

Chapter 3

# *In Vitro and In Vivo Comparison of 4 Different Matrix Systems for Chondrocyte Transplantation*

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## *Summary*

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**I**n recent years, a great variety of different matrix systems for cultivation of chondrocytes in order to treat cartilage defects have been developed. Although some of these scaffolds showed promising experimental results, their potential clinical value remains unclear. Because of different assay systems used and the use of chondrocytes of different origin, experimental findings are hard to compare. In this study, we propagated human articular chondrocytes on several different scaffolds under standardized *in vitro* and *in vivo* conditions. Scaffolds were investigated with regard to handling and biochemical properties. While collagen gel systems seem to be superior when treating deep cartilage defects, membrane systems might rather be useful in improving conventional autologous chondrocyte transplantation (ACT).

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## Introduction

Since Hunter's observation in 1743, that cartilage once „destroyed, is not repaired“, numerous techniques have been developed to treat cartilage defects. The poor self-healing capacity of cartilage limits the use of existing procedures. Regenerated tissue is only cartilage-like, with different biochemical and biomechanical characteristics in contrast to the surrounding cartilage and shows a more or less poor bonding. In recent years, Brittberg's method of transplanting autologous chondrocytes in suspension (1) has been improved by the use of different carriers, including agarose (2), alginate (3), matrices of polylactic acid (PLA) (4), meshes of different collagens (5), collagen gels (6) and many others. While some of the different scaffolds show promising results *in vitro*, the potential value for *in vivo* treatment of hyaline cartilage defects remains unclear. Assay parameters of the performed *in vitro* and *in vivo* studies vary to a great extent. Cultivation conditions, age, and used cell number differ as well as the origin of chondrocytes (cattle, rabbit, human or other). Recently, even mesenchymal stem cells have been used to regenerate hyaline-like cartilage (7). This variation of setup parameters lead to serious problems in estimating the potential clinical use of a given matrix system.

In this study, we cultivated human articular chondrocytes *in vitro* on different scaffolds. For the first time, we were able to compare handling, morphological and biochemical properties of important matrix systems. Additionally, scaffolds were investigated in nude mice.

## Materials and Methods

### Chondrocyte Isolation

Articular cartilage samples from patients suffering from knee disease were obtained under aseptic conditions and collected in DMEM medium containing 10% FBS, 100 U/ml penicilline, 100 µg/ml streptomycine. The tissue was cut into 1-2 mm<sup>3</sup> pieces, transferred into 50 ml tubes and digested with 1 mg/ml Collagenase B (Roche Diagnostics, Indianapolis, USA) for 16-24 hours at 37°C in a

humidified 5% CO<sub>2</sub> atmosphere. The released chondrocytes were seeded in a 10 cm culture dish and grown for 1-2 weeks until passage 2. The cells were released, and their total number and the number of viable cells were determined by trypan blue exclusion and counting in a cell counter (CASY1, Schärfe System, Reutlingen, Germany).

### *Chondrocyte Matrix Cultures*

Released chondrocytes were cultivated in four different matrix systems, namely CaReS (Ars Arthro, Esslingen, Germany), Atelocollagen (Koken, Tokyo, Japan), Chondro-Gide (Geistlich Biomaterials, Wolhusen, Switzerland), and Integra Dermal Regeneration Template (Integra LifeSciences, Plainsboro, USA). One vol. Ars Arthro collagen type I gel from rat tail was mixed with one vol. 2x DMEM/2 M HEPES (0.93:0.07), whereas eight vol. Atelocollagen (3% aqueous solution) were given to one vol 0,05 N NaOH/2,2% NaHCO<sub>3</sub>/200 mM HEPES solution. These collagen solutions were suspended with chondrocytes in a final concentration of 2x 10<sup>5</sup> cells/ml. 1.5 ml were allowed to gel in 12 well plates, overlaid with DMEM medium after one hour and cultivated under standard conditions (37°C, 5% CO<sub>2</sub>, 95% air) in a humidified incubator. Chondro-Gide, a collagen type I/III matrix, was cut into 1 cm<sup>2</sup> pieces under sterile conditions and seeded with 2x 10<sup>5</sup> cells/cm<sup>2</sup>. Integra Dermal Regeneration Template from bovine collagen type I was cut in 1 cm<sup>2</sup> pieces, washed for two hours with sterile isotonic NaCl and seeded with 2x 10<sup>5</sup> cells/cm<sup>2</sup>. All matrices were cultivated under standard conditions for up to 4 weeks and fed with fresh medium every three days.

### *Cultivation of Matrices in Nude Mice*

For the *in vivo* assay, samples of the different scaffolds were loaded with passage 2 chondrocytes and implanted subcutaneous in nude mice (BALB/c-nu/nu). Mice were sacrificed after three weeks. Recovered samples were fixed in formalin, and histological and immunological staining was performed as described below.

### *Histochemistry*

After cultivation for up to four weeks, matrices were fixed overnight in a phosphate-buffered solution of 4% paraformaldehyde and embedded in paraffin. Five µm sections were stained with hematoxylin & eosin (H&E), and Safranin O to stain for proteoglycans according to standard

protocols. Images were captured by a Leica microscope (Leica, Heerbrugg, Switzerland) and prepared using the Discus software by the same manufacturer.

### *Immunocytochemical Staining*

Cross-sectioned slices of formalin-fixed, paraffin embedded matrices were deparaffinized and incubated with polyclonal antibody to human collagen type II (Biotrend, Köln, Germany). Staining was visualized using the streptavidin/biotin technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, USA). Diaminobenzidine (DAB Peroxidase Substrate Kit, Vector Laboratories, Burlingame, USA) was used as the developing substrate leading to a brownish colour of the immunopositive cells. The specificity of the immunoperoxidase staining was verified by omission of the primary antibody and the use of matrices not seeded with cells, giving the background staining of the different matrix systems.

### *Proliferation and Cell Viability*

Proliferating cells were detected by indirect immunohistochemistry using the Ki-67 polyclonal antibody (NeoMarkers, Fremont, USA), while apoptotic cells were detected by TUNEL staining of fragmented DNA (Dead End Colorimetric Apoptosis Detection System, Promega, Madison, USA) according to the manufacturer's instruction.

## **Results**

The two tested collagen type I gel systems (first two matrix types) were easy to mix homogenously with the suspended chondrocytes and pourn into wells of different size as desired. Their viscosity generally was moderate. After gelling, samples of the first two matrix systems could be easily handled with foreceps. Chondro-Gide and Integra Dermal Regeneration Template matrix systems

were as easy to handle as the collagen gels. The initial transfer of cells on the matrices was quiet easy, propagating and handling with forceps did not cause any problems.

In the course of cultivation, loss of weight occurred in all tested systems. This loss of weight was most significant regarding the two gels (first two matrix systems). It was less distinct with respect to the membrane systems. When we were cultivating matrix samples and omitted cells as a control, we did not observe a significant loss of weight.

The observed shrinking of the matrix samples did not influence morphology and distribution of the chondrocytes (Fig.1).

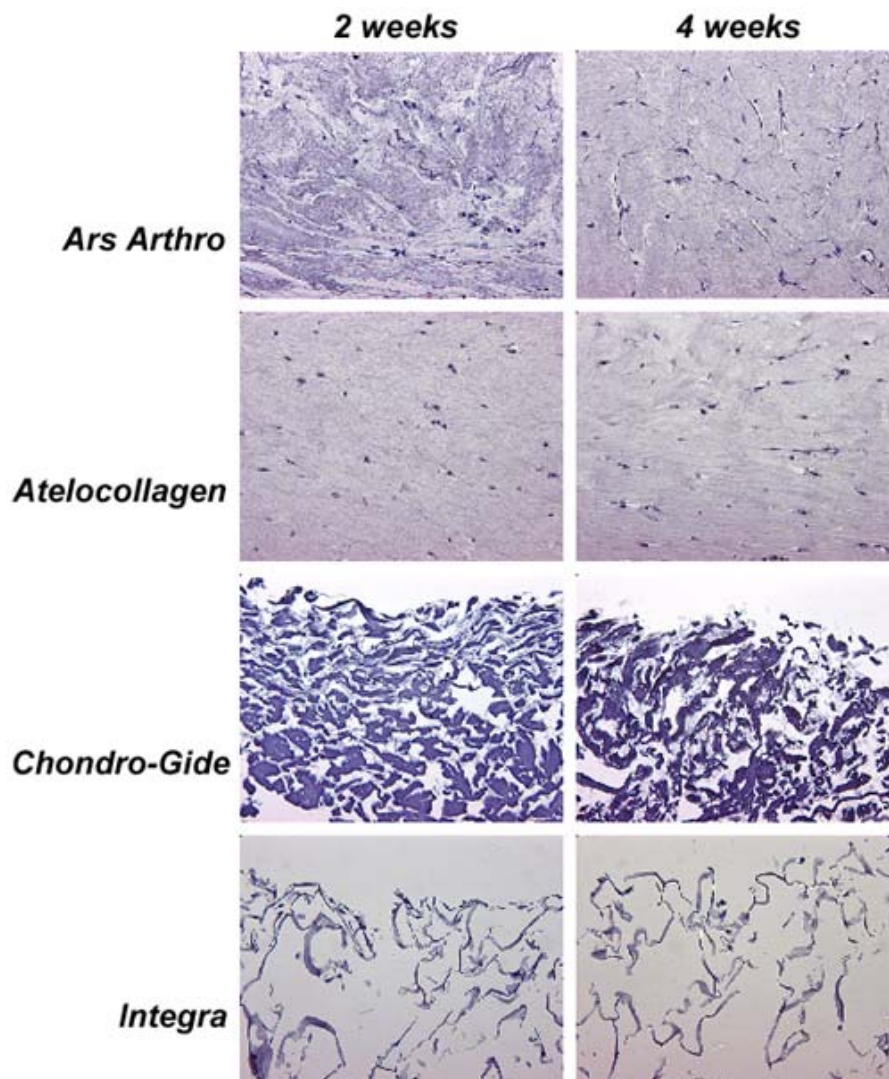


Fig. 1: Hematoxyline/Eosine staining of chondrocytes cultivated on different matrices for up to 4 weeks

Cells seeded on Chondro-Gide and Integra were observed mainly on the outer side of the membrane, while Ars Arthro and Atelocollagen showed a homogeneous distribution throughout the whole sample. The cell number in Ars Arthro and Atelocollagen matrix systems was nearly equal, while the cell number of Chondro-Gide was significantly higher, mainly on the outer side of the membrane where multilayers were formed. Proliferation on Integra was lower than proliferation on the other three investigated matrix systems, as H&E staining revealed the existence of less cells in comparison to the other matrix systems. Generally, cell number increased only slightly during cultivation, which is consistent with results we obtained from a proliferation assay. After two weeks, this assay (immunochemical staining of Ki-67, a protein specific for S-phase) stained only very few chondrocytes.

The general appearance of the cells was elongated and (fibroblast-like). Cultivation on none of the investigated matrix systems lead to a significant morphological redifferentiation of the chondrocytes, resulting in a spherical morphology of the cells.

With respect to production of components of the extracellular matrix (ECM), a partial redifferentiation of the chondrocytes was observed. Cultivation on all investigated matrix systems lead to a significant production of collagen type II (Fig.2).



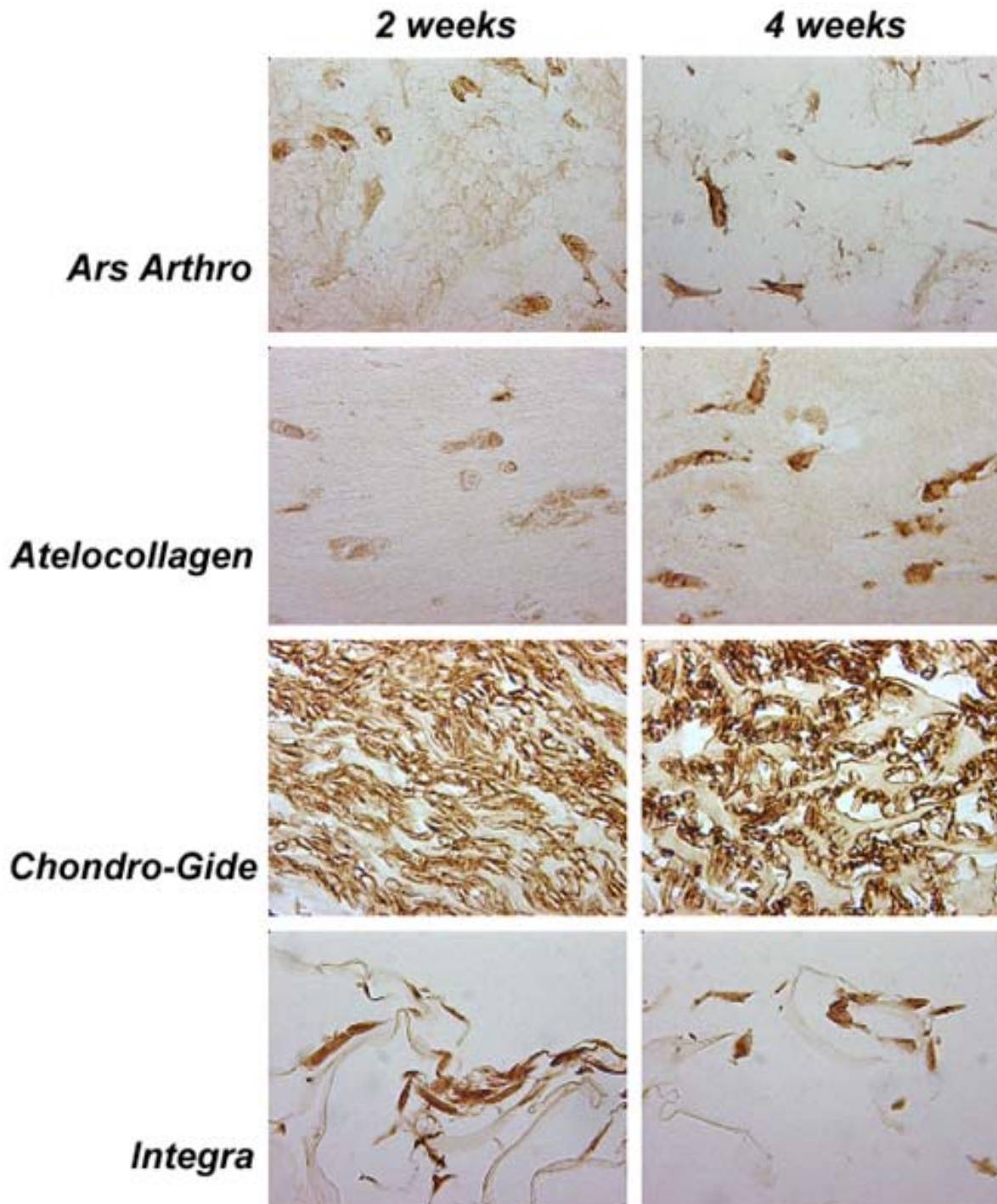


Fig. 2: Immunohistological detection of collagen type II in different matrix samples seeded with human articular chondrocytes and cultivated in vitro for up to four weeks



Collagen type II was synthesized early in cultivation and persisted during the whole period of cultivation. It was stored mainly in the pericellular region and did not build a territorial matrix characteristic for hyaline cartilage.

Histological and immunohistochemical examination of samples recovered from mice was done. The introduced changes in cultivation conditions lead to major improvements in cell proliferation and collagen type II production compared with *in vitro* cultivation (Fig.3).

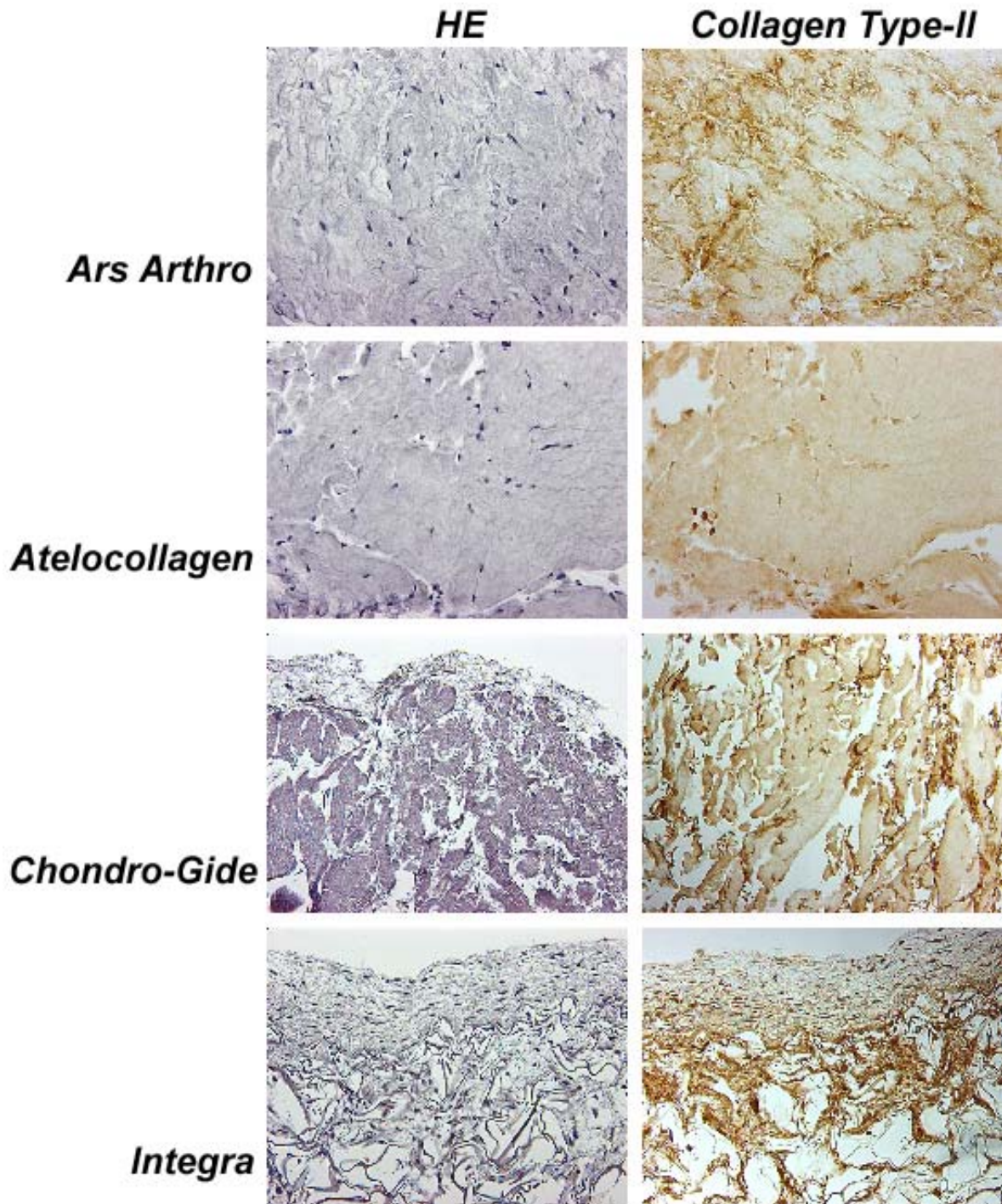


Fig. 3: HE and collagen type II staining of matrix samples cultivated in nude mice for 3 weeks

After three weeks *in vivo*, cell number was higher compared with the *in vitro* situation, especially with regard to Integra bilayer. Cultivating the matrices in nude mice generally enhanced collagen type II production. Cells started to build a territorial matrix, although it was significantly weaker than in hyaline cartilage. Loss of weight occurred to a similar degree as observed when cultivating the matrix samples *in vitro*.

## Discussion

The presented data highlight the advantages of chondrocyte cultivation in scaffolds, especially in combination with *in vivo* cultivation systems. During the course of cultivation of the investigated matrix samples, a loss of wet weight of matrices could be observed which was most significant with regard to the collagen gel systems. Because the observed shrinking was dependent of the number of cultivated cells, an intrinsic instability of the matrices can be excluded. When we were cultivating matrix samples and omitted cells as a control, we did not observe a significant loss of weight *in vitro*. Possibly, an enhanced production of matrix metalloproteases or other catabolic enzymes lead to this loss of weight. Bau *et al.* investigated the expression of various genes involved in cartilage degradation by quantitative PCR (8). When cultivating human articular chondrocytes in high density monolayer or alginate beads, the collagenases MMP-1 and MMP-13 were expressed to a much greater extent compared with normal or osteoarthritic (OA) cartilage. They stated that the expression of many enzymes involved in matrix metabolism is significantly different *in vitro* and *in vivo*.

As immunochemical detection of Ki-67 revealed, chondrocytes were proliferating only in the early part of cultivation, leading to a multilayer on the upper side of the membrane systems. Possibly, building of ECM early during the course of cultivation lead to this restriction in proliferation. Collagen type II, the main component of the hyaline cartilage matrix, was only detected pericellularly. It was synthesized early in cultivation and persisted until the end of the investigation

period. So catabolic effects, which might be responsible for the loss of wet weight, did not noticeably affect the newly-synthesized collagen type II in the interior of the matrix samples.

Although there was a distinct production of collagen type II detectable under *in vitro* conditions, both intensity and distribution was far from the situation normally seen in hyaline cartilage. Cultivation of matrix samples in nude mice lead to significant improvements with regard to collagen type II production and distribution. Cells showed an enhanced proliferation and started to build a territorial matrix. Haisch *et al.* transplanted samples of human nasal chondrocytes precultivated on poly-L-lactide/polactide-co-glycolide PLLA/PGLA/Fibrinogen scaffold subcutaneously in nude mice (9). After six to 12 weeks, histological staining revealed round-shaped clusters of homogeneously distributed chondrocytes. Collagen type II was found to a large extent, but predominantly pericellularly. Interestingly, control samples without chondrocytes had been absorbed completely after six to 12 weeks *in vivo*. Sittinger *et al.* reported, that 20% of PLLA/PGLA samples *in vitro* precultivated with human nasal chondrocytes had resorbed after cultivation in nude mice for up to 24 weeks (10). Rotter *et al.* examined whether biomechanical or biochemical properties of tissue-engineered human septal cartilage vary with donor age and *in vitro* precultivation time (11). Chondrocytes were seeded on PLA coated polyglycolide (PGA) discs and implanted subcutaneously in nude mice with or without *in vitro* precultivation. When omitting precultivation, samples of all investigated age groups showed a loss of wet weight of up to 50% of the scaffolds after 4 weeks cultivation in nude mice. This observation was not made when samples were precultivated *in vitro* for three weeks. Our data confirm a substantial loss of wet weight of collagen gels when seeded with human articular chondrocytes and implanted subcutaneously in nude mice. Regarding ECM formation, it is unknown which factors that *in vivo* compartment might be have provided for the observed improvements. Mechanical stimulation of the samples occurring in nude mice might be involved.

The investigated matrix systems revealed some significant differences. Both of the investigated membranes were easy to handle and suitable for the cultivation of chondrocytes. Chondro-Gide, a bilayer scaffold made of collagen I/III has a porous side where cells are able to attach. Fuß *et al.* cultivated human chondrocytes propagated in monolayer on Chondro-Gide and found morphologically no complete redifferentiation (5). Cells formed a multilayer on the rough side of the membrane and migrated into the scaffold occupying about one quarter of its thickness.

Frenkel *et al.* used another bilayer membrane, exclusively made of bovine collagen type I (Integra Dermal Regeneration Template) for the cultivation of chondrocytes (12). Chondrocytes from New Zealand white rabbits were precultivated in monolayer culture before they were seeded on the rough side of Integra membranes. After two weeks, they were implanted in rabbit femoral trochleas and followed up for six months. After 24 weeks, bonding, glycosaminoglycan (GAG) and collagen type II production in the membranes seeded with cells were superior to those seen with membranes that were not seeded with cells and to those defects that were left empty. Nevertheless, nonseeded matrices control showed 75% content of collagen type II compared with seeded matrices. Empty defects showed 20% collagen type II content compared with seeded matrices, but a significantly lower collagen type I production. Obviously, filling cartilage defects with this mesh has a positive effect on cartilage repair, but the participation of chondrocytes seeded in the matrices remains unclear. In our study, the number of chondrocytes increased to a significantly lesser degree in Integra Dermal Regeneration Template as compared with Chondro-Gide and the collagen gel systems *in vitro*.

Both Integra bilayer and Chondro-Gide have proven to be useful tools for chondrocyte cultivation, although concerns remain regarding the treatment of deep cartilage defects. Both membranes failed in building up a matrix of a substantial thickness *in vitro*. In addition, an uneven distribution of cells may lead to an inhomogeneous repair tissue. Both types of membranes might rather be advantageous in conventional ACT, when replacing the periosteal flap with a biodegradable membrane of controlled properties, than filling deep cartilage defects.

Both investigated collagen gels, Ars Arthro collagen type I and Atelocollagen, were easy to handle. The Atelocollagen samples that were available for us varied slightly in their viscosity. This inconsistency of quality might be due to the fact, that Atelocollagen is a side product of cattle not raised exclusively for Atelocollagen production. In addition, the bovine origin of Atelocollagen might be a problem for clinical application in general because it is xenogenic, although the most important antigenic determinants, the telopeptides, are usually removed from this type of collagen. At present, Atelocollagen is used clinically as a wrinkle treatment material in plastic surgery and dermatology. In recent years, several studies have been performed showing the possibility of cultivating chondrocytes in Atelocollagen gel. Katsube *et al.* (13) cultivated chondrocytes of



Japanese white rabbits for three weeks in Atelocollagen matrices. When they were applied to fill full-thickness defects in knee joints of these rabbits, cells cultivated in Atelocollagen maintained their round cell morphology and revealed a better proteoglycan production and bonding compared to conventional ACT procedure using cells propagated in monolayer culture, sealed with periosteal grafts or left as empty defects. The reparative tissue was not replaced by mesenchymal cells and showed no signs of immunologic rejection. However, some years ago, a low inflammatory reaction to Atelocollagen was confirmed by Kusaka *et al.* (14). Ochi *et al.* presented the results of a clinical trial, where conventional ACT (1) was improved by the use of cell/Atelocollagen implantation (15). Chondrocytes were cultured in Atelocollagen for 3-4 weeks, transplanted into focal cartilage defects in the knee joints of 26 patients and covered with periosteal flaps. After two years follow up, all 26 patients showed reduced swelling and pain. Eighty-eight percent of all investigated knees had a good to excellent outcome according to the Lysholm score (16). However, in nine cases, graft hypertrophy and in one case partial ossification were detected. Moreover, only young patients with knee defects were subject to this study. This type of young patients can usually be expected to have better healing capacity than older patients who suffer from degenerative diseases. Additionally, the use of sutured periosteal flaps to seal defects may lead to a damage of the surrounding healthy cartilage.

Ars Arthro collagen I, which is obtained from tails of inbred rats, offers a controlled quality. Experiments performed with Göttinger minipigs showed an excellent bonding when artificial knee defects were filled with this type of matrix. The gel is easy to handle and to use for filling cartilage defects, and a repair tissue with morphologically excellent quality can be achieved. Currently, in our center a clinical trial is running to verify these results.

In our study, well established four various collagen matrices for chondrocyte cultivation were compared for the first time using a comparative *in vitro* and *in vivo* assay. In our opinion, a homogeneous cell distribution is a crucial feature of cartilage repair and can most successfully be achieved in a gel matrix. Therefore, when dealing with deep cartilage defects, collagen gels seem to be superior to membranes. Integra bilayer, Chondro-Gide and other types of membrane matrix systems might be useful for other indications such as in improving conventional ACT.

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