

Scaffold-dependent differentiation of human articular chondrocytes

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Abstract. Matrix-associated autologous chondrocyte transplantation (MACT) is a tissue-engineered approach for the treatment of cartilage defects and combines autologous chondrocytes seeded on biomaterials. The objective of the study is the analysis of growth and differentiation behaviour of human articular chondrocytes grown on three different matrices used for MACT. Human articular chondrocytes were kept in monolayer culture for 42 days and then seeded on matrices consisting of either collagen type I/III, hyaluronan, or gelatine. During the culture time of 4 weeks the constructs were analyzed weekly. Morphological criteria were studied by scanning and transmission electron microscopy. The expression of the main type collagens was analyzed by real-time PCR. The collagen type I/III matrix supported a differentiation that closely resembled the tissue organisation of native cartilage, but cell number and type II collagen synthesis were low and differentiation occurred rather late in the cultivation period. The hyaluronan matrix and the gelatine-based matrix supported a rather rapid differentiation, with a high number of cells and a relatively high amount of type II collagen, but there was no spatial assembly that mimicked native cartilage. These facts indicate that the nature of the matrix is of great influence in the differentiation behaviour of dedifferentiated chondrocytes.

Introduction

Hyaline cartilage is a highly specialised tissue that lacks vascularization and innervation (1,2). Terminally differentiated chondrocytes are the only cellular component of adult hyaline

cartilage, accounting for 1-5% of the total volume and maintaining the matrix of the extracellular cartilage (3). When the cartilage layer is destroyed, there is no functionally appropriate tissue for *in vivo* replacement (4,5). Defects are covered with fibrous or fibrocartilaginous tissue, which does not withstand the demands of reversible deformation. Mechanical pressure is no longer hampered, but rather, transferred to the highly vascularized and well-innervated cartilage/bone border and evokes recurrent pain with every movement of the joint (6,7).

For the treatment of focal cartilage defects, biological methods have been developed with the *in vitro* expansion of patients' own chondrocytes (8,9). But, such chondrocytes that were propagated in monolayer culture for a specific time display different characteristics than do native chondrocytes (10). When re-entering the cell cycle *in vitro*, the chondrocytes lose their rounded shape and exhibit a flattened fibroblast-like appearance (11,12). One of the goals of tissue engineering is the expansion and transplantation of cells that match the original tissue as closely as possible. The combination of autologous chondrocytes seeded on biomaterials has been introduced as matrix-associated autologous chondrocyte transplantation (MACT) for the biological treatment of focal cartilage defects in the knee joint (10,11,13). Several scaffolds have been developed to support the redifferentiation process (14-20). These scaffolds are to fulfil two major functions. First, they serve as a carrier for chondrocytes and cover the defect; second, they are designed to support the redifferentiation of the cultivated cells (19,21,22).

The purpose of this study was to investigate the morphological and molecular biological changes of cultivated human articular chondrocytes on different scaffolds with regard to the change of the expression pattern of type I and type II as the hallmarks of cartilage differentiation.

Materials and methods

Cultivation of the cells. Human articular chondrocytes (HACs) were collected from the femoral head of patients with no history of joint disease, who were scheduled to undergo joint replacement following femoral neck fracture (approved by the Ethics Board of the University; code 184/98). The chondrocytes were pooled with a donor age of 26 to 94 years and

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Table I. Morphological criteria for differentiated and dedifferentiated chondrocytes.

Criteria for differentiated chondrocytes	Criteria for dedifferentiated chondrocytes
SEM	SEM
Spherical to ovoid cell shape	Filiform or polygonal, flat cell shape
Small cellular protrusions may be present for adhesion to the substratum or to neighbouring cells	Extensive cell processes
Numerous microvilli	Few microvilli
TEM	TEM
Spherical or lobed nucleus	Ovoid, probably lobed nucleus
Nucleus should be surrounded by a rime of matrix containing cell organelles - nucleus: cytoplasm relation >1:1	Nucleus is closely surrounded by the cell membrane in cross sections and on two sides in longitudinal sections
Missing of cell processes	Presence of elongated cell processes
Whirls of intermediar filaments in the cytoplasm or around the nucleus (facultative)	Rich in cytoskeleton; frequently strands of actin filaments and microtubuli underlie the cell membrane and support the cell processes

For the evaluation of the relation of the two cell types was done according to the following criteria: only cell sectioned in the plane of the nucleus have been considered peripheral sections (visible by tangential cut nucleus membranes) have been excluded from the evaluation cell processes have been excluded from the evaluation.

mixed gender. The cells were cultivated in monolayer culture for 42 days according to our standard operation procedure, as previously described (23). After day 42 of cultivation three biomaterials consisting of hyaluronan (HYAFF® 11 Fidia, FAB, Italy), a porcine collagen I/III fleece (Geistlich Pharma, Wolhusen, Switzerland) and a gelatine gel matrix (Ferrosan, Soeborg, Danmark) were seeded with a single-cell suspension that contained 5×10^5 cells. All membranes were used in an uncoated form. Culture medium for both, monolayer culture and 3D culture consisted of DMEM, containing 1% L-glutamine, 2 µg/ml Amphotericin B, 50 mg/l ascorbic acid, 100 mg/l gentamycin, 5 mg/l insuline and 10% FCS. During the culture time of four weeks in a stationary culture system, the tissue constructs were analysed in a weekly sequence at days 3, 10, 17, 24 and 31.

Electron microscopy. Morphological criteria were studied by scanning and transmission electron microscopy (SEM, TEM). Samples were prefixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.3 for 24 h at 4°C. The tissues were washed three times in the same buffer. Post-fixation was carried out in 1% buffered osmium tetroxide for 2 h. Afterward, the samples were washed again three times in the same buffer solution.

TEM samples were dehydrated in a graded ethanol series and infiltrated in Agar 100 resin with propyleneoxide as a medium and were then polymerised for three days at 60°C. Ultrathin sections of 70 nm were obtained with a diamond knife on Ultracut S (Leica) and collected on copper grids. Staining was performed with LKB 2168 Ultrastain with a conventional solution of uranyl acetate and lead citrate. The sections were examined on a Zeiss EM 902 electron microscope.

SEM samples were chemically dehydrated with 2.2 dimethoxypropane, dried with hexamethyldisilazane and

coated with gold in a Polaron 5800 sputter coater. Examination followed on a Philips XL 20 scanning electron microscope. The evaluation of the SEM and TEM samples was performed according to the criteria summarized in Table I.

Extraction and purification of total RNA from chondrocytes cultivated on scaffolds. Total RNA was obtained from cells being cultivated on 3D scaffolds. Cells were harvested every week. Scaffolds were put into liquid nitrogen and disrupted with a mortar and pestle. Using the RNeasy® plant mini kit (Qiagen, Germany), the disrupted material was transferred to a QIAshredder™ spin column sitting on a 2 ml collection tube. The further procedure for isolating total RNA was executed as described in the RNeasy Mini Handbook (Qiagen, Germany, 06/2001).

The purity and amount of RNA were determined by measurement of the OD_{260/280} ratio. All samples showed purity indices between 1.5 and 1.8.

cDNA synthesis. Total RNA (0.2-1 µg) was diluted to a volume of 12.5 and 1 µl of oligo(dt)18 primer (20 µM) was added. The RNA was heated for 2 min at 70°C and quenched on ice. Using the Advantage™ RT-for-PCR kit (Biosciences Clontech, CA, USA) the cDNA was synthesized according to the manufacturer's instructions.

Primers and probes for quantitative analyses. Primers and probes of collagen type I and II were designed using the Primer3 program (http://www-genome.wi.mit.edu/genome_software/other/primer3.html), to create oligonucleotides with similar melting temperatures and minimal self-complementarity (Table II). To avoid amplification of genomic DNA, the probes were placed at the junction of two exons. Gene specificity of the primers and probes and the absence of DNA polymorphism were confirmed by BLASTN searches. Primers

Table II. Description of the designed primers and probes for real-time PCR.

Gene	Forward and reverse primers (5'→3')	Primers concentration on used (nM)	Probe ^a (5'→3')	Probe concentration on used (nM)	Amplicon size (bp)
Collagen I	ATGCCTGGTGAACGTGGT	900	ACCAGCATCACCTCTGTCCACCCTT	200	87
	AGGAGAGCCATCAGCACCT	900			
Collagen II	GCCTGGTGTTCATGGGTTT	300	AAAGGTGCCAA <u>CG</u> GTGAGCCT	200	71
	GTCCCTTCTCACCAGCTTTG	300			

^aThe underlined bases in the probe sequence indicate the position of an intron in the corresponding genomic sequence.

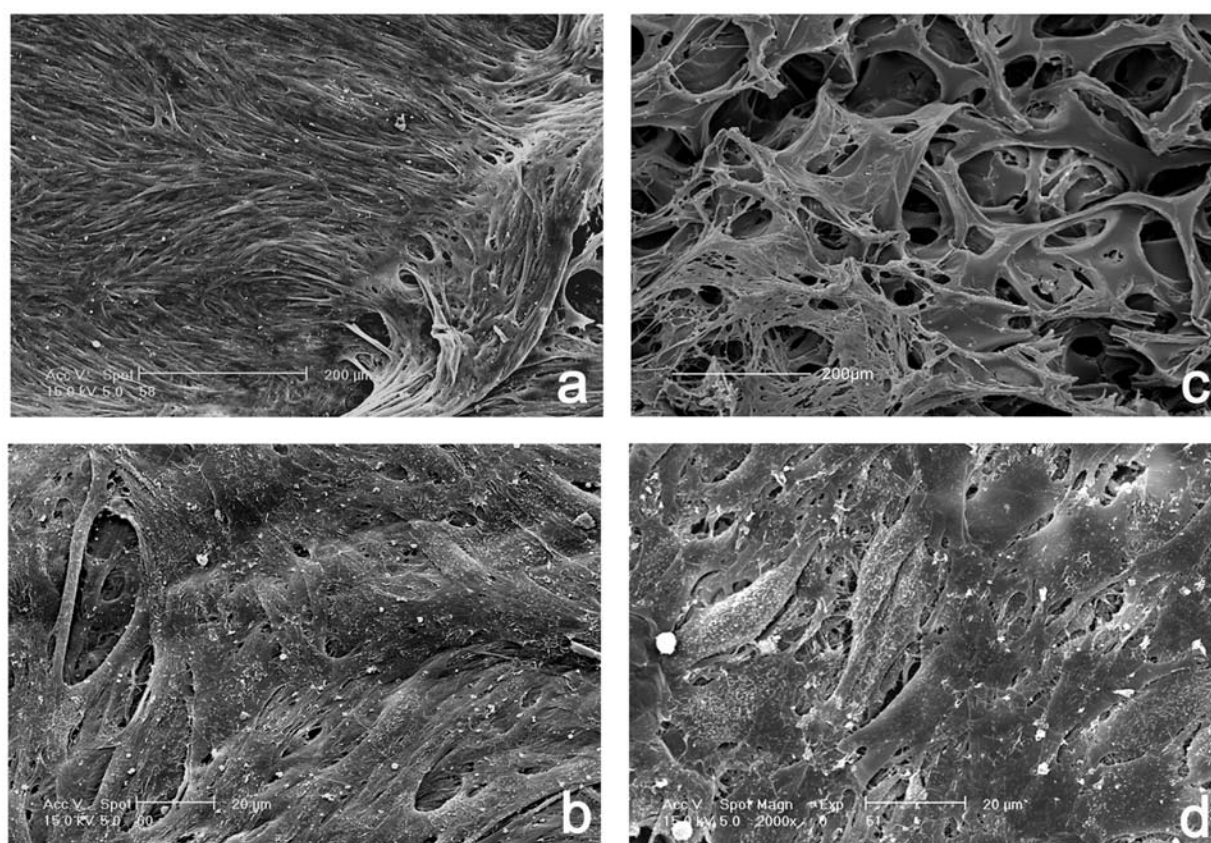


Figure 1. Collagen I/III fleece and Gelatin scaffold two days after seeding. The collagen I/III fleece presents with a confluent cell layer of elongate to polygonal, flat cells with a smooth surface (a and b); the Gelatin scaffold shows a cell layer locally (c), which consists of very different morphotypes, comprising flat polygonal to elongated cells, as well as some rounder cells with microvilli on the surface (d).

and probes were synthesised from GenXpress (Maria Wörth, Austria).

Real-time PCR amplification and analysis. For the quantification of mRNA, real-time PCR was performed. PCR amplification was performed and monitored using an ABI Prism® 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems, CA, USA). Master mix was based on Brilliant™ Quantitative PCR Core Reagent kit (Stratagene, CA, USA). The thermal cycling conditions comprised the initial steps at 50°C for 2 min and at 95°C for 10 min. Amplification of the cDNA products was performed with 40 PCR cycles, consisting of a denaturation step at 95°C for 15 sec and an extension step at 60°C for 1 min. All samples were normalised to β -actin, using the pre-developed

Taqman® assay (Applied Biosystems). All cDNA samples (5 in 25 μ l) were analysed in triplicate. The final numeric value was calculated as the ratio of the collagens to β -actin and expressed in arbitrary units. The standard curve for type 1 collagen was defined with human fibroblasts and the relative value 1 was assigned to this expression.

Results

Scanning electron microscopy (SEM). In the collagen type I/III membrane and the gelatine-based matrix two days after seeding a close cell layer of fibroblast-like cells covered the carrier surface nearly completely (Fig. 1a-d). Contrary to the collagen type I/III and gelatine-based matrices, the hyaluronan-based scaffold bore only small cell groups that either encircled

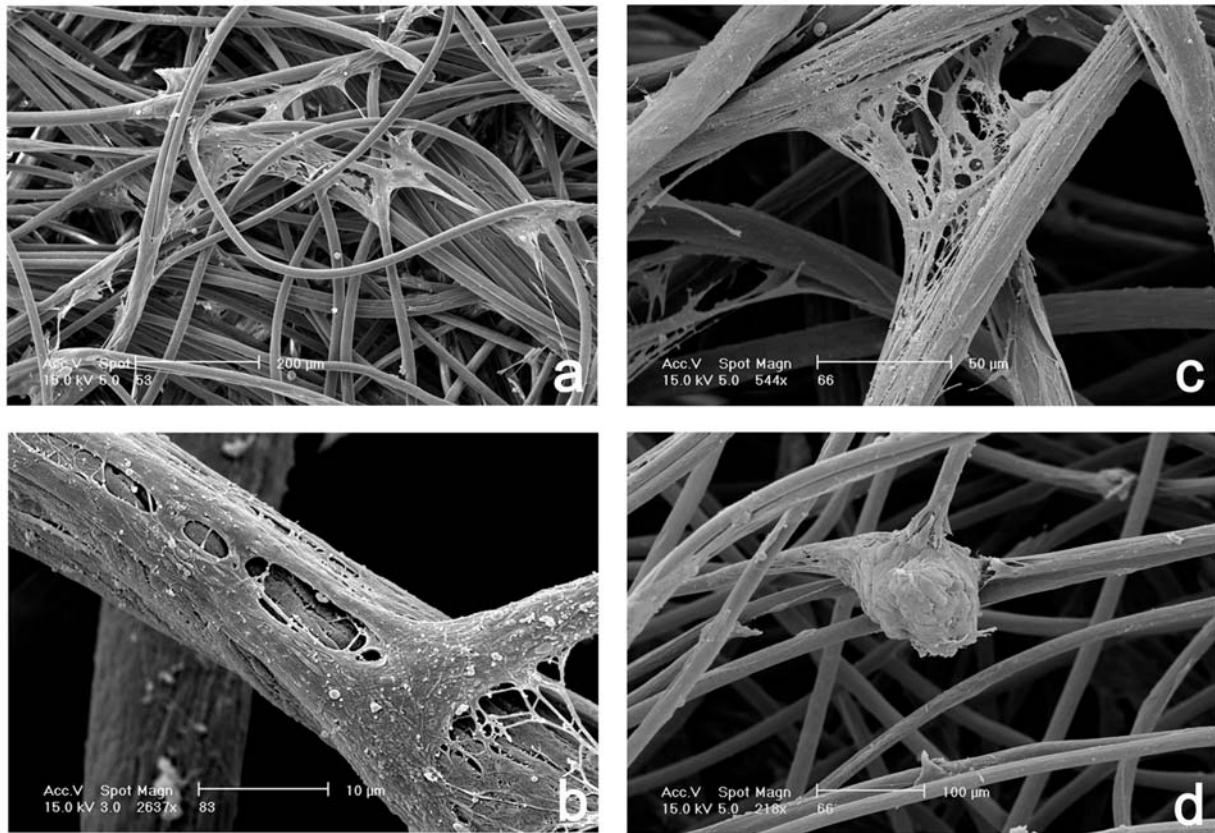


Figure 2. Hyaluronan scaffold two days after seeding: small cell groups are discernible on the fiber network (a). Higher magnification shows flat, elongated cells stretching along the carrier fibers (b), flat and polygonal cells spanning the fibers (c) and clots of spherical cells (d).

the scaffold fibers, spanned the fibers, or formed clot-like cell aggregates (Fig. 2a-d). Cells encircling the hyaluronan fibers were elongated (Fig. 2b) and those spanning between the fibers were polygonal and flat (Fig. 2c). Both cell types referred to dedifferentiated chondrocytes. Clot-like aggregates consisted of spheroidal cells, bearing microvilli on the surface, which indicated the differentiated phenotype of chondrocytes (Fig. 2d).

Within the following week, the cell layers on the collagen fleece and the gelatine scaffold became denser and homogeneous. On the hyaluronan fibre network, the cell nests enlarged and formed small cell layers. The number and extension of scaffold fibres encircled by elongated cells was increased. After four weeks of cultivation, cell layers were formed on the surface of all three scaffold types. Due to the scaffold texture, the cell layer on the collagen type I/III membrane showed a smoother surface than that on the gelatine sponge (Fig. 3a and b). On the hyaluronan carrier, an extended cell layer was formed, but often did not cover the whole surface (Fig. 3c).

Transmission electron microscopy - subcellular observations.

The subcellular characteristics confirmed the phenotype indicated by the outer cell morphologically. The spherical chondrocytes with microvilli had a round or lobed nucleus, some synthetically active cell organelles [such as rough endoplasmatic reticulum (rER)], a Golgi-apparatus and some mitochondria, infrequent cytoskeletal elements (Figs. 4a, 5a, 6a and b). These characteristics corresponded to chondrocytes

in native cartilage and therefore were indicative of the differentiated stage. The elongated cells with few microvilli possessed an ovoid nucleus and were rich in cytoskeletal elements, comprising microtubuli, actin filaments and intermediary filaments (Fig. 7c).

After three weeks of cultivation, signs of synthetic activity, such as glycogen accumulation visible as empty cytoplasmic spaces (EPS) and lipid droplets, became visible in both the differentiated and the dedifferentiated cells (Figs. 4a, 5a and b and 6a and b). In all cell cultures, electron-dense inclusions (EI) were present intracytoplasmically from the initial point of observation onward (two days after seeding) (Fig. 7a). The EIs derived from the Golgi apparatus and became highly numerous, with continuing cultivation. After two weeks, normal matrix vesicles detached from the Golgi apparatus and displaced the EI to the cell periphery. This process correlated with the appearance of fibrillar ECM in the extracellular space.

The cell layer on the collagen I/III scaffold, which was visible from the initial observation point onward (two days after seeding), initially consisted of one or two cell layers of dedifferentiated, fibroblast-like cells. After two weeks, the layers were extended to a cell multi-layer. The outermost cell layers were still fibroblast-like; the cells underneath displayed a chondrocyte-like appearance, and they were rather spherical, contained round or lobed nuclei, lipid droplets and empty cytoplasmic spaces. Flocculent and fibrous matrix filled the intercellular space (Fig. 4a). The morphology of the cells was elongated to polygonal (Fig. 4b). A cell multi-layer similar to that on the collagen carrier was formed on the gelatine scaffold

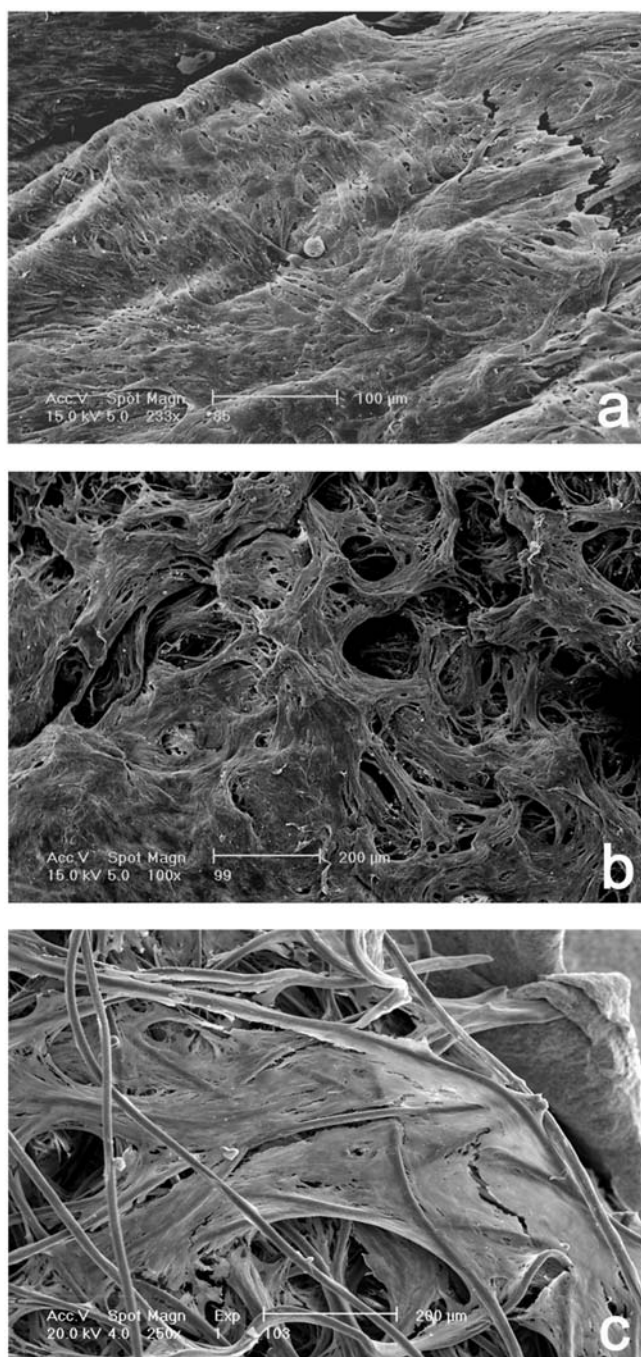


Figure 3. The cell-biomaterial constructs for weeks after seeding. A confluent cell layer of flat cells entirely covers the carrier surface of the collagen I/III fleece (a); an extended cell layer is locally present on the surface of the Gelatin scaffold (b); an extended cell layer stretches between the fibers of the hyaluronan scaffold (c).

(Fig. 5a). Inside the scaffold the cell number was higher than in the collagen type I/III matrix during the whole cultivation time. Apart from some spherical cells, the mainly morphology was polygonal to elongated (Fig. 5b).

The amount of ECM detected around single cells was minimal, regardless of culture time, culture system, or phenotype of the cell. Cell groups or cell multi-layers, in contrast, contained ECM in the intercellular space, which became denser with the cultivation time and thickness of the cell multi-layers.

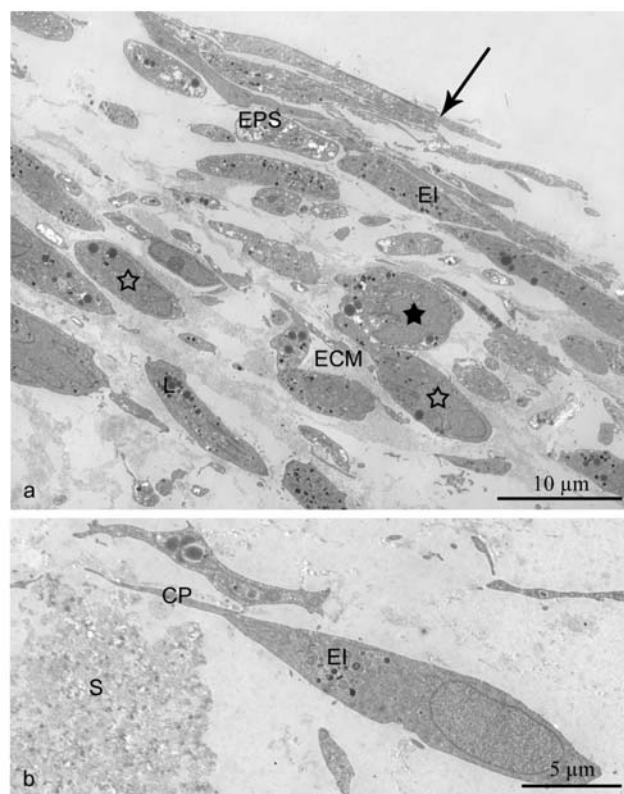


Figure 4. (a) TEM image of the cell multi-layer on the surface of the collagen type I/III scaffold after 31 days of cultivation. The outermost chondrocytes are fibroblast-like extending elongated cell processes (arrow). In deeper regions of the multi-layer a higher amount of ECM is present between the cells and the cells become ovoid (empty asterisk) or spherical (asterisk). The cells are separated by ECM. L, lipid droplets; EPS, empty cytoplasmic spaces and EI, electron dense inclusion are present in spherical as well as elongated cells. (b) Fibroblast-like chondrocyte spanning between the scaffold fibers (S) after 24 days of cultivation on the collagen type I/III carrier. CP, cell process and EI, electron dense inclusions.

Real-time PCR. With regard to the reference gene, relative collagen type I expression in cells grown on the hyaluronan matrix and on the gelatine gel matrix dropped from $\sim 6 \times 10^0$ to 2×10^{-1} (Fig. 8). Expression of collagen type I on the collagen I/III matrix showed a slight increase along the cultivation period from 2×10^{-1} to 6×10^{-1} . Thus, after a cultivation period of 31 days, all three culture systems showed a similar relative amount of collagen type I expression.

Similar to the expression of collagen type I, the expression of collagen type II on the hyaluronan matrices was decreased from 2×10^0 to 2×10^{-1} (Fig. 9). Gelatine gel matrix reduced collagen type II expression from 1.5×10^{-2} to 7×10^{-4} . Collagen type II expression on the collagen type I/III matrix showed a very low relative expression of 2.5×10^{-5} at day 3, reached a peak expression of 2.6×10^{-2} at day 17, showed a decline at day 24 of 5×10^{-4} and increased to 1×10^{-3} by day 31; this corresponds to a 100-fold increase. The expression pattern of collagen type II on the gelatine-based matrices showed a constant decline from 0.15×10^{-2} on day 3 to 2×10^{-4} on day 24 and increased to 7×10^{-4} on day 31.

The differentiation index, defined as the ratio of collagen type II to collagen type I, was calculated for all preparations (Fig. 10). This index stayed $\sim 1 \times 10^0$ for all cells cultivated on

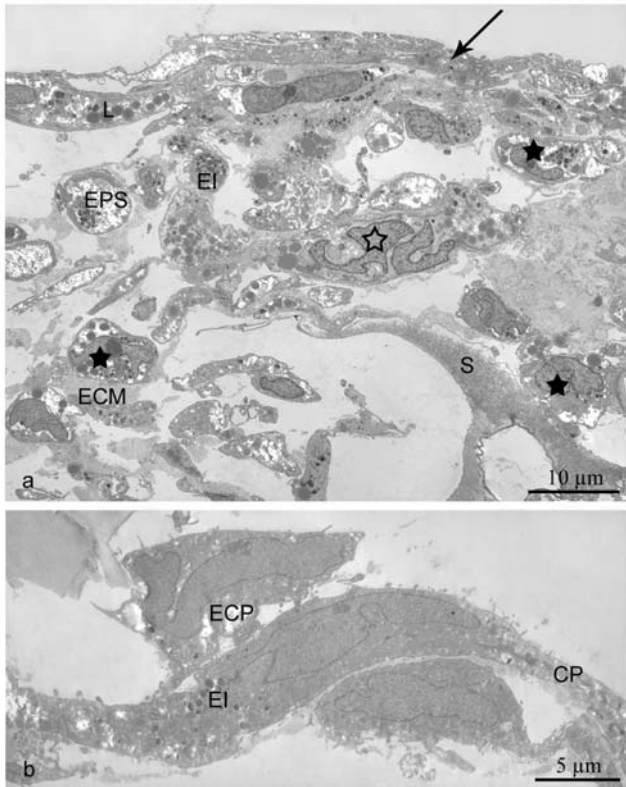


Figure 5. (a) Cell multi-layer on the gelatine sponge 31 days after seeding. Under the superficial fibroblast-like cells (arrow) some chondrocytes become ovoid (empty asterisk) and spherical (asterisk). ECM is present between the cells. L, lipid droplets; EPS, empty cytoplasmic spaces and EI, electron dense inclusion are visible in spherical as well as elongated cells. (b) Fibroblast-like chondrocyte spanning between the gelatine fibers (S) after 24 days of cultivation. CP, cell process; EI, electron dense inclusions and ECP, empty cytoplasmic space.

the hyaluronan scaffold and $\sim 1 \times 10^{-3}$ for cells on the collagen scaffolds and gelatine scaffold, with a peak in both preparations at day 17.

Discussion

Biological evaluation of different scaffolds and their effects on chondrocytes is an ongoing process and cannot

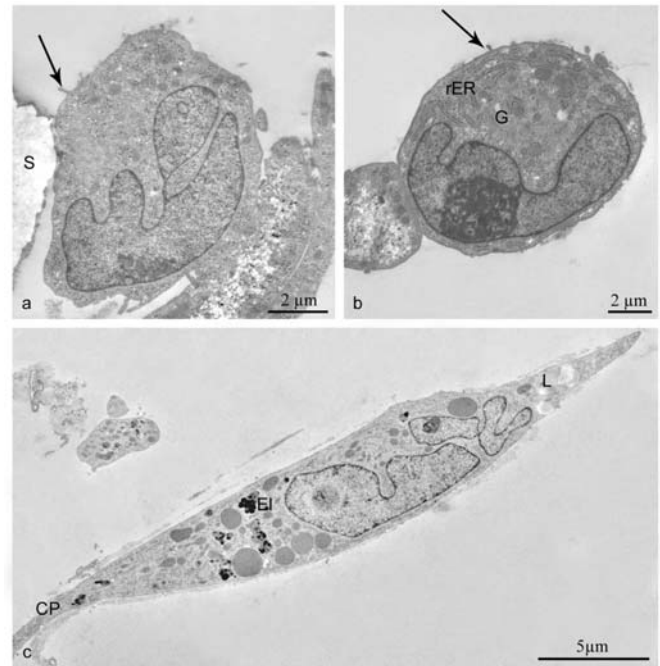


Figure 6. (a) Spherical chondrocyte attached to a hyaluronan scaffold fiber (S) and covered by cell processes (CP). Microvilli (arrow). (b) Spherical cell attached to a cell process without any contact to the scaffold fiber in this section. Microvilli (arrow), rough endoplasmic reticulum (rER), Golgi apparatus (G). (c) Elongated chondrocyte with extended cell processes spanning between the hyaluronan fibers. EI, electron dense inclusions and L, lipid droplets.

be judged definitively as yet (14,15,17,24-27). The situation is further complicated, because there is strong evidence that differentiation and dedifferentiation are at least, in some respects, species-specific, and, thus, the relevancy of animal data to human conditions may be problematic (28).

Each scaffold type that we used in our study has been extensively tested by the companies that developed those biomaterials (19,22,29). The aim of our study was to compare the influence of the most commonly used scaffolds to de-differentiated chondrocytes under identical conditions.

For our testing, we chose three scaffolds, one built up from hyaluronic acid, one scaffold consisting of a porcine

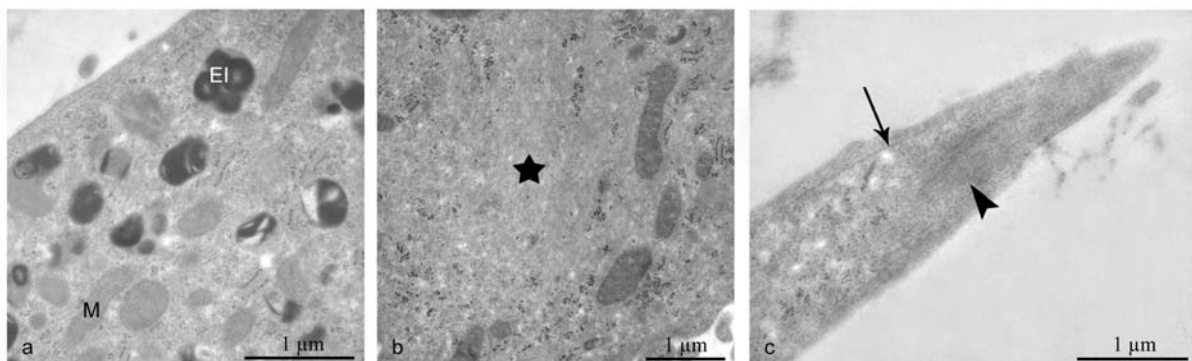


Figure 7. (a) Electron dense inclusions in the cytoplasm of a chondrocyte. These membrane bound vesicles contain electron dense structures in a fine granular or electron lucent matrix. They can be found in fibroblast-like and spherical cells on all three scaffold materials from the first observation point on. M, mitochondrion. (b) A bundle of cytoskeletal elements (asterisk) in the cytoplasm of a chondrocyte. (c) Cell process of a fibroblast-like chondrocyte with microtubuli (arrow) running along side the cell membrane and actin filaments (arrow head). The electron dense areas in the bundle of actin filaments refer to stress fibers.

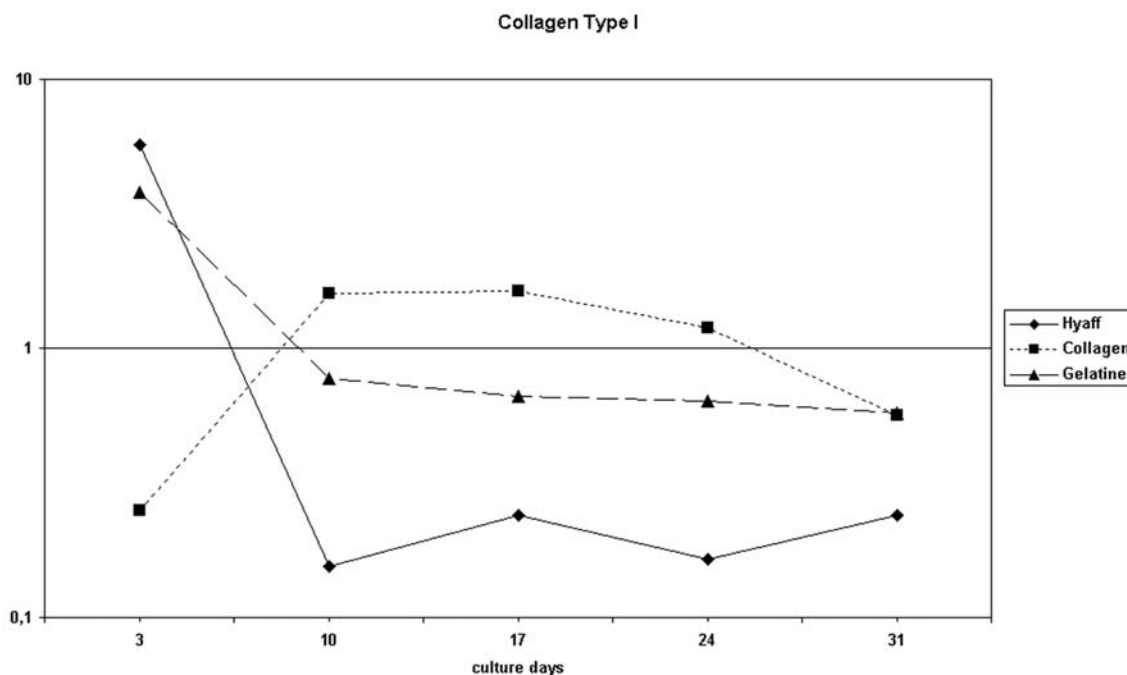


Figure 8. Real-time PCR of type I collagen expression: the synthesis of type I collagen is in particular reduced in cells grown on a hyaluronan scaffold within the first week and remains low over the entire cultivation period; cells grown on the gelatine scaffold show also a decrease in the expression of type I collagen whereas cells grown on collagen I/III membranes show an increase of type I collagen expression.

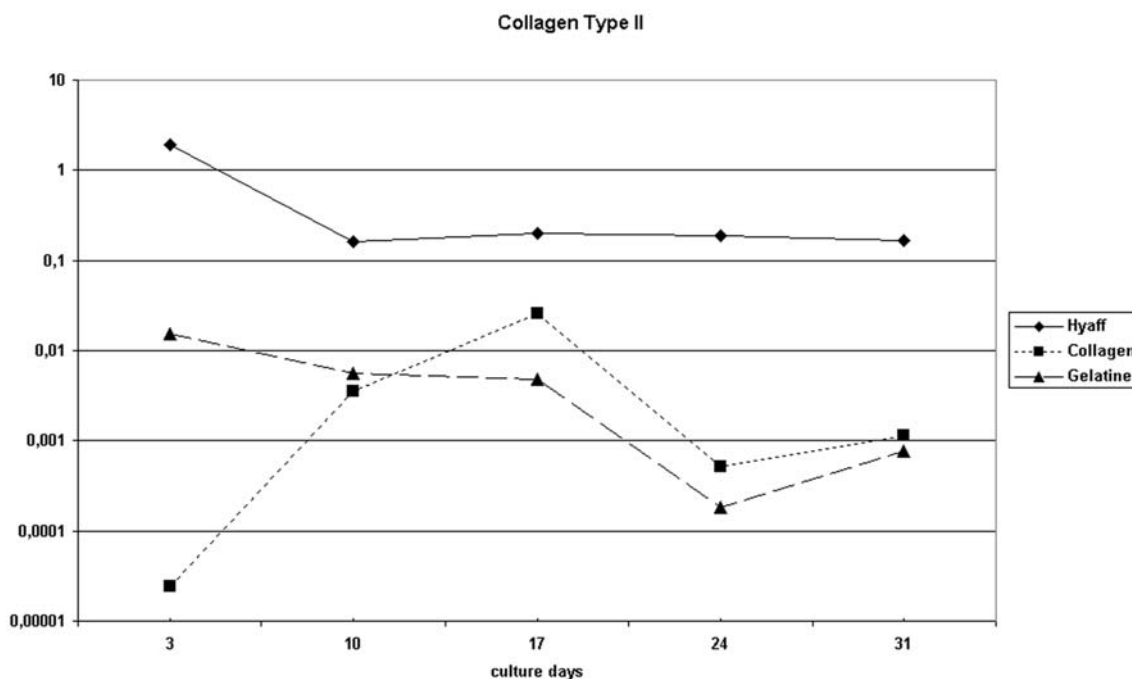


Figure 9. Real-time PCR of type II collagen expression: cells grown on a hyaluronan scaffold show a rather high amount of expression of type II collagen from the very beginning to the end of the cultivation period; cells grown on the collagen type I/III membrane display a smaller amount of type II collagen expression, but there is a 1000-fold increase in the course of the cultivation period.

type I/III collagen and one gelatine-based scaffold. If it were true that matrix composition has an impact on cellular behaviour, we wanted to determine whether these differences could be quantified and characterized by morphological and molecular biological means. There was good evidence that a three-dimensional environment

stimulates a redifferentiation process in chondrocytes, which should be judged morphologically as well as by molecular biological means (18,30).

Though many studies claim the importance of a variety of different markers, all authors agree on the fact that the most important criterion for chondrocyte differentiation is the

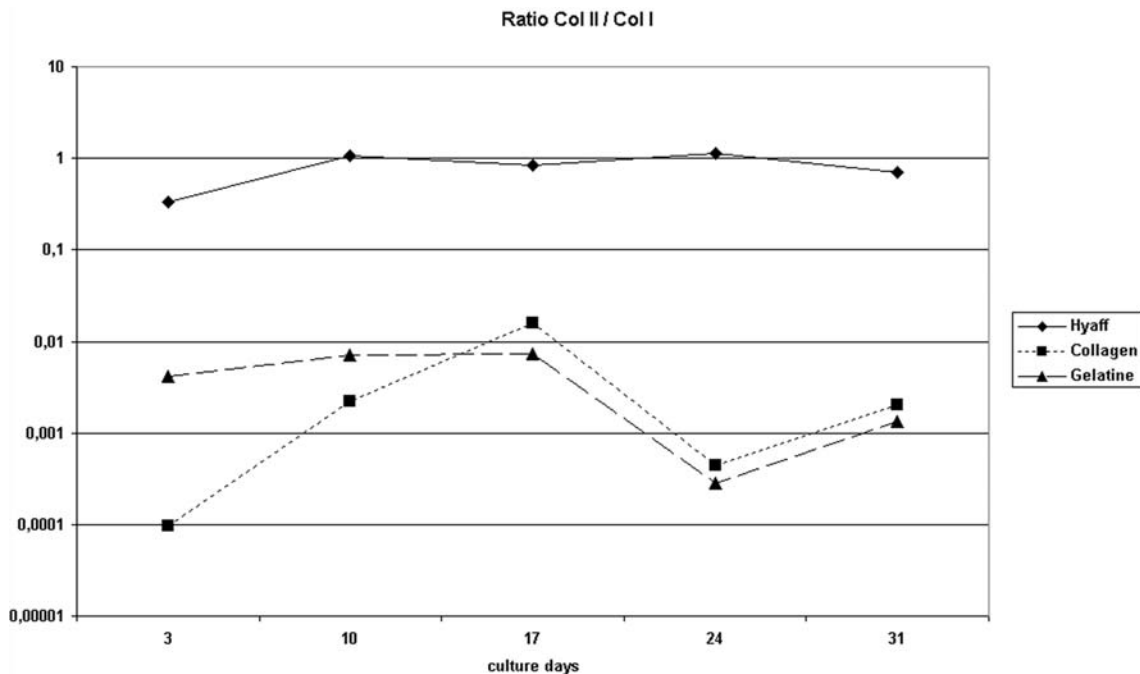


Figure 10. Real-time PCR and the differentiation index (ratio of collagen type II/collagen type I): on hyaluronan scaffold the index is high and mainly constant over the entire cultivation period; on gelatine and collagen I/III matrices the ratio is increased at the beginning in the first half of the cultivation period.

sufficient production of collagen type II (9,15,17,20,23,25,30-34). Therefore, we also refer to this protein as the benchmark for the differentiation status of the cell (26).

Our results indicate that there is a general difference in the differentiation pattern of chondrocytes grown on the various scaffolds.

In cells grown on the hyaluronan matrix, the differentiation index was ~ 1 and this index showed no major alterations during the whole period. Thus, this system seems to guarantee a stable environment for the maintenance of cell populations, which generate an equimolar amount of type I and type II collagen. In contrast to the cells on the hyaluronan matrix, cells grown on a collagen matrix revealed a differentiation index of only 1×10^{-4} after three days of cultivation. However, a 100-fold increase, with regard to the collagen type II expression, could be noticed within 14 days. The expression of type I collagen remained mostly stable during the whole period. Therefore, from a molecular biological point of view, the improvement of the index, which was evoked by the augmentation of the collagen type II expression, can be considered the most important benefit of this scaffold, although there was still a large difference between the indices of the different biomaterials.

The cells on the gelatine matrix behave between those on a hyaluronan matrix or a gelatine matrix. There is a decline in the collagens type I and II from day 3 to day 17, as is found on the hyaluronan membranes, but the amount of collagen type II is much lower on the gelatine-based scaffold than on the hyaluronan membrane. The fact that the decline in collagen type I expression is rather dramatic within the period from day 3 to day 10 results in an overall increase of the differentiation index for this period in both systems.

The use of transmission electron microscopy revealed that in contrast to the hyaluronan membrane, which obviously favours differentiation directly after seeding, the collagen membrane seemed to promote the generation of two distinct cell types by 14 days.

The hyaluronan membrane supported the rapid expression of a relatively high amount of type II collagen, as is found in typical hyaline cartilage, but the histological organisation of the construct as a whole has no similarity with native articular cartilage.

The collagen scaffold seemed to stimulate a differentiation process, generating cells of two different morphologies. These two cell types were not present from the very beginning of the cultivation period on the 3D scaffold. One cell type maintained a fibroblast-like appearance, while the other cell type seemed to develop a chondrocyte-like shape. TEM indicated that the inner cell layer consisted of cartilage-like cells, while the fibroblast-like outer cell layers confined the construct. This construct displayed a very well-organized structure and mimicked the composition of articular cartilage very closely. It was not the relative amount of expression, but rather, the increase in type II collagen in comparison to type I collagen, and further the organisation of the cells that may be responsible for the clinical value and favourable properties of the type I/III membranes.

Thus, our data indicate that different scaffolds favour different morphological and molecular biological properties of cell constructs. Collagen type I/III membranes support the formation of a relatively low number of tissue-like organized cells, with cartilage-like cells in the interior and fibroblast-like cells forming the outermost border of the construct. Contrary to this situation, hyaluronan matrices enable the formation of a relatively large amount of cartilage-like differentiated cells that lack three-dimensional

organisation. It remains to be determined which molecular biological mechanisms are responsible for this different growth and differentiation behaviour and what clinical consequences would result.

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