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Integrin expression on epiphyseal mouse chondrocytes in monolayer culture

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Summary. The expression of α1-, α3-, αv- and α5ß1-integrins and their specific ligand binding were investigated in monolayer cultures of chondrocytes from 17-day-old mouse embryos using morphological and immunomorphological methods. After a 1-day culture period αv-, α3- and α5ß1-integrins were observed on these cells. Immunoelectronmicroscopic investigation revealed localization predominantly in the contact areas with extracellular structures on the cell surface. α1-integrin could not be demonstrated on chondrocytes. After a 5-day culture period the number of fibroblast-like cells with α1-, αv- and α5ß1-integrins had increased. α3-integrin was hardly recognizable on these cells. Collagen type I and fibronectin could be shown as ligands on the cell surface. The number of chondrocytes with collagen type II on their surface continuously decreased. α3-integrin is obviously responsible for the binding of collagen type II, and α1-integrin for the binding of collagen type I. Therefore, it can be concluded that the changes of chondrocytes to fibroblast-like cells in monolayer culture are accompanied by changes of integrin genes.

Key words: Chondrocytes, Integrin expression, Monolayer culture, Immunoelectronmicroscopy, Dedifferentiation,

Introduction

The extracellular matrix in cartilage plays an essential role in the behaviour, differentiation and proliferation of chondrocytes (Kosher et al., 1973; Kosher and Church, 1975; Hewitt et al., 1982; Sommarin et al., 1989; Ramachandrula et al., 1992). Numerous cell adhesion molecules that mediate these interactions have in the meantime been discovered. They belong to the large integrin family (Hynes, 1987; Hemler, 1990; Ruoslahti, 1991). Being receptors, integrins bind matrix components, such as collagen, fibronectin, laminin, vitronectin and fibrinogen (Hynes, 1987, 1992; Ruoslahti and Pierschbacher, 1987; Hemler, 1990; Ruoslahti, 1991). Integrins are heterodimer adhesion molecules which consist of α- and ß-subunits. These subunits are linked with one another by non-covalent binding. Both subunits extend through the lipid double layer of the cell membrane; in other words, they have three domains, a short intracellular, a trans-membranous and a long extracellular domain which allows a close interaction between extracellular components and cytoskeleton during cell adhesion and cell migration (Hynes, 1987, 1992; Albelda and Buck, 1990; Hemler, 1990; Ruoslahti, 1991).

It is known that many integrins recognize and bind ligands via an RGD-sequence (Ruoslahti and Pierschbacher, 1987). For this purpose they must have both subunits, an α- and a ß-chain, and a binding site for divalent cations. So far, at least 8 different ß-and 14 α-subunits are known, each ß-subunit being able to bind any of these α-subunits (Cheresch et al., 1989; Hynes, 1992). Integrins α1ß1 and α2ß1 are known to be collagen and laminin receptors (Wayner and Carter, 1987; Languino et al., 1989; Defillippi et al., 1991; Albelda, 1993), integrin α3ß1 laminin, collagen and fibronectin receptors (Wayner et al., 1988), integrin α5ß1 fibronectin receptor (Argraves et al., 1987) and integrin α6ß1 laminin receptor (Sonnenberg et al., 1988).

Several authors have reported on proliferation, differentiation and dedifferentiation of chondrocytes in monolayer culture. After 4 to 8 days in monolayer culture the direction of differentiation of chondrocyte changes, which is expressed morphologically (the round or polygonal cells are changed to bipolar cells with stretching and formation of processes) as well as in a change of the synthesis programme (collagen type II not present in contrast to collagen types I and III, change in the proteoglycan pattern) (Abbott and Holtzer, 1966; Chacko et al., 1969; Horwitz and Dorfman, 1970; Lavietes, 1971; Layman et al., 1972; Benya et al., 1977; von der Mark et al., 1977; Grundmann et al., 1980; Merker et al., 1980).

According to Salter et al. (1992), Dürr et al. (1993),

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Loeser (1993) and Enomoto et al. (1993) ß1-integrins are involved in the binding of collagen type II of chondrocytes in vivo as well as in vitro. However, it is not exactly known which α-chain is involved in the binding of collagen type II on chondrocytes.

Morphological as well as immunomorphological means were applied to demonstrate the time-dependence of the transformation of chondrocytes to fibroblast-like cells and, at the same time, the change in the expression pattern of integrins and in matrix components. These methods also served to show which ligands preferentially bound to which integrins during this alteration in monolayer culture.

Materials and methods

Materials

1. Limb buds from 17-day-old mouse embryos (strain NMRI).

2. Antibodies:

a) Monoclonal mouse antibody P1B6 against α3 and VNR 147 against αv (Telios Pharmaceuticals, Inc., USA). Wayner et al. (1988) and Freed et al. (1989) have reported on the specificity of these antibodies.

b) The polyclonal antibody CPHBO1 against α5ß1 (Telios Pharmaceuticals, Inc., USA). Argraves et al. (1987) have reported on the specificity of this antibody. The polyclonal antibody against α1 was a kind gift from Prof. Reutter (Institute of Molecular Biology and Biochemistry, Free University of Berlin). This specificity of this antibody was tested using the ELISA technique (Löster et al., 1994).

c) The polyclonal antibody against collagen types I and II and fibronectin was produced in the rabbit. The specificity of this antibody was tested using the ELISA technique (Gosslau and Barrach, 1979).

d) GAR- and GAM-FITC: conjugated goat-anti-rabbit and -mouse immunoglobulin were purchased from Dianova (Hamburg, FRG).

e) GAR-10 nm and GAM-10 nm conjugated goat-anti-rabbit and goat-anti-mouse immunoglobulin with 10 nm gold particles were purchased from Amersham (Braunschweig, FRG).

3. Ham F12 nutrient mixture containing 1% FCS, 75 µg/ml ascorbic acid, 50 IU/ml streptomycin, 50 IU/ml penicillin and 2.5 µg/ml amphotericin B (Seromed, München, FRG).

4. Nuncleon dishes: 35 mm x 10 mm (Nunc Inter Med, Denmark); Epon, LR-White and thermanox (Plano, Marburg, FRG), Fluoromount mountant (BDH, England), HMDS: hexamethyl-disilazane (Sigma, München, FRG), trypsin (EG 3.4.21.4., Sigma, München, FRG), hyaluronidase (Serva, Heidelberg, FRG), collagenase (from *Clostridium histolyticum*, 0.15 IU/mg, Boehringer Mannheim, FRG), silver enhancement (IntenSE, Amersham, Braunschweig, FRG).

5. Several other chemicals (pure grade).

Methods

1. Chondrocyte cell culture.

Mouse embryos were removed from the uterus on day 17 of gestation (day 0=day of conception) and placed into Hank's balanced Salt Solution (HBSS). The upper and lower limb buds were dissected, hands and skin removed and the remaining parts rinsed in HBSS. Muscles and connective tissue of long bones were removed by trypsin treatment (twice, 0.2%) and by shaking in Ca^{2+}- + Mg^{2+}- free solution for 30 min at 37 °C. After inactivation of trypsin by addition of foetal calf serum (FCS) the bones were washed several times with HBSS. Epiphyseal cartilage was removed from the bone and shaken in 0.2% collagenase in HBSS for 60 min at 37 °C. After addition of medium the suspension was homogenized by pipetting and freed from non-dissociated tissue fragments by filtration through a nylon net. After centrifugation the cells were washed twice in HAM F12 growth medium.

The cells were grown for immunofluorescence microscopic investigations on glass plates, for scanning electron microscopic investigations on Thermanox plates and for transmission electron microscopic investigations in Petri dishes in monolayer culture. The cells were counted and their number was adjusted to 1.5×10^6/ml.

The cultures were grown in an incubator at 37 °C in 95% air and 5% CO_2. The cells were removed after 3 hrs, and 1, 2, 3, 4 and 5 days in culture. After appropriate fixation, they were investigated by light, electron and immunoelectron microscopy.

2. Immunofluorescence.

Cells were fixed with 3% paraformaldehyde, 0.25% glutaraldehyde in PBS for 5 min and treated whith collagenase (5000 U/ml) to remove the newly-produced matrix as prerequisite for the immunolabelling of the integrins, and with hyaluronidase (5000 U/ml) to unmask collagenous fibrils as prerequisite for the immunolabelling of collagen type II for 15 min at room temperature (RT). After washing, the cells were incubated with the primary antibody (anti-collagen type II 1:10; anti-α1 and anti-α5ß1 1:30 anti-αv and anti-α3 1:20 in PBS/1% BSA) in a moist chamber overnight at 4 °C. After washing, the samples were incubated with the secondary antibody (GAR-FITC 1:30 and GAM-FITC 1:30 in PBS/BSA) for 1 hr at RT. They were washed with aqua bidest. for 30 min, dried and covered with Fluoromount mountant and the samples inspected under a Zeiss Axiophot 100 light microscope.

3. Transmission electron microscopy (TEM).

The cultures were fixed in 1% glutaraldehyde and 1% tannic acid in 0.1M phosphate buffer, pH 7.4.

Table 1. Chodrocytes and fibroblasts proportions during days 1, 3 and 5. The experiments were repeated four times.

	EXPERIMENT 1		EXPERIMENT 2		EXPERIMENT 3		EXPERIMENT 4	
	C	F	C	F	C	F	C	F
1st Day	73%	27%	80%	20%	79%	21%	74%	26%
3rd Day	85%	15%	84%	16%	82%	18%	84%	16%
5th Day	11%	89%	9%	91%	15%	85%	8%	92%

Subsequently, they were post-fixed in a 2% OsO_4 solution. After dehydration in the ascending alcohol series, the specimens were embedded in Epon. Ultathin sections were contrasted with 2% uranyl acetate and lead citrate and investigated under a Zeiss EM10 transmission electron microscope.

4. Immunoelectron microscopy.

4a. Pre-embedding technique for TEM

The chondrocyte culture was immunolabelled as follows:

The cells were treated with hyaluronidase (5000 U/ml) or in combination with collagenase (5000 U/ml) for 15 min at RT. After washing, the cells were incubated in medium with primary antibodies (collagen type II 1:10; fibronectin 1:10 and integrins α1 and α5ß1 1:30; αv and α3 1:20 in PBS/BSA) for 5 min at RT. The cells were washed and then incubated with the secondary antibody (GAR-10 nm 1:30, GAM-10 nm 1:30) for 5 min at RT. Subsequently, they were fixed with 2% glutaraldehyde for 15 min. Embedding and microscopic investigation were performed as described above.

4b. Post-embedding technique for TEM

The cells were washed after fixation (3% paraformaldehyde, 0.25% glutaraldehyde in PBS, 1hr) and dehdydrated in ethanol. This was followed by embedding in LR-White (London Resin). Sections were cut on an Ultracut E (Reichert). The LR-White ultrathin sections were immunolabelled as follows: incubation with hyaluronidase (5000 U/ml) for 30 min; washing and blockage with PBS/1% BSA/Tween for 3 x 10 min; incubation with the primary antibodies (anti-collagen I, II and fibronectin 1:10; anti-integrins α1 and α5ß1 1:30, α3 and αv 1:20 in PBS/BSA) overnight at 4 °C; washing with PBS/BSA/Tween for 3x10 min; incubation with the secondary antibody (GAR-10 nm/GAM-10 nm 1:30) for 60 min at RT; washing with PBS for 3 x 5 min; contrasting with 1% tannic acid for 10 min and uranyl acetate for 15 min; and washing with aqua bidest. The preparations were investigated using a Zeiss EM10.

5. Scanning electron microscopy

5a. Coverslips with attached chondrocytes were fixed with 1% glutaraldehyde for 5 min, treated with collagenase, dehydrated with ethanol, air-dryed with HMDS, gold-coated with a Technics Hummer V, and examined with a Cambridge stereoscan microscope.

5b. Part of the specimens was immunolabelled with anti-αv, -α3-, α1- and α5ß1-integrins. Immunolabelling was performed as in the case of pre-embedding for TEM. After addition of the secondary antibody, the gold particles were silver-enhanced for 20 min. Subsequently, the samples were washed, fixed with 1% glutaraldehyde for 5 min and processed as described above.

Results

1. Light microscopy

After a 3-hr culture period of chondrocytes from 17 day-old mouse embryos, numerous cells had already adhered in the form of a monolayer. During the first 4 days, the number of the cells had increased, a matrix become perceptible. After a 5 day culture period the flat fibroblast-like cells, often of bipolar shape, increased in number at the cost of the chondrocytes. However, some fibroblast-like cells could always be demonstrated from the beginning of cultivation onwards.

2. Immunofluorescence

Immunomorphological investigations during the first 4 days of the culture period revealed a diffuse distribution of the surface receptors α3, α5ß1 (Fig. 1e,f), αv (not shown) and collagen type II (Fig. 1d) on chondrocytes. The round to oval chondrocytes could not be immunolabelled with anti-α1-integrin. At the same time, integrin α1 could be shown on the few fibroblast-like cells present (Fig. 1a,b). Integrin α3 was only weakly expressed on culture, which now consisted predominantly of fibroblast-like cells, integrin α1 (Fig. 1c), α5ß1 and αv (not shown) could be demonstrated on the surface of these cells.

3. Scanning electron microscopy

3a. After 1, 2, 3, 4 and 5 days in culture the chondrocytes exhibited a round to oval shape and numerous small cuspidal processes of the surface (Fig. 2b). After a culture period of 2 days, scanning electron microscopy revealed the formation of collagen fibrils. After 3 days the chondrocytes were embedded in a network of collagenous fibrils. Therefore, their surface was recognizable in certain areas only (Fig. 2a). During the first 4 days of the culture period more than 70% of the cells were chondrocytes (Fig. 2d,e); on day 5 more than 80% of the cells resembled fibroblasts (Fig. 2f). These

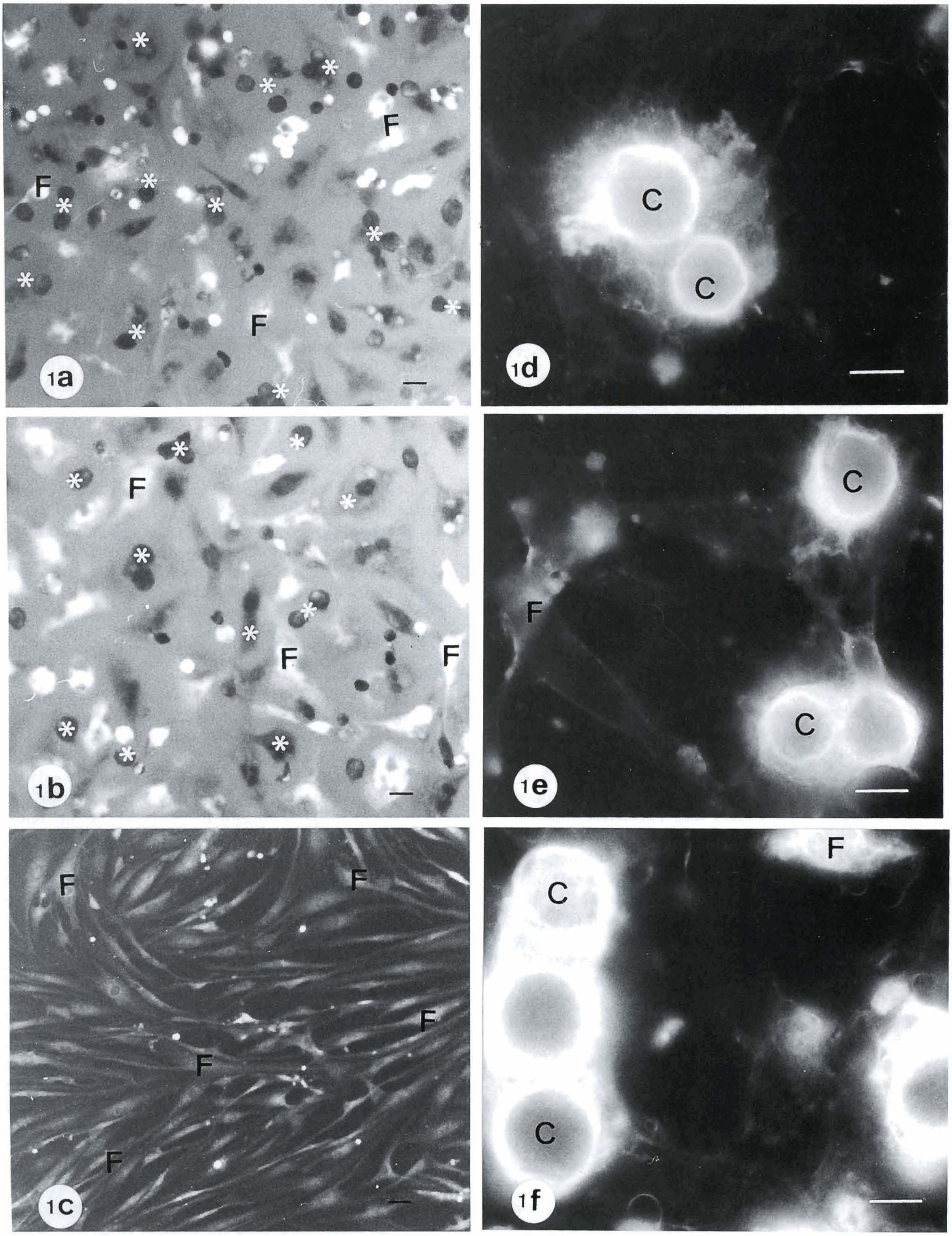
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Fig. 1. Immunofluorescence microscopic investigations of chondrocytes in monolayer culture. **a, b, c.** Immunofluorescence localization of integrins in epiphyseal chondrocytes in monolayer culture. a, b. After a 1- to 3-day culture period only the round to oval chondrocytes (*) are not yet immunolabelled with α1-integrin. In contrast, some bipolar, fibroblast-like cells (F) are positively labelled. x 200; bar= 20 μm. c. After a 5-day culture period only fibroblast-like cells (F) are observed which can be labelled with α1-integrin. x 200. bar= 20 μm. **d, e, f.** Immunofluorescence labelling of chondrocytes after 2 days cultivation with anti-collagen type II antibodies (d), anti- α3-integrin antibodies (e) and anti-α5ß1-integrin antibodies (f). x 480; bar= 20 μm. Collagen type II was observed directly around chondrocytes (C); integrin α3 was located directly on chondrocytes (C) and weakly expressed on fibroblast-like cells (F), and α5ß1-integrin was located on chondrocytes as well as fibroblast-like cells.

experiments were repeated four times (Table 1).

3b. For the immunolabelling of the surface receptors for the scanning electron microscopic investigation the chondrocytes were first treated with collagenase. Subsequently, the cells were immunolabelled against integrins α1, α3, αv and α5ß1. In some experiments the cells were scratched off the plates using a pair of tweezers before imunolabelling was carried out. The surface of the chondrocytes showed positive labelling of αv-, α3- and α5ß1-integrins. Those areas where the cells had been scratched off the plates showed diffuse positive immunolabelling of the integrins on the adhesion area (Fig. 2c).

4. Transmission electron microscopy

4a. After 1 day in culture the cartilage cells had assumed a round to oval shape and contained a large nucleus and numerous cell organelles, such as a well-developed rough endoplasmic reticulum and a large Golgi apparatus. Their surface showed small cuspidal processes. After a 2-day culture period a thin matrix rim consisting of fine irregularly-running filaments could be observed extracellularly on the surface (Fig. 3a,b). After 3 days, the cells were embedded in a matrix of typical collagenous fibrils (18-22 nm). The amount of these extracellular structures increased further during the next few days. On day 5 we observed fibroblast-like cells, the surface of which showed thicker (20-28 nm), mainly bundled fibrils (Fig. 4a). Some chondroblasts were still recognizable showing thin, isolated and irregulary-running collagenous fibrils.

4b. Immunomorphological investigations using the pre-embedding technique with secondary gold-conjugated antibodies against primary collagen and fibronectin antibodies showed that chondrocytes were surrounded by collagen type II and fibronectin which was located between collagenous fibrils (Fig. 3c,d). This could already be observed after a 2-day culture period. Collagen type I could not be demonstrated in the cartilage matrix by immunomorphology. Integrins αv (not shown), α3 and α5ß1 were observed on the surface of chondrocytes (Fig. 3e,f). To demonstrate integrins on the cell surface with the above-mentioned technique, we tried to remove collagenous material using collagenase before immunolabelling of the integrins. For the demonstration of the integrins in the presence of ligands we employed the post-embedding technique. Immuno-labelling against collagen type I was detected on day 5 in the region of the thick fibrils on the surface of fibroblast-like cells (Fig. 4b). Integrin α1 was especially pronounced in the contact area between ligand and cell membrane (Fig. 4c). Integrins α5ß1 and αv were also seen in fibroblast-like cells (Fig. 4d,e).

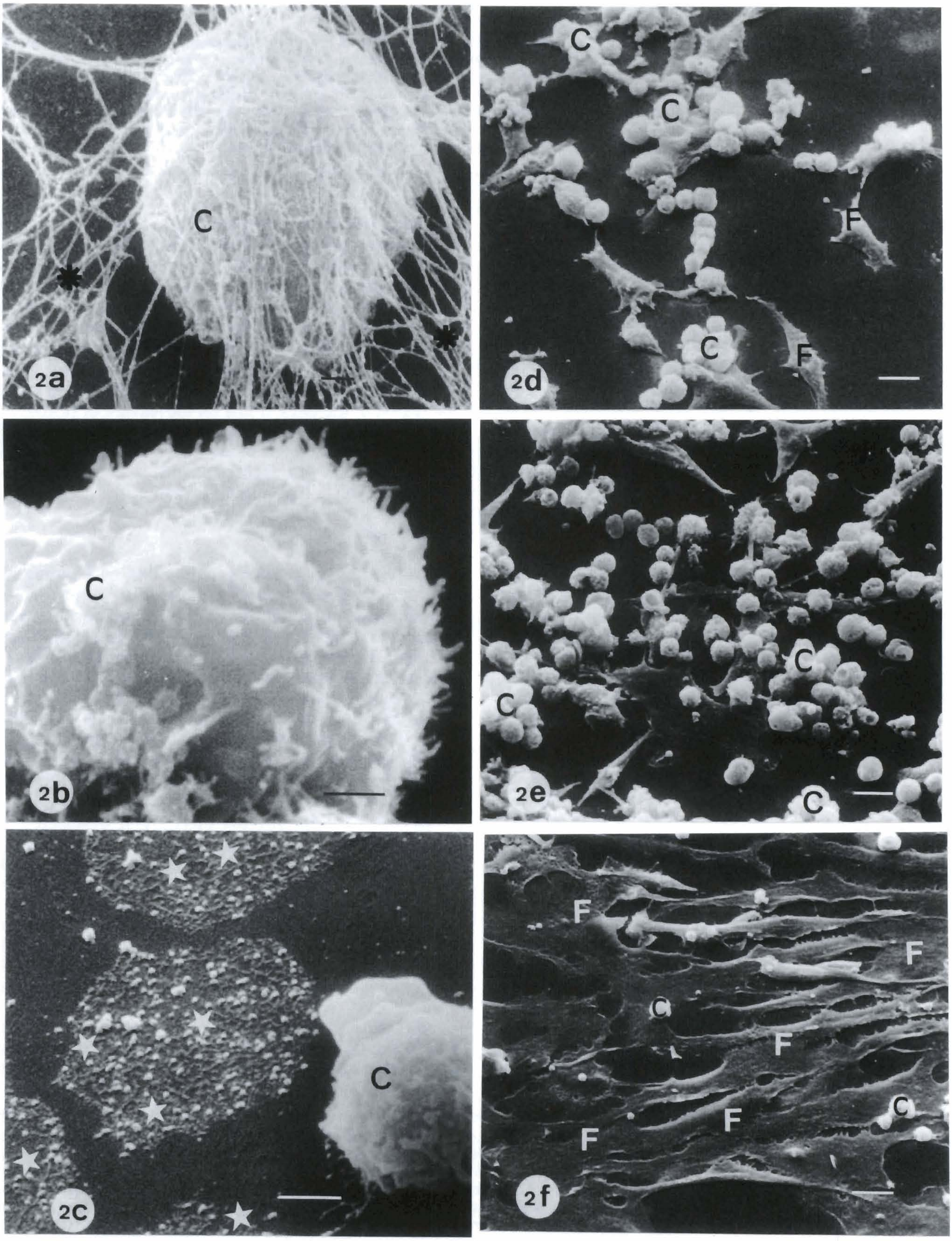
Discussion

This study describes the expression of ß1-integrins and their specific ligand binding on the surface of chondrocytes in monolayer culture. Chondrocytes were isolated from limb buds of 17-day-old mouse embryos. A large amount of the cultured cells formed collagen type II and fibronectin; the morphology of some cells corresponded to that of fibroblast-like cells. The surface of the chondrocytes showed integrin αv, α3 and α5ß1. After 5 days in culture many of these chondrocytes dedifferentiated into fibroblast-like cells. These cells bound to collagen type I and fibronectin and additionally exhibited integrin α1 on their surface.

Different mechanisms have been discussed as responsible for the dedifferentiation of chondrocytes in monolayer culture: (1) loosening of the connection between chondrocytes and matrix leading to de-differentiation (Merker et al., 1978; Grundmann et al., 1980; Shakibaei et al., 1993a); (2) cell maturation (Chacko et al., 1969; Sokoloff, 1976); (3) ageing of cells (Mayne et al., 1976; Moskalewski et al., 1979); and (4) contamination and overgrowth by fibroblasts (Norby et al., 1977).

We cannot decide as yet which of these four mechanisms is responsible for the dedifferentiation observed in our culture system. We only know that there

Fig. 2. Scanning electron microscopic investigations of chondrocytes after 1, 3 and 4 days in monolayer culture. **a.** A chondrocyte (C) of typical round to oval shape after 3 days in culture is embedded in a network of collagenous fibrils (*). x 3,000; bar= 2 μm. **b.** A round chondrocyte after collagenase treatment; its surface without collagenous fibrils; numerous small cuspidal surface processes. x 11,000; bar= 1 μm. **c.** Part of a chondrocyte was scratched off the plate and immunolabelled with anti-α5ß1-integrin. The adhesion area (*) of the removed chondrocytes shows positive immunolabelling against α5ß1-integrin. x 5,500; bar= 2 μm. **d, e, f.** Chondrocyte after 1, 3 and 5 days in monolayer culture. After 1 day (d) chondrocytes (c) can be distinguished from fibroblast-like cells (F). After 3 days (e) the cells have increased in number and density. After a 5 day (f) culture period fibroblast-like cells (F) of bipolar shape increase in number. The number of chondrocytes is also increased. x 750; bar= 10 μm.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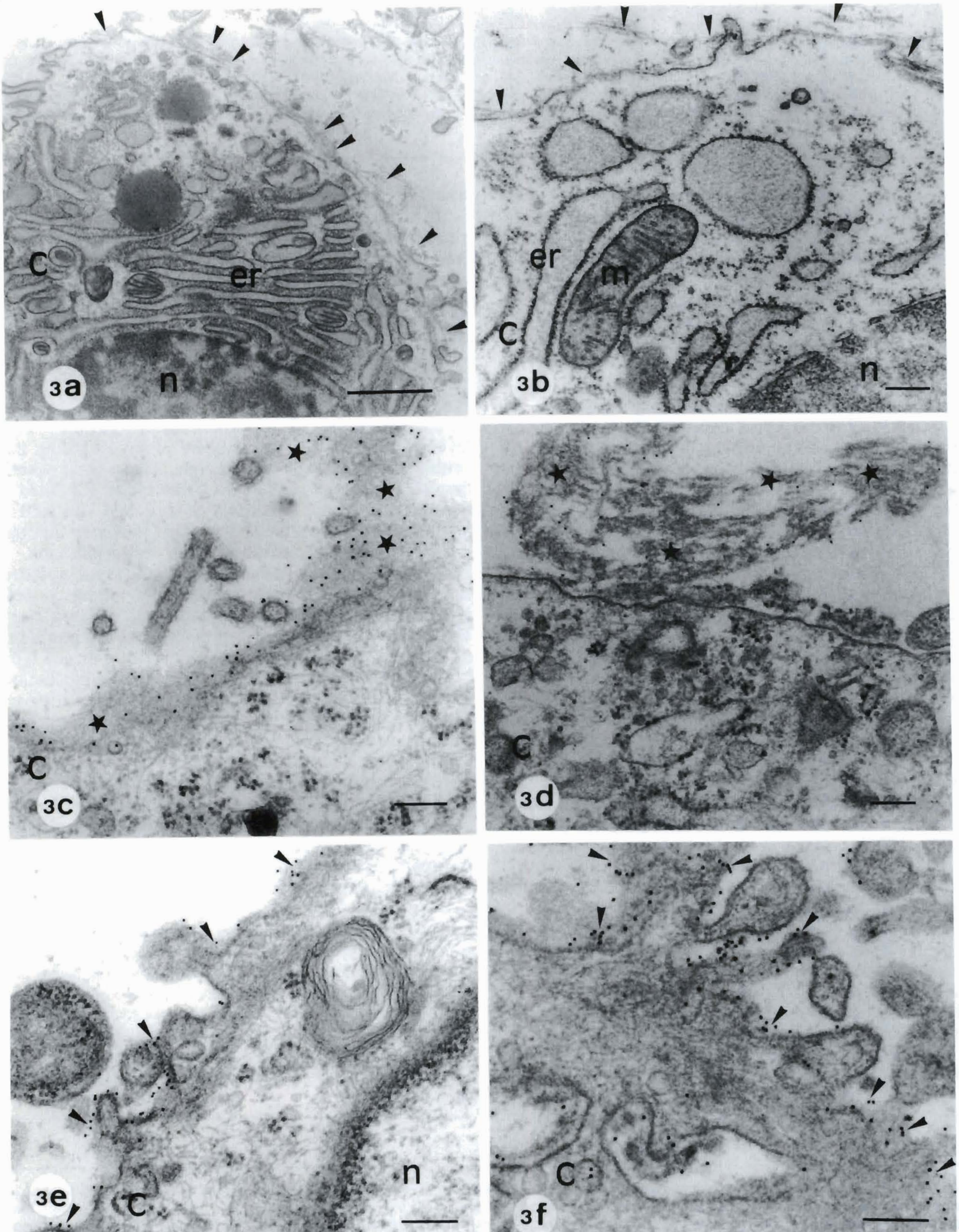
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Fig. 3. Transmission and immunotransmission electron microscopic investigations of chondrocytes after 2 days in monolayer culture. **a, b.** After 2 days in culture the chondrocytes (C) exhibit a round to oval shape. A matrix develops on their surface (arrowheads). n: nucleus, m: mitochondrium, er: endoplasmic reticulum. a, x 15,000; bar = 1 μm; b, x 40,000; bar= 0.2 μm. **c, d.** Immunolabelling against collagen type II (c) and fibronectin (d) in the formed matrix (*) shows that these are chondrocytes. c, x 50,000; bar= 0.2 μm; d, x 40,000; bar= 0.2 μm. **e, f:** Immunolabelling of chondrocytes against integrins α3 (e) and α5ß1 (f). Dense labelling on the cell surface of the two integrin types (arrowheads). e, x 50,000; bar= 0.2 μm; f, x 60,000; bar= 0.2 μm.

are two cell populations: one cell type which does not express α1 but clearly expresses α3 (chondroblasts); and another cell type which expresses α1 but only little α3 (fibroblasts). After 5 days this second type of fibroblasts had increased considerably. It remains to be the elucidated whether this shift is brought about by the fact that there is a transformation from chondroblasts to fibroblasts or whether fibroblasts are overgrown. Findings of previous authors (Abbott and Holtzer, 1966; Horwitz and Dorfman, 1970; Grundmann et al., 1980; Shakibaei et al., 1993a) appear to indicate a transformation instead of an overgrowth. In this case dedifferentiation is accompanied by a changed matrix binding due to the occurrence of other matrix components and a changed integrin expression.

Immunohistochemical investigations of human cartilage (Salter et al., 1992; Dürr et al., 1993) and chondrocytes from chick embryos (Enomoto et al., 1993) have demonstrated the presence of ß1-integrins. The present immunohistochemical as well as immunoelectron microscopic investigations have shown that ß1-integrins (αv, α3, and α5ß1) can be expressed in monolayer cultures of chondrocytes from mouse embryos. The formed cells express integrin α1 only after dedifferentiation of chondrocytes in culture, suggesting that this ß1 integrin subunit (α1) is responsible for the binding of newly-formed collagen type I of fibroblast-like cells in vitro. In accordance with these observations several authors have reported that integrin α1ß1 is responsible for the binding of collagen and laminin (Ruoslahti, 1991; Defilippi et al., 1991; Hynes, 1992; Albelda, 1993). After a 2- to 3-weeks culture period (mass culture) of limb bud cells from 12-day-old mouse embryos collagen type I occurs in increasing amounts on the surface of chondrocytes (Schröter-Kermani et al., 1991; Shakibaei et al., 1993a). At the same time new receptors of the integrin α1ß1 and α2ß1 types are expressed which bind collagen type I on the cell surface(Shakibaei et al., 1993b).

Integrin α5ß1 is a widespread receptor. It occurs in numerous cell types, e.g. on epithelial cells, leukocytes and fibroblasts. It is known that this integrin type only binds fibronectin (Argraves et al., 1987; Hemler et al., 1987; Adams and Watt, 1990). The appearance of fibronectin receptors on the surface of chondrocytes seems to be important for the behaviour of these cells. Fibronectin plays an important role in normal and pathological conditions of cartilage and in the dedifferentiation of chondrocytes to fibroblast-like cells in culture. Under pathological conditions an increase in fibronectin is often observed (Pennypacker et al., 1979; West et al., 1979; Grundmann et al., 1980; Labat-Robert, 1986).

Immunolabelling of chondrocytes for scanning electron microscopy has shown that integrins α3, αv and α5ß1 are expressed on the cell surface. This technique and the cultivation of chondrocytes in monolayer culture offer the possibility of investigating the adhesion area of cells on integrins. Those areas where the cells had been scratched off the plates exhibited a massive and dense labelling of αv-, α3- and α5ß1-integrins directly on the plates. This indicates that the integrins are actively involved in cell-matrix adhesion.

After 5 days cultivation in monolayer culture chondrocytes change their synthesis programme as well as their shape because of their maturation and/or ageing. The ensuing new cell type expresses other receptors, i.e. α1-integrin, which leads to the binding of collagen type I. This is an indication of a change in the differentiation of the original cartilage cells. The behaviour of chondrocytes in long-term monolayer culture can thus be compared with the changes of cartilage cells in high density culture (Shakibaei et al., 1993a).

We assume that integrin α3 is also responsible for the binding of collagen type II and integrin α1 also for the binding of collagen type I. The changes in the shape of chondrocytes, their synthesis programme with a new surface receptor and new ligands in culture, might be due to integrin-gen activity under in vitro conditions.

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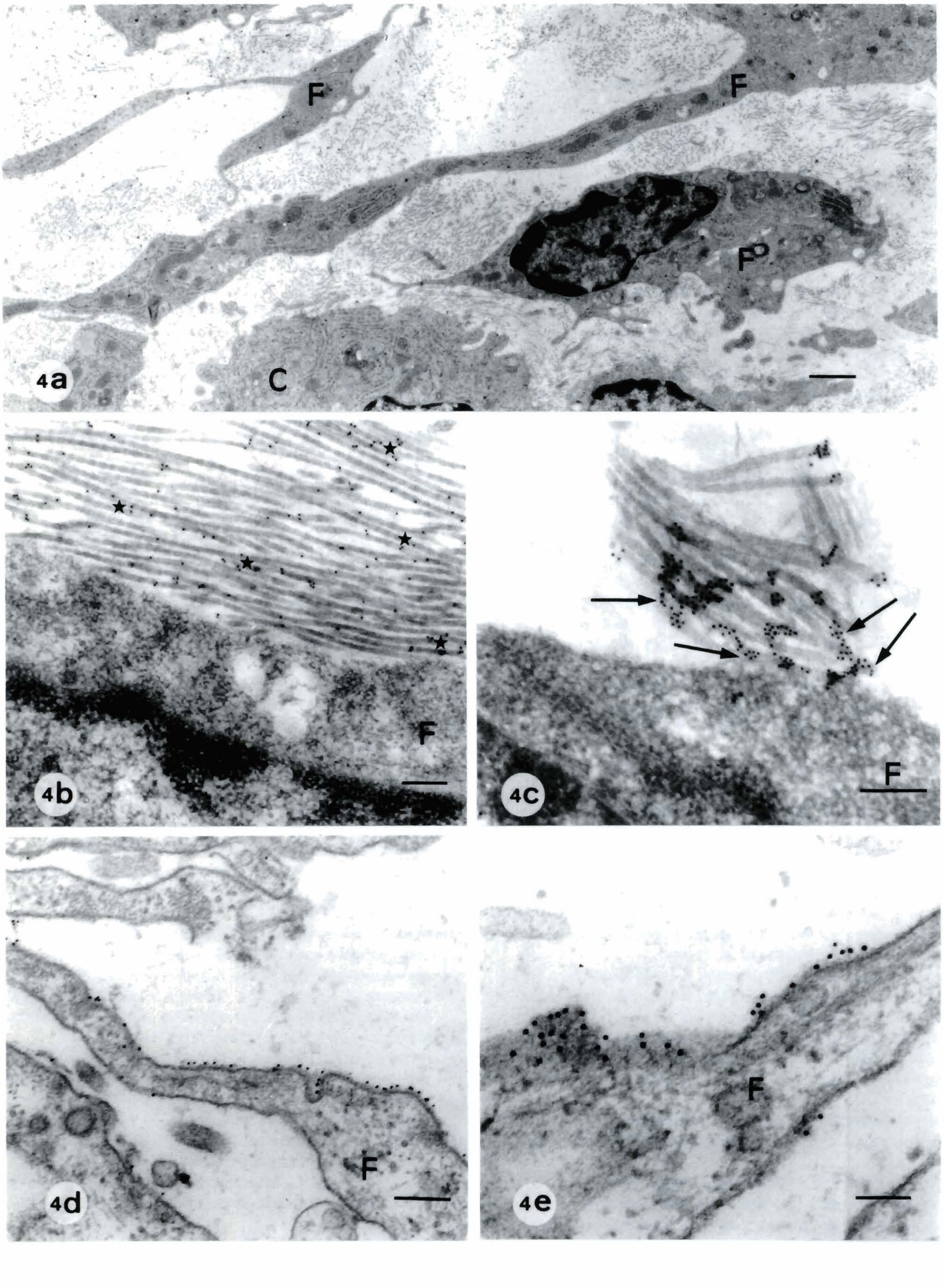
Fig. 4. Transmission and immunotransmission electron microscopic investigations of chondrocytes after 5 days in culture. **a.** After a 5-day culture period fibroblast-like cells (F) of bipolar shape and with long pseudopodia increase at the cost of chondrocytes (C). a, x 8,000; bar= 1 μm. **b.** They form a matrix with new collagenous fibrils on type I (*). b, x 40,000; bar= 0.2 μm. **c.** In addition, they express integrin α1 (arrows) on the surface, thus being able to bind collagen type I. Their surface also show integrin αv **(d)** and α5ß1 **(e)**. c, x 60,000; bar= 0.2 μm; d, x 50,000; bar= 0.2 μm; e, x 95,000; bar= 0.1 μm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References

Abbott J. and Holtzer H. (1966). The loss of phenotypic traits by differentiated cells. III. The reversible behaviour or chondrocytes in primary cultures. J. Cell Biol. 28, 473-487.

Adams J.C. and Watt F.M. (1990). Changes in keratinocyte adhesion during terminal differentiation: reduction in fibronectin binding precedes α5ß1 integrin loss from the cell surface. Cell 63, 425-435.

Albelda S.M. (1993). Biology of disease, role of integrins and other cell adhesion molecules in tumor progression and metastasis. Lab. Invest. 68, 4-17.

Albelda S.M. and Buck C.A. (1990). Integrins and other cell adhesion molecules. FASEB J. 4, 2868-2880.

Argraves W.S., Suzuki S., Arai H., Thompson K., Pierschbacher M.D. and Ruoslahti E. (1987). Amino acid sequence of the human fibronectin receptor. J. Cell Biol. 105, 1183-1190.

Benya P.D., Padilla S.R. and Nimni M.E. (1977). The progeny of rabbit articular chondrocytes synthesis collagen types I, III and type I trimer, but not type II. Verifications by cyanogen bromide peptide analysis. Biochemistry 16, 865-872.

Chacko S., Abbott J., Holtzer S. and Holtzer H. (1969). The loss of phenotypic traits by differentiated cells. VI. Behaviour of the progeny of a single chondrocyte. J. Exp. Med. 130, 417-441.

Cheresh D., Smith J., Cooper H. and Quaranta V. (1989). A novel vitronectin receptor integrin (αvßx) is responsible for distinct adhesive properties of carcinoma cells. Cell 57, 59-69.

Defilippi P., van Hinsbergh V., Bertolotto A., Rossino P., Silengo L. and Tarone G. (1991). Differential distribution and modulation of expression of α1/ß1 integrin on human endothelial cells. J. Cell Biol. 114, 855-863.

Dürr J., Goodman S., Potocnik A., von der Mark H. and von der Mark K. (1993). Localization of ß1-integrins in human cartilage and their role in chondrocyte adhesion to collagen and fibronectin. Exp. Cell Res. 207, 235-244.

Enomoto M., Leoboy P.S., Menko A.S. and Boettiger D. (1993). ß1-integrins mediate chondrocyte interaction with type I collagen, type II collagen, and fibronectin. Exp. Cell Res. 205, 276-285,

Freed E., Gailit J., van der Geer P., Ruoslahti E. and Hunter T. (1989). A novel integrin ß subunit is associated with the vitronectin receptor α subunit (αv) in a human osteosarcoma cell line and is a substrate for protien kinase C. EMBO J. 8, 2955-2965.

Gosslau B. and Barrach H.J. (1979). Enzyme-linked immunosorbent microassay for quantification of specific antibodies ot collagen type I, II, and III. J. Immunol. Methods 29, 71-77.

Grundman K., Zimmermann B., Barrach H.J. and Merker H.-J. (1980). Behaviour of epiphyseal mouse chondrocyte populations in monolayer culture. Virchows Arch. (A) 389, 167-187.

Hemler M.E., Huang C. and Schwartz L. (1987). The VLA protein family. Characterization of five distinct cell surface heterodimers each with a common 130,000 weight beta subunit. J. Biol. Chem. 262, 3300-3309.

Hemler M.E. (1990). VLA proteins in the integrin family: structures, functions and their role on leukocytes. Annu. Rev. Immunol. 8, 365-400.

Hewitt A.T., Varner H.H., Silver M.H., Dessau W., Wilkes C.M. and Martin G.R. (1982). The isolation and partial characterization of chondronectin, an attachment factor for chondrocytes. J. Cell Biol. 257, 2330-2334.

Horwitz A.L. and Dorfman A. (1970). The growth of cartilage cells in soft agar and liquid suspension. J. Cell Biol. 45, 434-438.

Hynes R.O. (1987). Integrins: a family of cell surface receptors. Cell 48, 549-554.

Hynes R.O. (1992). Integrins: Versatility, modultion, and signaling in cell adhesion. Cell 69, 11-25.

Kosher R.A. and Church R.L. (1975). Stimulation of in vitro somite chondrogenesis by procollagen and collagen. Nature 258, 327-330.

Kosher R.A., Lash J.W. and Minor R.R. (1973). Environmental enhancement of in vitro chondrogenesis. IV. Stimulation of somite chondrogenesis by exogenous chondromucoprotein. Dev. Biol. 35, 210-220.

Labat-Robert J. (1986). Structural glycoproteins and cell-matrix interactions in normal and pathological conditions. Fron. Matrix Biol. 11, 17-29.

Languino L.R., Gehlsen K.R., Wayner E.A., Carter W.G., Engvall E. and Ruoslahti E. (1989). Endothelial cells use α2ß1 integrin as a laminin receptor. J. Cell Biol. 109, 2455-2462.

Layman D.L., Sokoloff L. and Miller E.J. (1972). Collagen synthesis by articular chondrocytes in monolayer culture. Exp. Cell Res. 73, 107-112.

Lavietes B.B. (1971). Kinetics of matrix synthesis in cartilage cell cultures. Exp. Cell Res. 68, 43-48.

Loeser R.F. (1993). Integrin-mediated attachment of articular chondrocytes to extracellular matrix proteins. Arthritis Reum. 36, 1103-1110.

Löster K., Voight S., Heidrich C., Hofman W. and Reutter W. (1994). Cell-colagen adhesion is inhibited by monoclonal antibody 33.4 against the rat α_1-integrin subunit. Exp. Cell. Res. 212, 155-160.

Mayne R., Vail M.S., Mayne P.M. and Miller E.J. (1976). Changes in the type of collagen synthesized as clones of chick chondrocytes growth and eventually lose division capacity. Proc. Natl. Acad. Sci. USA 73, 1674-1678.

Merker H.-J., Günther Th. and Krüger U. (1978). Effect of 4- methyl-umbelliferryl-ß-D-xylopyranoside on the morphology of embryonic cartilage in limb bud cultures. Teratology 18, 291-310.

Merker H.-J., Zimmermann B. and Grundmann K. (1980). Differentiation of isolated blastemal cells from limb bunds into chondroblasts. In: Tissue culture in medical research. Vol. II. Richards R.J. and Rajan K.T. (eds). Pergamon Press. Oxford, New York. pp 31-39.

Moskalewski S., Adamiec I. and Golaszewska A. (1979). Maturation of rabbit auricular chondrocytes grown in vitro in monolayer culture. Am. J. Anat. 155, 339-348.

Norby D.P., Malemud C.J. and Sokoloff L. (1977). Differences in the collagen types synthesized by lapine articular chondrocytes in spinner and monolayer culture. Arthritis Rheum. 20, 709-715.

Pennypacker J.C., Wassell J.R., Yamada K.M. and Pratt R.M. (1979). The influence of an adhesive cell surface protein on chondrogenic expression in vitro. Exp. Cell Res. 121, 411-415.

Ramachandrula A., Tiku K. and Tiku M.L. (1992). Triopeptide RGD-dependent adhesion of articular chondrocytes to synovial fibroblasts. J. Cell Sci. 101, 859-871.

Ruoslahti E. (1991). Integrins. J. Clin. Invest. 87, 1-5.

Ruoslahti E. and Pierschbacher M.D. (1987). New perspectives in cell adhesion: RGD and integrins. Science 238, 491-497.

Salter D.M., Hughes D.E., Simpson R. and Gardner D.L. (1992). Integrin expression by human articular chondrocytes. Brit. J. Rheumatol. 31, 231-234.

Schröter-Kermani C., Hinz N., Risse P., Zimmermann B. and Merker H.-

J. (1991). The extracellular matrix in cartilage organoid culture: biochemical immunomorphological and electron microscopic studies. Matrix 11, 428-440.
Shakibaei M., Schröter-Kermani C. and Merker H.-J. (1993a). Matrix changes during long-term cultivation of cartilage (organoid or high-density cultures). Histol. Histopathol. 8, 463-470.
Shakibaei M., Abou-Rebyeh H. and Merker H.-J. (1993b). Integrins in ageing cartilage tissue in vitro. Histol. Histopathol. 8, 715-723.
Sokoloff L. (1976). Articular chondrocytes in culture. Matrix production and hormonal effects. Arthritis Rheum. 19, 426-429.
Sommarin Y., Larsson T. and Heinegard D. (1989). Chondrocyte-matrix interactions: attachment to proteins isolated from cartilage. Exp. Cell Res. 184, 181-192.
Sonnenberg A., Modderman P.W. and Hogerborst F. (1988). Laminin receptor on platelets is the integrin VLA-6. Nature 336, 487-489.
von der Mark K., Guss V., von der Mark H. and Müller P. (1977). Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. Nature 267, 531-532.
Wayner E.A. and Carter W.G. (1987). Identification of multiple cell adhesion receptors for type VI collagen and fibronectin in human fibrosarcoma cells possessing unique α and common ß subunits. J. Cell Biol. 105, 1873-1884.
Wayner E.A., Carter W.G., Piotrowicz R.S. and Kunick T.J. (1988). The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoprotein Ic-IIa. J. Cell Biol. 107, 1881-1891.
West C.M., Lanza R., Rosenbloom J., Lowe M. and Holtzer H. (1979). Fibronectin alters the phenotypic properties of cultured chick embryo chondroblasts. Cell 17, 491-501.

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