Histol Histopathol (2006) 21: 467-476

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Development and phenotypic characterization of a high density *in vitro* model of auricular chondrocytes with applications in reconstructive plastic surgery

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Summary. Cultivation of phenotypically stable auricular chondrocytes will have applications in autologous chondrocyte transplantation and reconstructive surgery of cartilage. Chondrocytes grown in monolayer culture rapidly dedifferentiate assuming a fibroblast-like morphology and lose their cartilage-specific pattern of gene expression. Three-dimensional high-density culture models mimic more closely the *in vivo* conditions of cartilage. Therefore, this study was undertaken to test whether the high-density cultures might serve as a suitable model system to acquire phenotypically and functionally differentiated auricular chondrocytes from porcine cartilage.

Freshly isolated porcine auricular chondrocytes were cultured for 7 passages in monolayer culture. From each passage (passage 0 and 1-7) cells were introduced to high-density cultures and examined by transmission electron microscopy. Western blotting was used to analyse the expression of cartilage-specific markers, such as collagen type II and cartilage specific proteoglycan, fibronectin, cell adhesion and signal transduction receptor ß1-integrin, matrix metalloproteinases (MMP-9, MMP-13), cyclo-oxygenase (COX)-2 and the apoptosis commitment marker, activated caspase-3.

When dedifferentiated auricular chondrocytes from monolayer passages 0-4 were cultured in high-density culture, they recovered their chondrocytic phenotype and formed cartilage nodules surrounded by fibroblast-like cells and synthesised collagen type II, proteoglycans, fibronectin and ß1-integrins. However, chondrocytes from monolayer passages 5-7 did not redifferentiate to chondrocytes even when transferred to high-density culture, and did not synthesize a chondrocyte-specific extracellular matrix. Instead, they produced increasing amounts of MMP-9, MMP-13, COX-2, activated caspase-3 and underwent apoptosis.

Three-dimensional high-density cultures may therefore be used to obtain sufficient quantities of fully differentiated auricular chondrocytes for autologous chondrocyte transplantation and reconstructive plastic surgery.

Key words: Auricular chondrocytes, High-density culture, Redifferentiation, Dedifferentiation, Apoptosis, Reconstructive surgery

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Introduction

In reconstructive surgery, auricular cartilage is often harvested to augment anatomical defects of the nose, ear and trachea. However the major limitation of this approach is the available quantity of cartilage and donor site morbidity (Chang et al., 2004; Anderl and Haid, 2005; Brodland, 2005). Therefore, alternative methods have been developed, particularly with hyaline cartilage from articular, nasal and costal origin, to circumvent limitation of cartilage, using small cartilage biopsies which is followed by enzymatic chondrocyte isolation and proliferation in monolayer culture (Kuettner et al., 1982b). Monolayer culture of articular chondrocytes is a major method of interest in chondrocyte transplantation because of its importance for chondrocyte expansion and amplification of cell density. Chondrocytes isolated from fully developed subjects have a limited capacity for cell division unless they are grown in monolayer cultures supplemented with serum and growth supplements. Growing chondrocytes in monolayer culture for a limited number of passages serves to increase the

number of dividing chondrocytes for subsequent transplant procedures but the major drawback of this approach is that chondrocytes dedifferentiate rapidly during monolayer proliferation (Moskalewski et al., 1979; Kuettner, 1982a,b; Shakibaei, 1995; Shakibaei et al., 1997; Schulze-Tanzil et al., 2002) and shift their differentiated phenotype to an undifferentiated fibroblast-like phenotype (Kuettner et al., 1982a). An altered cellular metabolism is an indication of chondrocyte dedifferentiation which is accompanied by reduced synthesis of cartilage-specific markers such as collagen type II and cartilage-specific proteoglycan (Benya and Shaffer, 1982). Dedifferentiation also changes the composition and abundance of key receptors and signalling molecules on the cell surface (Shakibaei, 1995). For these reasons, in prolonged monolayer culture, dedifferentiated chondrocytes continue to proliferate but appear to lose their chondrogenic potential irreversibly (Martin et al., 1999, Schulze-Tanzil et al., 2002). However, recent studies in our laboratories and several other laboratories have shown that monolayer cultured articular and auricular chondrocytes can regain their chondrogenic potential if they are transferred from an early monolayer passage to high-density culture. High-density and organoid cultures appear to promote chondrogenic redifferentiation since the 3-dimensional environment supports cell-cell interactions and closely mimics the physiological conditions of cartilage *in vivo* (Ahrens et al., 1977; Gay and Kosher, 1984; Solursh et al., 1987; Denker et al., 1999; Haas and Tuan, 1999; Naumann et al., 2004). These interactions are mediated by cell adhesion molecules such as integrins (Shakibaei, 1998; Haas and Tuan, 1999; Loeser, 1993; Schulze-Tanzil et al., 2001). Integrins are cell membrane protein dimers which are composed of one α and one ß-subunit. They bind components of the matrix thus regulating the behaviour of the cells (Hynes, 1992; Ruoslahti and Pierschbacher, 1987; Albelda and Buck, 1990).

Auricular cartilage plays an important role in reconstructive surgery due to the fact that the auricular region is easy accessible and possesses distinct characteristics of elastic cartilage. Despite this, only a limited number of studies have focused on the regenerative potential of auricular cartilage *in vitro* and *in vivo*. One such study performed on elastic cartilage used juvenile rabbit auricular chondrocytes as a model and focused on morphological characteristics of the cells such as microscopic cell size and extracellular matrix deposits within *in vitro* monolayer culture. In this model elastic chondrocytes maintained their ability to redifferentiate and to express elastic fibres *in vitro* and *in vivo* until 4 cell population doublings. Longer periods of cultivation resulted in a significant decline in the ability of chondrocytes to produce an extracellular matrix (Moskalewski et al., 1979). By comparing the collagen expression of hyaline and elastic rabbit cartilage during *in vitro* monolayer culture, the authors could demonstrate that auricular chondrocytes change their cellular phenotype more rapidly after transfer to monolayer culture than hyaline chondrocytes. Further studies revealed that high cell density promotes the increase of cartilage-specific proteins like tropoelastin expression in developing bovine elastic tissue which is closely linked to the expression of type II collagen and proteoglycans (Lee et al., 1994). Thus far explicit investigation of elastic cartilage in high-density culture has only been performed by Naumann and co-workers in 2004, demonstrating the capability of human nasal and auricular cartilage to develop new cartilage within a macro-aggregate culture system without a biomaterial carrier. The investigation focused on histological and immunohistological analysis, especially concentrating on the extracellular expression of collagens and elastin.

The first human auricular (elastic cartilage) chondrocyte cell culture model was presented by Quatela and co-workers in 1993. In this model auricular cartilage was obtained fresh from human cadavers for cell culture and investigated for the production of collagen type II and alkaline phosphatase both of which were found to be produced in monolayer culture in response to transforming growth factor-beta (TGF-ß) and basic fibroblast growth factor (bFGF). In contrast to hyaline nasal septal cartilage, auricular chondrocytes exhibited a significant response by increasing proliferation rate and matrix production (Quatela et al., 1993).

Since these studies were published, the mainstream research on the field of tissue engineering has focused on the three-dimensional reorganisation of the chondrocytes within different biomaterials and the ability to express cartilage-like proteins *in vivo* using immuno-competent and immuno-compromised animal models (Cao et al., 1998; Rodriguez et al., 1999; Arevalo-Silva et al., 2000, 2001Vacanti et al., 2000; Yang et al., 2000; van Osch et al., 2001). To date the cellular physiology and behavior of auricular cartilage in 3-dimensional culture systems have not been investigated for potential use of auricular chondrocytes in autologous cartilage transplantation or subcutaneous cartilage generation for applications in reconstructive surgery. Very limited basic information about the redifferentiation capability and extracellular matrix forming capacity of ear chondrocytes in high-density cultures is currently available from the research of Naumann and co-workers (Naumann et al., 2004). Accordingly, the aim of this study was to gather fundamental information about the *in vitro* behaviour of porcine auricular chondrocytes in monolayer and high-density culture and analyse intracellular, transmembrane and extracellular cartilage-specific proteins, and correlates the findings with cellular morphology by transmission electron microscopic analysis.

Material and methods

Antibodies, growth medium and chemicals

Collagen type II- (AB746), the monoclonal anti-cartilage proteoglycan (MAB 2015) and ß1-integrin-

antibodies (MAB1977), the secondary alkaline phosphatase-conjugated anti-mouse-IgG (AP326A) and the alkaline phosphatase-conjugated anti-rabbit-IgG (AP304A) antibodies were obtained from Chemicon International (Temecula, CA, USA). The anti-cartilage proteoglycan antibody is reported to react specifically with proteoglycans such as keratan sulfate from articular cartilage (Cs-Szabo et al., 1995). Vinculin (V9131) antibody was provided by Sigma (Munich, Germany). Cyclo-oxygenase-2 antibody was purchased from Cayman Chemical (CX229) (Ann Arbor, MI, USA). The active caspase-3 antibody (AF835) was obtained from R&D Systems (Abingdon, UK).

The chondrocyte growth medium consisted of Ham's F-12/DMEM Medium (50/50) supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Germany), 25 µg/mL ascorbic acid (Sigma, Munich, Germany), 1% essential amino acids (Biochrom AG, Berlin, Germany), 50 IU/mL penicillin (Biochrom AG, Berlin, Germany), 50 IU/mL streptomycin (Biochrom AG, Berlin, Germany) and 2.5 µg/mL amphotericin B (Biochrom AG, Berlin, Germany).

Chondrocyte culture

Primary chondrocytes were isolated from porcine auricular cartilage. The skin, connective tissue and perichondrium were removed from auricular cartilage. Cartilage was cut into small slices of approximately 2x2 mm and washed in growth medium. The cartilage slices were digested using 10 000 units collagenase type CLS II (Biochrom AG, Berlin, Germany), 30 units collagenase P (Roche, Mannheim, Germany) and 1000 units hyaluronidase (Sigma-Aldrich, Taufkirchen, Germany) dissolved in RPMI 1640 medium (PAA, Austria) containing 10% fetal calf serum (FCS), 50 IU/mL penicillin, 50 IU/mL streptomycin (Biochrom AG, Berlin, Germany) in a spinner flask (Wheaton, Milleville NJ, USA) for 16-18 hours at 37°C and 5% CO_2. The isolated cells were suspended in growth medium and a homogeneous suspension was prepared by repeated pipetting. The percentage of viable cells was determined by Trypan blue (0.5%) dye exclusion assays using a haemocytometer.

High-density culture

Primary chondrocytes (passage 0) were cultured as monolayers and passaged 7 times. The cultures were passaged when they reached 80-90% confluence. Cells were passaged by trypsinization, counted and reseeded in monolayer culture at a cell density of $5.1x10^3$ cells per cm^2. From each monolayer passage (passage 0-7) high-density cultures were prepared as previously described (Shakibaei et al., 1993, 1995; Shakibaei and de Souza, 1997). For the high-density cultures 10 µl cell pellets containing about $2x10^6$ cells were pipetted on a membrane filter with a pore diameter of 0.2 µm (Satorius, Göttingen, Germany) and placed on the top of a stainless steel grid at the medium-air interface in a Petri dish. This method has been described in detail in past publications from our laboratory (Zimmermann et al., 1992; Schulze-Tanzil et al., 2002). The cultures were grown for 7 days at 37°C in a humidified atmosphere with 5% CO_2 and growth medium was changed every 3 days.

Western blot analysis

Total protein was extracted from high-density cultures (passage 0-7) using lysis buffer [50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% (v/v) sodium dodecyl sulfate] on ice for 30 minutes. Total protein concentration was determined with the bicinchoninic acid system using bovine serum albumin (BSA) as a standard. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and were transferred for 60 minutes at 120 volts onto nitrocellulose membranes. After blocking the membranes at room temperature (RT) for 30 minutes in blocking buffer [5% (w/v) low fat skim milk powder, in phosphate buffered saline with 0.1% (v/v) Tween 20], they were incubated with the primary antibody for 1 hour at RT followed by washing with blocking buffer. The membranes were labelled with alkaline-phosphatase conjugated secondary antibody for 60 minutes. The membranes were washed with 0.1 M TRIS, pH 9.5 containing 0.05 M $MgCl_2$ and 0.1 M NaCl. Nitroblue tetrazolium and 5-bromo-chloro-3-indoylphosphate (p-toluidine salt; Pierce Rockford, IL) were used as substrates for alkaline phosphatase.

Transmission electron microscopy

The high-density cultures were fixed in Karnovsky-fixative and post-fixed in 1% tannic acid (0.1 M phosphate buffer) and 1% OsO_4 solution (0.1 M phosphate buffer) followed by a dehydration in ascending alcohol series and contrasting with 2% uranyl acetate/lead citrate. They were embedded in Epon and cut on a Reichert Ultracut. For examination of the cultures a transmission electron microscope (TEM 10, Zeiss, Germany) was used.

Results

Chondrocyte and their proliferation in monolayer culture

Freshly isolated primary auricular chondrocytes (P0) maintained in monolayer culture lost their chondrocytic phenotype and exhibited a fibroblast-like appearance. This monolayer culture was passaged every three days until passage P7. The chondrocytes proliferated rapidly (data not shown). Every third day the confluent monolayer culture cells were passaged, finally reaching passage 7. These cells increasingly assumed a polymorphic, fibroblast-like form. During monolayer

culturing, the proliferation rate of the fibroblast-like cells increased continuously. After culturing for 1, 8, 16 and 32 h (P3) on Petri dishes, the total number of cells increased by about 17% (p=0.0041), 58% (p=0.0014), 120% (p=0.00024) and 193% (p=0.0043), respectively, relative to the number present after 1 h (data not shown).

Ultrastructure of high-density cultures prepared from monolayer chondrocytes

High-density cultures were prepared from chondrocytes of monolayer passages 0, 1-7 and cultivated for 1 to 7 days under identical conditions, before they were fixed and prepared for transmission electron microscopy. High-density cultures from early monolayer passages (passage 0 and 1 until passage 4) exhibited nodules containing viable, large and round cells, a large euchromatic nucleus, several nucleoli, small vacuoles and a well structured cytoplasm surrounded by a few layers of flattened cells like a perichondrium. In the perichondrium thick collagen fibrils could be seen. On the first day of high-density culture, cells lay in intimate contact exhibiting intensive cell-cell contacts. The cells in the surface layer assumed an increasingly spherical shape, cells of the deeper layer had a flattened shape (Fig. 1A). On day seven large cartilage nodules could be observed in high density cultures derived from passages 0-4 high-density cultures (Fig. 1B-F). Chondrocytes were embedded in an extensive fine fibrillar matrix tightly attached to the cytoplasmic membrane. Rounded auricular chondrocytes had a well structured cytoplasm containing well developed cell organelles such as rER, mitochondria, glycogen granules, free cytoplasmic ribosomes, Golgi apparatus and prominent large euchromatic nuclei with nucleoli. Cell populations from passage 4 (Fig. 1F) transferred to high-density culture revealed matrix embedded chondrocytes that showed early signs of degeneration such as dilated rER, some areas of condensed chromatin in their nuclei and vacuoles. Cells from passage 5-7 (Fig. 1G-H; passage 7: data not shown) transferred to high-density culture showed clear signs of cell lysis, extensive matrix loss and some characteristic features of apoptotic cell death such as apoptotic bodies

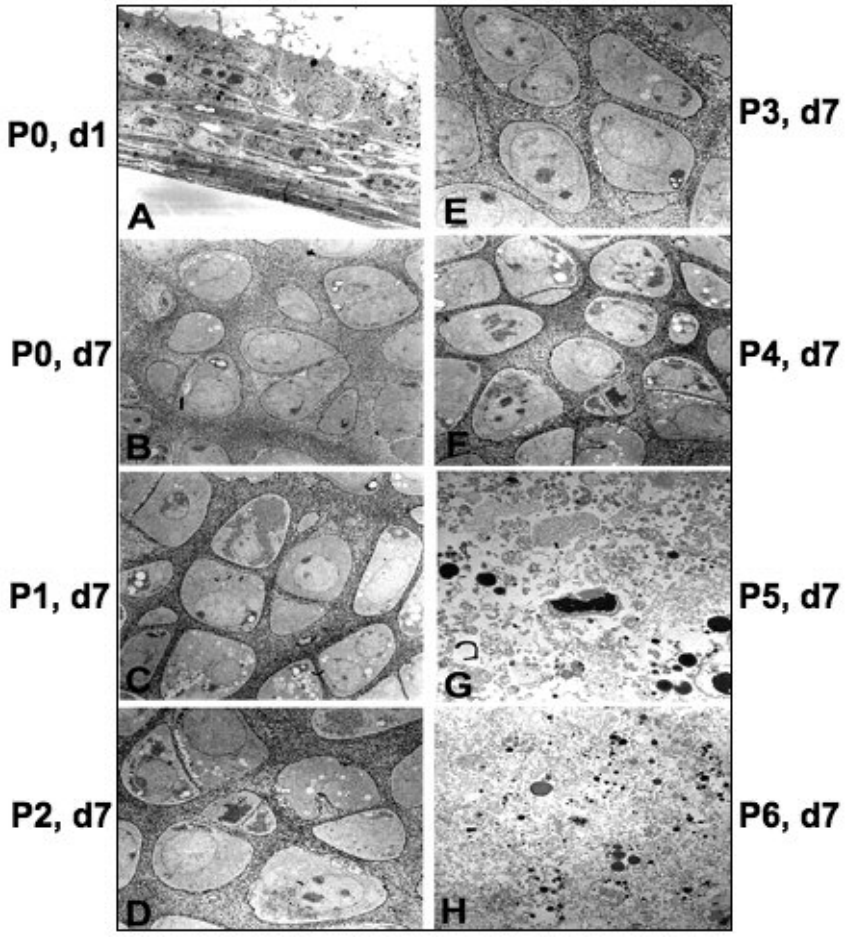

Fig. 1. Electron microscopic demonstration of high-density cultures of auricular chondrocytes. Electron micrographs of chondrocytes (C) in 1-day (passage 0) **(A)** or 7-day old high-density cultures **(B-H)** that derived from passages 0 and 1-7. Chondrocytes from passage 0-4 **(B-F)** had a typical round or oval profile. At day seven they were embedded in an extensive fibrillar cartilage matrix. Redifferentiating chondrocytes exhibited a well structured cytoplasm containing a well-developed rough endoplasmic reticulum, Golgi apparatus, the usual complement of mitochondria, vacuoles and cytoplasmic granules. Cells from passage 4 **(F)** had slightly degenerated and dilated rER and areas of condensed chromatin in chondrocyte nuclei could be demonstrated. Cells from passage 5-6 **(G-H)** introduced to high-density culture revealed clearly cell lysis, extensive matrix loss and some features of apoptotic cell death such as apoptotic bodies and chromatin condensation. x 4,000

and annular chromatin condensation around the nuclear membrane.

Deposition of phenotypic markers in auricular chondrocytes in high-density culture

Western blot analysis was performed to determine the synthesis of key proteins in auricular chondrocytes in high-density culture. Specimens from each passage (passage 0, 1-7) were analysed. The deposition of collagen type II and cartilage proteoglycan in the high-density cultures was continuously reduced from passage 4 onwards (Fig. 2A,B). Fibronectin, and ubiquitous extracellular matrix proteins revealed a similar synthesis pattern showing a markedly decreased expression from passage 4 onwards (Fig. 2C). Expression of the transmembrane cell-matrix receptor protein ß1-integrin was also investigated and found to continuously decrease after passage 4 (Fig. 2D).

Irreversibly dedifferentiated auricular chondrocytes introduced in high-density culture show evidence of commitment to apoptosis by expressing activated caspase-3

Expression of the apoptosis marker activated caspase-3 which indicates cell commitment to apoptosis was evaluated to establish whether dedifferentiating chondrocytes undergo apoptotic cell death in high-density culture. From passage 0 and 1 until passage 2 there was no signal for the activated caspase-3 in Western blots. However, in passage 3 cells the first traces of activated caspase-3 could be seen which increased until passage 7 (Fig. 3A). Densitometric evaluation of a representative Western blot experiment to analyse the appearance of activated caspase-3 expression by human chondrocytes in high-density culture is shown in Fig. 3B.

Expression of matrix degrading enzymes and inflammatory markers in auricular chondrocyte high-density culture

In order to explain the pericellular matrix loss observed by electron microscopy and to test the hypothesis that irreversible dedifferentiation may correlate with enhanced synthesis of matrix degrading enzymes the production of matrix metalloproteinases was investigated. Expression of MMP-9 and MMP-13 (Fig. 4A) was detected in high-density cultures from passage 3 onwards and this expression increased in later passages. To establish whether the synthesis of inflammatory markers increases in later passages we studied the expression of the COX-2 enzyme.

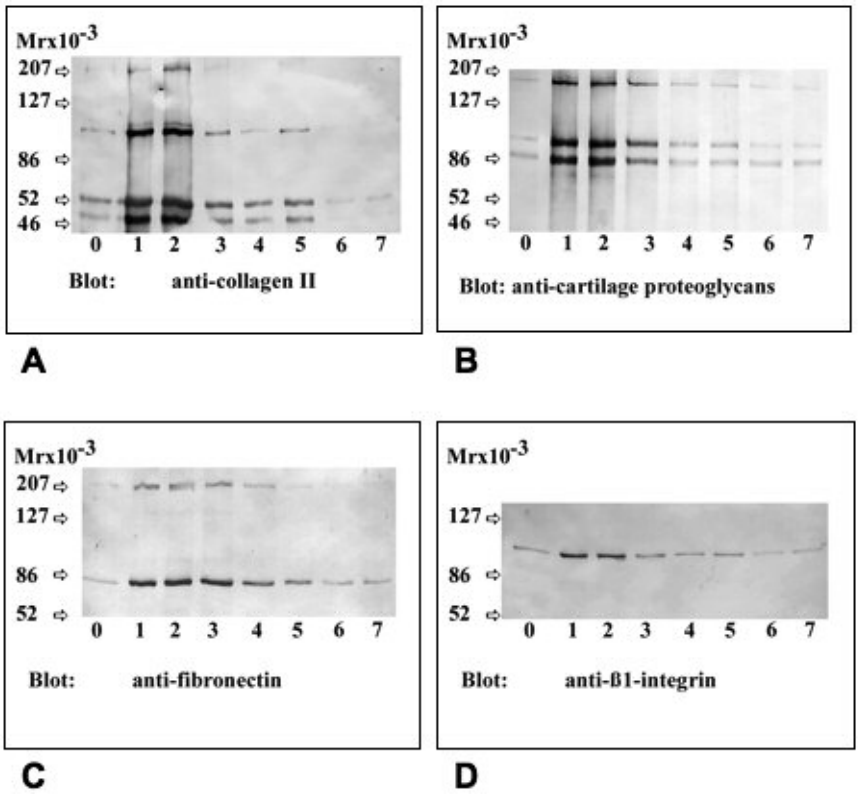

Fig. 2. Demonstration of extracellular matrix proteins in high-density cultures by Western blot analysis. The deposition of collagen type II **(A)** and cartilage proteoglycan **(B)** in the high-density cultures was continuously reduced from passage 4 onwards. Fibronectin **(C)** revealed a similar synthesis pattern showing a markedly decrease from passage 4 onwards. Synthesis of the transmembrane cell-matrix receptor protein ß1-integrin **(D)** continuously decreased after passage 4. Data shown are representative of three independent experiments.

Interestingly, elevated COX-2 synthesis was observed in high-density cultures from later passages (passage 3 onwards; Fig. 4A). Densitometric evaluation of a representative caspase-3 Western blot experiment is shown in Fig. 4B.

Discussion

In the present study we established an *in vitro* model of porcine auricular chondrocytes and evaluated the behaviour of the cells in monolayer and high-density cultures. Three-dimensional high-density cultures for cultivation of chondrocytes are gaining increasing popularity because they reflect the *in vivo* cartilage conditions more closely than monolayer cultures. The results we have presented in this paper are summarized as follows: (1) Auricular chondrocyte cultures maintained up to the fourth monolayer passage (P4), dedifferentiated but were able to redifferentiate when introduced to high-density culture and produced cartilage-specific markers (collagen type II and CSPG) after a few days in the new culture conditions. (2) Cells from monolayer passages P5-P7 did not redifferentiate in high-density culture. These cells underwent apoptotic cell death and exhibited typical morphological features of apoptosis (or chondroptosis) and showed evidence of activation of the apoptosis commitment marker caspase-3. (3) These irreversibly dedifferentiated auricular chondrocytes from later passages produced matrix degrading enzymes such as MMP-9 and MMP-13 and expressed the pro-inflammatory enzyme COX-2 whereas cells from earlier passages did not.

Pioneering studies have demonstrated that after only a few days in monolayer culture, primary articular and elastic ear chondrocytes completely dedifferentiate to fibroblast-like cells (Benya and Shaffer, 1982; Shakibaei, 1995; Shakibaei et al., 1997; Mandl et al., 2004). This property of primary, freshly isolated chondrocytes presents researchers and clinicians with a real challenge: in order to repair and cover cartilage lesions fairly large quantities of fully differentiated chondrocytes are needed for autologous chondrocyte transplantation (ACT). It is therefore advisable to avoid using irreversibly dedifferentiated chondrocytes which have been cultivated for prolonged periods of time in monolayer cultures as such cells are not able to produce a biomechanically competent and biochemically specific extracellular matrix with sufficient load bearing resistance akin to that found in cartilage *in vivo* and, therefore, are not suitable for chondrocyte transplantation.

The results of this study confirm that porcine auricular chondrocytes from monolayer passages P0, P1-P4 (as opposed to cells from P5-P7) redifferentiate into chondrocytes after transfer to high-density culture. These cells partly redifferentiated into cartilage specific matrix producing chondrocytes localized in the nodules of high-density cultures and partly into prechondrogenic mesenchymal cells, which form the internodular space, as previously shown in articular chondrocytes (Shakibaei, 1998; Schulze-Tanzil, et al., 2002). In contrast cells from monolayer passages P5-P7 were not able to redifferentiate in high density cultures. These cells remained fibroblast-like or underwent cell death

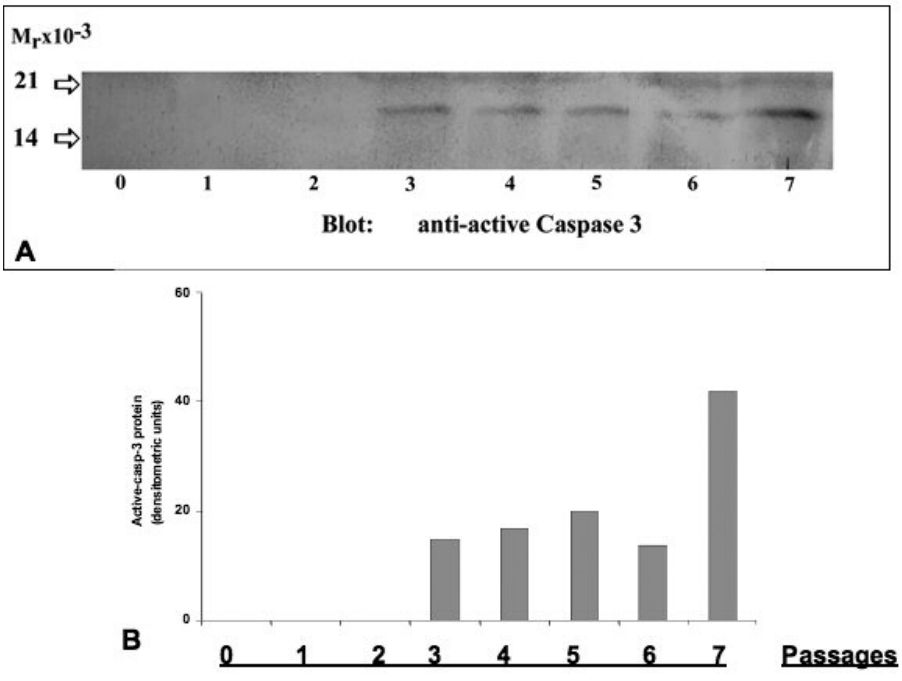

Fig. 3. Western blot analyses of the apoptosis marker activated caspase-3 proteins in high-density cultures. **A.** From passage 1 to passage 3 there was no detectable activated caspase-3. In contrast, in passage 4 cells the first traces of activated caspase-3 could be demonstrated. Caspase-3 expression increased up to passage 8. **B.** Densitometric evaluations of the Western blot analyses. Data shown are representative of three independent experiments.

and did not produce any cartilage-specific markers, neither in monolayer culture nor in high density culture. It appears that these cells lose their chondrogenic potential at or around the 5th monolayer passage demonstrating the loss of redifferentiation capacity. The ultrastructural features of chondrocytes from these later monolayer passages revealed extensive loss of extracellular matrix, cell lysis and typical apoptotic features. The occurrence of apoptotic cell death in later passages was demonstrated by electron microscopy and confirmed at the molecular level by Western blot analysis of caspase-3 activation which is a molecular marker of cell commitment to apoptosis (Cohen, 1997).

It is well known that cell-cell contacts play a major role during the first phase of chondrocyte differentiation in chondrogenesis, a phase known as the cell condensation stage (Denker et al., 1999). This stage also has been observed at early stages of high density culture. Cell-matrix interactions are essential for chondrocyte survival and differentiation (Hirsh et al., 1997; Yang et al., 1997). Accordingly, inhibition of ß1-integrin-collagen interaction has also been shown to lead to chondrocyte death (Cao et al., 1999; Pulai et al., 2002) and additionally, inhibition of ß1-integrins by integrin antibodies has been shown to prevent chondrogenesis (Shakibaei, 1998). In this study we have demonstrated the presence of the adhesion molecule, ß1-integrin signaling proteins in auricular chondrocytes in the first 5 passages in high density cultures. It is well accepted that integrins play a central role in cell/cell and cell/extracellular matrix interactions as adhesion receptors in various tissues including cartilage (Albeda and Buck, 1990; Loeser et al., 1995; Enomoto et al., 1993; Shakibaei et al., 1995, 1997; Shakibaei, 1998). The MAPkinase pathway is an essential signal transduction pathway mediating cell survival and differentiation (Shakibaei et al., 1999, 2001). Inhibition of MAPkinase pathway leads directly to chondrocyte apoptosis (Shakibaei et al., 2001). Integrins have been shown to mediate activation of MAPkinase pathway.

The signalling pathways responsible for apoptosis induced by dedifferentiation also remain unclear, but chondrocyte apoptosis seems to correlate closely with loss of cartilage-specific matrix production. The interaction between chondrocytes and cartilage-specific matrix components has been shown to be a prerequisite of chondrocyte survival (Yang et al., 1997). In previous investigations with articular cartilage we have shown that inhibition of the MAPkinase pathway during irreversible dedifferentiation of chondrocytes, leads to

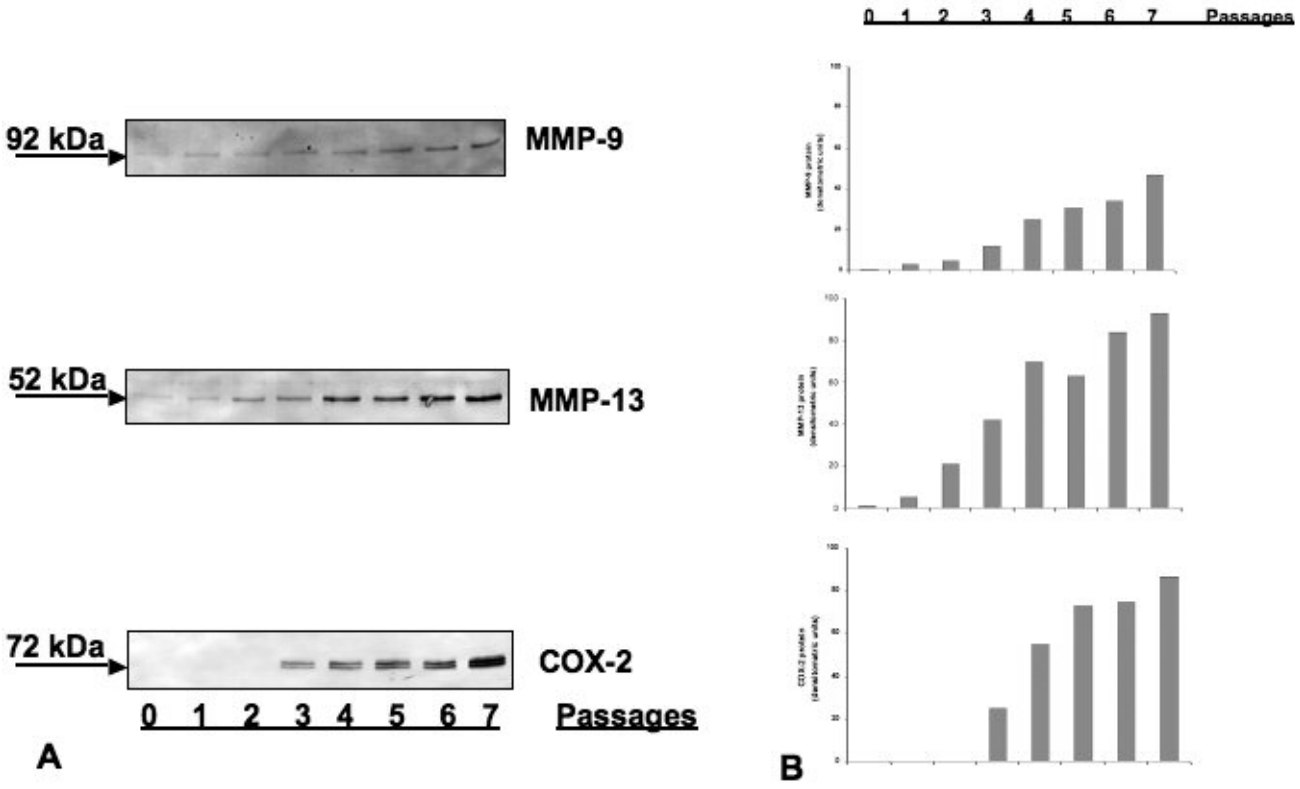

Fig. 4. Western blot analysis of MMP-9, MMP-13 and COX-2 proteins in high-density cultures. **A.** Synthesis of MMP-9, MMP–13 and COX-2 was detectable in high-density cultures in passage 3 and increased in passages 4-7. **B.** Densitometric analysis of the Western blot shown in A. Data shown are representative of three independent experiments.

apoptosis. This suggests that this important signaling pathway is involved in the maintenance of chondrogenic potential (Schulze-Tanzil et al., 2004).

To clarify whether enhanced cartilage matrix degradation may also contribute to the loss of extracellular matrix, the expression of matrix degrading enzymes such as matrix metalloproteinases was studied by Western blot analysis. Matrix metalloproteinases (MMPs) mediate extracellular matrix remodelling/turn over by degrading extracellular matrix macromolecules under physiological conditions (Schmitz et al., 1996). They are expressed as inactive zymogens and require activation. Activation of MMPs occurs by the removal of an amino-terminal domain by auto-activation, by other proteinases or by other MMPs (Schmitz et al., 1996; Horton et al., 1998; Yong et al., 1998). Expression and activity of MMP-1, MMP-9 and MMP-13 has been reported to be increased in osteoarthritic cartilage (Freemont et al., 1997; Horton et al., 1998; Saito et al., 1998). It has been reported, that proteolysis of ECM proteins alters integrin-mediated anchorage, the organization of focal adhesions, cytoskeletal architecture and signalling molecules like focal adhesion kinase (FAK), paxillin, talin, etc. Binding of cleaved ECM fragments by integrins also activates different subcellular signalling pathways (Werb, 1997). In many pathophysiological cartilage conditions when chondrocyte homeostasis is disturbed MMP production increases; MMP-13 seems to play a pivotal role in type-II collagen degradation observed in arthritis (Billinghurst et al., 2000; Neuhold et al., 2001). Increased production of matrix metalloproteinases MMP-9 and MMP-13 was seen in later passages in the present study and may be interpreted as a feature that subsequently follows cell lysis and apoptosis induced by irreversible chondrocyte dedifferentiation.

The inflammatory mediator cyclooxygenase-2 (COX-2) is known to lead to prostaglandin E_2 (PGE_2)-production and further inflammation and cartilage damage in a variety of arthritic conditions. For this reason, we also investigated the expression of COX-2 in auricular chondrocytes in high density cultures and found an increased expression of COX-2 in the present study which could be interpreted as an inflammatory feature that subsequently follows the lysis and apoptosis induced in a proportion of cells by irreversible dedifferentiation.

In summary, the three-dimensional high-density culture adopted in this study seems to be a suitable culture model for promoting auricular chondrocyte redifferentiation as an approach for obtaining fully differentiated auricular chondrocytes for tissue engineering applications and reconstructive plastic surgery.

Acknowledgments. Ms. Angelika Steuer, Mrs. Angelika Hartje, Mr. Benjamin Kohl and Mr. Jörg Romahn are gratefully acknowledged for their excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (HA 2729/1-5).

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Accepted November 22, 2005