Mechanical Quality of Tissue Engineered Cartilage: Results after 6 and 12 Weeks *In Vivo*

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Abstract: Traumatic events are a primary cause for local lesions of articular cartilage. If treated early, restoration of the initial joint geometry and integrity may be achieved. In large defects, sufficient material is not available to bridge the affected area. Heterologeous transplantation is not well accepted due to the risk of infection and immune response. Alternatives are cartilage-like structures, which may be cultured *in vitro* and transplanted into the defect site. Critical to the success of these new tissues are their mechanical properties. Goals of this study were to generate a hyaline-like cartilage structure, to evaluate its performance *in vivo* and to verify that its cellular and material properties meet those of native cartilage. Hyaline-like cartilage specimens were generated *in vitro* and implanted in the backs of nude mice. Specimens were explanted after 6 and 12 weeks, mechanically tested using an indentation test and histologically examined. In mechanical testing, stiffness and failure load significantly increased between weeks 6 and 12. At 12 weeks, mechanical properties of the hyaline-like cartilage were comparable to those of native nasal septal cartilage. Compared to native articular cartilage, the engineered tissue achieved up to 30–50% in strength and mechanical stiffness. In histological examination, specimens showed neocartilage formation. The mechanical testing procedure proved to be sufficiently sensitive to identify differences in properties between cartilage specimens of different origin and at different stages of healing. As an adjunct to histological analysis, mechanical testing may be a valuable tool for judging the utility of engineered cartilage prior to a broad clinical usage. © 2000 John Wiley & Sons, Inc. J Biomed Mater Res (Appl Biomater) 53: 673–677, 2000

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INTRODUCTION

Traumatic events in sports or vehicle accidents are a main cause of local lesions of articular cartilage.[1-4] The initial damage produced in a traumatic event may be a disruption of the articular surface. Another possibility is that the underlying bone may be compacted, resulting in a bone bruise and derangement of the subchondral structure.[2-4] Injuries or illnesses may furthermore lead to a softening of the cartilage composite structure.[5]

In such instances, the cartilage composite structure may be irreversibly destroyed. Self-repair processes may not be adequately able to restore the articular joint surface and integrity. Without further surgical treatment, the cartilage lesions or defects lead in the long term to large-scale degenerative changes and osteoarthritis.[6]

Currently the damaged tissue is replaced and defects are filled. Material for defect filling may be obtained from another, nominally unloaded region and transferred to the defect site.[7] However, suitable autologous tissue is only available to a limited extent. Heterologous transplantation is not well accepted due to the risk of infection.[8] Alternatively, engi-

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neered cartilage tissue, which may be cultured for orthopedic purposes from nonarticular locations, provides the potential of ample amounts to fill defects and span large distances.[9-11] As an alternative to the replacement of defect tissue by cartilage or cartilage-like tissue, such voids may be filled with a substance that matures *in vivo* and potentially may bear full weight in the long term.[12]

If post-traumatic lesions are treated early, restoration of the initial joint geometry and integrity may be achieved.[12] The long-term outcome of the available clinical treatment methods are, however, still unknown; data from double-blind randomized clinical studies are scarce, and results are usually limited to single clinical centers or surgeons.[12]

A crucial factor for the initial as well as long-term performance of these surgical treatment methods are the mechanical properties of the defect fillings.[12] A number of methods exist that allow static[13] and dynamic behavior of articular cartilage to be determined.[14] Methods have been introduced to describe the viscoelastic behavior of cartilage specimens.[15,16] To this point, however, it remains unclear to what degree these surgical treatment methods are at all capable of producing mechanically stable regenerates and if this mechanical quality changes during the different regenerate stages.

Goals of this study were (a) to generate a hyaline-like cartilage structure, (b) to evaluate its performance *in vivo*, and (c) to verify that its cellular and material properties meet those of native cartilage.

MATERIALS AND METHODS

The methodology of cell isolation and cultivation has been previously reported and is here only briefly described.[9-11] A local slaughterhouse supplied freshly slaughtered adult bovine forelimbs. Pieces of articular cartilage of the humeral head were surgically removed under sterile conditions. The cartilage specimens were enzymatically digested with 1 mg/mL collagenase P (Boehringer), 0.1 mg/mL Hyaluronidase (Serva, Frankfurt, Germany) and 1.5 mg/mL DNAse (Paesel, Frankfurt, Germany) in RPMI 1640 Medium (Seromed, Berlin, Germany) to obtain the cellular contents of the tissue. The cells were passed through a nylon filter (Reichelt Chemie, Heidelberg, Germany) and washed three times in Hanks solution. The vital cells were assessed by haemocytometer counting, using Trypan blue exclusion staining.

A commercially available fibrin glue system was used as the first component of the carrier composite (Tissucol Duo S 1, Immuno, Heidelberg, Germany). The fibrin sealant consisted of a fibrinogen component (Human Plasma Protein Fraction 80–120 mg; Fibrinogen 70–110 mg; Factor XIII 10–50 E; Plasminogen 0.02–0.08 mg; Aprotinin 3000 KIE), and a thrombin compound (Human Thrombin 500 IE; Calciumchlorid 2 H_2O 5.88 mg). Thrombin was diluted in a ratio of 1:10 with RPMI 1640 medium. The chondrocytes were suspended in the fibrinogen component in a ratio of 1:1. As a second component of the carrier composite served a lyophilized bioresorbable co-polymer fleeces of vicryl (PGLA), Ethisorb 210, Ethicon, Norderstedt, Germany). The 2-mm thick fleeces were cut into 10 × 10 mm slices and soaked with the chondrocyte-fibrinogen suspension.[10]

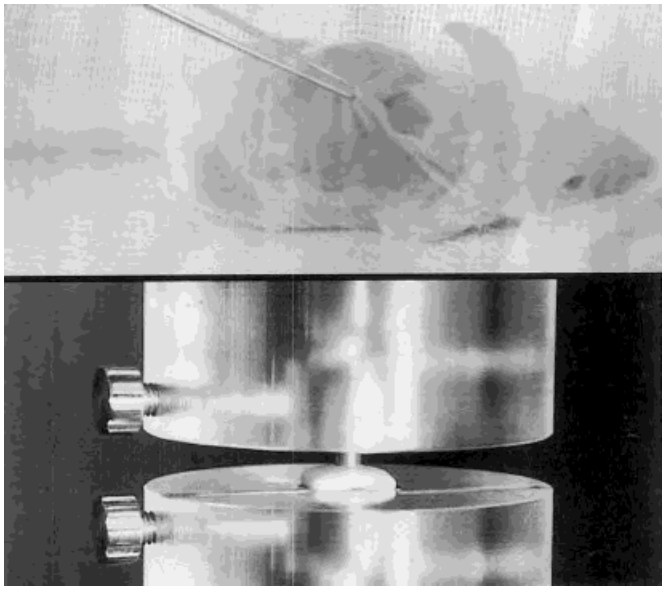

Figure 1. Implantation of the specimens in a subcutaneous pocket in the back of (top) a male homocygotic athymic nude mouse and (bottom) the specimen after 6 weeks *in vivo* during testing.

Cell amplification was performed in culture flasks (Nunc, Naperville, USA) with modified RPMI 1640 Medium (10% FCS, 2% Hepes, 1% Penicillin / Streptomycin): $4–5 \times 10^6$ cells were plated on 75 cm^2 culture flasks and incubated under standard culture conditions. The medium was renewed every other day.

A native, freshly isolated chondrocyte population, which had been cultivated for 3–4 weeks over several passages, was used to prepare three-dimensional chondrocyte cultures. Cells were suspended in the fibrinogen solution and transferred into separate wells of a 24-well tissue plate (Becton Dickinson, NJ). Cell density was $30 \times 10^6/cm^3$. Next, chondrocytes were loaded into the polymer constructs by soaking the biomaterial with the chondrocyte-fibrinogen suspension. Adding 50 μL/cm^3 thrombin solution polymerized the fibrin. Cell culture was performed under standard conditions with continuous medium supply and with separate cell perfusion chambers (Minucell, Regensburg, Germany) over a period of 8 days.

For transplantation, subcutaneous pockets were prepared in the back of 33 anesthetized male homozygotic athymic nude mice (Fig. 1; CD 1 nu/nu, Charles River Wiga, Sulzfeld, Germany; age: 60–80 days, weight: 35–45 g). All animal experiments were carried out according to the policies and principles established by the Animal Welfare Act, the NIH Guide for Care and Use of Laboratory Animals and the national animal welfare guidelines. The experiments were approved by the local legal representative.* Animals were

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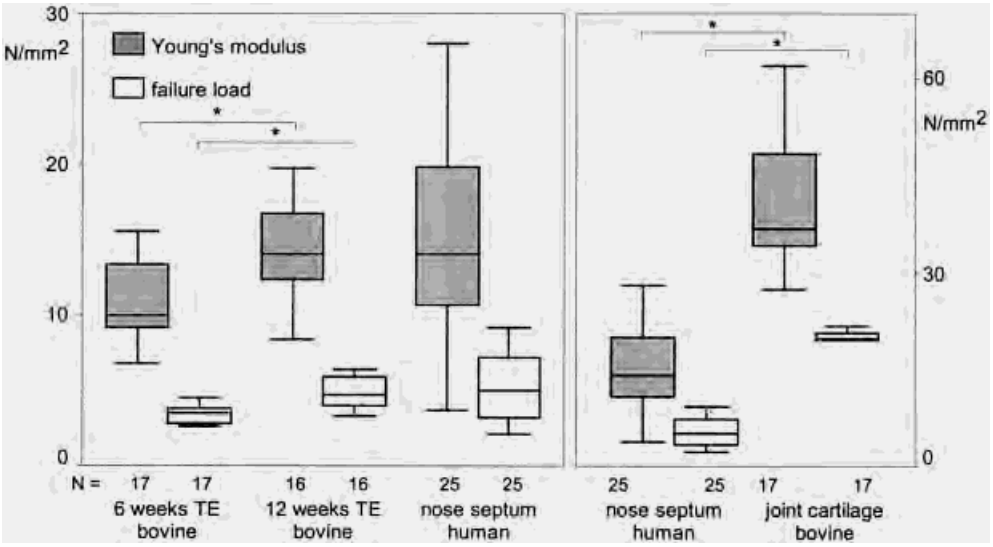

Figure 2. Young's modulus and failure load for 6 weeks and 12 weeks specimens (origin: bovine articular cartilage) in comparison to human nasal septal and bovine articular cartilage specimens (*$p <$ 0.001; Mann–Whitney U-Test).

anesthetized with 5 mg/kg of Xylazinhydrochloride 2% (Rompun®, Bayervital, Leverkusen, Germany) and 100 mg/kg Ketaminehydrochloride (Ketanest® 50, Parke–Davis, Freiburg, Germany) in a ratio of 1:6. After disinfection of the back of the mice with iodine, skin incision was performed with a scalpel, followed by subcutaneous pocket preparation. Cartilage implant was inserted and skin closure was performed with a 4–0 monofil non-reabsorbable bond (Ethicon, Norderstedt, Germany). After 6 and 12 weeks, the mice were sacrificed by an overdose of ether (Hoechst, Frankfurt, Germany) and the cartilage implant was separated from the surrounding fibrous tissue (Fig. 1) and transferred to macroscopic and microscopic analysis.

After written consent, similarly sized nasal septum cartilage specimens were obtained from patients (ages 25–45), who had undergone therapeutic reconstructive surgery of the nasal septum. Only the cartilage pieces that were not designated for re-implantation were collected for the *in vitro* testing. The perichondrium was surgically removed and the cartilage specimens were cut into pieces of 1×1 cm^2. Bovine articular cartilage served as an additional control group. A local slaughterhouse supplied freshly slaughtered adult bovine forelimbs. Pieces of articular cartilage (1×1 cm^2) of the humeral head were surgically removed under sterile conditions and separated from the underlying bone.

Within 3 h after sacrifice, biomechanical testing was performed on all engineered and native tissue. Throughout the experiment, specimens were kept moist (Ringer's solution). Mechanical indentation tests were performed on a materials testing machine (Zwick 1455, Ulm, Germany) using a calibrated load cell (accuracy 0.15 N). Testing was carried out in displacement control with a speed of 5 mm/min (LVDT, accuracy 0.02 mm). The diameter of the indentor was selected to be 4 mm; with this diameter, local variations in material properties do not drastically influence the test result as the indentor diameter is relatively small compared to the specimen dimensions. For testing, specimens were placed unconstrained on a rigid metallic surface to allow free lateral deformation, thereby eliminating possible effects of surrounding specimen holders. The Young's modulus was determined from the linear slope of the load-deflection curve. The failure load was calculated from the maximum force divided by the cross-section of the indentor. The deflection was defined as displacement divided by the thickness of the specimens. Prior to testing, the zero position of the LVDT was determined by positioning the indentor on the rigid metallic surface. At a preload of 1 N, the thickness of the specimens was determined from the LVDT reading. Nonparametric statistical analysis was performed on Young's modulus and failure load data (Mann–Whitney U-Test).

After biomechanical testing and fixation with formalin, cartilage transplants were embedded in paraffin. 5 μm sections were prepared for a series of histochemical examinations. For morphological measurements, haematoxilin and eosin (HE) staining was performed. Proteoglycan synthesis was analyzed by alcian blue (AB) staining. Masson trichrome (MT) staining was applied to investigate collagen matrix formation.

RESULTS

At explantation, the tissue-engineered specimens appeared whitish in color, not unlike normal hyaline cartilage (Fig. 1). No infection in any of the mice was observed throughout the experiment. After 6 and 12 weeks, implants appeared elastic and of solid consistency. At sacrifice, the implants were attached to the subcutaneous pocket, due to a superficial infiltration of adjacent tissue.

In biomechanical testing, the 12-week implants proved to have superior mechanical properties compared to those at 6 weeks ($p < 0.001$; Fig. 2), both in Young's modulus and failure load. The mechanical stiffness of the pure polymer fleece was below the sensitivity of the biomechanical test setup. During maturation, the hyaline-like specimens showed an increase in their mechanical properties. No statistical difference, however, was found in material properties between tissue-engineered specimens 12 weeks and native human nasal septal transplants. Even though the standard deviation of the human tissue probes was rather large compared to the tissue-engineered samples (Fig. 2), both probes appeared to have a similar consistency. In contrast to the nasal septal transplants, bovine articular cartilage appeared much stronger in failure load and much stiffer in elasticity compared with the tissue-engineered samples (Fig. 2). Young's modulus as well as failure load of the 12 weeks tissue-engineered specimens was only 25–30% of the normal bovine articular cartilage. A significant increase in elasticity and failure load was found between human nasal septal probes and native bovine articular cartilage specimens.

Tissue-engineered specimens showed neo-cartilage formation in macroscopical and histological examination using haematoxilin and eosin staining. The neo-cartilage showed typical histologic features of normal cartilage with a chondron-like cell-matrix formation. At 6 and 12 weeks, round-shaped chondrocytes were homogeneously embedded within the cartilage matrix. Using HE staining, specimens demonstrated at 12 weeks *in vivo* only slight differences in matrix formation compared with specimens after 6 weeks *in vivo* (Fig. 3). At 12 weeks, the pericellular matrix appeared more compact than at 6 weeks. Thin layers of fibrous tissue covered the cartilage transplant. Due to the nonspecific process of wound healing, it is difficult to conclude to what extent the infiltrating fibrous tissue led to the slight resorption of engineered cartilage at the margins of the implants at both time periods. Alcian blue staining demonstrated homogeneous purple staining, indicating the presence of sulfate glycosaminoglycans such as chondroitin sulfate. Masson′s trichrome stain exhibited a green-blue stained pericellular matrix, indicating abundant collagen formation. At both time points, no pieces of the polymeric fleece were detectable within the tissue engineered specimens.

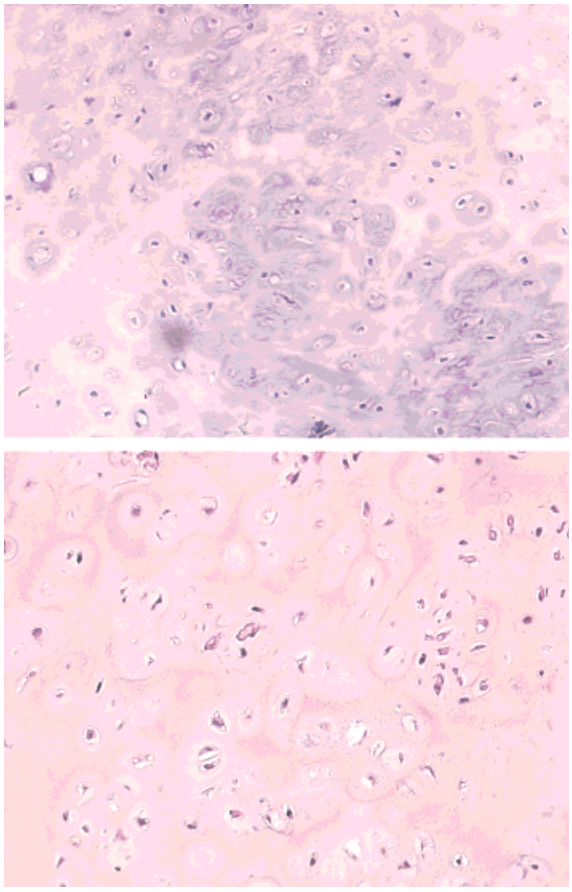

Figure 3. Tissue engineered specimens showing neo-cartilage formation in standard haematoxilin and eosin staining. At (top) 6 and (bottom) 12 weeks, round shaped chondrocytes were homogeneously embedded within the cartilage matrix.

DISCUSSION

Goals of this study were to generate a hyaline-like cartilage structure, to evaluate its performance *in vivo*, and to verify that its cellular and material properties meet those of native cartilage.

The macroscopical examination as well as histological staining indicated the hyaline-like nature of the tissue-engineered cartilage specimens after 6 and 12 weeks. Neocartilage showed typical features of native cartilage. Cells appeared round in shape and homogeneously distributed within a compact pericellular cartilage matrix. During the course of healing, the increasing homogeneity of the pericellular matrix staining indicated further development of a more compact pericellular matrix. Due to the remodeling nature of wound healing, signs of resorption with infiltration of fibrous tissue were observed at the margin areas after 6 and 12 weeks.

The *in vivo* experiments showed that the mechanical properties of the engineered cartilage match those found in native cartilage of the human nasal septum. The mechanical properties of native bovine articular cartilage, however, were clearly superior. The significant gain in mechanical stability between the 6- and 12-week groups showed a positive influence of the *in vivo* maturation process on the quality of the tissue. This is especially remarkable, since the polymeric fleece, which determined the stability at implantation, had basically no mechanical stiffness compared to the values measured at 6 and 12 weeks. After 6 and 12 weeks, no pieces of the polymeric fleece were histologically detectable.

The normal maturation process in these mice may have been additionally stimulated by the tissue implantation, which may have supplied a moderate mechanical stimulus to the engineered specimens and thereby additionally stimulated the tissue formation.[17]

The experimental findings illustrate that simple mechanical indentation testing possesses enough sensitivity to differ-

entiate the mechanical quality of the healed cartilage implants at 6 and 12 weeks (Fig. 2). Indentation testing thereby represents a first approximation of the mechanical quality of the specimens, which is fully described by a complex set of mechanical parameters; using the bi-phasic theory, the material behavior is described by the elasticity and relaxation of the solid phase as well as by the fluid flow.[16] Due to the lack of availability, none of these mechanical parameters has been determined for tissue engineered cartilage-like specimens up to now. Beside a morphological comparability to native cartilage, tissue engineered specimens should be capable to withstand certain mechanical loads. The mechanical quality of the engineered tissue is of critical importance for its prospects clinically in a mechanically demanding environment. Based on the presented findings on the potential of these newly developed materials, further analysis of their mechanical comparability in respect to native tissue (e.g., fluid flow, relaxation) are necessary.

To reliably demonstrate sufficient quality of *in vitro* generated tissues, easy-to-use and highly reproducible testing protocols are needed. The materials testing protocol introduced in this study appears to provide a useful experimental foundation for such evaluations preceding wide clinical application.

The mechanical testing, which lends itself well to standardization, is also suitable to identify factors that may influence cartilage generation. The mechanical quality of the tissue-engineered cartilage studied here proved to be sufficient for various indications in plastic surgery, e.g., reconstruction of saddle nose deformity and external ear reconstruction. These areas are not exposed to repeated impact forces as in articular cartilage. However, further studies are needed to generate tissue-engineered cartilage that is comparable in histology and mechanics to normal articular cartilage.

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