

ADHESION RATES AND CHONDROCYTE PHENOTYPE IN MONOLAYER CULTURES UNDER THE INFLUENCE OF COLLAGEN TYPE II AND IGF-I

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Abstract - When grown in monolayer culture at low density and with the addition of serum, cartilage cells dedifferentiate or are overgrown by fibroblast-like cells. The aim of this study was to optimize the cultivation of chondrocytes in monolayer culture and to slow down their transformation or their overgrowth by fibroblast-like cells. The interaction between chondrocytes and cartilage-specific matrix is mediated largely by the $\beta 1$ subfamily of integrin receptors. This interaction can be regulated or synergized by growth factors, such as IGF-I, which stimulates many chondrocyte functions.

For this reason isolated chondrocytes of cartilage anlagen from 17-day old mouse embryos were grown on collagen type II-coated substrates with or without the addition of IGF-I (Insulin Like Growth Factor-I). Using this model chondrocytes grown on collagen type II with or without IGF-I maintained their round phenotype until the end of cultivation. The neutral red assay revealed that chondrocytes cultivated on collagen type II in the presence of exogenous IGF-I, showed a significantly higher density of chondrocyte adhesion from the beginning of cultivation onwards in comparison to chondrocytes cultivated on collagen type II without IGF-I treatment. The cartilage-specific surface receptors (integrins of the $\beta 1$ -group) could also be demonstrated on the membrane of these cells. Chondrocytes cultivated on plastic with or without treatment with IGF-I, resulted in mixed cultures consisting of fibroblast-like cells and round chondrocytes as observed from the beginning of cultivation onwards. After a 3 to 5 days culture period, flat fibroblast-like cells predominated.

Hence, collagen type II and IGF-I prevent chondrocyte dedifferentiation to fibroblastlike cells and this model allows a pure chondrocyte culture.

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dedifferentiate to fibroblast-like cells. They lose their polygonal phenotype and become spindle-like shape that resembles that of fibroblasts. Under these conditions, they produce collagen type I instead of type II and fewer proteoglycans (1,8).

Differentiation of chondrocytes in culture is supposed to be dependent on the culture conditions, such as cell density and medium, as well as on hormones and growth factors (8,11). It is known that hormones (12,14) and growth factors (15,17), interleukins (18), prostaglandins (19,20) and bone morphogenetic protein (BMP) (21,24) may influence differentiation, metabolism and activity of chondrocytes or stabilize their phenotype. The matrix, too, is of great significance for the regulation of differentiation and function (25,26). The IGF-I (Insulin Like Growth Factor-I) used for our experiments also stimulates proliferation and differentiation of chondrocytes in vivo and in vitro (27,34) and matrix synthesis (incorporation of sulphate into proteoglycans).

Hence, the morphology of cartilage cells is the result of an intensive interaction between cells and their surrounding. This interaction is partly mediated by cell membrane receptors, e.g. integrins which are heterodimeric transmembrane molecules composed of an α - and a β -subunit. They consist of a large extracellular, a transmembranous and a short cytoplasmic domain. The extracellular domain is able to bind to various extracellular matrix ligands, such as collagens, fibronectin, laminin and vitronectin (35,37). Integrins of the $\beta 1$ -group have been demonstrated in cartilage tissue in vivo as well as in vitro (38,44).

The phenotype of cells is an important indication of the function of cells in vitro. Flattening of the mostly round chondrocytes with the formation of a fibroblast-like phenotype is accompanied by a change of the synthesis programme, e.g. synthesis of collagen types I and III (45,46). Rounding of the cells, however, is associated with the synthesis of collagen type II and cartilage-specific proteoglycans (45,47,48).

The results of this study indicate that collagen type II-coating and addition of the growth factor IGF-I influence proliferation, the adhesion behaviour of cells and the maintenance of the chondrocyte phenotype in vitro.

INTRODUCTION

When chondrocytes are grown in monolayer culture on dishes coated with collagen type I or on plastic, they

MATERIALS AND METHODS

Antibodies: (a) Polyclonal antibody against $\beta 1$ was obtained from Chemicon International, Inc., Temecula, CA, USA. Collagen types I and II were isolated and purified according to Engvall and Ruoslahti (49). Polyclonal antibodies against these collagens were raised in rabbits; specificity was controlled by the ELISA technique (50). (b) GAR-10 nm-conjugated goat-anti-rabbit immunoglobulin with 10 nm gold particles was obtained from Amersham (Braunschweig, Germany).

Ham F-12 nutrient mixture containing 15% FCS, 75 μ g/ml ascorbic acid, 50 IU/ml streptomycin, 50 IU/ml penicillin and 2.5 μ g/ml amphotericin B (Seromed, Munchen, Germany). Nuncleon dishes: 35 mm \times 10 mm; microtiter plates (96-well plates) (Nunc Inter Med, Denmark); Epon and Thermanox (Piano, Marburg, Germany). HMDS: hexamethyldisilazan, neutral red, trypsin (EG 3.4.21.4), IGF-I and Collagen type II for coating (Sigma, Munchen, Germany), collagenase (from clostridium histolyticum, 0.15 IU/mg, Boehringer, Mannheim, Germany).

1. Chondrocyte cultures

Chondrocyte cultures of epiphyseal cartilage have been described in detail by Grundmann et al (5) and Shakibaei (7). Briefly, skin of upper and lower limb buds from 17-day-old mouse embryos were removed and the remaining parts placed into Hanks salt solution. Muscle and connective tissue were removed by trypsin treatment (0.2%, twice for 30 min each at 37°C). Epiphyseal cartilage was separated from the anlage of the long bones and shaken in 0.2% collagenase in Hanks BSS for 60 min at 37°C. After centrifugation, the cells were washed twice with Ham F-12 growth medium. Subsequently, the washed chondrocytes were resuspended in Ham F-12, counted and their number adjusted to 1.5×10^6 /ml. The cells were grown in monolayer culture, for scanning electron and light microscopic investigations on Thermanox and glass plates, for transmission electron microscopic investigations in Petri dishes and for the neutral red assay on microtiter plates. Cultivation was performed as follows: on collagen type II coated substrates with/without IGF-I (100 ng/ml). The cultures were grown in an incubator at 37°C and with 5% CO₂. The medium was changed every 3 days. Cells of day 1 to day 17 of the culture period served for our investigations.

2. Collagen coating

Thermanox, glass and microtiter plates (96-well plates) and Petri dishes were coated with collagen type II (0.5 - 2 mg/ml in 0.25% acetic acid), air-dried and placed under UV-light over night. The plates were

washed three times with PBS prior to use.

3. Transmission electron microscopy (TEM)

The cartilage cultures were fixed in Karnovsky's solution or in a mixture of 1% glutaraldehyde plus 1% tannic acid in PBS followed by post-fixation in a 2% OsO₄ solution. After rinsing and dehydration the material was embedded in Epon and cut using an Ultracut (Reichert). The ultrathin sections were contrasted with 2% uranyl acetate and lead citrate and examined under a Zeiss EM10 transmission electron microscope.

4. Immunoelectron microscopy

Pre-embedding technique for TEM: For the immunolabelling of collagen type II and fibronectin the cells were incubated with hyaluronidase (5000 IU/ml) for 15 min for 3 min at RT. After two times rinsing with medium, the cells were incubated in medium with the primary antibodies (collagen type II 1:30; fibronectin 1:30 and integrin $\beta 1$ 1:30 in medium) for 10 min at 37°C. This was followed by rinsing with medium for 3 \times 5 min and incubation with the secondary antibody (GAR-10 nm 1:30) for 10 min at 37°C. Subsequently, the cells were washed with medium and fixed with 2% glutaraldehyde for 5 min. Contrasting, dehydration, embedding, sectioning and microscopic examination were performed as described above.

5. Scanning electron microscopy (SEM)

The chondrocytes grown on Thermanox plates were fixed with 1% glutaraldehyde for 5 min, followed by post-fixation with 1% OsO₄ for 5 min, dehydration with ethanol in the ascending alcohol series, 5 min incubation in HMDS and then air-dried. The samples were gold-coated with a Technics Hummer V and examined under a Cambridge Stereoscan (250 MK2) scanning electron microscope at 20 kV.

6. Neutral red assay

The freshly isolated chondrocytes were grown on microtiter plates coated with collagen type II. One part of the holes was treated with IGF-I (100 ng/ml) from the beginning of cultivation onwards. The adherent chondrocytes were quantified every 24 hours using the neutral red assay. After rinsing with medium (3 times), 100 μ l neutral red medium (0.4% neutral red in medium without serum) were placed into each hole and incubated for 2 hours at 37°C. Only adherent vital chondrocytes stained. After incubation, the medium was removed, the cells were fixed with 2% paraformaldehyde for 2 min and incubated with ethanol and 1% acetic acid. The microtiter plate was shaken for 20 min and the released stain measured using a microplate reader (SLT-Labinstruments, Austria) and a 540 nm filter.

RESULTS

1. Scanning electron microscopy

The chondrocytes grown on collagen type II with or without IGF-I always exhibited a round to oval shape and numerous small ridge-like or cuspidal surface processes from the beginning of cultivation onwards (Fig. 1a). After 3 to 5 days, the number and density of the chondrocytes had increased. After a culture period of 17 days the remaining chondrocytes had maintained their round to oval shape. They were embedded in a network of newly formed collagenous fibrils. In the second week of the culture period, more and more cells detached from the substrate leaving a network of fibrillar material, partly in the form of a pericellular capsule. Chondrocytes cultivated on collagen type II in presence of exogenous IGF-I, showed a significantly higher density of chondrocyte adhesion from the beginning of cultivation onwards in comparison to chondrocytes cultivated on collagen type II without treatment with IGF-I (Fig. 1b). In experiments without collagen type II and with or without IGF-I, a mixed

culture consisting of fibroblast-like cells and round chondrocytes was observed from the beginning onwards. After a 3 to 5 days culture period, flat fibroblast-like cells predominated (7).

2. Transmission electron microscopy

(a) One day after the beginning of cultivation the cartilage cells exhibited a round shape and contained a big nucleus, numerous cell organelles, a well-developed rough endoplasmic reticulum and a large Golgi apparatus. Their surface showed small ridge-like and cuspidal processes. After a 2 days culture period a thin, pericellular matrix rim consisting of fine (<10 nm) irregularly running filaments could be demonstrated extracellularly on the cell surface (Fig. 2a). In the following days the quantity and thickness of fibrils in this extracellular rim increased, but the morphology of the cells did not change until the end of cultivation. The cells were surrounded by a capsule of typical collagenous fibrils which were arranged mainly singly and irregularly but more or less parallel to the surface.

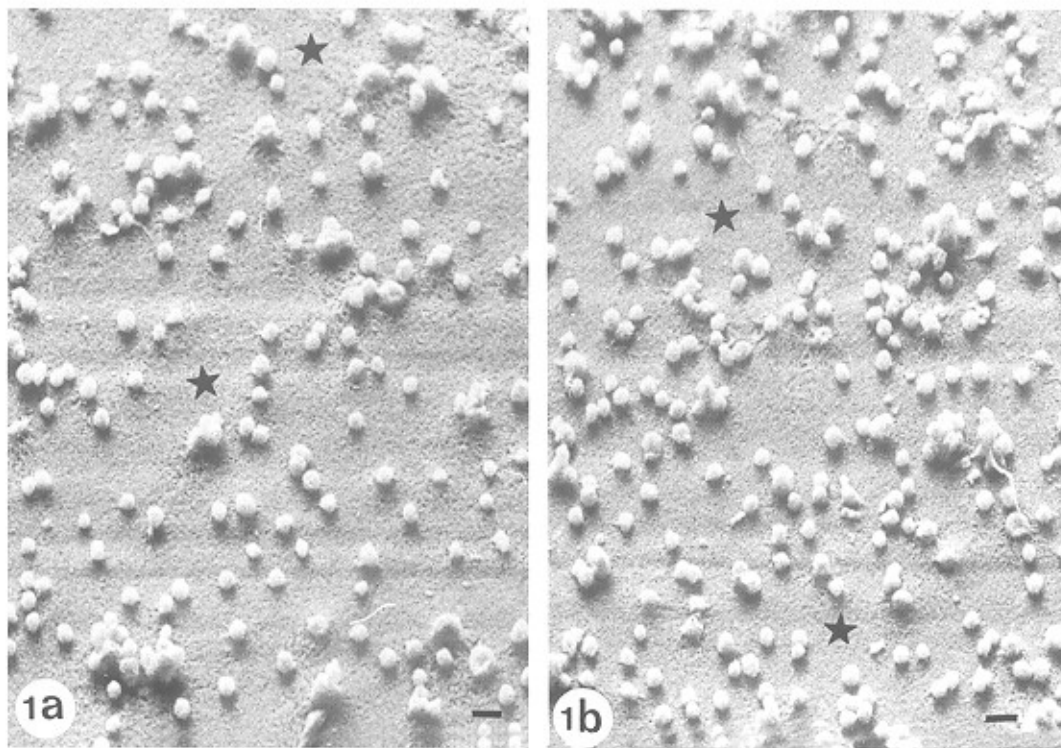


Fig. 1a-b. Scanning electron microscopic investigations of chondrocytes in monolayer culture.

a: Culture period: 7days, when the chondrocytes are cultured on collagen type II (*), the dedifferentiation did not occur and the cells maintained their round phenotype.

b: Culture period: 7days, when the chondrocytes are cultivated on collagen type II with addition of IGF-I, it revealed a higher density of the chondrocytes showing an improved adhesion and higher proliferation rate. a= x 350, bar = 20 μ m; b = \times 350, b = 20 μ m.

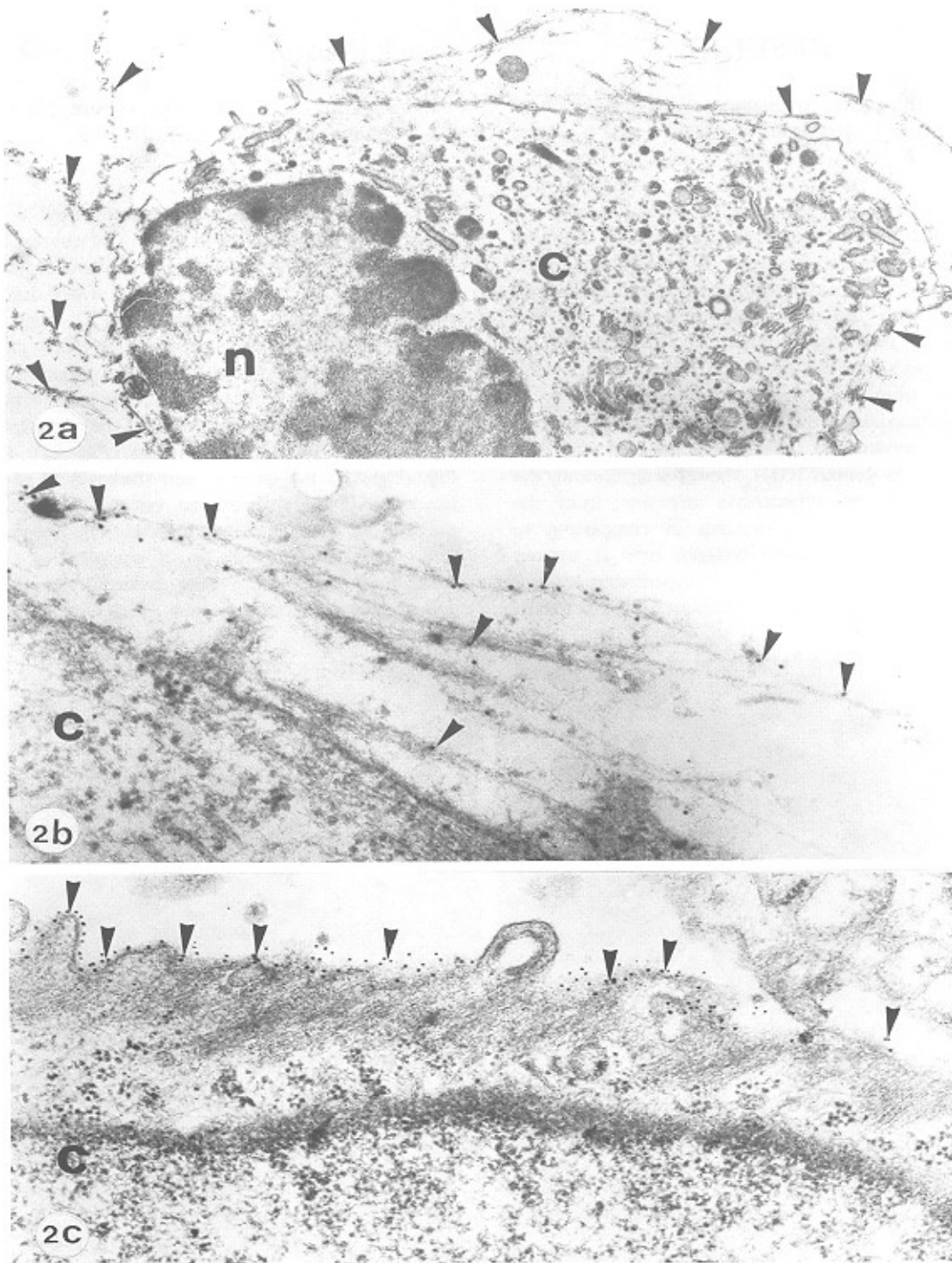


Fig. 2a-c.Transmission and immunotransmission electron microscopic investigations of chondrocytes after a 2 days culture period in monolayer culture.

a: Chondrocytes (C) showed a round to oval shape and formed a thin matrix rim on their surface (arrowheads), n = nucleus, X 15000, b= 0.5 μ m.

b: Immunolabelling against collagen type II (arrowheads) on the surface of a chondrocyte revealed a cartilage-specific matrix. X 60000, bar = 0.2 μ m.

c: Immunolabelling of chondrocytes against integrin β_1 . A dense labelling (arrowheads) is seen on the surface of the chondrocytes. X 45000, b = 0.2 μ m.

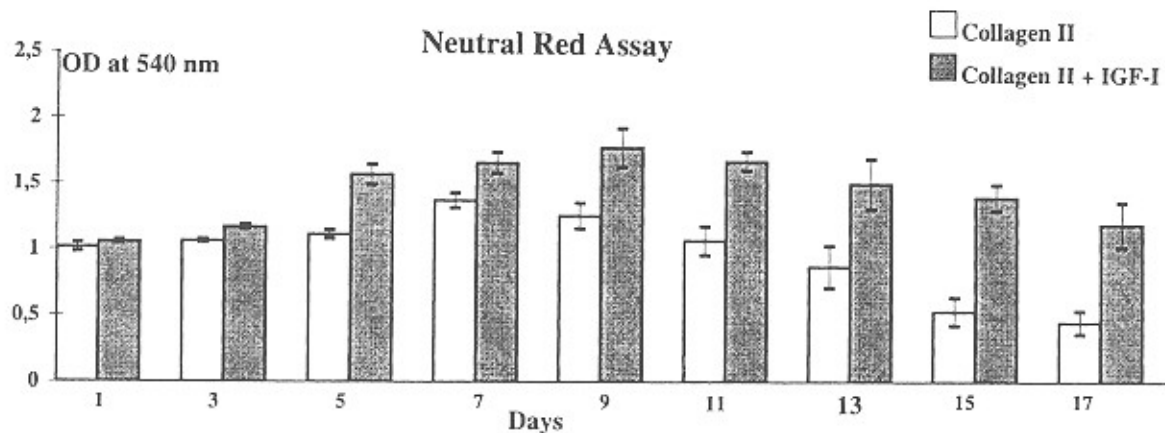


Fig. 3. Neutral red adsorption at 540 nm. The effect of collagen type II-coating and IGF-I on the number of chondrocytes in monolayer culture. The adherent chondrocytes grown on collagen type II without IGF-I showed a weaker absorption of neutral red than those chondrocytes treated with IGF-I. In both experiments an absorption of neutral red was achieved up to a 17 days culture period. The mean values and standard deviation from three independent experiments are indicated.

OD = Optical density.

(b) Immunomorphological investigations using the "pre-embedding technique" and secondary gold-conjugated antibodies against primary collagen and fibronectin antibodies showed that the pericellular fibrils consisted of collagen type II (Fig. 2b) and that fibronectin was located between the collagenous fibrils (not shown). This observation could be made as early as after a 2 days culture period. Immunomorphological investigations did not reveal collagen type I in the cartilage matrix when grown on a collagen type II substrate. Integrins β_1 (Fig. 2c) were observed on the surface of the chondrocytes from the first until the last day of the culture period.

3. Neutral red assay

The experiments with collagen type II coating but without IGF-I revealed an increase in neutral red content, i.e. in the number of cells (see Discussion), until days 8/9. Thereafter, the neutral red content decreased until the end of the culture period (day 17). Chondrocytes on collagen type II which had been treated with the growth factor IGF-I showed an increase in the cell number from the beginning of cultivation onwards as compared with the cells not treated with IGF-I (Fig. 3). The experiments without collagen type II coating shall not be considered in this context, since we observed a mixed population or a pure population of fibroblast-like cells.

Taken together, the neutral red assay is based on an active staining of lysosomes (51,55). Any changes in the storage of neutral red might, therefore, may be due to an inhibition or stimulation of the lysosomal system.

However, assuming a constant activity of the lysosomal system, the changes in the values can be attributed to alterations in the cell number. These results indicate a positive effect of collagen type II and IGF-I on the stabilisation of chondrocyte differentiation and adhesion.

DISCUSSION

Chondrocytes grown in monolayer culture at low density and with the addition of serum either dedifferentiate, whereby their cell shape changes, or they are overgrown by fibroblast-like cells (1,8,55,60).

It was shown that chondrocytes, when grown on collagen type II with the addition of IGF-I, are able to survive for a long period without changes in their morphology and the secretion product. Under these conditions the chondrocytes produce collagen type II and express a pattern of surface receptors (integrins of the β_1 -group). The interaction between extracellular matrix and cells via receptors can regulate the behaviour of chondrocytes. It leads to an activation and stabilisation of cartilage cells (40,41,61,64). Moreover, it is known that IGF-I influences differentiation, proliferation and matrix formation in vivo and in vitro, thus being a regulator of cartilage behaviour (28,30,31,33,34,65).

The neutral red assay is based on an active staining of lysosomes (51,55). Any changes in the storage of neutral red might, therefore, be due to an inhibition or

stimulation of the lysosomal system. However, at constant activity the changes in the values can be attributed to alterations in the cell number. Electron microscopic inspection did not confirm an influence on the lysosomal system so that a regular staining could be assumed and the finding be ascribed only to a change in the cell number. The data obtained 24 hours after beginning of cultivation and after addition of the stain must mainly be ascribed to the adhesion behaviour, since it takes at least 24 hours before the chondrocytes have overcome the isolation and cultivation shock and proliferation is triggered. This explanation is confirmed by the data obtained as early as after 1 hour after the beginning of cultivation (not shown here). In contrast, the changes in the values measured after 2 to 17 days could be attributed predominantly either to proliferation or to necrosis.

When grown on plastic substrates, numerous fibroblast-like cells adhere from the beginning of cultivation onwards. Cartilage from 17-day-old mouse fetuses undoubtedly consist of a heterogeneous cell population (5,7,66). Fibroblasts attached to the outside of cartilage and perichondral cells are certainly also released due to enzymatic treatment. A great number of the fibroblast-like cells which increase in number in the absence of collagen coating derives from this source, i.e. we are dealing with impurities and later on with overgrowth. However, it could be shown with certainty that round chondrocytes grow on plastic substrates dedifferentiate after a couple of days (5,7,56,58). Hence, some of the fibroblast-like cells might have been formed in this way. It has to be considered that due to fibroblast-like cells other collagen types, proteoglycans, factors and matrix-degrading enzymes get into the medium and additionally influence the behaviour or dedifferentiation of the chondrocytes. Hence, the absence of fibroblast-like cells on collagen coating after 10 days *in vitro* largely depends on the missing overgrowth and its possible consequences. On the other hand, a positive effect of collagen type II on the stabilisation of chondrocyte differentiation can, apart from the better adhesion and higher proliferation rate, not be excluded.

The preferential adhesion of chondrocytes on collagen type II-coated substrates and the missing adhesion of fibroblast-like cells indicate a specific mechanism. Some integrins of the 131-group are available on the surface of chondrocytes for a specific binding. This integrin pattern occurs in cartilage *in vivo* and *in vitro* (7,38,44,64). Consequently, any blockage of integrins of this group should fundamentally influence the adhesion behaviour. And indeed, adhesion on collagen type II can clearly be reduced by antibodies against integrins. Integrin 131- antibodies block the adhesion of chondrocytes to collagen type II and these speak for a participation of integrins in the adhesion

process (40,41,67).

The positive effect of IGF-I on proliferation, differentiation and function of chondrocytes is well known (30,33,34,65,68). When cartilage cells are grown on plastic substrates this effect is not seen, in other words, 5 days after the beginning of cultivation and the use of IGF-I only fibroblast-like cells still occur. However, when grown on collagen type II with or without IGF-I, all cells show a round shape and cartilage-specific functions, as revealed by electron microscopy and immunomorphology. After 9 days, more and more cells become necrotic without any indication of a preceding dedifferentiation, i.e. flattening. The effect of IGF-I is shown morphologically by the greater number of chondrocytes. Since after 1 or 24 hours without the addition of IGF-I the cell numbers are distinctly lower, a positive effect of this substance on the adhesion behaviour can be assumed. Since, on the other hand, after 9 days the effect of IGF-I on the cell number is larger, an increase in the proliferation rate can additionally be supposed. Later on, from day 9 to 17 with or without IGF-I the cell numbers continuously decrease which can be attributed to necrotic processes. The mechanism of necroses, however, cannot be assessed with certainty, since most of the cells detach from the substrate at this stage. The few cells which could be demonstrated during necrosis showed indications of a swelling of the organelles and fragmentation of the cell membrane. Therefore, apoptosis most probably does not occur. The difference in the IGF-I effect between plastic and collagen-coated substrates cannot be explained with morphological means. Obviously, the chondrocytes must have fully differentiated in order to respond to IGF-I and other cell types (fibroblast-like cells) must not counteract with factors etc. The absence of dedifferentiation processes of chondrocytes grown on collagen type II in monolayer culture and the immediate transition to necrosis are unusual findings. The collagen/cell contact apparently stabilizes differentiation until the 10 onset of necrosis. The rate of necrosis with and without IGF-I addition then runs parallel. Since, however, more cells are present after the addition of IGF-I, more cells are still available at the end of cultivation (e.g. day 17) and the culture can be used for a longer period.

The different behaviour of the cells after IGF-I treatment in dependence on the substrate can hardly be explained by our present knowledge of the IGF-I mode of action. IGF-I activates tyrosine receptor kinase via its binding to the receptor. This tyrosine receptor kinase, on its part, activates other protein kinases. During these processes enzymes or transcription factors are activated or inactivated and glucose homeostasis, proliferation and differentiation processes are regulated (69,70).

Thus, it is possible to preserve a pure chondrocyte

monolayer culture for more than 1 week on collagen type II-coated substrates. Treatment with IGF-I increases the amount of chondrocytes and extends its usability to 3 weeks. These findings can be explained by the different adhesion behaviour of chondrocytes and fibroblast-like cells on collagen type II-coated substrates and the higher adhesion and proliferation rates after addition of IGF-I.

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