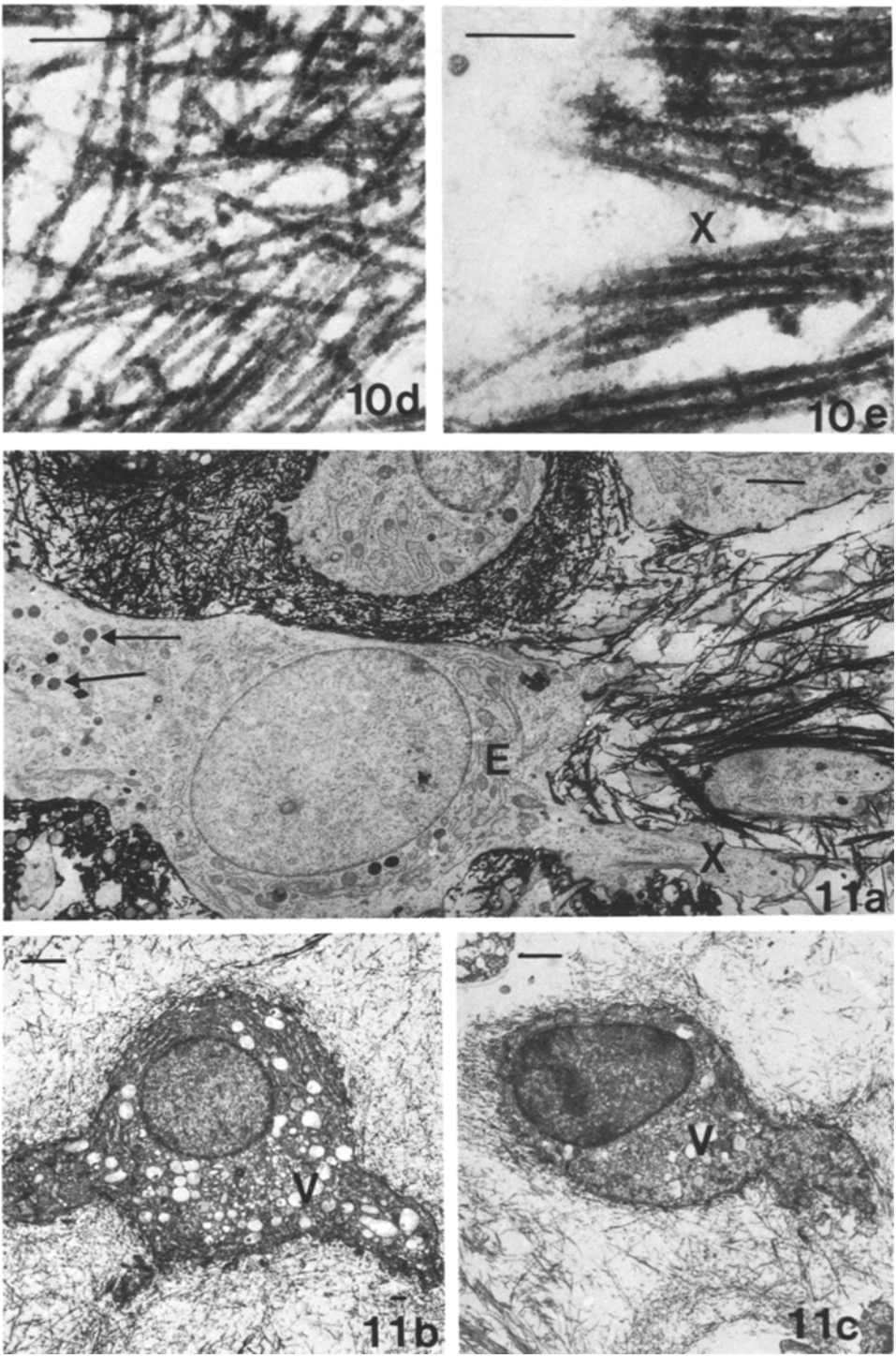

Fig. 11 a–c. Chondrocyte monolayer after twelve days in culture. **a** An elongated fibroblast embedded in filamentous matrix with rough ER (*E*), phagolysosomes (→) and intracellular filaments (*x*) is shown. ×3,900, bar = 2 μm. **b and c** More intensely stained cells can be identified as transitional stages, which contain vacuoles (*V*) and show pseudopodia (**b**) as well as an elongation of cellular form (**c**). These cells correspond to those shown in Fig. 5b and 6a. ×3,200, bar = 2 μm

(Fig. 10d), and collagen bundles sometimes are in contact with the cell membrane (Fig. 10e).

After twelve days, bipolar cells embedded in dense collagen fibres are mainly seen. More strikingly, collagen fibres appear now in parallel arrangement and microfilaments can be shown intracellularly (Fig. 11a). In some regions of the culture we can demonstrate all transitional stages from typical chondrocytes to FLC, e.g., those which look like chondrocytes, but form pseudopodia (Fig. 11b), or those which already show the bipolar fibroblast shape (Fig. 11c).

3. Biosynthesis of Collagen

The immunofluorescent staining method with FITC-conjugated anti-collagen antibodies is used to demonstrate the collagen biosynthesis of chondrocytes in vitro. In vivo chondrocytes produce only type II collagen (Fig. 12), whereas chondrocytes in monolayer culture are described to lose their differentiated state when changing their collagen type. With our experiments we can establish that in mouse chondrocytes an alteration in the biosynthesis of collagen types occurs after four days in culture.

After one day in monolayer culture, chondrocytes have settled down and begin to synthesize procollagen type II (Fig. 13a). A reaction with anti-procollagen type II antibodies can be shown in the cytoplasm and in a small rim outside the cells but not in the region of the nucleus. This result suggests that procollagen type II is present primarily inside but also on the surface of chondrocytes.

The anti-procollagen type II antibodies used are directed against antigenic determinants on the additional peptide extensions at both ends of the proα chain. They do not cross-react with anti-type II collagen antibodies and anti-procollagen type III antibodies, as was tested with the ELISA. The anti-type II collagen antibody fraction, however, is directed against the helical region of the molecule. With these antibodies it is therefore possible to detect the type II collagen as well as the procollagen type II α-helix.

Figure 13b shows the distribution of type II collagen inside and outside of chondrocytes, as expected. A filamentous halo has been formed around the cells, which cannot be seen when stained with anti-procollagen type II antibodies. After four days in monolayer culture, this halo has become an amorphous fibrillar coat with several fibres which connect the chondrocytes and stain intensely with anti-type II collagen antibodies (Fig. 14a). A biosynthesis of procollagen type II is still present (Fig. 14b). After one day in culture, no type I collagen is detectable. Even after four days we do not find any staining of chondrocytes with anti-type I collagen antibodies, but a distinct reaction of some FLC (Fig. 14c). In these early stages of culture (day four), we can also identify chondrocytes which have just begun to synthesize procollagen type III (Fig. 14d). They show a comparable pattern to that seen with anti-type II collagen antibodies at day one of culture (Fig. 13b). It seems evident that these round or polygonal cells are typical chondrocytes in shape, but nevertheless produce type III procollagen.

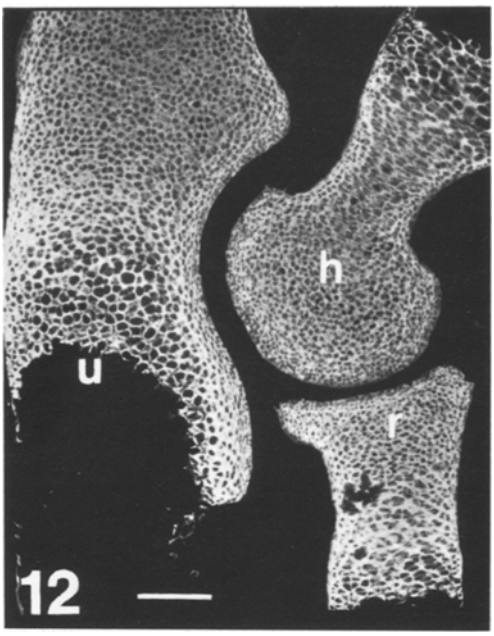

Fig. 12. Section of an elbow joint of a mouse embryo limb at day 16 of gestation. h = humerus, r = radius, u = ulna. Immunofluorescent staining with FITC-conjugated, anti-type II collagen antibodies. ×48, bar = 200 μm

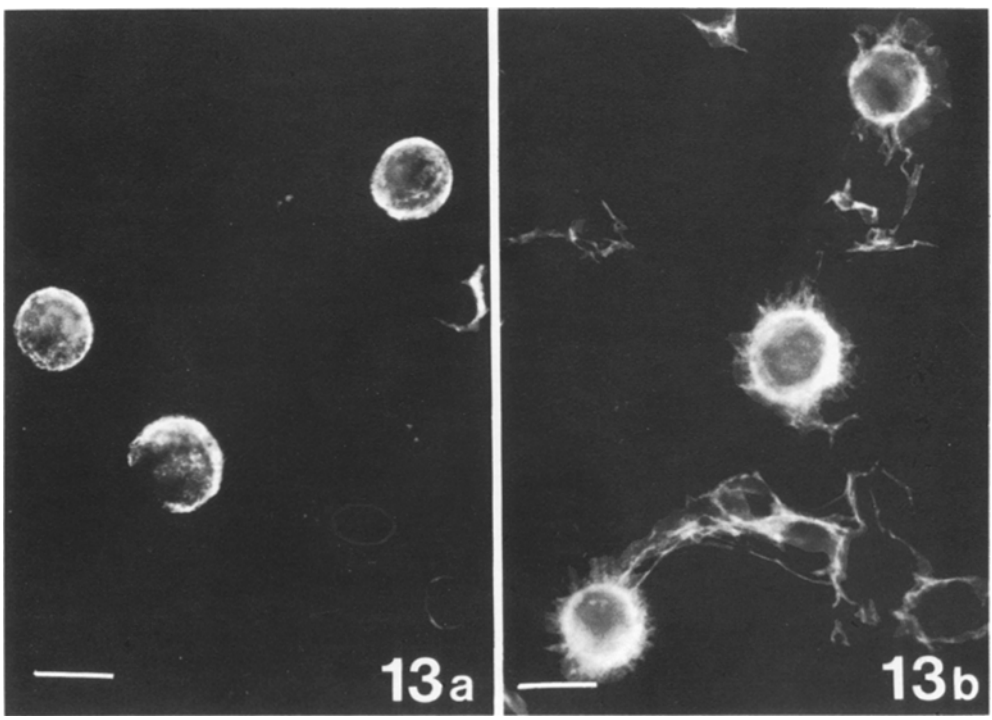

Fig. 13a and b. Immunofluorescent staining of a one-day chondrocyte monolayer culture with FITC-conjugated, anti-procollagen type II antibodies (**a**) and anti-type II collagen antibodies (**b**). ×480, bar = 20 μm. Procollagen type II is found only intracellularly (**a**), whereas with anti-type II collagen antibodies a filamentous matrix halo can be detected (**b**)

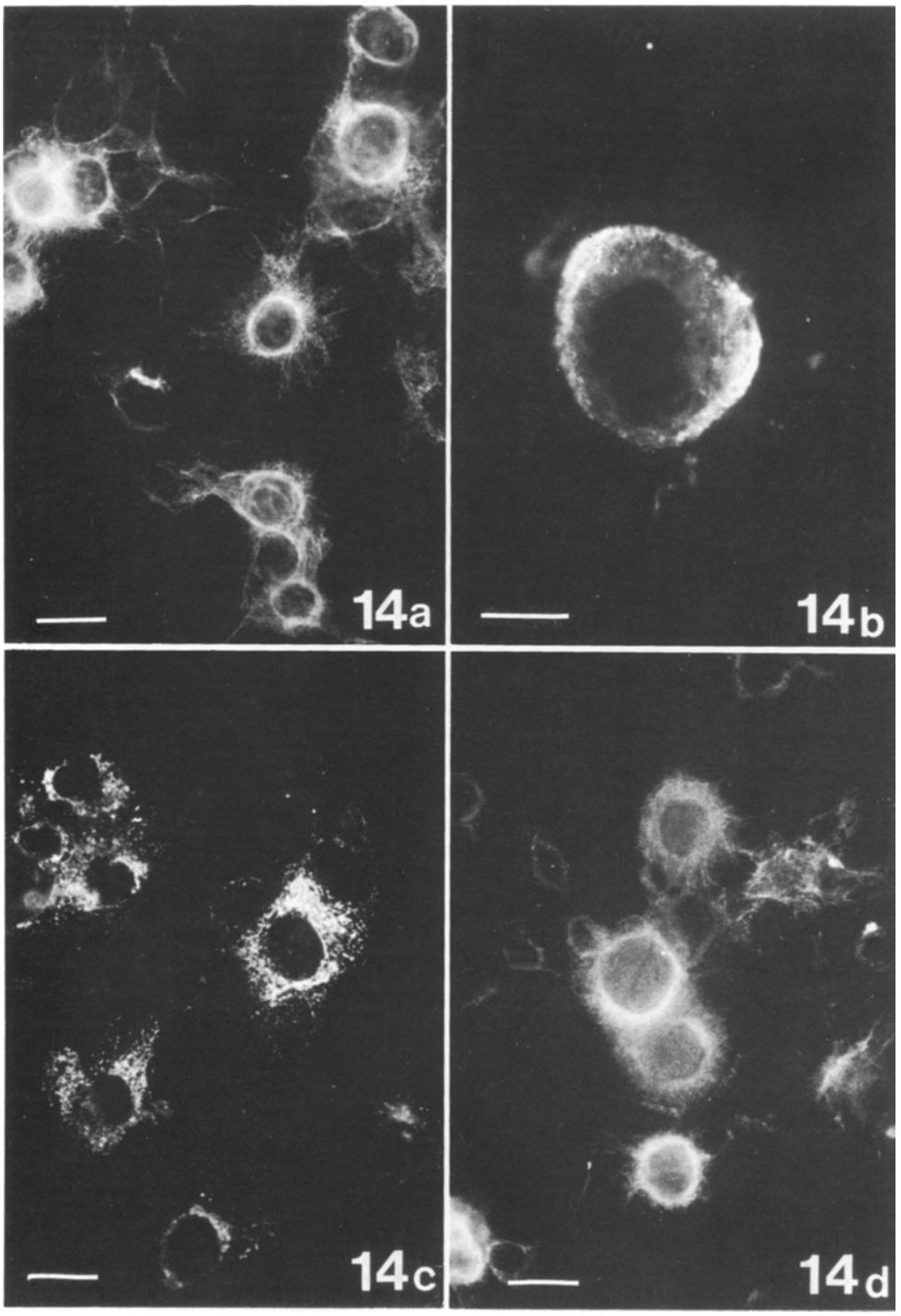

Fig. 14a–d. Immunofluorescent staining of a four-day chondrocyte monolayer culture with FITC-conjugated, anti-type II collagen antibodies (**a**), anti-procollagen type II antibodies (**b**), anti-type I collagen antibodies (**c**) and anti-procollagen type III antibodies (**d**). Cells and matrix stain intensely with anti-type II collagen antibodies (a, ×480) and newly synthesized procollagen type II can be found (b, ×1,200) simultaneously. Type I collagen is present only in fibroblast-like cells (c, ×480), but a weak staining with procollagen type III can be detected in chondrocytes (d, ×480). bars for a, c and d = 20 μm, bar for b = 10 μm

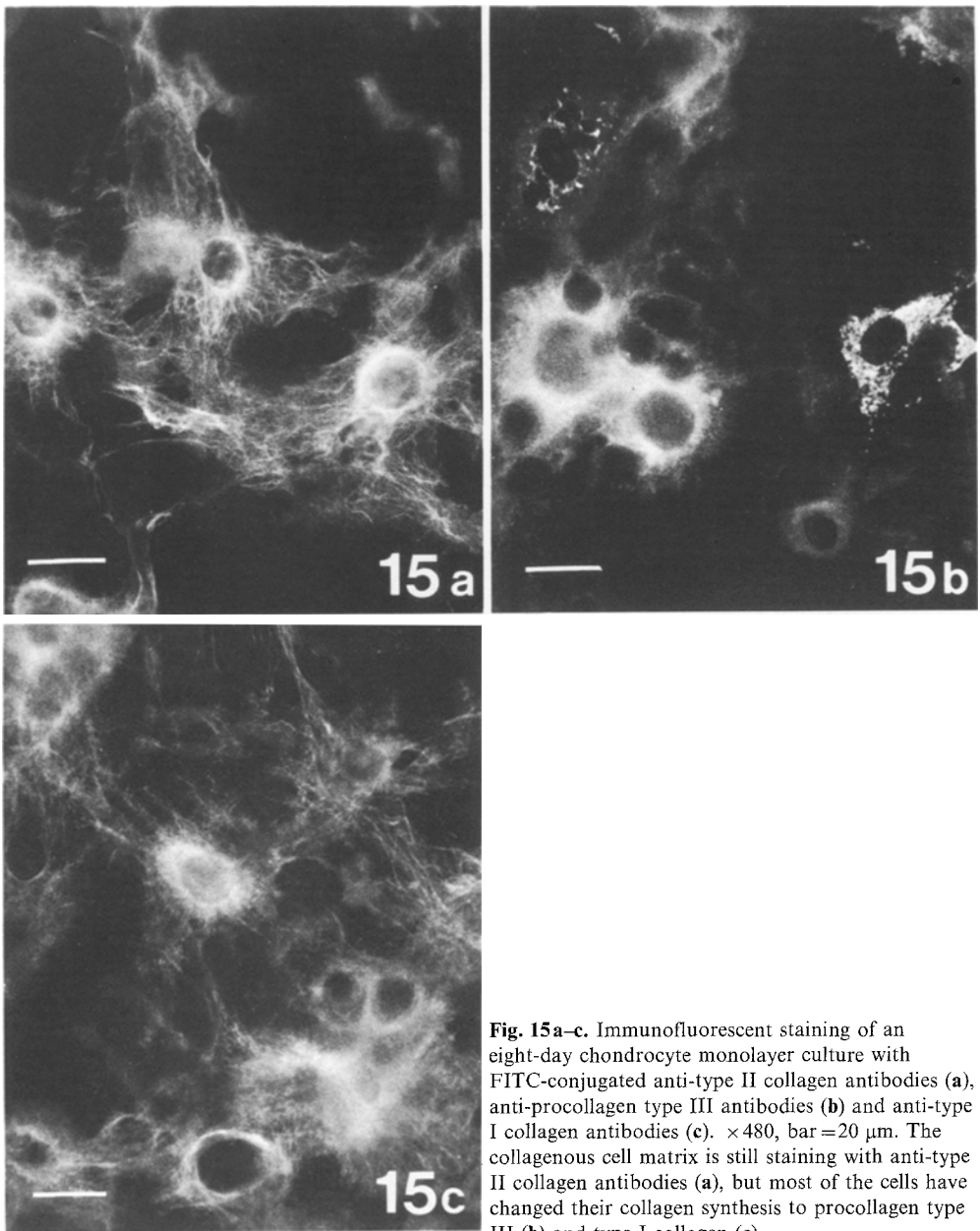

Fig. 15a–c. Immunofluorescent staining of an eight-day chondrocyte monolayer culture with FITC-conjugated anti-type II collagen antibodies (**a**), anti-procollagen type III antibodies (**b**) and anti-type I collagen antibodies (**c**). ×480, bar = 20 μm. The collagenous cell matrix is still staining with anti-type II collagen antibodies (**a**), but most of the cells have changed their collagen synthesis to procollagen type III (**b**) and type I collagen (**c**)

The loss of differentiated state is completed after eight days in monolayer culture. When stained with anti-type II collagen antibodies, the chondrocytes appear embedded in a dense fibrillar matrix (Fig. 15a). At this stage of culture no further type II collagen synthesis can be established; the staining with anti-procollagen type II antibodies is negative. On the other hand, these chondrocytes

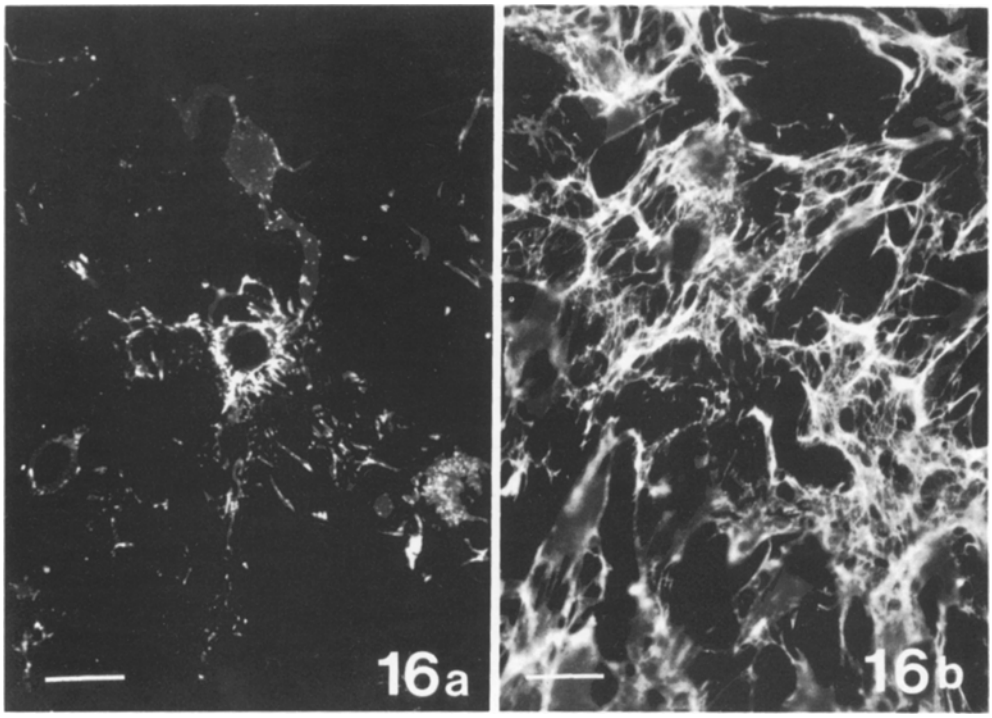

Fig. 16. Immunofluorescent staining of chondrocyte monolayer cultures with FITC-conjugated anti-fibronectin antibodies. ×480, bar = 20 μm. Only a weak reaction with anti-fibronectin antibodies can be identified at day one of culture (**a**), whereas at day eight a close layer of fibronectin is present (**b**)

have increased the production of procollagen type III (Fig. 15b). Referring to cell shape chondrogenic cells can be distinguished from FLC both synthesizing the same collagen type. Moreover, it can be demonstrated that after eight days in culture chondrocytes synthesize type I collagen (Fig. 15c). They are surrounded by a fibrillar network which stains with anti-type I collagen antibodies.

At day twelve of culture only a few round chondrocytes can be detected. The culture has become multilayered and shows a dense population of FLC, which can be stained with anti-procollagen type III antibodies as well as with anti-type I collagen antibodies (not shown).

To compare the immunofluorescence pattern of collagen and fibronectin, mouse chondrocytes have been stained with FITC-conjugated anti-fibronectin antibodies at day one and day eight of culture. As shown in Fig. 16a, in early culture stages only a few fibronectin fibres can be seen in the area surrounding the chondrocytes (Dessau et al., 1978). After eight days a thick multilayer of fibronectin has been formed (Fig. 16b) simultaneously with the increase in FLC.

Discussion

It has not been clearly established to date how dedifferentiation of chondrocytes in vitro takes place. It has been proposed by previous authors that this phenomenon may be the result of the alteration of chondrocytes themselves to fibroblast-like cells (Chacko et al. 1969; Schiltz et al. 1973; Sokoloff 1976) or of the senescence of chondrocytes (Mayne et al. 1976; Moskalewski et al. 1979) under culture conditions. Overgrowth by contaminating fibroblasts may also give the appearance of dedifferentiation (Bryan 1968; Hough and Sokoloff 1975; Norby et al. 1977).

Collagen type I and III is considered to be a good marker of chondrocyte dedifferentiation. The change of collagen types has been demonstrated to occur after some days in culture (Benya et al. 1977; Cheung et al. 1976; Mayne et al. 1976; Müller et al. 1975; Müller et al. 1977; von der Mark et al. 1977). All authors emphasize the importance of working with chondrocyte cultures which are free of fibroblasts, thus, from the alteration of collagen types, they conclude that they are observing the dedifferentiation of mature chondrocytes. However, they neglect the possibility of the existence of a non-homogeneous cell population.

The existence of a heterogeneous chondrocyte population in embryonic cartilage, which consists of proliferating chondroblasts as well as mature chondrocytes and all transitional stages, can easily be shown by histological methods. Various authors have demonstrated (Kim and Conrad 1977; Levenson 1970; Schiltz 1979; Shulman and Meyer 1968) that several chondrocyte cell populations with different behaviours are present in vitro, too.

On the whole the results of our studies with monolayer cultures of epiphyseal mouse chondrocytes are in agreement with those of other authors who have worked with chondrocyte monolayer cultures of different animal species (Coon 1966; Lavietes 1971; Mayne et al. 1976; von der Mark et al. 1977). Under our culture conditions cells derived from mouse epiphyseal cartilage behave like typical chondrocytes. They show the chondrogenic cell shape and produce type II collagen and a metachromatically staining matrix. After four to eight days in culture, they begin to dedifferentiate: they lose their matrix; collagen synthesis is switched first to type III then to type I collagen; and morphologically, they change to FLC. With electron microscopic investigations, we can demonstrate that, in these cells, phagocytosis and intracellular glycogen, occur simultaneously with the alteration of cell shape.

It is a fact that the chondrocyte preparations of embryonic epiphyses consist of mature and hypertrophic chondrocytes as well as immature chondroblasts. Moreover, in spite of light microscopic controls of trypsinized epiphyses, we cannot exclude scarce contaminations with perichondral fibroblasts. Therefore, we postulate that chondrocytes which have synthesized their typical matrix and type II collagen at day one of culture (Figs. 2, 13b) are part of the population of mature chondrocytes. They die after a short culture period and can be detected as necrotic cells (Figs. 8c, 9). Even at day eight and twelve of culture necroses can be seen. We conclude from these late necroses that a subpopulation of cells undergoes all differentiation stages as under in vivo conditions.

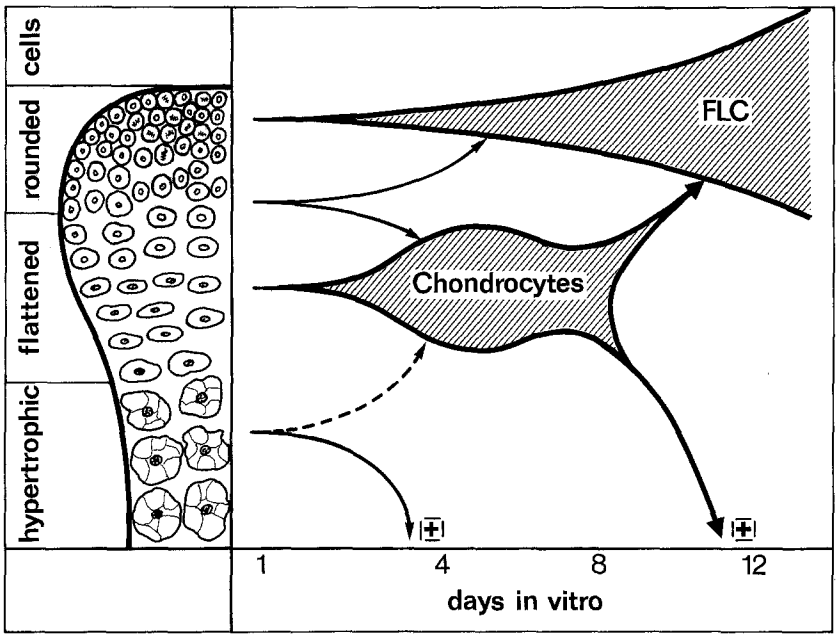

Fig. 17. Behaviour of chondrocytes in monolayer culture in relation to embryonic epiphyseal cartilage in vivo. The schematic diagram on the left side shows heterogeneity of chondrocytes in the epiphyses. Only next to the joint are immature chondroblasts and mitotic figures present. We propose that these cells grow in monolayer culture as fibroblast-like cells (*FLC*) (right side of the diagram). Cells derived from the rounded and flattened cell zone are young chondrocytes and develop in culture to mature chondrocytes, before they dedifferentiate to FLC or die. Cells of the hypertrophic zone are alive and active only for a limited time in culture

Most of the chondrocytes in culture belong to a subpopulation of less mature cells which correspond to the round cells of epiphyseal cartilage. They first appear as polygonal cells and differentiate in vitro to mature chondrocytes which represent the main part of matrix and collagen-producing cells at day four of culture (Figs. 4, 14). These chondrocytes begin to dedifferentiate. Simultaneously with the reduction of matrix (Figs. 5, 6), they show cell elongation, formation of pseudopodia and intracellular filaments (Figs. 10, 11) indicating cell movement. Phagocytosis (Fig. 10c) and the production of type III and type I collagen (Fig. 15) refer to FLC, but they have retained glycogen granules (Fig. 10b, c). We cannot establish the order of events exactly, but it is evident that at first procollagen type III (day four) and later type I collagen (day eight) occur, whereas the type II collagen synthesis is stopped.

Cartilage differentiation in vivo is terminated by calcification which begins with the appearance of alkaline phosphatase (Cabrini 1961; Pritchard 1952). As indirect proof of the transformation of chondrocytes themselves, we can show the activity of alkaline phosphatase first only in chondrocyte plaques, but later on in FLC, which are embedded in the remaining matrix (Zimmermann, unpublished).

Immature chondroblasts represent a third subpopulation of our chondrocyte preparation. It seems unlikely that these cells are able to differentiate into

chondrocytes in monolayer culture; however, they appear as FLC. These interconnections are explained in Fig. 17.

Recent experiments of Pennypacker et al. (1979) and West et al. (1979) show that fibronectin seems to play a key role in the dedifferentiation of chondrocytes. The addition of fibronectin to chondrocyte cultures leads to a fibroblast-like phenotype of the cells. When cultured on collagen substrate, the fibronectin concentration in 10% fetal calf serum is shown to be sufficient to induce this change (Pennypacker et al. 1979). In our chondrocyte population, with a time-dependent increase in the number of FLC, an increase of fibronectin production takes place after some time in culture, as is demonstrated in Fig. 16b. It seems possible that the fibronectin, which is produced additionally to the fibronectin content in the medium, could initiate an accelerated dedifferentiation of chondrocytes in vitro.

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