Integrin expression on epiphyseal mouse chondrocytes in monolayer culture

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Summary. The expression of α1-, α3-, αv- and α5β1-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 α5β1-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 α5β1-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 β-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 transmembranous 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 β-chain, and a binding site for divergent cations. So far, at least 8 different β- and 14 α-subunits are known, each β-subunit being able to bind any of these α-subunits (Cheresch et al., 1989; Hynes, 1992). Integrins α1β1 and α2β1 are known to be collagen and laminin receptors (Wayner and Carter, 1987; Languino et al., 1989; Defilippi et al., 1991; Albelda, 1993), integrin α3β1 laminin, collagen and fibronectin receptors (Wayner et al., 1988), integrin α5β1 fibronectin receptor (Argarves et al., 1987) and integrin α6β1 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 Holtzner, 1966; Chack et al., 1969; Horwitz and Dorfman, 1970; Lavites, 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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Löser (1993) and Enomoto et al. (1993) B1-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 α5β1 (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 Ca²⁺ + Mg²⁺-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 Thermonax plates and for transmission electron microscopic investigations in Petri dishes in monolayer culture. The cells were counted and their number was adjusted to 1.5x10⁶/ml.

   The cultures were grown in an incubator at 37 °C in 95% air and 5% CO₂. The cells were removed after 3 hrs, 1 and 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 with 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-α5β1 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.
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Table 1. Chondrocytes and fibroblasts proportions during days 1, 3 and 5. The experiments were repeated four times.

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<thead>
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<td>1st Day</td>
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<td>3rd Day</td>
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Subsequently, they were post-fixed in a 2% OSO₄ solution. After dehydration in the ascending alcohol series, the specimens were embedded in Epon. Ultrathin sections were contrasted with 2% uranyl acetate and lead citrate and investigated under a Zeiss EM10 transmission electron microscope.

4. Immuno-electron 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 α5β1 1:30; αβ and α3 1:20 in PBS/BSA/Tween) 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 dehydrated 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 α5β1 1:30, α3 and αβ 1:20 in PBS/BSA) overnight at 4 °C; washing with PBS/BSA/Tween for 3 x 10 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-dried 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-αα, -α3-, α1- and α5β1-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, α5β1 (Fig. 1e,f), αα (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), α5β1 and αα (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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integrins

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 α5β1. In some experiments the cells were scratched off the plates using a pair of tweezers before immunolabelling was carried out. The surface of the chondrocytes showed positive labelling of αv-α3- and α5β1-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 irregularly-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 α5β1 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. Immunolabelling 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 chondrocytes in contact with the cell membrane (Fig. 4c). Integrins α5β1 and αv were also seen in fibroblast-like cells (Fig. 4d,e).

Discussion

This study describes the expression of β1-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 α5β1. After 5 days in culture many of these chondrocytes were observed as 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 differentiation (Merker et al., 1978; Grundmann et al., 1980; Shikibai 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...
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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 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 B1-integrins. The present immunohistochemical as well as immunoelectron microscopic investigations have shown that B1-integrins (αα, α3, and α5β1) 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 B1 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 α1β1 is responsible for the binding of collagen and laminin (Ruoslathi, 1991; Defilippi et al., 1991; Hynes, 1992; Albeda, 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 (Schüür-Kermani et al., 1991; Shakibaei et al., 1993a). At the same time new receptors of the integrin α1β1 and α2β1 types are expressed which bind collagen type I on the cell surface (Shakibaei et al., 1993b).

Integrin α5β1 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, αα and α5β1 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 αα, α3- and α5β1-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. αα-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. 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: mitochondrion, 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 α5β1 (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.

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 fibres on type I (⁎). b, x 40,000; bar= 0.5 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 αα (d) and α5β1 (e). c, x 80,000; bar= 0.2 μm; d, x 50,000; bar= 0.2 μm; e, x 95,000; bar= 0.1 μm.
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