BIOCHEMICAL PHARMACOLOGY 72 (2006) 1382–1395

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Igf-I extends the chondrogenic potential of human articular chondrocytes *in vitro*: Molecular association between Sox9 and Erk1/2☆

Mehdi Shakibaei [a,*], *Claudia Seifarth* [a], *Thilo John* [b], *Masyar Rahmanzadeh* [c], *Ali Mobasheri* [d]

[a] *Institute of Anatomy, Ludwig-Maximilians-University, Pettenkoferstrasse 11, 80336 Munich, Germany*
[b] *Charité Medicine University, Berlin, Germany*
[c] *Joint Surgery Center Berlin, Berlin, Germany*
[d] *Division of Veterinary Medicine, The School of Veterinary Medicine and Science, The University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, United Kingdom*

ARTICLE INFO

Article history:
Received 13 July 2006
Accepted 22 August 2006

Keywords:
Chondrocyte
Igf-I
Apoptosis
Sox9
Erk1/2
High-density culture
Immunoprecipitation

ABSTRACT

Expansion of articular chondrocytes in monolayer culture leads to loss of the unique chondrocyte phenotype and the cells' redifferentiation capacity. Dedifferentiation of chondrocytes in monolayer culture is a challenging problem for autologous chondrocyte transplantation (ACT). It is well established that Igf-I exerts positive anabolic effects on chondrocytes *in vivo* and *in vitro*. Accordingly, in this study, we examined whether the anabolic insulin-like growth factor-I (Igf-I) is capable of extending the chondrogenic potential of dedifferentiated chondrocytes *in vitro*. Chondrocyte monolayers were cultured up to 10 passages. At each passage chondrocytes were stimulated with Igf-I (10 ng/ml) and introduced to high-density cultures for up to 7 days. Expression of collagen type II, cartilage-specific proteoglycans, activated caspase-3, integrin β1, extracellular signal-regulated kinase (Erk) and Sox9 was examined by Western blotting, immunoprecipitation and immunomorphological techniques. Monolayer chondrocytes rapidly lost their differentiated phenotype. When introduced to high-density cultures, only chondrocytes from P1–P4 redifferentiated. In contrast, Igf-I treated cells from P1 up to P7 redifferentiated and formed cartilage-like tissue in high-density culture. P8–P10 cells exhibited apoptotic alterations and produced significantly less matrix. Igf-I markedly increased expression of integrin β1, Erk and Sox9. Immunoprecipitation revealed that phosphorylated Erk1/2 physically interacts with Sox9 in chondrocyte nuclei, suggesting a previously unreported functional association which was markedly enhanced by Igf-I. Treatment of chondrocyte cultures with Igf-I stabilizes chondrogenic potential, stimulates Sox9 and promotes molecular interactions between Erk and Sox9. These effects appear to be regulated by the integrin/MAPK signaling pathways.

© 2006 Elsevier Inc. All rights reserved.

☆ The research was conducted in part for the doctoral thesis of Claudia Seifarth to be submitted to Fachbereich Humanmedizin, Charite Medicine University, Berlin, Germany.

* *Corresponding author at*: Institute of Anatomy, Musculoskeletal Research Group, Ludwig-Maximilians-University Munich, Pettenkoferstrasse 11, D-80336 Munich, Germany. Tel.: +49 89 5160 4827; fax: +49 89 5160 4828.
E-mail address: mehdi.shakibaei@med.uni-muenchen.de (M. Shakibaei).

0006-2952/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2006.08.022

1. Introduction

OsteoArthritis (OA) is a degenerative disorder which is characterized by degradation of extracellular matrix macromolecules and loss of joint function. In the last decade, the technique of autologous chondrocyte transplantation (ACT) has been introduced for cartilage repair. ACT relies on isolation of chondrocytes from the patient, *ex vivo* culture and expansion in the laboratory and implantation into the cartilage defect [1]. The success of this approach correlates with the capacity of implanted chondrocytes to produce a functionally and mechanically competent hyaline cartilage matrix consisting of collagen type II and cartilage-specific proteoglycans (CSPG) [2]. However, the main drawback associated with ACT is that during proliferation in monolayer culture chondrocytes rapidly dedifferentiate [3–7]. Dedifferentiated chondrocytes develop a fibroblast-like appearance and produce collagen type I, a matrix component not normally found in articular cartilage [8,9]. Recent studies have shown that dedifferentiated human chondrocytes are able to redifferentiate toward the chondrogenic lineage upon transfer to a three-dimensional culture system [10]. However, prolonged proliferation in monolayer culture leads to irreversible loss of differentiated functions and chondrogenic capacity resulting in cells that are biochemically incompetent for implantation and cartilage defect repair [4,5]. It is therefore of considerable practical and clinical interest to prolong the redifferentiation capacity of chondrocytes *in vitro* and to increase the pre-requisite cell yield for improving the chances of ACT success. In recent studies we have shown that dedifferentiated human chondrocytes from P1 to P4 maintain the capacity to redifferentiate if transferred to high-density or alginate culture system and form a cartilage-specific matrix. We have also shown that loss of chondrogenic potential is accompanied by reduced expression of key signaling proteins in the mitogen-activated proteins (MAP) kinase (MAPK) pathway and apoptosis [11,12].

It is becoming increasingly accepted that anabolic growth factors influence important cellular processes including differentiation, growth and survival [13–15]. For example, transforming growth factor-β (TGF-β) is abundantly expressed in normal articular cartilage [16] and augments cell proliferation and production of matrix proteins [17]. Fibroblast growth factor-2 (FGF-2) has been demonstrated to maintain the chondrogenic potential during chondrocyte expansion in monolayer culture [18] and induces growth arrest and differentiation [19]. Insulin like growth factor-I (Igf-I) is capable of stimulating chondrocyte differentiation and survival and stimulating matrix synthesis *in vivo* and *in vitro* [14,20–22]. In contrast to other growth factors Igf-I more potently influences the differentiation and proliferation state of chondrocytes; Igf-I strongly promotes chondrogenesis in progenitor cells *in vitro* [23]. We have previously shown that Igf-I plays a role in chondrocyte differentiation; in response to Igf-I stimulation the Igf-I receptor activates key signaling proteins of the MAPK pathway [24]. Adult human articular chondrocytes *in vivo* contain high Sox9 mRNA levels [25]. Sox9 has been strongly implicated in the regulation of cartilage-specific matrix components in chondrocytes (such as collagen type II and aggrecan) and may play an important role in chondrocyte differentiation [26–29], but other co-factors are also important for collagen type II promotor activation [25,30,31]. Indeed, it has been reported that Sox9 expression does not positively correlate with collagen type II expression in adult articular chondrocytes [25]. However, Sox9 is implicated in prevention of hypertrophic terminal differentiation of chondrocytes [25,30].

Thus, in this study we tested the hypothesis that Igf-I extends the redifferentiation capacity of dedifferentiated chondrocytes by modulating the expression of components of the MAPK pathway and promoting the function of the chondrogenic transcription factor Sox9.

2. Materials and methods

2.1. Antibodies

Antibodies against collagen type II (AB746), cartilage-specific proteoglycan (MAB 2015) and β1-integrin (MAB1977) were purchased from Chemicon International (Temecula, CA, USA). Antibodies to active caspase-3 (AF835) were from R&D Systems, Inc., (Heidelberg, Germany). Sox9 antibody was purchased from Acris Antibodies GmbH (Hiddenhausen, Germany). Antibodies to phospho-p42/p44 Erk1/2 were purchased from Promega (Mannheim, Germany). Anti-pan Erk1/2 antibodies were purchased from Transduction Laboratories (Heidelberg, Germany). Gold particle conjugated secondary antibodies were purchased from Amersham (Braunschweig, Germany).

2.2. Cell culture and growth media

Growth medium (Dulbecco's modified Eagle's/Ham's F-12 medium (50/50) containing 0.1/10% fetal calf serum, 25 μg/ml ascorbic acid, 50 IU/ml streptomycin, 50 IU/ml penicillin and 2.5 μg/ml amphotericin B, 1% glutamine and 1% essential amino acids) was obtained from Seromed (Munich, Germany). Alginate, collagenase, O.C.T. compound embedding medium and trypsin/EDTA (EG 3.4.21.4) were purchased from Sigma (Munich, Germany). Plano (Marburg, Germany) provided Epon. Igf-I was purchased from Biomol (Hamburg, Germany). The APAAP-Kit was derived from Dako (Carpinteria, CA, USA).

2.3. Chondrocyte isolation and culture

Primary cultures of human chondrocytes were prepared from articular cartilage of femoral heads obtained during joint replacement surgery for femoral neck fractures as described previously [24]. Twelve samples of cartilage derived from the same donor were used for these investigations. Cartilage samples were derived from the human patient with full informed consent and local ethics committee approval.

2.4. Igf-I treatment

Chondrocytes in monolayer culture were washed three times with serum-free medium. Following stimulation with Igf-I (10 ng/ml in serum-free medium) for 24 h, every third day the confluent monolayer culture cells were passaged, finally reaching passage 10. Cells from each monolayer passage were

grown in high-density cultures. Briefly, cells were washed twice in growth medium and pelleted by centrifugation (600 rpm for 10 min). Ten microliter aliquots of the sedimented cells ($\sim 2 \times 10^6$ cells/10 μl) were pipetted onto a membrane filter with a pore diameter of 0.2 μm (Sartorius, Göttingen, Germany) on top of a stainless steel grid at the medium-air interface in a Petri dish. The same procedure was used for untreated controls. Cultures were grown at 37 °C in a humidified atmosphere with 5% CO_2 and growth medium was changed every third day.

2.5. Light microscopy

One-micrometer-thick sections were prepared from the Epon-embedded high-density culture, stained with toluidine blue (Merck, Darmstadt, Germany), rinsed with distilled water, and air-dried. The slices were cover-slipped with Kaisers' glycerol gelatin before examination under a light microscope (Axiophot, Zeiss, Germany) and digital photomicroscopy.

2.6. Alkaline phosphatase anti-alkaline phosphatase (APAAP) technique

Alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was performed as described earlier [5,6,32].

2.7. Isolation of chondrocyte nuclei

Cells were trypsinized and washed 2× in 1 ml ice cold PBS buffer. After removal of the supernatant, the pellet was resuspended in 400 μl hypotonic lysis buffer containing protease inhibitors and incubated on ice for 15 min. 12.5 μl of 10% NP-40 were added and the cell suspension vigorously mixed for 15 s. The extracts were centrifuged for 1.5 min. The supernatants were removed and 25 μl ice cold nuclear extraction buffer were added to the pellets and incubated for 30 min with intermittent mixing. Extracts were centrifuged and the supernatant (nuclear extracts) transferred to pre-chilled tubes for storage at −70 °C. The purity of the preparations was determined by transmission electron microscopy.

2.8. Transmission electron microscopy

Transmission electron microscopy was performed as described earlier [5,6,32]. For statistical analysis, ultrathin sections of the samples were prepared and evaluated with an electron microscope (Zeiss EM 10). The number of cells with morphological features of apoptotic cell death was determined by scoring 100 cells from 20 different microscopic fields.

2.9. Immunoelectron microscopy

A detailed description of the culture technique used for immunoelectron microscopy has been published [4,33]. After fixation in 3% formaldehyde (freshly prepared from paraformaldehyde plus 0.25% glutaraldehyde) in PBS for 1 h, the cultures were washed with PBS/1% BSA, dehydrated in ethanol and embedded in LR-white. Ultrathin sections were cut and treated with the following solutions: (1) 1% BSA at AT for 30 min; (2) testicular chondroitinase (5000 U/ml) for 5 min at AT to unmask epitopes; (3) PBS/1% BSA/0.5% Tween 20 2× 5 min at AT; (4) primary antibodies (1:50 in PBS/1% BSA/0.5% Tween 20) overnight at 4 °C; (5) PBS/BSA/Tween for 2× 5 min at AT; (6) secondary antibodies conjugated with goat anti-rabbit immunoglobulin with 10 nm gold particles (1:50 for 30 min) at AT; (7) after rinsing for 2× 5 min at AT; (8) contrasting was carried out with 1% tannic acid for 20 min at AT, with OsO_4 for 10 min and with 2% uranyl acetate for 30 min. Finally, the sections were rinsed and examined under a transmission electron microscope (TEM 10, Zeiss, Germany).

2.10. Immunoblotting

Western blotting was performed as described earlier [24,32].

2.11. Immunoprecipitation of Sox9 and Erk

For immunoprecipitation, the extracts were precleared by incubation with 25 μl of normal rabbit IgG-serum or normal mouse IgG-serum and *Staphylococcus aureus* (*S. aureus*) cells, incubated with primary antibodies diluted in wash buffer (0.1% Tween 20, 150 mM NaCl, 50 mM Tris–HCl, pH 7.2, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF) for 2 h at 4 °C followed by *S. aureus* cells for 1 h at 4 °C. Control immunoprecipitations were performed by incubating the samples with rabbit anti-mouse IgG alone. *S. aureus* cells were washed five times with wash buffer and once with 50 mM Tris–HCl, pH 7.2 followed by boiling in SDS-PAGE sample buffer. Samples were separated on SDS-polyacrylamide gels under reducing conditions as described above.

2.12. Statistical analysis

The results are expressed as the means ± S.D. of a representative experiment performed in triplicate. The means were compared using student's *t*-test assuming equal variances. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Chondrocyte proliferation with or without Igf-I treatment in monolayer culture

Primary human chondrocytes were cultivated in alginate beads. During the first 4–7 days of culture, some cells migrated from alginate beads and adhered to Petri dishes reaching confluence 3 days later. At this point it is important to highlight the rationale for adopting this approach which is a critical aspect of our experimental design. We have previously shown that alginate migrated cells are fully differentiated chondrocytes and uniquely possess chondrogenic potential. Our ultrastructural studies have demonstrated that alginate culture may be used as a selective filter to separate motile cells with chondrogenic potential from dying and degenerating cells trapped within the alginate bead [11,12]. This monolayer (P1) was passaged every 3 days until passage P10 with or without Igf-I. The alginate migrated chondrocytes exhibited a typical chondrocytic round shape and proliferated rapidly (data not shown). During Igf-I-stimulation, the cells did not

show any morphological features indicative of degeneration at the light and electron microscopic levels.

3.2. *Igf-I is able to extend the viability of chondrocytes in culture*

High-density cultures were prepared from chondrocytes of monolayer passages 1–10 (untreated control and Igf-I treated chondrocytes) and cultivated for 7 days under identical conditions, before they were fixed and prepared for transmission electron microscopy. During the first day of the high-density culture period, chondrocytes from monolayer culture formed blastema-like nodules and made tight contacts (Fig. 1A). They revealed round to oval shapes, prominently large euchromatic nuclei, free cytoplasmic ribosomes, mitochondria and endoplasmatic reticulum (ER) as well as vacuoles. In contrast, high-density cultures of Igf-I treated cells produced more distinct and robust cell–cell-contacts from day 1 (such as desmosomes and gap-junctions) (Fig. 1B).

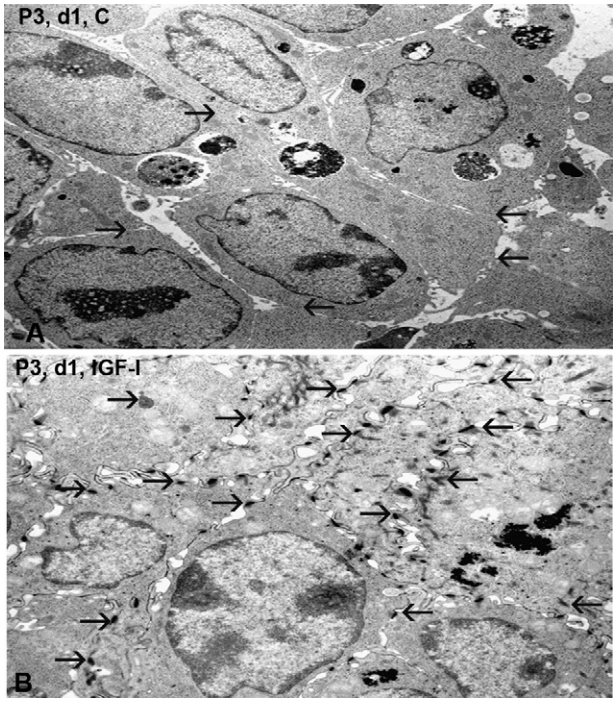

Fig. 1 – Electron microscopic demonstration of early high-density cultures of Igf-I treated chondrocytes. Serum starved chondrocytes were cultured and incubated with or without Igf-I (10 ng/ml) for 24 h; every third day the confluent monolayer culture cells were passaged, finally reaching passage 10. Cells from each monolayer passage were then grown in high-density cultures. Electron micrographs of chondrocytes (C) in 1-day old high-density cultures that were derived from passage P3 either treated with 10 ng/ml Igf-I (B) or left untreated (A). Chondrocytes had a typically round or oval profile and had intimate contact. These chondrocytes contained a well-developed rough endoplasmic reticulum, a Golgi apparatus, mitochondria, vacuoles and cytoplasmic granules. In Igf-I-treated chondrocyte cultures many cell–cell contacts were evident (arrows). 15 000×; bars = 1 μm.

Transmission electron microscopy revealed that high-density cultures of untreated cells (control) appear as viable chondrocytes exhibiting characteristic morphological features and they formed a dense and regular fibrillar extracellular matrix (Fig. 2, P1–P4: A). From P5, control cells developed unequivocally less matrix and exhibited characteristic signs of cellular degeneration, organelle destruction and apoptosis; morphological alterations included appearance of condensed material in the nucleus, formation of apoptotic bodies and bleb formation at the cell surface (Fig. 2, P5–P10: A). The few surviving cells that remained in the periphery were fibroblast-like and had completely lost their chondrocytic appearance. Up to P10 apoptotic cell death was apparent throughout the high-density cultures and only a few viable cells were still recognizable.

In contrast, high-density cultures treated with Igf-I exhibited significantly more viable cells featuring cytoplasmic glycogen accumulation. Igf-I treated chondrocytes formed cartilaginous nodules and these conditions were maintained up to P6 (Fig. 2, P1–P6: B). At that time point, fibroblast-like cells could be seen in the periphery and cartilaginous nodules featured abundant extracellular matrix at the center. P6 appeared to be the turning point; high-density cultures exhibited some viable chondrocytes, which produced an irregular matrix with non-specific proteoglycans around the cells and a pericellular matrix free space was visible. Chondrocytes showed signs of degeneration and apoptosis including annular condensation of nuclear chromatin, multiple vacuoles with fatty deposits accumulating in the cell periphery. Although some fibrillar matrix was detectable, it seemed disjointed and disorganized. Most of the cells featured a swollen ER. At P9 and P10 the majority of the cells were already dead or dying (Fig. 2, P8–P10: B).

Scoring the frequency of apoptotic cells and statistical evaluation of the data clearly highlighted changes in the number of apoptotic and viable cells during high-density culture in the presence and absence of Igf-I. The frequency and total number of apoptotic cells increased in control cultures from P5. The data confirm the findings observed by immunomorphology and electron microscopy (Fig. 3).

3.3. *Igf-I treated chondrocytes produced more collagen type II and CSPG*

Monolayer cultures derived from each initial passage (control and Igf-I treated cells) were transferred to high-density cultures and maintained for up to 7 days. They were then stained with APAAP-coupled antibodies against collagen type II and CSPG. Collagen type II and CSPG labeling were found in the extracellular matrix.

No differences in synthesis were seen with respect to these extracellular matrix proteins between the untreated control and the Igf-I stimulated chondrocytes until P4 (Fig. 4, P1–P5: A and B, C and D). The P4 samples yielded abundant and evenly distributed staining for collagen type II and CSPG in the cartilage nodules. From P5 onwards staining of the control cells became significantly weaker and nearly disappeared by P8–P10 (Fig. 4, P6–P10: A and C). At the same time a similar signal to the earlier time points could be observed in Igf-I treated chondrocytes (Fig. 4, P6–P10: B and D). The intensity of the staining was still high in P9 and P10, but had remarkably

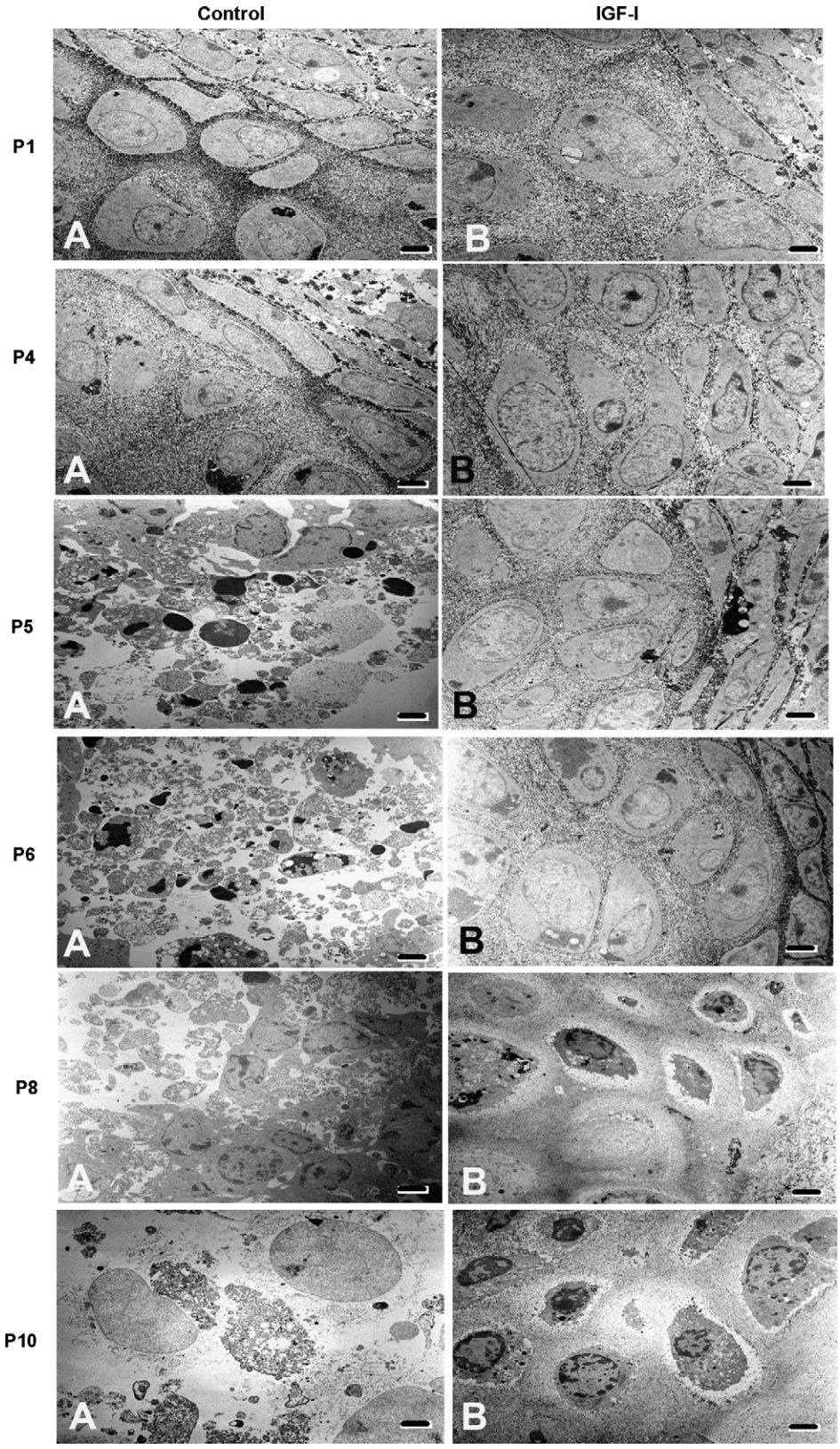

Fig. 2 – Electron microscopic demonstration of 7-day old high-density cultures of Igf-I treated chondrocytes. Serum starved chondrocytes were cultured and incubated with or without Igf-I (10 ng/ml) for 24 h; every third day the confluent monolayer culture cells were passaged, finally reaching passage 10. Cells from each monolayer passage were then grown in high-density cultures. Electron micrographs of chondrocytes in 7-day old high-density cultures derived from passages P1, P4, P5, P6, P8 and P10 either treated with Igf-I (B) or left untreated (A). Round nodules consisting of typical round chondrocytes that were surrounded by several layers of flattened fibroblast-like cells were evident in control cells of P1–P4. The cells had

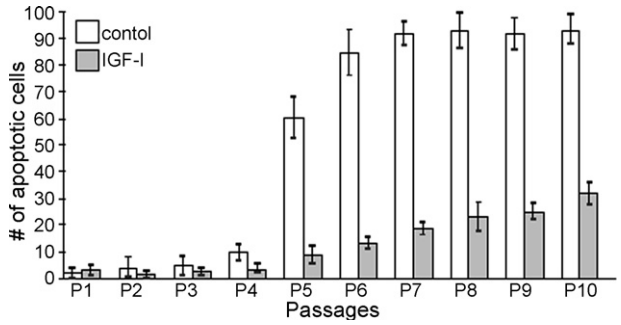

Fig. 3 – Statistical evaluation of data obtained by electron microscopy: the frequency of apoptosis in control and Igf-I-treated cultures was evaluated by scoring 100 cells from 20 different microscopic fields in each situation. The graph highlights the number of pathologically altered cells in control cells compared to Igf-I-treated cells; mean values are shown with standard deviations ($n = 3$ independent experiments).

decreased. Similar expression was found for both collagen type II and CSPG.

3.4. *Demonstration of collagen type II and activated caspase-3 in high-density cultures treated with and without Igf-I*

Immunoblotting was performed to determine the effect of Igf-I treatment on protein synthesis in human chondrocytes. Specimens from each passage were analysed in triplicate. We focused primarily on the cartilage-specific extracellular matrix protein collagen type II (Fig. 5A) as the presence of this extracellular matrix protein correlates with the chondrogenic potential of chondrocytes and exemplifies the basic assumption that cartilage matrix is being produced. Densitometric evaluation of a representative Western blot experiment performed in triplicate (Fig. 5C) revealed that collagen type II expression in untreated control was decreased from P5. In contrast, Igf-I-treated cells exhibited increased collagen type II expression during the culture period compared to chondrocytes maintained in the absence of Igf-I.

Western blot analysis was also used to examine the expression of the apoptosis marker activated caspase-3 in Igf-I treated and control chondrocytes. Our objective was to underline the correlation of decreasing matrix production with increasing apoptotic cell death. From P1 to P4 activated caspase-3 was not detectable in control or Igf-I treated cells (Fig. 5D). However, control cultures began expressing activated caspase-3 at P5 with increasing amounts up to P10. In contrast, Igf-I treated chondrocytes showed the first sign of activated caspase-3 expression at P7 which only marginally increased to P10 (Fig. 5D). Densitometric analysis of Western blot experiments (performed in triplicate) showed an increased expression of caspase-3 in untreated control cells from P5 (Fig. 5F). In contrast expression of activated caspase-3 was detected in Igf-I treated chondrocyte from P7 with minor increase to P10 (Fig. 5F).

3.5. *Igf-I stimulates expression of β1-integrins in chondrocytes*

Thus far our observations confirm that Igf-I-treated cells in monolayer cultures survive longer after being introduced to high-density cultures. We then proceeded to determine the molecular mechanisms and downstream signaling induced by Igf-I in chondrocytes.

It is well established that Igf-I receptor associates with β1-integrins in chondrocytes and stimulates the MAPK pathway which is an important signaling cascade for chondrocyte differentiation and survival [24]. To investigate the hypothesis that integrin-dependent intracellular signaling proteins were influenced by Igf-I in chondrocytes, human chondrocytes were grown in monolayer culture from P3 in the presence or absence of Igf-I and the expression of β1-integrin was examined by immunoelectron microscopy. As shown in Fig. 6, a marked increase in expression of β1-integrin was seen in Igf-I-treated chondrocytes, (Fig. 6B) compared to chondrocytes cultivated without Igf-I treatment (Fig. 6A).

To clarify the role of β1-integrins in this process, human chondrocytes were cultured in the presence or absence of Igf-I, lysed and immunoblotted with antibodies raised against-β1 integrin. Western blot analysis of Igf-I-treated chondrocytes showed that the expression of β1-integrins increased significantly (Fig. 6C) compared to untreated chondrocytes. Densitometric evaluation of the results indicated (Fig. 6D) a significant increase in the expression of the β1-integrin in response to Igf-I (10 ng/ml) compared to chondrocytes in the absence of Igf-I. These findings confirm the immunomorphological results described above.

3.6. *Igf-I induces activation of Erk1/2 in chondrocytes*

It has been shown that a reduction in the Erk signaling pathway stimulates the apoptotic pathway in different cell types (26). Serum-starved human chondrocytes were examined for the Erk1/2 protein after stimulation with 10 ng/ml Igf-I for 24 h (Fig. 7A and B). Samples from each passage were analysed in triplicate. Western blot analysis with anti-activated Erk1/2 from chondrocytes stimulated with Igf-I demonstrated a significant increase in Erk phosphorylation (a more intense 42 kDa band compared with the 44 kDa band), compared with chondrocytes in the absence of Igf-I (Fig. 7A). The expression of activated Erk1/2 was completely abolished from passage 5 in control cultures but in cultures stimulated with Igf-I it was still detectable until passage 7. In contrast, Western blot analysis with a pan-Erk antibody which recognizes both the phosphorylated and non-phosphorylated form of Erk1 and 2 did not reveal any change in response to Igf-I treatment (Fig. 7B).

intimate contact to a well developed fibrillar matrix. The same scenario was observed in Igf-I-treated cells until P6. In control cells from P6 until P10 no specific matrix could be seen. Chondrocytes exhibited cell lysis. Chondrocytes of P8 until P10 treated with Igf-I had a rounded shape, appeared viable and were embedded in a fibrillar matrix but exhibited pericellular areas of low matrix density. Nuclei of P8–P10 Igf-I-treated chondrocytes showed areas of condensed heterochromatin at the nuclear membrane. 5000×; bars = 1 μm.

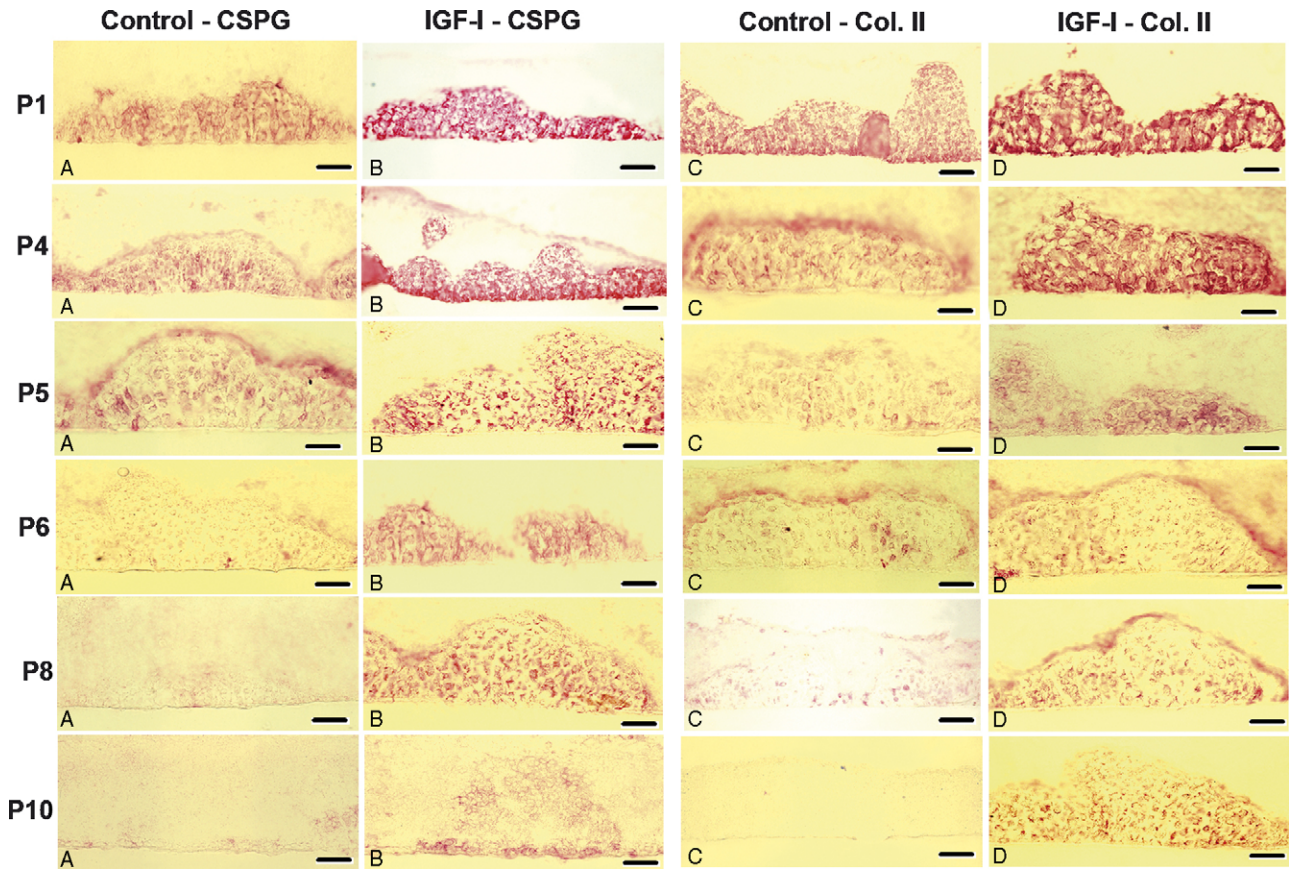

Fig. 4 – Immunolabeling for collagen type II and CSPGs of chondrocyte high-density cultures treated with Igf-I. Serum starved chondrocytes were cultured and incubated with or without Igf-I (10 ng/ml) for 24 h; every third day the confluent monolayer culture cells were passaged, finally reaching passage 10. Cells from each monolayer passage were grown in high-density cultures. Immunolabeling of chondrocytes (P1, P4, P5, P6, P8, P10) that were either treated with Igf-I (B and D) or were left untreated (A and C) for CSPG and for collagen type II according to the APAAP method. Immunolabeling for CSPG and collagen type II (A and C) was clearly observed around control cells derived from passages P1–P4 or P1–P5, but was barely visible around those obtained from passages P6–P10. In cultures treated with Igf-I collagen type II (D) and CSPG (B) expression could be seen up to P8. 160×, bars = 50 μm.

3.7. *Effects of Igf-I-induced expression of Sox9 in the chondrocyte nucleus*

It has been shown that the MAPK pathway mediates the up-regulation of Sox9 expression, a master regulator of the chondrocyte lineage by growth factors in mouse chondrocytes [34]. To test the hypothesis that Igf-I is able to activate the transcription factor Sox9 in human chondrocytes, monolayer cultures (P1–P10) were either left unstimulated (Fig. 8A) or stimulated with Igf-I (Fig. 8B). Cells were labelled with APAAP-coupled antibodies against Sox9. Positive immunolabeling was found in chondrocyte nuclei. Control chondrocytes revealed positive Sox9 labeling from P1 to P5 (Fig. 8A, P1–P5). From P6 onwards immunolabeling of control cells became weaker and almost disappeared by P8–P10 (Fig. 8A, P6–P10). A similar expression of Sox9 in the earlier time points could be observed in Igf-I treated chondrocytes (Fig. 8B, P1–P5), but the immunolabeling was still present in P8 and P9. By P10 Sox9 labeling had remarkably decreased (Fig. 8B; P6–P10).

Sox9 is a specific transcription factor that controls the expression of chondrocyte-specific extracellular matrix protein genes and plays a pivotal role in chondrocyte differentiation [35]. To confirm the immunomorphological results described above and to show more precisely the influence of Igf-I on the expression of Sox9 in chondrocytes, nuclear extracts of serum-starved human chondrocytes were probed for Sox9 expression after the chondrocytes had been stimulated with 10 ng/ml Igf-I for 24 h (Fig. 9A and B). Specimens from each passage were analysed in triplicate. Treatment of chondrocytes with Igf-I at different passages revealed increased Sox9 expression in the nuclear extracts (Fig. 9A). Densitometric analysis of a typical Western blot experiment performed in triplicate (Fig. 9B) showed that Sox9 expression increased in Igf-I treated chondrocytes compared to untreated chondrocytes.

3.8. *Association between translocated Erk1/2 with Sox9 in the chondrocyte nucleus*

We hypothesized that activation of Erk and its translocation to the nucleus has a direct connection with Sox9 because the MAPK pathway mediates up-regulation of Sox9 and its gene products. To test this hypothesis, nuclear extracts of serum-

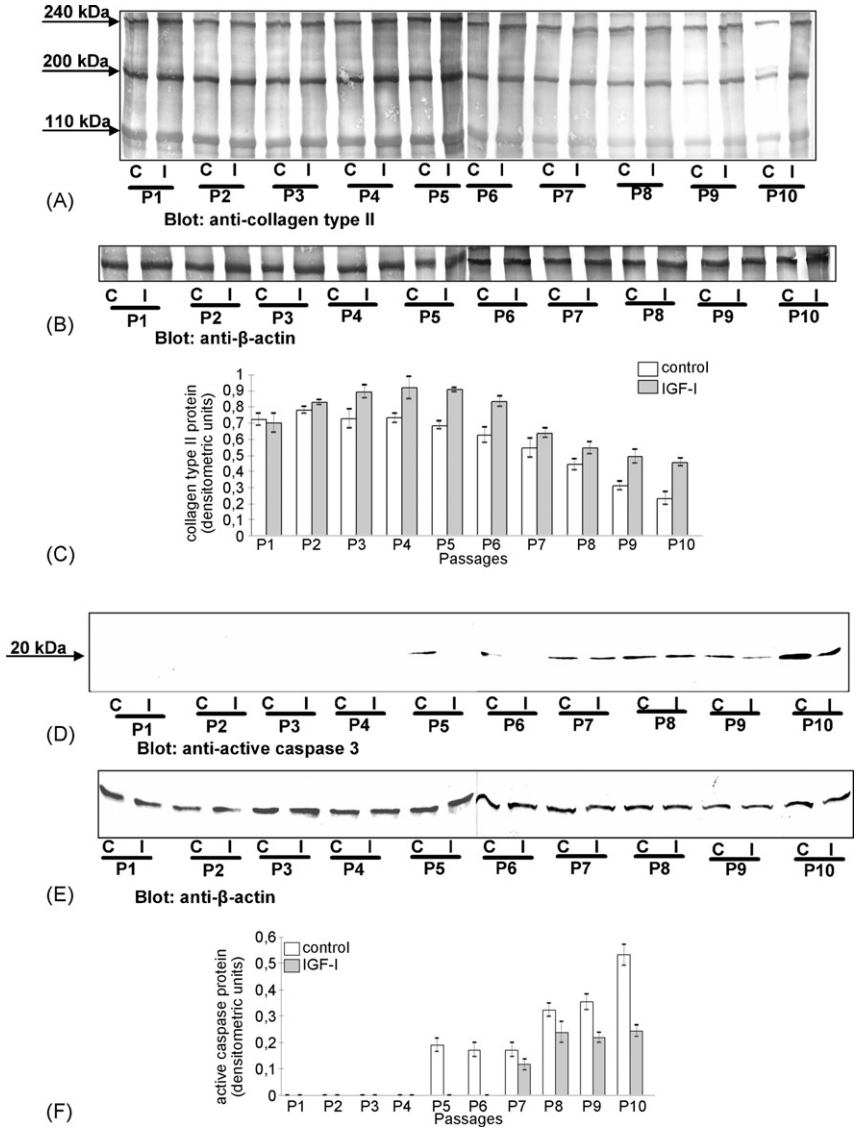

Fig. 5 – Demonstration of collagen type II and activated caspase-3 in high-density cultures treated with or without Igf-I by Western blot analysis. Serum starved chondrocytes were cultured and incubated with or without Igf-I (10 ng/ml) for 24 h; every third day the confluent monolayer culture cells were passaged, finally reaching passage 10. Cells from each monolayer passage were grown in high-density cultures. Synthesis of collagen type II in 7-day old high-density cultures treated with Igf-I vs. control as revealed by Western blotting. A decrease in collagen type II synthesis could be demonstrated after P6 in control and Igf-I treated cultures. In P7 until P10 Igf-I-treated cultures showed a significantly higher synthesis of collagen type II compared to untreated cultures as revealed by densitometric evaluation (A). Expression of the house-keeping gene β-actin was unaffected by Igf-I treatment (B). Quantitative densitometry (C) demonstrated increasing expression of collagen type II in the presence of Igf-I and showed a significantly higher expression from the beginning of the Igf-I cultivation period in comparison to those cultivated without with Igf-I. The mean values and standard deviations from three independent experiments are indicated. Activation of caspase-3 could be shown in untreated high-density cultures of P5 until P10. Cultures treated with Igf-I showed caspase-3 activation in P7 until P10. Caspase-3 activation was significantly higher in control cultures compared to Igf-I treated cultures as revealed by densitometric evaluation (D). The mean values and standard deviations from three independent experiments are indicated. Expression of the house-keeping gene β-actin was not affected by Igf-I treatment (E). Quantitative densitometry (F) demonstrated increasing expression of caspase-3 protein in the absence of Igf-I and showed a significantly higher density from P5. The mean values and standard deviations from three independent experiments are indicated.

starved human chondrocytes were probed for the presence of the Sox9 protein after stimulation of chondrocytes with 10 ng/ml Igf-I for 24 h. Specimens from each passage were analysed in triplicate by performing co-immunoprecipitation assays. After immunoprecipitation of nuclear lysates with anti-Sox9 antibodies, the SDS-PAGE resolved samples were probed by immunoblotting with antibodies to Erk1/2. Results indicated that Sox9 interacted with Erk1/2 up to P4 in both Igf-I-treated

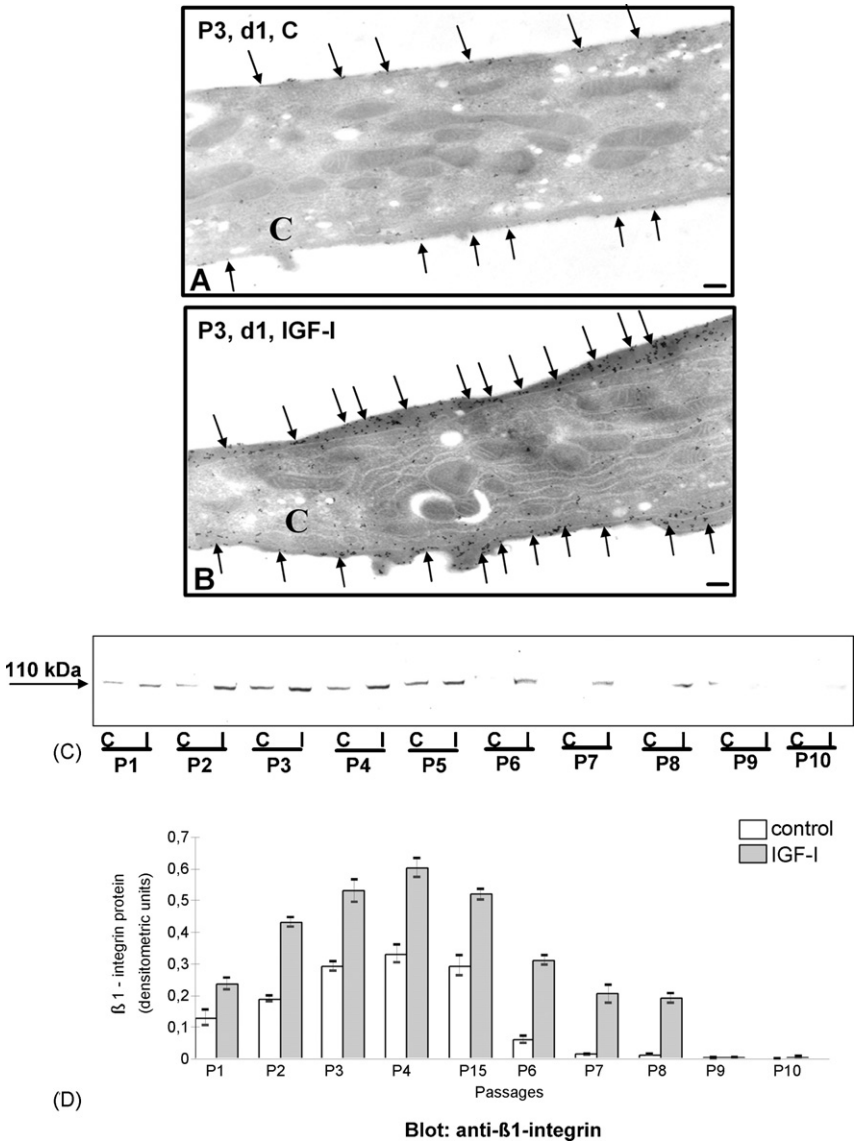

Fig. 6 – The influence of Igf-I on the expression of adhesion molecules in chondrocytes *in vitro*. (A and B) Immunoelectron microscopic demonstration of integrin-β1 in monolayer cultures of chondrocytes. Unstimulated chondrocytes (A) and Igf-I-stimulated chondrocytes (B) labeled with anti-integrin-β1 antibodies and gold-coupled secondary antibodies. Gold particles (10 nm) were detectable at the plasma membrane (arrows) and cytoplasmic processes of Igf-I-stimulated chondrocytes (Fig. 6B) were clearly increased compared to control cells (Fig. 6A). 50 000×; bars = 0.1 μm. (C) To confirm the results from immunoelectron microscopy, serum starved chondrocytes were cultured and incubated with or without Igf-I (10 ng/ml) for 24 h. Total cell lysates were prepared and analyzed by Western blot using antibodies that recognize the integrin-β1 receptor (C). Molecular weight markers are indicated in kDa. Quantitative densitometry (D) demonstrated increasing expression of integrin-β1 receptor protein in the presence of Igf-I and showed a significantly higher density from the beginning of the Igf-I cultivation period in comparison to those cultivated without treatment with Igf-I. The mean values and standard deviations from three independent experiments are indicated.

and control cells. The interaction of Sox9 with Erk1/2 was enhanced in chondrocytes treated with Igf-I. However this interaction could only be shown in Igf-I-treated cells up to P6 (Fig. 10).

4. Discussion

In this study we investigated the effect of Igf-I on the redifferentiation capacity of dedifferentiated human chondrocytes using a high-density redifferentiation model system previously established in our laboratory. Chondrocytes were passaged up to 10 times in monolayer culture, followed by transfer to high-density cultures for each passage. The results of this study demonstrate that loss of redifferentiation capability and apoptosis are significantly delayed up to passage 7 by Igf-I treatment of monolayer cultured chondrocytes. Indeed, it seems that the Igf-I stabilizes chondrogenic potential by MAPK signaling pathway, Sox9 stimulation

and interaction between Erk and Sox9 and strongly suggest that these effects are regulated by the integrin signaling pathway. Therefore, the redifferentiation capability of dedifferentiated monolayer chondrocytes may be substantially extended (up to a further three monolayer passages) by Igf-I stimulation.

4.1. *Dedifferentiation delay tactics: use of anabolic growth factors to stabilize the chondrogenic potential of dedifferentiated chondrocytes*

Exposing chondrocytes in monolayer culture to anabolic growth factors may present a novel strategy of ''dedifferentiation delay'' to postpone, albeit briefly, the rapid loss of chondrogenic potential and cartilage-specific matrix production during monolayer expansion *in vitro*. This approach may also partially protect chondrocytes from apoptotic cell death. Recently, several investigators have reported on the beneficial effects of various growth factors such as FGF-2, BMP-2 and TGF-β on chondrocyte differentiation or redifferentiation in culture [15,17,18,36,37]. We have demonstrated the positive effects of Igf-I on the maintenance of chondrogenic potential in chondrocytes during redifferentiation in high-density culture. In addition, the present study confirms and extends the findings of previous investigations carried out by our group and others [5,10–12,18] by demonstrating that dedifferentiated chondrocytes or mesenchymal cells are able to (re)differentiate after transfer into high-density or alginate cultures, but irreversibly lose their chondrogenic potential in response to prolonged passaging (e.g. more than four passages) in monolayer culture. In the present study the redifferentiation capability of human chondrocytes could be further extended by Igf-I treatment for at least 2–3 passages up to passage 7. This delay may result in significant increases in chondrocyte yield especially if the starting primary cell population was already low. However, the present study and results of others support the hypothesis that growth factors have a central role in the interplay of these signaling pathways associated in this process. Indeed, one of the very essential signaling pathways in chondrocytes is the integrin-dependent intracellular signaling pathway, which obviously, as shown here, can be influenced by Igf-I. Cell–matrix interaction is mediated by integrin receptors [6,24,38–40] which play a crucial role in early cartilage differentiation, since the presence of anti-integrin antibodies is able to inhibit differentiation of blastemal cells to chondroblasts [33].

4.2. *Synergistic action of Igf-I and high-density cultures: a case for cell–cell contacts*

The high-density culture system employed here has been successfully used as a model for chondrogenic differentiation and redifferentiation of dedifferentiated chondrocytes [11,41–44]. During the first phase of chondrocyte differentiation in chondrogenesis cells go through a ''condensation'' phase which is symbolized by strong cell–cell contacts [41,45]. This phase can clearly be observed in the early stages of high-density cultures [11,45]. The three-dimensional architecture of densely packed cells in high-density culture provides many cell–cell contacts. One form of cell–cell contact is the direct

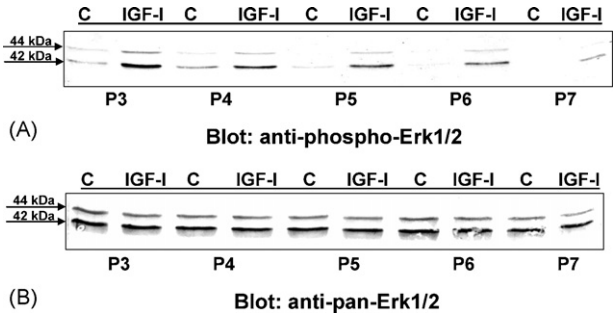

Fig. 7 – Effect of Igf-I on MAPK pathway in chondrocyte monolayer cultures. Activity profiles of Erk1/2 with or without Igf-I in monolayer cultures. Serum starved chondrocytes were cultured and incubated with or without Igf-I (10 ng/ml) for 24 h. Total cell lysates were prepared and analyzed by Western blot using antibodies that recognize phosphorylated Erk1/2 and pan-Erk1/2 to verify equal loading. Igf-I treatment increased p-Erk1/2 protein levels significantly compared to untreated controls and the expression of activated Erk1/2 was completely abolished from passage 5 in control cultures, but in cultures stimulated with Igf-I Erk1/2 expression was still apparent until passage 7 (A). In contrast, Western blot analysis with a pan-Erk antibody which recognizes both the phosphorylated and non-phosphorylated forms of Erk1 and 2 showed that total Erk did not change in response to Igf-I treatment (B). The results shown are representative of three identical experiments.

coupling between the cytoplasm of adjacent cells through gap-junctions, which have been demonstrated in primary cultures of bovine articular chondrocytes and in rat and human chondrocyte high-density cultures [45,46]. The gap-junction protein connexin 43 mediates the condensation phase of chondrogenesis and plays an important role in the (re-) differentiation of chondrocytes [45]. We have used electron microscopy to demonstrate that chondrocytes on day 1 of P1–P4 feature more gap-junctions after treatment with Igf-I than control cells, underlining the influence of Igf-I on the differentiation of chondrocytes and vital junctional contacts. Indeed, this phase also occurs at the early stages of high-density culture. During the condensation phase of chondrogenesis enhanced expression of the HMG family transcription factor Sox9 has been reported [26,27,47]. Sox9 is also strongly expressed in high-density cultures [44]. Sox9 has been implicated together with other co-factors such as Sox5 and Sox6 in the regulation of expression of cartilage-specific matrix components such as collagen type II and aggrecan by several authors [26,27,29,30,47]. Igf-I is known to stimulate collagen type II expression in chondrocytes [25,48]. However, Aigner et al. [25] reported also that Sox9 expression does not positively correlate with type II collagen expression in adult human articular chondrocytes. This observation seems somehow contradictory since Sox9 has been directly implicated in collagen type II expression and a Sox9 regulatory element is known to reside in the type II collagen gene enhancer [49].

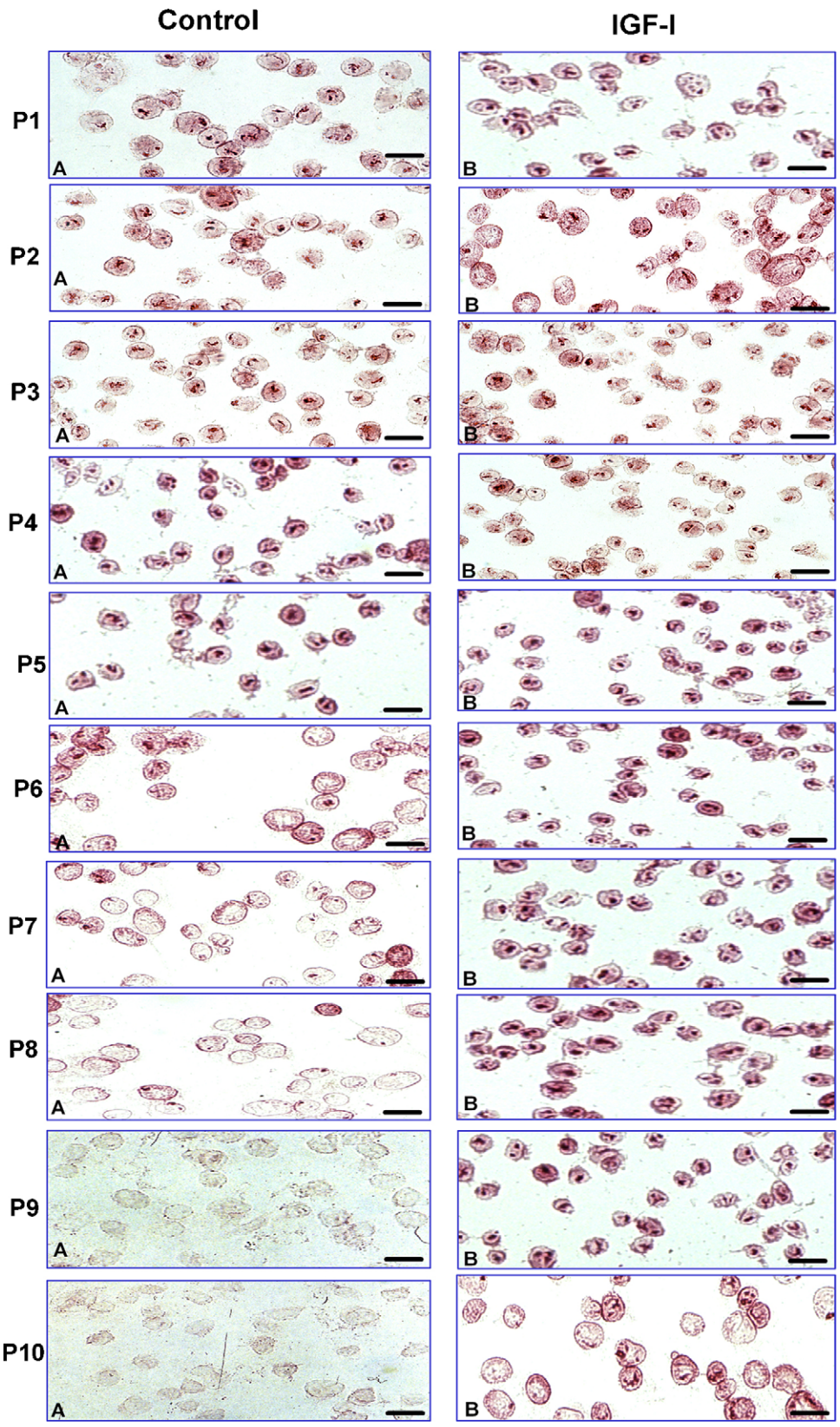

Fig. 8 – (A and B) Immunohistochemical detection of Sox9 in chondrocyte nuclei in monolayer cultures treated with or without Igf-I *in vitro*. Immunolabeling of chondrocytes (P1–P10) treated with 10 ng/ml Igf-I (B) or untreated (A) for Sox9 according to the APAAP method. Immunolabeling for Sox9 (A) was clearly observed in the nuclei of chondrocytes from passages P1–P5, but was barely visible around those obtained from passages P6–P10. In cultures treated with Igf-I the expression of Sox9 (B) could be seen up to P9. 160×, bars = 50 μm.

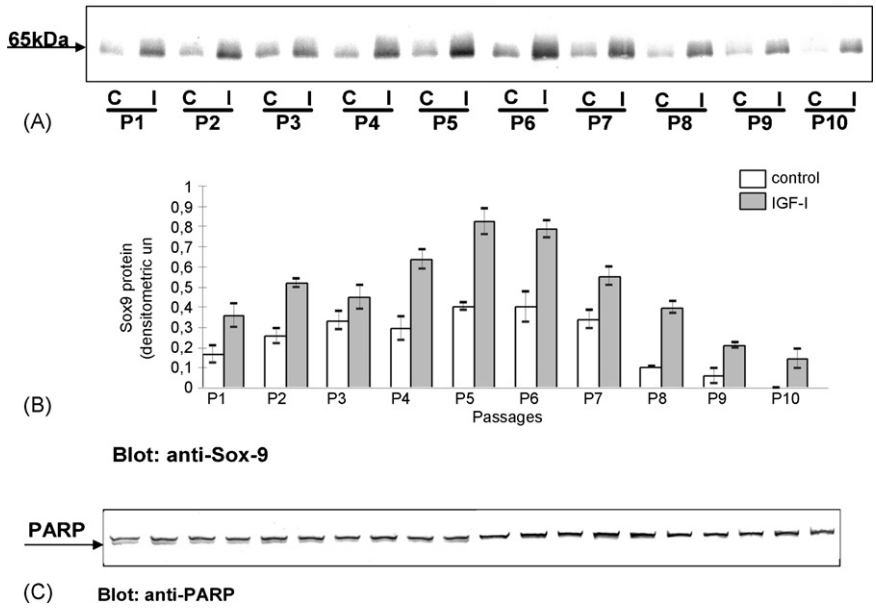

Fig. 9 – Igf-I stimulated expression of Sox9 in nuclear extracts of chondrocytes in monolayer cultures. Serum-starved human chondrocytes were plated and then treated with Igf-I (10 ng/ml) for 24 h (A). The chondrocytes were lysed and equal amounts of total protein separated by 10% SDS-PAGE and analyzed by immunoblotting with anti-Sox9 antibodies. Densitometric quatitation (B) demonstrated an increase in Sox9 synthesis after P1 in Igf-I treated cultures compared to controls. In P4–P8 Igf-I-treated cultures showed a significantly higher synthesis of Sox9 compared to untreated cultures. Values are means ± S.D. of a representative experiment performed in triplicate. Synthesis of the gene product PARP remained unaffected in nuclear extracts (C). Data shown are representative of three independent experiments.

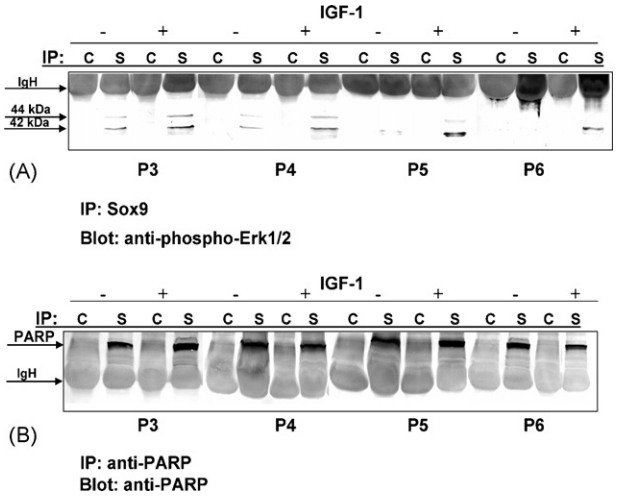

Fig. 10 – Association of Sox9 protein with activated Erk1/2 in monolayer cultures with or without Igf-I treatment (nuclear extracts). Serum-starved human chondrocytes were cultured and then treated with Igf-I (10 ng/ml) or left untreated for 24 h. Equal amounts of total protein were lysed and immunoprecipitated using anti-Sox9 antibody (S) and control normal IgG-serum (C). Immunoprecipitates were separated by 10% SDS-PAGE and analyzed by immunoblotting with anti-activated Erk1 (44 kDa) and Erk2 (42 kDa) antibodies. Synthesis of the gene product PARP remained unaffected in nuclear extracts (B). Data shown are representative of three independent experiments. IgH: immunglobulin heavy chain.

4.3. *Effects of Igf-I and high-density cultures on chondrocyte apoptosis, caspase activation and matrix markers*

Chondrocyte apoptosis (revealed by activation of caspase-3) was observed in control monolayer cultures for the first time in passage 5. This was confirmed by electron microscopy which morphologically revealed apoptotic features characteristic of apoptotic chondrocytes in high-density cultures of monolayer P5–P10. In Igf-I treated cultures activated caspase-3 was evident later at P7. Deposition of cartilage-specific matrix proteins such as collagen type II and CSPGs as revealed by Western blot and immunohistochemical analysis showed a significant decrease in collagen type II and CSPG deposition in passages 7–10 of Igf-I treated cultures.

4.4. *Rationale for effectiveness of Igf-I*

Igf-I has been shown to support cell survival since inhibition of the type 1 Igf-receptor (Igf-IR) leads to caspase activation and increased apoptosis [14]. Since dedifferentiation-induced apoptosis accompanies the loss of expression of cartilage-specific matrix proteins such as collagen type II and cartilage-specific proteoglycans one can assume that inhibition of cell–matrix interaction may significantly contribute to chondrocyte apoptosis. Many investigators agree that interaction between chondrocytes and cartilage-specific matrix components stimulates chondrocyte survival, since inhibition of cell–matrix interaction leads to apoptosis [32,50,51]. Cell matrix receptors such as β1-integrins play an important role in cell–matrix interactions and inhibition of such interactions

leads to cell death. Inhibition of β1-integrins has been shown to prevent chondrogenesis in high-density cultures [33]. The Igf-IR has been shown to associate with β1-integrins in chondrocytes and stimulates the MAPK pathway [24], an important signaling cascade for chondrocyte differentiation and survival. Indeed, it has been reported that the MAPK pathway increases the chondrogenic factor Sox9 in chondrocytes and this process is improved by fibroblast growth factor [34]. In a previous study the expression and interaction of key signaling proteins of the MAPK pathway were shown to correlate with the maintenance of chondrogenic potential [12]. Interestingly, inhibition of MAPK pathway has been shown to lead to chondrocyte apoptosis and further inhibits the expression of Sox9 [32,34]. Furthermore, Sox9 is a well known chondrogenic transcription factor for chondrocyte differentiation and expression of chondrocyte specific genes. One explanation might be that Igf-I treatment stabilizes chondrogenic potential and chondrocyte survival via Integrin/MAPK signaling. Therefore, Igf-I and the adhesion/signal transduction receptor β1-integrin play an essential role in chondrocyte differentiation and survival. Further work is in progress concerning the Igf-I stimulated signaling pathways during redifferentiation of chondrocytes.

4.5. *Functional association between Sox9 and Erk1/2*

We surmised that functional activation of Erk and its nuclear translocation may have a direct connection with Sox9 since the MAPK pathway mediates the up-regulation of Sox9 and its gene products. The results presented here indicate that Sox9 interacts with Erk1/2 up to P4 in both Igf-I-treated and control cells. Thus, Igf-I markedly enhances Sox9 expression and this effect appears to be mediated by the integrin signaling pathway thereby stabilizing chondrocyte differentiation.

Acknowledgements

Ms. Angelika Steuer, Mrs. Karoline Fischer, Mr. Benjamin Kohl and Ms. Ursula Schwikowski are gratefully acknowledged for their excellent technical assistance and Ms. Gundula Schulze-Tanzil and Agnes Ellinghaus for additional support. This work was supported by the Deutsche Forschungsgemeinschaft (DFG Grants Sh 48/2-4 and Sh 48/2-5).

REFERENCES

[1] Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889–95.
[2] Peterson L, Minas T, Brittberg M, Nilsson A, Sjogren-Jansson E, Lindahl A. Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. Clin Orthop Relat Res 2000;212–34.
[3] Grundmann K, Zimmermann B, Barrach HJ, Merker HJ. Behaviour of epiphyseal mouse chondrocyte populations in monolayer culture. Morphological and immunohistochemical studies. Virchows Arch A Pathol Anat Histol 1980;389:167–87.
[4] Shakibaei M. Integrin expression on epiphyseal mouse chondrocytes in monolayer culture. Histol Histopathol 1995;10:339–49.
[5] Shakibaei M, De Souza P. Differentiation of mesenchymal limb bud cells to chondrocytes in alginate beads. Cell Biol Int 1997;21:75–86.
[6] Shakibaei M, De Souza P, Merker HJ. Integrin expression and collagen type II implicated in maintenance of chondrocyte shape in monolayer culture: an immunomorphological study. Cell Biol Int 1997;21:115–25.
[7] van der Kraan PM, Buma P, van Kuppevelt T, van den Berg WB. Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. Osteoarthritis Cartilage 2002;10:631–7.
[8] Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215–24.
[9] Loty S, Forest N, Boulekbache H, Sautier JM. Cytochalasin D induces changes in cell shape and promotes in vitro chondrogenesis: a morphological study. Biol Cell 1995;83:149–61.
[10] Barbero A, Ploegert S, Heberer M, Martin I. Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum 2003;48:1315–25.
[11] Schulze-Tanzil G, de Souza P, Villegas Castrejon H, John T, Merker HJ, Scheid A, et al. Redifferentiation of dedifferentiated human chondrocytes in high-density cultures. Cell Tissue Res 2002;308:371–9.
[12] Schulze-Tanzil G, Mobasheri A, de Souza P, John T, Shakibaei M. Loss of chondrogenic potential in dedifferentiated chondrocytes correlates with deficient Shc-Erk interaction and apoptosis. Osteoarthritis Cartilage 2004;12:448–58.
[13] Loeser RF, Pacione CA, Chubinskaya S. The combination of insulin-like growth factor 1 and osteogenic protein 1 promotes increased survival of and matrix synthesis by normal and osteoarthritic human articular chondrocytes. Arthritis Rheum 2003;48:2188–96.
[14] Loeser RF, Shanker G. Autocrine stimulation by insulin-like growth factor 1 and insulin-like growth factor 2 mediates chondrocyte survival in vitro. Arthritis Rheum 2000;43:1552–9.
[15] Mandl EW, van der Veen SW, Verhaar JA, van Osch GJ. Serum-free medium supplemented with high-concentration FGF2 for cell expansion culture of human ear chondrocytes promotes redifferentiation capacity. Tissue Eng 2002;8:573–80.
[16] Morales TI, Joyce ME, Sobel ME, Danielpour D, Roberts AB. Transforming growth factor-beta in calf articular cartilage organ cultures: synthesis and distribution. Arch Biochem Biophys 1991;288:397–405.
[17] Kim SE, Park JH, Cho YW, Chung H, Jeong SY, Lee EB, et al. Porous chitosan scaffold containing microspheres loaded with transforming growth factor-beta1: implications for cartilage tissue engineering. J Control Rel 2003;91:365–74.
[18] Martin I, Vunjak-Novakovic G, Yang J, Langer R, Freed LE. Mammalian chondrocytes expanded in the presence of fibroblast growth factor 2 maintain the ability to differentiate and regenerate three-dimensional cartilaginous tissue. Exp Cell Res 1999;253:681–8.
[19] Dailey L, Laplantine E, Priore R, Basilico C. A network of transcriptional and signaling events is activated by FGF to induce chondrocyte growth arrest and differentiation. J Cell Biol 2003;161:1053–66.
[20] Trippel SB, Corvol MT, Dumontier MF, Rappaport R, Hung HH, Mankin HJ. Effect of somatomedin-C/insulin-like growth factor I and growth hormone on cultured growth plate and articular chondrocytes. Pediatr Res 1989;25:76–82.

[21] van Osch GJ, van den Berg WB, Hunziker EB, Hauselmann HJ. Differential effects of IGF-1 and TGF beta-2 on the assembly of proteoglycans in pericellular and territorial matrix by cultured bovine articular chondrocytes. Osteoarthritis Cartilage 1998;6:187–95.
[22] van Osch GJ, van der Veen SW, Buma P, Verwoerd-Verhoef HL. Effect of transforming growth factor-beta on proteoglycan synthesis by chondrocytes in relation to differentiation stage and the presence of pericellular matrix. Matrix Biol 1998;17:413–24.
[23] Fukumoto T, Sperling JW, Sanyal A, Fitzsimmons JS, Reinholz GG, Conover CA, et al. Combined effects of insulin-like growth factor-1 and transforming growth factor-beta1 on periosteal mesenchymal cells during chondrogenesis in vitro. Osteoarthritis Cartilage 2003;11:55–64.
[24] Shakibaei M, John T, De Souza P, Rahmanzadeh R, Merker HJ. Signal transduction by beta1 integrin receptors in human chondrocytes in vitro: cllaboration with the insulin-like growth factor-I receptor. Biochem J 1999;342:615–23. Pt 3.
[25] Aigner T, Gebhard PM, Schmid E, Bau B, Harley V, Poschl E. Sox9 expression does not correlate with type II collagen expression in adult articular chondrocytes. Matrix Biol 2003;22:363–72.
[26] Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev 2002;16:2813–28.
[27] Akiyama H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, Zhang Z, et al. Interactions between Sox9 and beta-catenin control chondrocyte differentiation. Genes Dev 2004;18:1072–87.
[28] Lefebvre V, Behringer RR, de Crombrugghe B. L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis Cartilage 2001;9(Suppl A):S69–75.
[29] Schaefer JF, Millham ML, de Crombrugghe B, Buckbinder L. FGF signaling antagonizes cytokine-mediated repression of Sox9 in SW1353 chondrosarcoma cells. Osteoarthritis Cartilage 2003;11:233–41.
[30] Ikeda T, Kamekura S, Mabuchi A, Kou I, Seki S, Takato T, et al. The combination of Sox5, Sox6, and Sox9 (the Sox trio) provides signals sufficient for induction of permanent cartilage. Arthritis Rheum 2004;50:3561–73.
[31] Ng LJ, Wheatley S, Muscat GE, Conway-Campbell J, Bowles J, Wright E, et al. Sox9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. Dev Biol 1997;183:108–21.
[32] Shakibaei M, Schulze-Tanzil G, de Souza P, John T, Rahmanzadeh M, Rahmanzadeh R, et al. Inhibition of mitogen-activated protein kinase induces apoptosis of human chondrocytes. J Biol Chem 2001;276:13289–94.
[33] Shakibaei M. Inhibition of chondrogenesis by integrin antibody in vitro. Exp Cell Res 1998;240:95–106.
[34] Murakami S, Kan M, McKeehan WL, de Crombrugghe B. Up-regulation of the chondrogenic Sox9 gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. Proc Natl Acad Sci USA 2000;97:1113–8.
[35] de Crombrugghe B, Lefebvre V, Behringer RR, Bi W, Murakami S, Huang W. Transcriptional mechanisms of chondrocyte differentiation. Matrix Biol 2000;19:389–94.
[36] Jakob M, Demarteau O, Schafer D, Hintermann B, Dick W, Heberer M, et al. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro. J Cell Biochem 2001;81:368–77.
[37] van Osch GJ, Marijnissen WJ, van der Veen SW, Verwoerd-Verhoef HL. The potency of culture-expanded nasal septum chondrocytes for tissue engineering of cartilage. Am J Rhinol 2001;15:187–92.
[38] Durr J, Goodman S, Potocnik A, von der Mark H, von der Mark K. Localization of beta 1-integrins in human cartilage and their role in chondrocyte adhesion to collagen and fibronectin. Exp Cell Res 1993;207:235–44.
[39] Enomoto M, Leboy PS, Menko AS, Boettiger D. Beta 1 integrins mediate chondrocyte interaction with type I collagen, type II collagen, and fibronectin. Exp Cell Res 1993;205:276–85.
[40] Mobasheri A, Carter SD, Martin-Vasallo P, Shakibaei M. Integrins and stretch activated ion channels; putative components of functional cell surface mechanoreceptors in articular chondrocytes. Cell Biol Int 2002;26:1–18.
[41] Denker AE, Haas AR, Nicoll SB, Tuan RS. Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells. I. Stimulation by bone morphogenetic protein-2 in high-density micromass cultures. Differentiation 1999;64:67–76.
[42] Healy C, Uwanogho D, Sharpe PT. Expression of the chicken Sox9 gene marks the onset of cartilage differentiation. Ann N Y Acad Sci 1996;785:261–2.
[43] Healy C, Uwanogho D, Sharpe PT. Regulation and role of Sox9 in cartilage formation. Dev Dyn 1999;215:69–78.
[44] Kulyk WM, Franklin JL, Hoffman LM. Sox9 expression during chondrogenesis in micromass cultures of embryonic limb mesenchyme. Exp Cell Res 2000;255:327–32.
[45] Loty S, Foll C, Forest N, Sautier JM. Association of enhanced expression of gap junctions with in vitro chondrogenic differentiation of rat nasal septal cartilage-released cells following their dedifferentiation and redifferentiation. Arch Oral Biol 2000;45:843–56.
[46] Donahue HJ, Guilak F, Vander Molen MA, McLeod KJ, Rubin CT, Grande DA, et al. Chondrocytes isolated from mature articular cartilage retain the capacity to form functional gap junctions. J Bone Miner Res 1995;10:1359–64.
[47] Akiyama H, Kamitani T, Yang X, Kandyil R, Bridgewater LC, Fellous M, et al. The transcription factor Sox9 is degraded by the ubiquitin-proteasome system and stabilized by a mutation in a ubiquitin-target site. Matrix Biol 2005;23:499–505.
[48] Kolettas E, Muir HI, Barrett JC, Hardingham TE. Chondrocyte phenotype and cell survival are regulated by culture conditions and by specific cytokines through the expression of Sox-9 transcription factor. Rheumatology (Oxford) 2001;40:1146–56.
[49] Klooster AR, Bernier SM. Tumor necrosis factor alpha and epidermal growth factor act additively to inhibit matrix gene expression by chondrocyte. Arthritis Res Ther 2005;7:R127–38.
[50] Goggs R, Carter SD, Schulze-Tanzil G, Shakibaei M, Mobasheri A. Apoptosis and the loss of chondrocyte survival signals contribute to articular cartilage degradation in osteoarthritis. Vet J 2003;166:140–58.
[51] Yang C, Li SW, Helminen HJ, Khillan JS, Bao Y, Prockop DJ. Apoptosis of chondrocytes in transgenic mice lacking collagen II. Exp Cell Res 1997;235:370–3.