Histol Histopath (1993) 8: 715-723

Histology and Histopathology

Integrins in ageing cartilage tissue *in vitro*

M. Shakibaei[1], H. Abou-Rebyeh[2] and H.-J. Merker[1]

[1]Institute of Anatomy and [2]Institute of Molecular Biology and Biochemistry, Free University of Berlin, Berlin, Germany

Summary. Matrix-cell interactions are of great importance for numerous cell functions whereby integrins play an essential role as transmitters of extracellular signals. In cultures of ageing cartilage tissue (organoid or high density cultures) cartilage cells occur on the surface of which thick fibrils of collagen type I are deposited. Since integrins, in their role as receptors, cause an interaction between matrix components and cell membrane, we tried to demonstrate immunomorphologically (light and electron microscopically) the corresponding integrin receptors for collagen type I ($ß_1\alpha_1$ and $ß_1\alpha_2$) on the surface of these ageing cartilage cells. Cultures of normal, i.e. young cartilage tissue exhibit only $ß_1\alpha_3$- and $ß_1\alpha_5$-receptors; labelling against the integrins $ß_1\alpha_1$ and $ß_1\alpha_2$ is not possible in this case. Our results show that after the occurrence of thick fibrils cartilage cells express new receptors ($ß_1\alpha_1$ and $ß_1\alpha_2$) on the cell membrane. Thus, in ageing or dedifferentiating cartilage tissue it is not only the synthesis programme of matrix components (e.g. instead of collagen type II >> collagen type I) which changes but also the integrins (instead of $\alpha_3/ß_1$, $\alpha_5/ß_1$ >> $\alpha_1/ß_1$, $\alpha_2/ß_1$) so that new collagen types can be bound. These findings may also serve for a better understanding and interpretation of cartilage changes *in vivo* during ageing and under pathological conditions.

Key words: Ageing cartilage, Organoid culture, Immunomorphology, Integrin receptors ($ß_1\alpha_1$ and $ß_1\alpha_2$)

Introduction

Interactions between cells and matrix are important for numerous cell functions; e.g. for proliferation, migration, adhesion and differentiation. They are transmitted by specific receptors (Buck and Horwitz, 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Akiyama et al., 1989; Albelda and Buck, 1990). Many of these matrix receptors belong to the family of integrins, especially of the $ß_1$-group (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Akiyama et al., 1989). Integrins are heterodimer molecules; i.e. they consist of different, non-covalently-linked polypeptide chains which are designated as α- and ß-subunits (Hynes, 1987, 1992; Albelda and Buck, 1990). The size of the α-subunit ranges from 120 to 180 Kd. It consists of an extracellular domain with several divalent cationic binding sites and a transmembranous domain as well as a cytoplasmic domain. The cationic binding sites are essential for the binding of ligands. The size of the ß-subunit varies between 90 and 110 Kd. It also consists of an extracellular, a transmembranous and a cytoplasmic domain (Hynes, 1987, 1992; Ruoslahti and Pierschbacher, 1987).

Electron microscopic investigations of isolated integrin receptors have shown that the two subunits of the integrins have a common extracellular globular end and two separate cytoplasmic ends (Carell et al., 1985; Kelly et al., 1987; Nermut et al., 1988). They function as transmembrane connections thus causing an interaction between the actin cytoskeleton in the cell and the extracellular matrix components (Ruoslashti, 1991).

At present 8 ß-subunits and 14 α-subunits are known. This allows numerous combinations which recognize many different extracellular matrix components on the surface of other cells (Hynes, 1992).

Apart from their biological significance for essential cellular processes, integrins also play a great role in the medical field; e.g. in the aggregation of blood platelets, in the immune system, during embryogenesis, tissue repair, wound healing, and in the formation of metastases (Albelda and Buck, 1990; Ruoslahti, 1991).

Using an organoid *in vitro* model, cartilage tissue can be produced within a few days (Zimmermann et al., 1990). After a 2- to 3-week culture period thick collagenous fibrils occur on the surface of the cartilage cells. According to light as well as electron microscopic findings, these collagenous fibrils consist of collagen type I. These cartilage cells lose, or have already lost, their contact with the cartilage matrix (Shakibaei et al.,

Offprint requests to: Dr. M. Shakibaei, Institut für Anatomie, Freie Universität Berlin, Königin-Luise-Str. 15, D-14195 Berlin, FRG

1993a). It is known that after loss of the connection with the matrix dedifferentiation of the cartilage cells occurs and they assume a fibroblast-like appearance (Merker et al., 1978; Grundmann et al., 1980).

The occurrence of thick collagenous fibrils on the surface of cartilage cells and the identification of these collagenous fibrils as collagen type I give rise to the assumption that in ageing cartilage tissue not only the synthesis programme of these cells is changed but also the spectrum of surface receptors, so that new collagens (type I) can be bound on the surface of the de-differentiating cartilage cells.

It was the aim of this study to demonstrate the presence of integrin receptors for collagen type I ($ß_1\alpha_1$ and $ß_1\alpha_2$) in ageing cartilage tissue using biochemical, as well as immunomorphological means. In young cartilage tissue only the integrins $\alpha_3/ß_1$ and $\alpha_5/ß_1$ can be demonstrated (Shakibaei et al., 1993b). We used monoclonal and polyclonal antibodies and the double labelling technique for the demonstration of integrin receptors at the light and electron microscopic level.

Materials and methods

Limb bud mesenchymal cells grow at high density (at least 2,000,000 cells/10 µl cell suspension) at the medium/air interphase comparable to the organ culture (Zimmermann, 1987; Zimmermann et al., 1988, 1990) described by Trowell in 1959.

Cell culture

The upper and lower limb buds of day-12 mouse embryos (day 0 = day of conception) were excised, rinsed in Hank's salt solution and shaken in 0.2% dispase in Ca- and Mg-free solution. After the addition of medium the suspension was homogenized by pipetting and separated from non-dissociated tissue fragments by filtration through a nylon mesh with a pore width of 20 µm. For sedimentation of the cells the cell suspension was centrifuged at 600 rotations for 10 min.

After removal of the foam the centrifugation tubes were decanted and the cells resuspended using an Eppendorf pipette. Ten µl each of a cell sediment with an average density of 2 x 10^6 cells were pipetted onto a membrane filter. The cells rested as a dense cell mass, similar to the Trowell culture (1959), on a membrane filter at the medium/air interphase. The high cell density caused extensive cell contacts and reorganization of the cells (sorting out) accompanied by the formation of organoid structures, and cellular differentiation. The cells were grown in an incubator under 5% CO_2 at 37°C. After corresponding fixation they were investigated morphologically and immunomorphologically after 7, 14, 21, 24 and 35 days of cultivation.

Western Blot Analysis

For Western Blot Analysis we used monoclonal antibodies of the mouse (Telios Pharmaceuticals, Inc., USA) and Polyclonal antibodies of the rabbit (Chemicon International, Inc., USA) which had been produced against various human integrins. The protein samples were separated electrophoretically on a 7.5% SDS-polyacrylamide gel using a 4% collecting gel. Subsequently, they were transferred for 2 hrs at 200 V to a nitrocellulose membrane (Schleicher and Schüssel, Dassel, FRG) using a transblot electrophoresis apparatus (Mini Trans Blot™, Bio Rad Laboratories, Richmond, USA). The membranes were then incubated with anti-integrin antibodies (diluted in 80 mM $Na_2H(PO_4)_3$, 100 mM NaCl, 1% bovine serum albumin, pH 7.4, overnight at 4 °C. The samples were specifically detected using an anti-mouse or anti-rabbit immuno-globulin, conjugated with peroxidase and a chemi-luminescence reaction and photographed on a Kodak XAR (England) film.

Immunofluorescence

The cultures were placed into an O.C.T. solution (Bayer, Munich, FRG) and frozen in liquid nitrogen. For the immunolabelling of the integrins 10 µm-thick sections were prepared and labelled with double immunofluorescence; i.e. the two integrin subunits of one section were differently labelled. GAR-FITC and GAM Rodamin (Dianova, Hamburg, FRG) served for the labelling of specific antibodies (Shakibaei et al., 1993a).

Electron microscopy

The cartilage cultures were fixed in 1% glutaraldehyde plus 1% tannic acid in 0.1 M phosphate buffer at pH 7.4 and post-fixed in 1% OsO_4 solution in phosphate buffer. After corresponding rinsing and dehydration in ascending alcohol series, the preparations were embedded in Epon, cut on a Reichert Ultracut and contrasted with 2% uranyl acetate/lead citrate. The sections were then inspected under a Zeiss EM10 transmission electron microscope.

Immunoelectron Microscopy

Immediately after fixation (3% paraformaldehyde, 0.25% glutaraldehyde in PBS, 1 hr) the cells were washed overnight in PBS/BSA and gradually dehydrated in ethanol (50%-100%). Subsequently, the cells were infiltrated for 12 hrs with a mixture of LR-White/100% ethanol (1:1) and then for 2 days with 100% LR-White. The medium was changed 4 to 5 times during this period. After two days, the material was placed in plastic capsules containing LR-White (Plano, Marburg, FRG) together with an accelerator (1 drop acclerator per 10 ml LR-White). Polymerization was achieved on ice for 30-60 min in the deep freeze. Subsequently, the capsules were stored for 2 hrs at 4 °C. After 24 hrs, the capsules were removed at room

temperature.

Double Immunolabelling of the LR-White Preparations

The LR-White ultrathin sections were transferred to Formvar coated grids and immunolabelled as follows:

1. Blocking	PBS/BSA/0.5% Tween	30 min
2. Incubation with 1st primary AB	anti-integrin AB 1:40 in PBS/BSA	overnight
3. Washing	PBS/BSA/Tween	3x15 min
4. Incubation with 1st secondary AB	GAR 10 nm 1:30 in PBS/BSA/Tween	60 min
5. Washing	PBS/BSATween	3x15 min
6. Incubation with 2nd primary AB	anti-integrin AB 1:40 in PBS/BSA	overnight
7. Washing	PBS/BSATween	3x15 min
8. Incubation with 2nd secondary AB	GAM 5 nm 1:30 in PBS/BSA/Tween	60 min
10. Washing	PBS/BSA/Tween	3x15 min
10. Fixation	1% glutaraldehyde	10 min
11. Washing	PBS	3x15 min
12. Contrasting	1% tannic acid in PBS	10 min
13. Washing	PBS	3x15 min
14. Contrasting	2% uranyl acetate	20 min
15. Washing	Aqua bidest.	several times

The preparations were inspected under a Zeiss EM10.

Antibodies

Collagen type I was obtained from foetal mouse skin (Trelstad et al., 1976); antibodies were produced in the rabbit. The specificity of the antibodies was determined using the ELISA technique (Gosslau and Barrach, 1979). The polyclonal antibodies against integrins were purchased from Chemicon (Chemicon International, Inc., USA) and the monoclonal antibodies from Telios (Telios Pharmaceuticals, Inc., USA). The secondary gold-labelled antibodies were purchased from Amersham (Brunswick, FRG).

Results

After a 7-day culture period of limb bud blastemal cells from 12-day-old mouse embryos at high density, cartilage nodules and plates had developed.

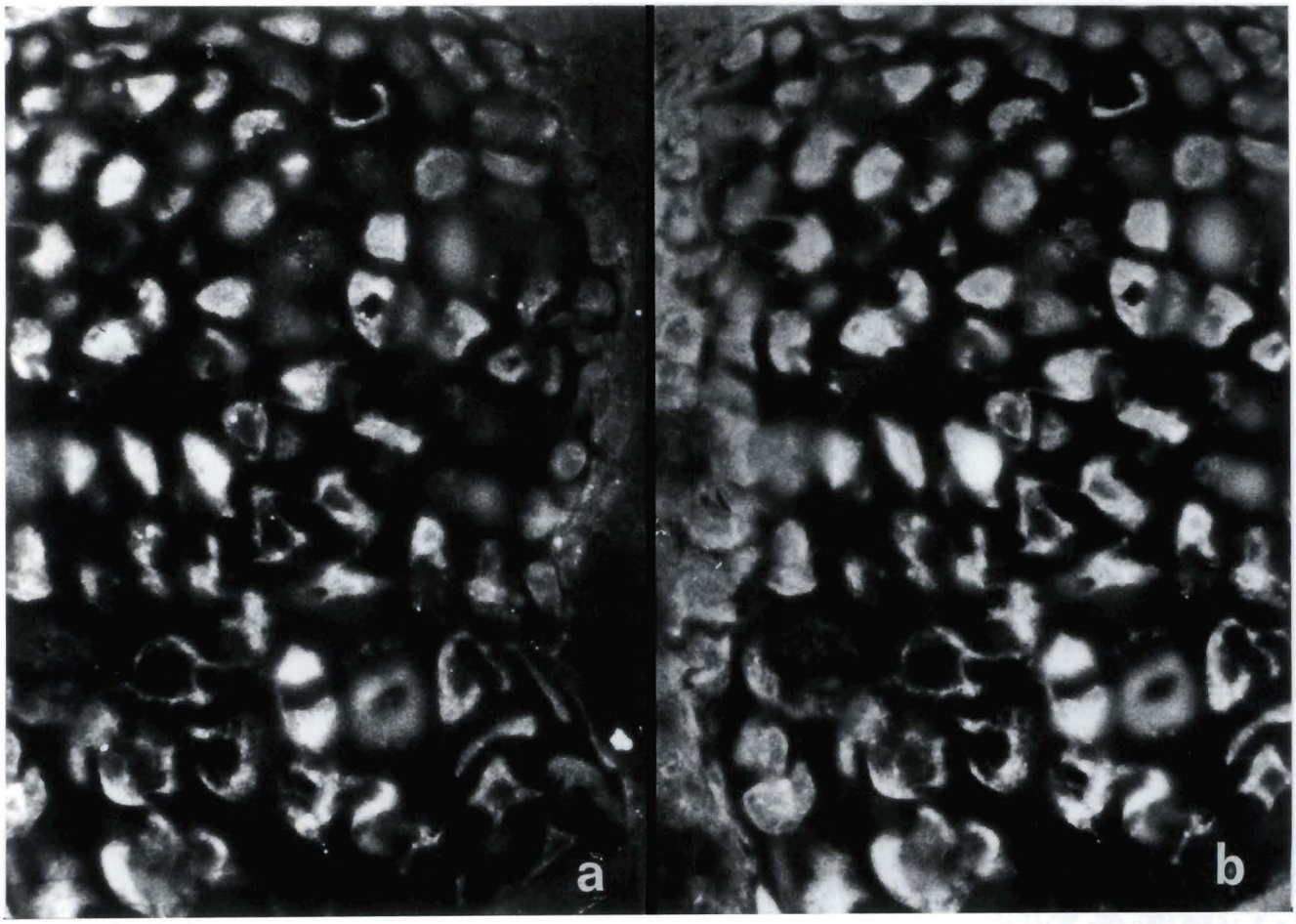

Fig. 1. Immunofluorescence microscopic demonstration of integrin $\beta_1\alpha_2$ in cartilage grown in organoid culture. **a.** 14-day-old culture; demonstration of the β_1-chain (FITC). x 140. **b.** 14-day-old culture; demonstration of the α_2-chain (Rodamin). x 140

These structures were delimited by a peri-chondrium consisting of 2 to 5 layers of elongated, bipolar fibroblast-like cells. Loose mesenchyme of polymorphous cells were located between them. The cartilage cells were mainly round with small cuspidal processes at their surface. They were characterized by a well-developed rough endoplasmic reticulum, a large Golgi apparatus and the occurrence of other organelles or structures (mitochondria, small vacuoles, vesicles, granules and some components of the cytoskeleton). The matrix was closely attached to the cell membrane. The matrix in the cartilage tissue consisted of thin (18-22 nm) collagenous fibrils which proceeded singly and irregularly. After tannic acid fixation some of these fibrils fused with the surface coat of the cell membrane. The cells flattened towards the perichondrium. The matrix of the perichondrium

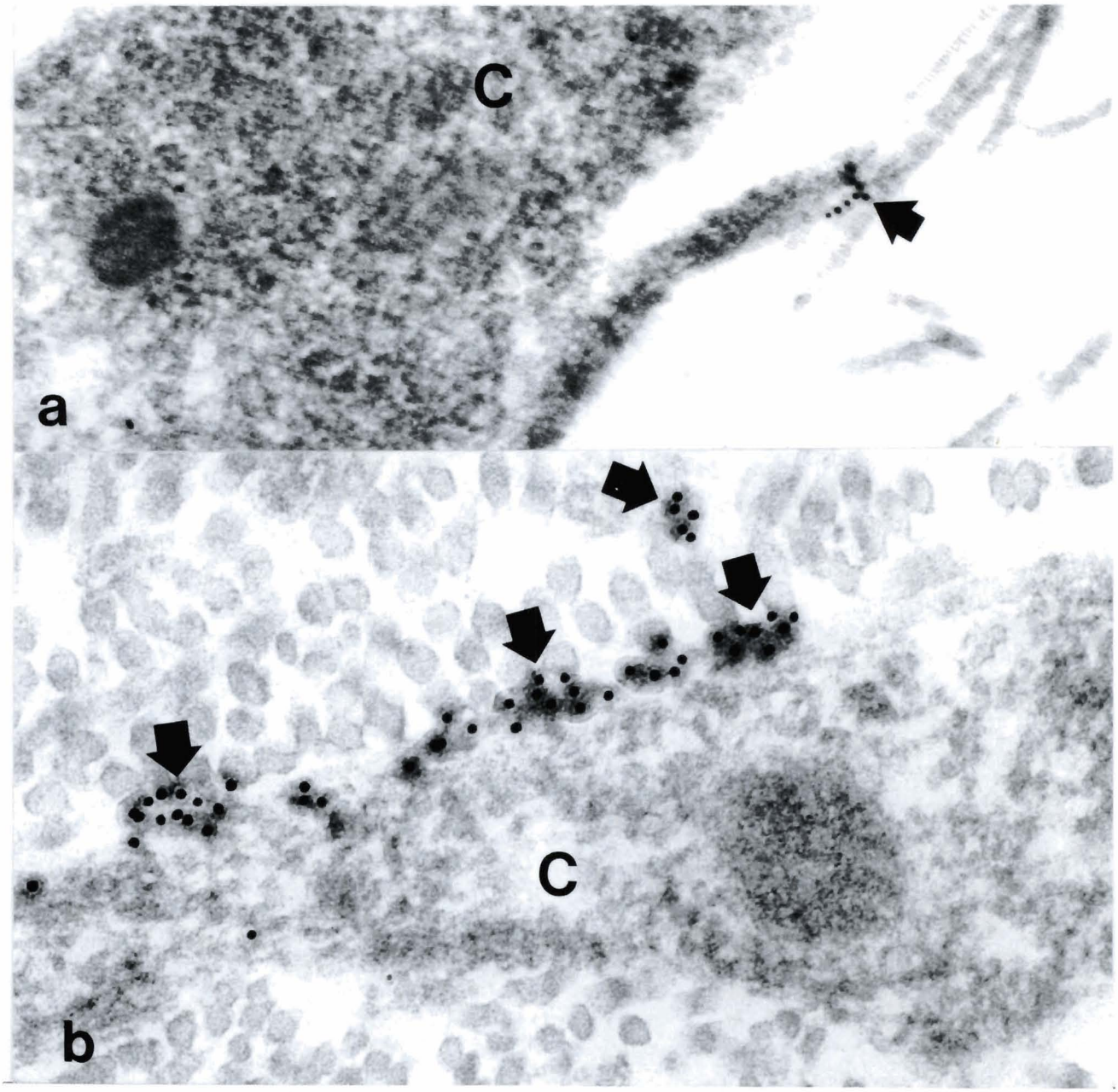

Fig. 2. 14-day-old culture. Numerous thick collagenous fibrils (>) resting on the surface of a chondroblast (C); typical thin collagenous fibrils (18-22 nm) at some distance to the cell (*). x 60,000. **b.** 14-day-old culture. Gold labelling against collagen type I only in the region of thick collagenous fibrils (>) in the vicinity of chondroblasts (C). No immunolabelling in the cartilage matrix (*). x 63,700

contained thicker fibrils (20-30 nm) which occurred mainly in bundles and almost parallel to the surface of the cartilage incorporations.

After a culture period of 2 weeks cells occurred which were characterized by two properties that were observed separately or jointly.

1) The cell membrane of some cartilage cells detached from the matrix over distances of varying length; i.e. an optically empty space developed between cell membrane and matrix (Fig. 1).

2) Cross-striated fibrils rested on the surface of the cells which were clearly thicker than the fibrils of the cartilage matrix (35-55 nm).

The number of cartilage cells showing these two changes became larger over the next few weeks (after 3-5 weeks of cultivation). The thick fibrils on the surface of the cartilage cells also increased in number.

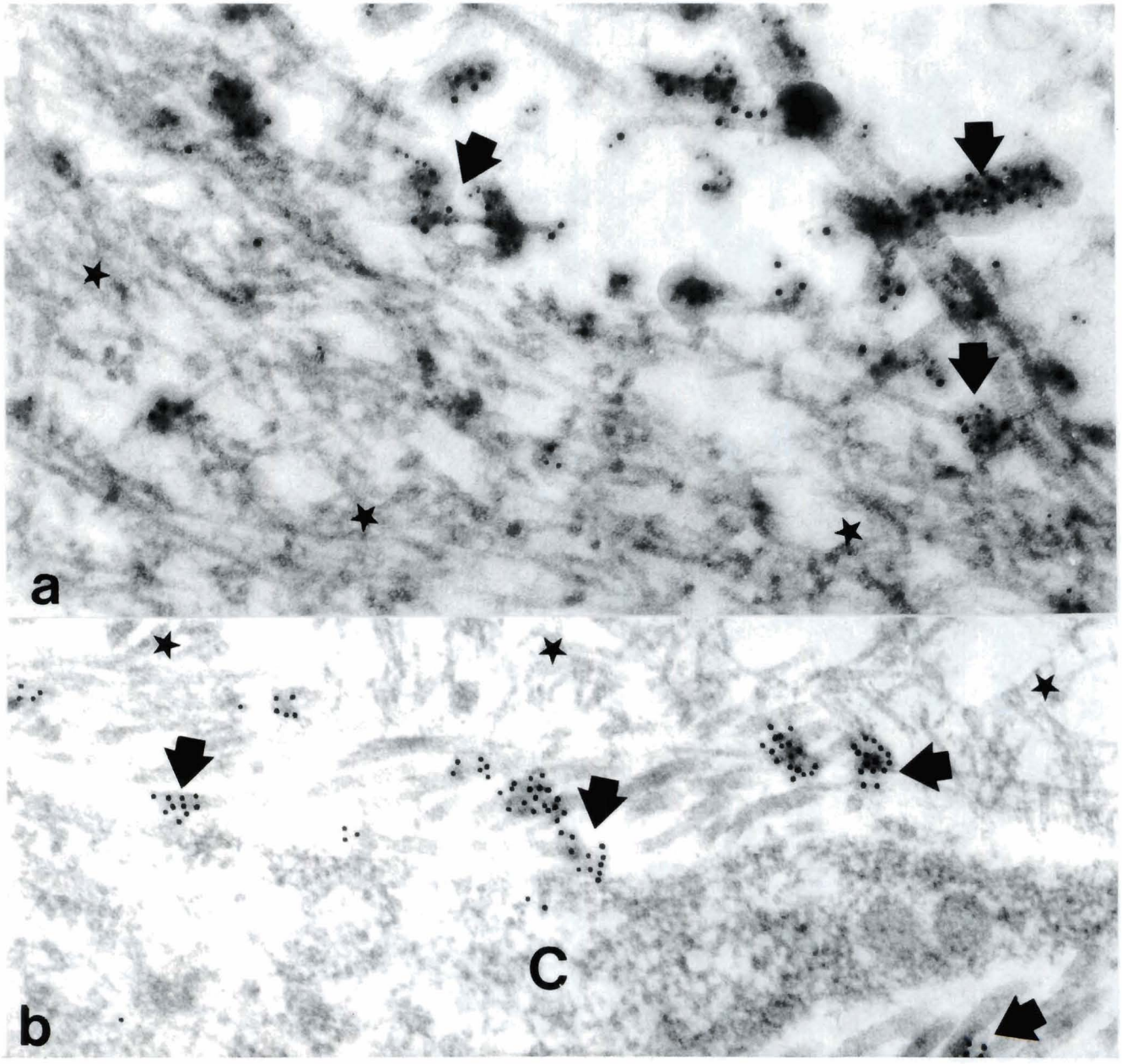

Fig. 3. 21-day-old culture. Demonstration of integrin receptors β_1 and α_2 (double labelling) in cartilage tissue. **a.** Gold particles only over thick collagenous fibrils (>). No labelling in the typical cartilage matrix (*). β-chain = 5 nm gold granules; α-chain = 10 nm gold granules. x 127,500. **b.** Simple immunolabelling against integrin α_2. Gold particles on the surface of chondrocytes (C) and in the region of thick collagenous fibrils (>). No immunolabelling in the cartilage matrix (*). x 100,000

Immunomorphological findings obtained at light and electron microscopic level showed that positive labelling could be achieved on the surface of cartilage cells in the regions of thick fibrils by the use of antibodies against collagen type I. Labelling against collagen type II could no longer be demonstrated (Shakibaei et al., 1983a) (Fig. 2). Thus, the thick fibrils represent collagen type I.

In normal «young» cartilage only integrins of the $ß_1α_3$- and $ß_1α_5$-types could be shown on the surface of cells as well as in the matrix of cartilage tissue. During the ageing process receptors for the newly-synthesized collagenous fibrils of type I were also expressed on the surface (i.e. integrins of the $ß_1α_1$- and $ß_1α_2$-types; Figs. 3-5). These integrins could be demonstrated light and electron microscopically. These new cell surface receptors were also observed on

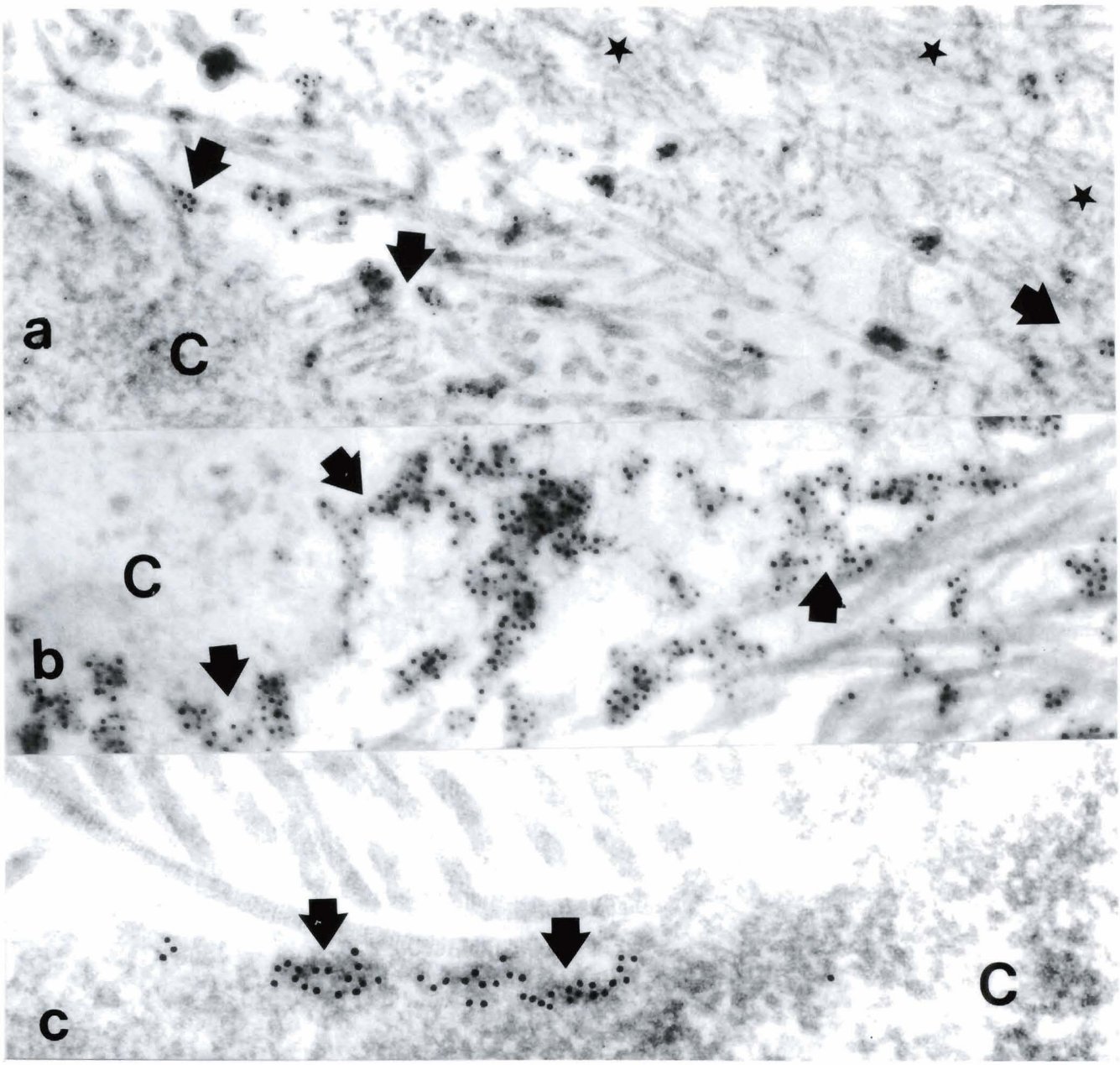

Fig. 4. 21-day-old culture. Demonstration of integrin receptors $ß_1α_1$, $ß_1α_2$ and $α_1$ in cartilage tissue. **a.** Double immunolabelling of integrin $ß_1α_1$. Gold labelling on the surface of chondroblasts (C) and in the region of thick collagenous fibrils (>). No labelling of $α_1$ in the cartilage matrix proper (*). ß-chain = 5 nm gold granules; α-chain = 10 nm gold granules. x 80,000. **b.** Double immunolabelling of integrin $ß_1α_2$ on the surface of chondroblasts (C) and in the region of thick collagenous fibrils (>). ß-chain = 5 nm gold granules; α–chain = 10 nm gold granules. x 100,000. **c.** Simple immunolabelling of integrin $α_1$. Gold particles only on the surface of chondroblasts. x 127,500

the thick collagenous fibrils in the matrix. The two integrin types ($ß_1\alpha_1$ and $ß_1\alpha_2$) also occurred in perichondral cells and in the matrix of the perichondrium *in vitro*.

The findings were confirmed by immunoblots (Fig. 6) of cartilage cell lysate in the presence of integrin receptors of the $ß_1\alpha_1$- and $ß_1\alpha_2$-types using antibodies which were also employed for immunomorphological investigations.

Discussion

After a 2-week culture period of blastemal cells from day-12 mouse limb buds in high density organoid cultures collagen type I occurs at an increasing extent on the surface of cartilage cells. This collagen type can be demonstrated light and electron microscopically by the use of specific antibodies (Schröter-Kermani et al., 1991; Shakibaei et al., 1993a).

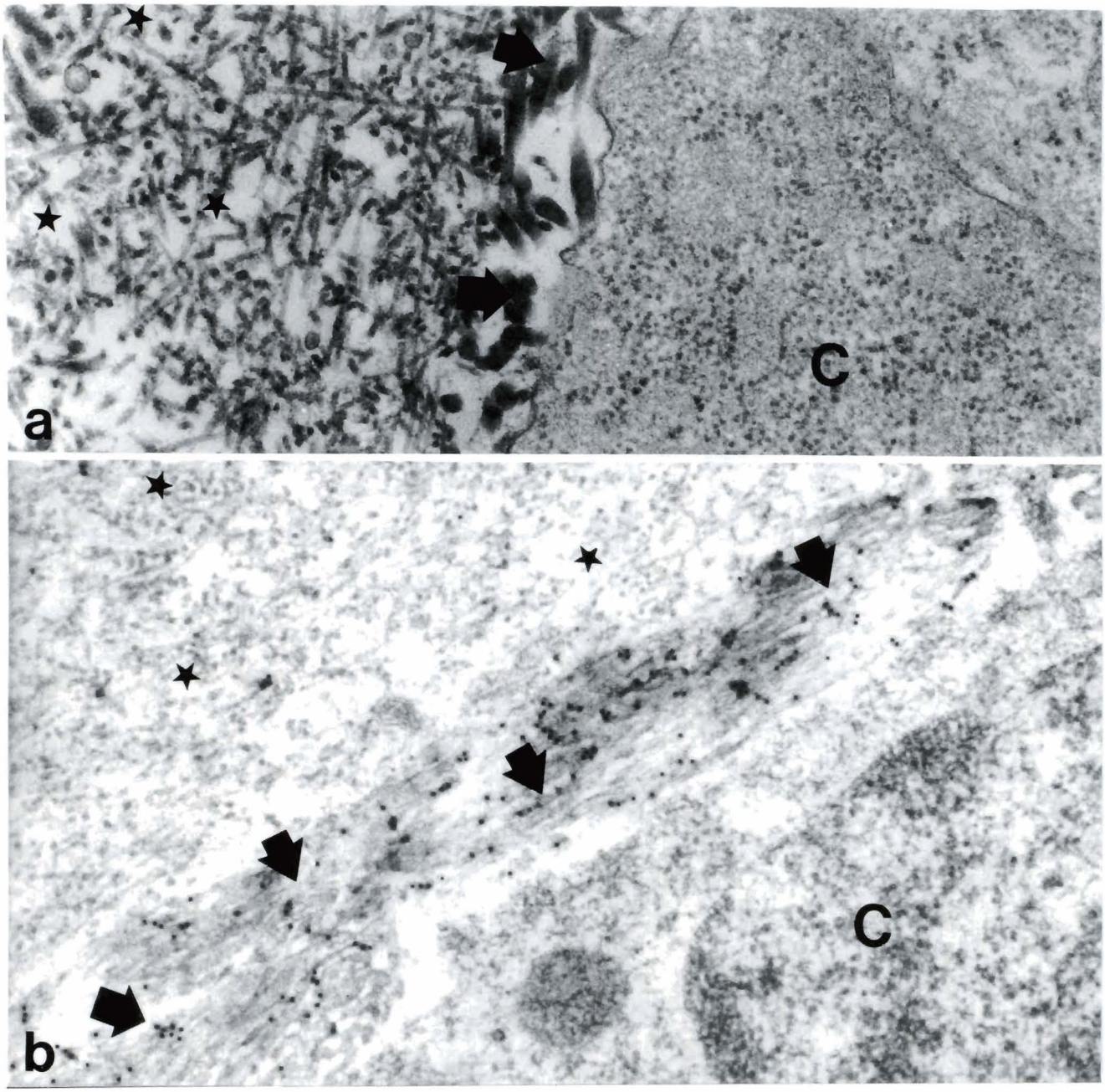

Fig. 5. 14-day-old culture. Demonstration of integrins α_1 and α_2 in the perichondrium using the gold immunolabelling technique. **a.** Immunolabelling against integrin α_2 only at the contact site of a cell process with thick collagenous fibrils. x 80,500. **b.** Immunolabelling against integrin α_1. Gold particles directly on the surface of the cells (C) or at thick collagenous fibrils (>). x 127,500

The occurrence of thick fibrils of collagen type I is in most cases preceded by a detachment of the cell membrane from the matrix; i.e. from the inside of the cartilage cell cavity. The thick type I fibrils are occasionally closely attached to the cell membrane again.

Hence, these changes in the composition of the cartilage matrix represent an alteration in the synthesis programme, as it has been described for the dedifferentiation of cartilage cells and degenerative cartilage diseases. Such modifications have also been observed during skeletogenesis *in vivo* (von der Mark et al., 1976; von der Mark and von der Mark, 1977; Shakibaei et al., 1993a).

The findings clearly show that the matrix binding of cartilage cells is initially lost. This is probably due to the loss of receptors for the cartilage matrix. The developing new cell type then expresses other receptors ($ß_1\alpha_1$, $ß_1\alpha_2$) which are able to bind collagen type I or other associated matrix components; e.g. fibronectin.

The question arises whether the occurrence of integrins in the matrix represents an artefact or a cross-reaction. Two reasons speak against an artefact or cross-reaction:

- For immunolabelling of the α- and the ß-chains we used the double immunolabelling technique at light and electron microscopic level; i.e. each chain was imunolabelled with different antibodies (mono- and polyclonal antibodies). There was always a close co-localization of the two chains and an identical localization of the two antibodies.
- Immunoblotting caused a specific staining of the integrins.
- Using the immunolabelling technique, the integrin receptors $ß_1\alpha_1$ and $ß_1\alpha_2$ were only found in the region of ageing cartilage and the perichondrium; i.e. only in areas containing collagen type I. Normal or young cartilage exhibited the integrin receptors $ß_1\alpha_3$ and $ß_1\alpha_5$.

Therefore, a specific binding between integrin receptors and their ligands can be assumed. It is, however, not easy to interpret the localization of integrin receptors in the matrix of cartilage and perichondrium. Secretion and shedding may be the mechanisms responsible for the presence of integrins in the matrix. Other membrane receptors of the family of syndecanes (heparan sulphate proteoglycans) are known to be secreted by the cell (Höök et al., 1984; Rapraeger et al., 1986, 1987; Hassel et al., 1986). Due to changes in the cell shape and/or loss of the binding with the matrix, the extracellular domain of the cytoplasm membrane receptors is detached and released into the medium («shedding»). Thus, the cell loses its anchorage and stabilization at the matrix (Jalkanen et al., 1987). Therefore, it is quite possible that also in the case of integrins of chondroblasts the extracellular part is detached and then adheres to the receding collagen fibrils. Secretion or activation of enzymes might be the reason for this event.

Secretion cannot be excluded because of the occurrence of integrin-positive vacuoles (Shakibaei et al., 1993b). When interpreting the function of integrins in the matrix, interlinkage of matrix structures can be assumed.

Hence, integrins play a great role in changing the direction of differentiation in ageing cartilage. However, the sequence of the processes is not yet fully understood. Must the integrin-matrix connections first be dissolved before the cells change their synthesis programme or is the integrin-matrix detachment a result of dedifferentiation? Furthermore, it is not known yet whether the new integrins represent a secondary response to the newly-formed collagens or whether their occurrence is already fixed in the new synthesis programme.

Certain is that a change of the synthesis programme of the matrix components is accompanied by changes in the cell-matrix interactions and that they establish a different matrix contact via new integrins.

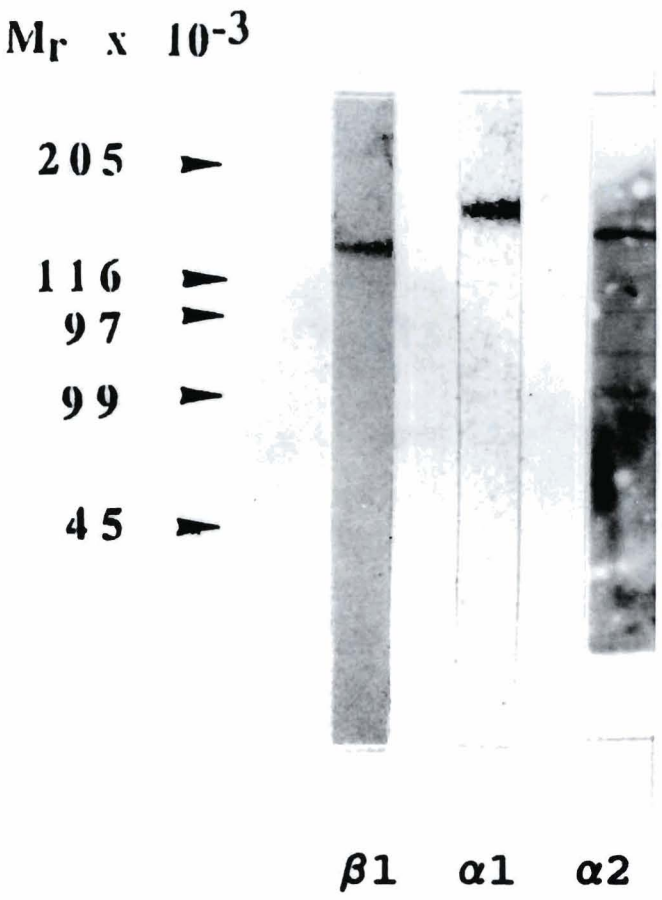

Fig. 6. Immunoblotting. Integrins $ß_1\alpha_1$ and $ß_1\alpha_2$ recognizable in the immunoblot due to antibody binding which is also used in immunomorphology.

Acknowledgements. This work was supported by grants from the Deutsche Forschungsgemeinschaft awarded to Sfb 174. The authors are indebted to Mrs. Barbara Steyn for preparation of the manuscript. The photographic work of Mrs. Simone Baar is gratefully acknowledged.

References

Akiyama S.K., Yamada S.S., Chen W.-T. and Yamada K.M. (1989). Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. J. Cell Biol. 109, 863-875.

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

Buck C.A. and Horwitz A.F. (1987). Cell surface receptors for extracellular matrix molecules. Annu. Cell Biol. 3, 179-205.

Carell N.A., Fitzgerald L.A., Steiner B., Erickson H.P. and Philips D.R. (1985). Structure of human platelet membrane glycoproteins IIb and IIIa as determined by electron microscopy. J. Biol. Chem. 260, 1743-1749.

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

Grundmann 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.

Hassel J.R., Kimura H. and Hascail V.C. (1986). Proteoglycan core protein families. Annu. Rev. Biochem. 55, 539-567.

Höök M., Kjellen L., Johnson S. and Robinson J. (1984). Cell surface glycosaminoglycans. Annu. Rev. Biochem. 53, 847-869.

Hynes R.O. (1987). Integrins: A family of cell surface receptors. Cell 40, 549-554.

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

Jalkanen M., Rapraeger A., Saunders A. and Bernfield M. (1987). Cell surface proteoglycan of mouse mammary epithelial cells is shed by cleavage of its matrix-binding ectodomain from its membrane-associated domain. J. Cell Biol. 105, 3087-3096.

Kelly T., Molony L. and Burridge K. (1987). Purification of two smooth muscle glycoproteins related to integrin. J. Biol. Chem. 262, 17189-17199.

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

Nermut M.V., Green N.M., Eason P., Yamada S.S. and Yamada K.M. (1988). Electron microscopy and structural model of human fibronectin receptor. EMBO J. 7, 4093-4099.

Rapraeger A., Jalkanen M. and Bernfield M. (1986). Cell surface proteoglycan associates with the cytoskeleton at the basolateral cell surface of mouse mammary epithelial cells. J. Cell Biol. 103, 2683-2696.

Rapraeger A., Jalkanen M. and Bernfield M. (1987). Integral membrane proteoglycans as matrix receptors: roles in cytoskeleton and matrix assembly at the epithelial cell surface. In: Biology of extracellular matrix. Vol. II. Biology of proteoglycans. Wight T.N. and Mecham R.P. (eds). Academic Press. New York. pp 129-154.

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, 491-487.

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, 428-441.

Shakibaei M., Schröter-Kermani C. and Merker H.-J. (1993a). Matrix changes during long-term cultivation of cartilage. Histol. Histopath. 8, 463-470.

Shakibaei M., Abou-Rebyeh H. and Merker H.-J. (1993b). Integrins of the $ß_1$-type in cartilage tissue *in vitro* (an immunomorphological study). Cell Tissue Res. submitted.

Trelstad R.L., Catanese V.M. and Rubin D.F. (1976). Collagen fractionation: Separation of native types I, II, and III by differential precipitation. Ann. Biochem. 71, 114-118.

Trowell O.A. (1959). The culture of mature organs in synthetic medium. Exp. Cell Res. 16, 118-147.

von der Mark K. and von der Mark H. (1977). Immunological and biochemical studies of collagen type transition during *in vitro* chondrogenesis of chick limb mesodermal cells. J. Cell Biol. 73, 736-747.

von der Mark H., von der Mark K. and Gay S. (1976). Study of differential collagen synthesis during development of the chick embryo by immunofluorescence. Dev. Biol. 48, 237-249.

Zimmermann B. (1987). Lung organoid culture. Differentiation 36, 86-109.

Zimmermann B., Wachtel H.C., Somogyi H., Merker H.-J. and Bernimoulin J.-P. (1988). Bone formation by rat calvarial cells grown at high density in organoid culture. Cell Differ. Dev. 25, 145-154.

Zimmermann B., Wachtel H.C. and Somogyi H. (1990). Endochondrial mineralization in cartilage organoid culture. Cell Differ. Dev. 31, 11-22.

Accepted May 14, 1993