Precultivation of Engineered Human Nasal Cartilage Enhances the Mechanical Properties Relevant for Use in Facial Reconstructive Surgery

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Objective: To investigate if precultivation of human engineered nasal cartilage grafts of clinically relevant size would increase the suture retention strength at implantation and the tensile and bending stiffness 2 weeks after implantation.

Summary Background Information: To be used for reconstruction of nasal cartilage defects, engineered grafts need to be reliably sutured at implantation and resist to bending/tension forces about 2 weeks after surgery, when fixation is typically removed.

Methods: Nasal septum chondrocytes from 4 donors were expanded for 2 passages and statically loaded on 15 × 5 × 2-mm size nonwoven meshes of esterified hyaluronan (Hyaff-11). Constructs were implanted for 2 weeks in nude mice between muscle fascia and subcutaneous tissue either directly after cell seeding or after 2 or 4 weeks of preculture in chondrogenic medium. Engineered tissues and native nasal cartilage were assessed histologically, biochemically, and biomechanically.

Results: Engineered constructs reproducibly developed with culture time into cartilaginous tissues with increasing content of glycosaminoglycans and collagen type II. Suture retention strength was significantly higher (3.6 ± 2.2-fold) in 2-week precultured constructs than in freshly seeded meshes. Following in vivo implantation, tissues further developed and maintained the original scaffold size and shape. The bending stiffness was significantly higher (1.8 ± 0.8-fold) if constructs were precultured for 2 weeks than if they were directly implanted, whereas tensile stiffness was close to native cartilage in all groups.

Conclusion: In our experimental setup, preculture for 2 weeks was necessary to engineer nasal cartilage grafts with enhanced mechanical properties relevant for clinical use in facial reconstructive surgery.

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Autologous cartilage grafts are frequently used in reconstructive and esthetic surgery of the nose. The main disadvantages of this approach are the limited availability of tissue, morbidity at the donor site[1,2] and time-consuming surgery. Indeed, the external ear and nasal septum provide only limited quantities of cartilage, and harvesting larger amounts of costal cartilage may lead to acute or delayed complications such as pneumothorax or chest wall deformities.[3] Tissue engineering offers the possibility of producing large quantities of cartilage of autologous origin, starting from a small tissue biopsy and thus with minimal donor site morbidity. Recent studies have shown that human nasal chondrocytes released form a tissue biopsy and de-differentiated by expansion in monolayers, have the capacity to redifferentiate and generate cartilaginous tissue structures when cultured at high density (eg, in micromasses or pellets[4,5]) or in a variety of porous scaffolds.[6,7]

To be used in a clinical setting for nasal reconstructive surgery, engineered cartilage grafts need to have sufficient mechanical integrity (ie, suture retention strength) at the time of implantation, to allow for reliable suturing at the recipient site, and sufficient mechanical stability (ie, tensile and bending stiffness) when fixation is typically removed (ie, 2 weeks after implantation), to resist contraction by scar tissue formation and by exposure to local or external forces in the recipient bed. So far, however, both native cartilage tissue and engineered cartilage grafts have been mostly characterized biomechanically in terms of compressive stiffness,[7–9] which would be of limited relevance for nasal reconstruction.

Another important issue to be addressed toward the clinical use of engineered cartilage grafts is the extent of precultivation of cell-scaffold constructs prior to their implantation. In this context, previous studies indicated that precultivated engineered cartilage tissues not only reach a superior quality, but display a higher capacity to further develop upon implantation than scaffolds implanted directly after seeding,[10,11] possibly due to less fibrous tissue and blood vessel ingrowth. However, an independent study reported that in vitro culture time had only a minor influence on construct development[7]: the issue is thus still controversial and is likely related to the scaffold used and the specific precultivation conditions.

Based on the above design considerations, the aim of this study was to investigate if precultivation of human tissue engineered nasal cartilage grafts of clinically relevant size would increase the suture retention strength before implantation and the tensile and bending stiffness at 2 weeks postimplantation. To address this question, the selected model system consisted of human nasal chondrocytes, de- and redifferentiated using previously identified culture medium supplements,[4,10] and loaded into nonwoven meshes made of esterified hyaluronic acid (Hyaff-11, Fidia Advanced Biopolymers, Abano Terme, Italy), already in clinical use for the repair of articular cartilage.[12] The in vivo model consisted of ectopic implantation in nude mice, in a pocket between excised muscle fascia and subcutaneous tissue, resembling the environment where nasal cartilage grafts would be clinically implanted (ie, highly vascularized mucosal tissue on one side, and thin layer of subcutaneous tissue on the other side).

MATERIALS AND METHODS

Cartilage Biopsies

Human nasal septal cartilage biopsies from 4 patients (mean age, 48.8 years; range, 34–61 years) were harvested at the Institute of Pathology and Forensic Medicine in Basel, following protocol approval by the local ethical committee (Ref.-No.: EK 40/03 and EK 263/03). Tissue harvesting was performed prior to the autopsy (within 36 hours postmortem) under sterile conditions, with meticulous care to minimize mechanical trauma to the specimens. The incisions were made according to standard principles of plastic surgery to avoid disfigurement. A sample of about 1.5 × 2 cm of cartilage was removed by an interseptocollumelar approach and careful separation from mucosa and perichondrium. The specimen was divided into 2 pieces: one part for cell isolation and tissue engineering, and the other for histologic, biochemical, and biomechanical characterization, as detailed below.

Chondrocyte Isolation and Expansion

Chondrocytes were isolated by 22-hour incubation at 37°C in 0.15% type II collagenase and resuspended in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum, 4.5 mg/mL D-glucose, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 100 mmol/L HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.29 mg/mL L-glutamine (complete medium). Chondrocytes were plated in plastic dishes at a density of 10^4 cells/cm^2 in complete medium further supplemented with 1 ng/mL transforming growth factor-β1 (TGF-β1), 5 ng/mL fibroblast growth factor-2 (FGF-2), and 10 ng/mL platelet-derived growth factor-bb (PDGF-bb) (all from R&D Systems, Minneapolis, MN). This specific combination of growth factors was previously shown to enhance human nasal chondrocyte proliferation and post expansion differentiation ability.[4] When subconfluent, cells were detached by sequential treatment with 0.3% type II collagenase and 0.05% trypsin/0.53 mmol/L EDTA, and replated at 5×10^3 cells/cm^2. Before reaching again confluence, cells were detached and seeded on scaffolds as described below.

Chondrocyte Seeding and Culture on Three-Dimensional Scaffolds

Cells were statically seeded at a density of 6.7E + 07 cells/cm^3 on nonwoven meshes (15 mm width × 5 mm length × 2 mm thickness fleeces) made of esterified hyaluronic acid (Hyaff-11, Fidia Advanced Biopolymers, Abano Terme, IT). Scaffolds were placed on dishes coated with a thin film of 1% agarose to prevent cell attachment to the dish bottom, and a cell suspension (1E + 07 cells in 60 μL) was distributed on the top surface. Constructs were statically cultured for 2 or 4 weeks in complete medium supplemented with 10 μg/mL insulin, 0.1 mmol/L ascorbic acid, and 10 ng/mL TGF-β3, with culture medium completely replaced twice a week. These supplements were previously shown to enhance chondrogenesis of de-differentiated human chondrocytes during culture into Hyaff-11 nonwoven meshes.[10] Constructs (N = 3 per condition for each donor), immediately after cell seeding or following preculture, were either processed for histologic, biochemical, and biomechanical characterization or implanted in nude mice, as described below.

Construct Implantation

Freshly seeded scaffolds, constructs precultured for 2 weeks, for 4 weeks, and cell-free scaffolds as control were implanted in the back of nude mice (CD-1 nu/nu, athymic, 6- to 8-week-old females) in a pocket between excised muscle fascia and subcutaneous tissue. All animals in this study were cared for and treated according to institutional guidelines. Each mouse received 2 grafts, and grafts from the same experimental group were implanted in different mice. Constructs were harvested after 2 weeks, corresponding (in the clinical setting) to the time when fixation is typically removed and thus when the constructs shall have reached adequate biomechanical properties.

Histologic Analysis

Tissue constructs were fixed in 4% formalin for 24 hours at 4°C, dehydrated, embedded in paraffin, and cross-sectioned (7 μm thick). Sections were stained with Safranin-O for sulfated glycosaminoglycans (GAG).

Biochemical Analysis

Native cartilage samples and tissue constructs were weighed and digested with proteinase K (1 mg/mL protease K in 50 mmol/L Tris with 1 mmol/L EDTA, 1 mmol/L iodoacetamide, and 10 μg/mL pepstatin-A) for 15 hours at 56°C.[13] GAG contents were measured spectrophotometrically using dimethylmethylene blue dye,[14] with chondroitin sulfate as a standard. The GAG content was expressed as percentage of tissue wet weight. Total collagen and type II collagen contents were determined respectively by measurement of hydroxyproline and by inhibition enzyme-linked immunosorbent assay (ELISA), as previously described.[15]

Biomechanical Testing

Specimens were maintained in phosphate-buffered solution prior to all mechanical tests and kept moist during the tests. The width and thickness of the specimens were measured with a Vernier caliper. All tests were performed by

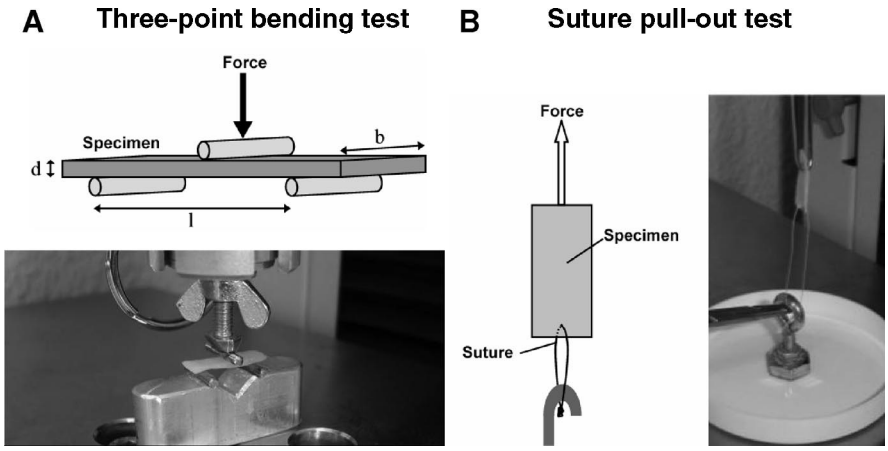

FIGURE 1. Biomechanical setup. Schematic diagrams and macroscopic views of setup configurations for the 3-point bending test (A) and the suture pull-out test (B).

applying deflections and measuring the corresponding forces using a standard miniature mechanical testing instrument (MTS Synergie 100, MTS Systems Corporation, Eden Prairie, MN), with data transmitted to a standard personal computer for subsequent calculations. Tests were conducted at room temperature.

Three-Point Bending Test

This test produces data showing the relationship between force and deflection (bending) of the specimen in the direction of the force, from which it is possible to obtain the bending stiffness of the material, a key property for materials required to maintain the shape of a structure such as the nasal septum. Generally, point loading occurs only if specimens have a cylindrical cross section, whereas for rectangular cross-section specimens, like those used in these studies, line loading is performed (Fig. 1A). The approximate bending stiffness or modulus E_B of the specimen at the deflection rate of 20 mm/min was calculated based on the assumption that the specimen is homogenous, using the formula:

$$E = \frac{\Delta F}{\Delta fm} \cdot \frac{l^3}{48I} \quad (1)$$

where F is the applied force, fm is the bending deflection in the direction of the force, and I is the second moment of area, which measures the efficiency of a specific shape in resisting bending in the direction of loading. For a rectangular section of width b and height d, the second moment of area is:

$$I = \frac{bd^3}{12} \quad (2)$$

For this study, the support span was 10.2 mm and 1 mm was chosen as the maximal deflection. Only data from deflections in the range between 0.4 mm and 0.9 mm, where the measured forces increased almost linearly with increasing displacement, were used to calculate the slope of the load/deflection regression line. Each specimen was deflected 11 times, turned over, and deflected 11 times again. Data from the first bending cycle and those during unloading were excluded. For each specimen, the bending stiffness was calculated as the mean of average values obtained in both orientations (before and after turnover).

Tensile Test

This test determines the elastic modulus in tension, E_T, of the specimen as the ratio of tensile stress to strain in the elastic region, as follows:

$$E_T = (F/A) \quad /(\Delta l/l_O) \quad (3)$$

where A is the specimen cross-sectional area at the original length l_O and Δl is the change in length between the zero force and the maximum recorded force, F. The specimens were secured at their lower end with a suture of a type that would be used clinically to secure a construct to the surrounding tissue (POLYSORB 5-0, coated, braided lactomer 9-1, synthetic absorbable suture with a P-13 needle, Syneture). The suture was inserted 1 to 2 mm above the lower end of the specimen (Fig. 1B) and fixed with a flying triple knot to the holding hook affixed to the base plate of the mechanical test machine. The knot from the suture was additionally held by a needle holder to reduce the internal sliding from the knot. The upper end of the specimen was gripped with an Allis tissue forceps (15 cm, 4 × 5 teeth), hung from a rigid metal hook attached to the load cell of the test instrument. On each specimen, different points were marked with a pen to identify a fixed initial specimen "gauge" length, whose changes were analyzed from video-sequences recorded during the force/elongation tests. The gauge length was between 5 and 10 mm in all cases. The video images were calibrated by recording a plastic scale placed at the same distance relative to the camera as the sample. Each specimen was first elongated 3 times to create a maximum length change of 1.2 mm, returning the actuator to the original position after each elongation. Subsequent additional sets of 3 elongations were performed with maximal length changes of 1.8, 2.4, and 3 mm, unless the resultant force produced specimen failure (ie, the suture tearing the tissue). The rate of elongation was set at 24 mm/min. The calculated value of E_T is approximate for several reasons, including 1) the assumption that the specimen is homogeneous in composition and structure, 2) the use

of an arbitrary rate of strain, 3) the use of the initial rather than instantaneous area of the cross-section in the computation, and 4) the assumption of linear force/displacement behavior over the data range used in the computations. Thus, the test was not performed to obtain precise material property data but rather to derive relative comparisons on the tensile performance of specimens in a test resembling some aspects of in vivo loading.

Suture Pull-out Test

The suture pull-out test was performed to measure the maximal force that could be applied on a suture in the axial direction before pulling out from the construct. The setup was the same as in the tension test described above: at the end of the tension test, the actuator was programmed to continue elongation at a rate of 24 mm/min until the suture pulled out of the specimen. The maximal applied force was normalized to the specimen thickness, measured prior to performing the tests, and is reported as N/mm.

Statistical Analysis

Data are presented as mean ± SD of results obtained from at least 3 constructs generated for each of the 4 donors. Mean values were compared using Mann-Whitney *U* tests. Statistical analyses were performed using the Sigma Stat software (SPSS Inc., version 13), with $P < 0.05$ as the criteria for statistical significance.

RESULTS

Engineered Tissues Following In Vitro Culture

Nasal chondrocytes from all donors could be reproducibly expanded in monolayers and underwent an average of 9.6 doublings in 14 days. After cell loading into the scaffolds, the resulting constructs maintained the original size and shape, and already after 2 weeks of culture acquired a typical cartilaginous glossy appearance, with a firmer consistency than the original fleece (Fig. 2).

Histologic cross sections of the generated constructs indicated progressive temporal development of cartilaginous tissues (Fig. 3A–C). Freshly seeded scaffolds consisted only of a network of fibroblastic cells among Hyaff-11 fibers (Fig. 3A). Extracellular matrix was abundant after 2 and 4 weeks of culture, with an increasing intensity of Safranin-O staining for GAG with time (Fig. 3B, C). Cells in precultured constructs appeared chondrocytic, with a round morphology and embedded in large lacunae.

Biochemical analysis (Fig. 4A–C) of the precultured constructs was consistent with their histologic appearance.

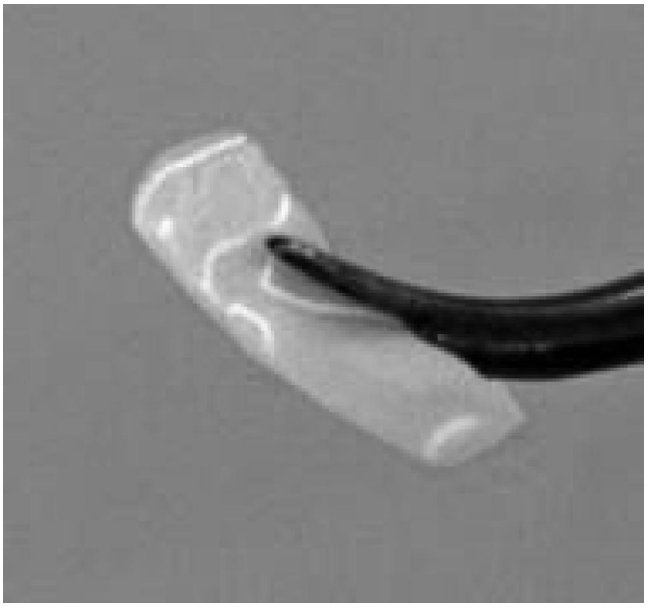

FIGURE 2. Macroscopic appearance of engineered cartilage. Glossy appearance and firm consistency of a typical engineered nasal cartilage graft after 2 weeks of preculture, prior to implantation.

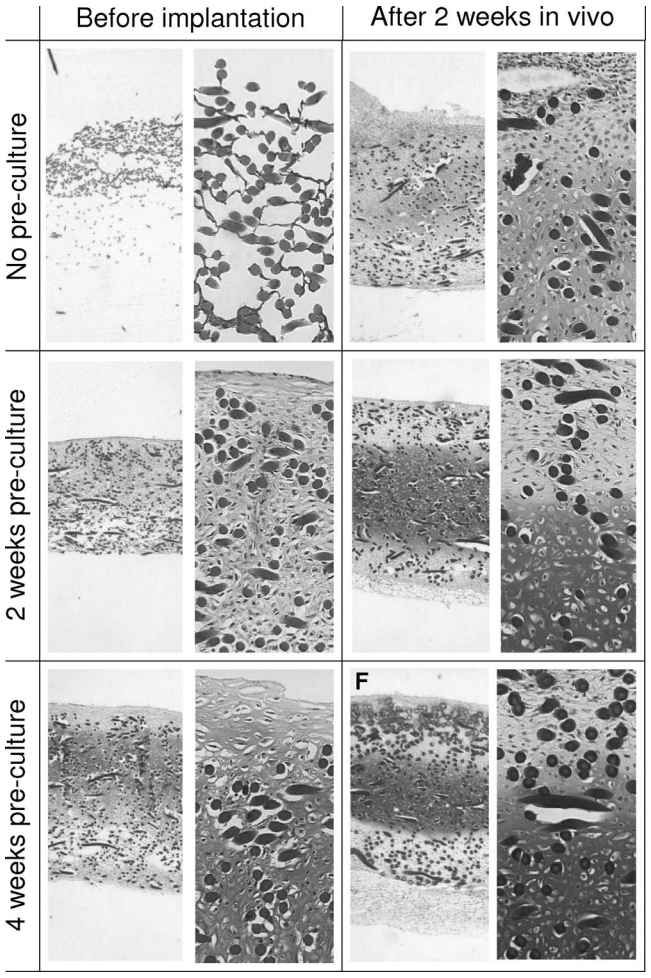

FIGURE 3. Histologic appearance of the grafts. Representative Safranin O-stained cross sections of constructs generated by nasal chondrocytes freshly seeded (A, C), precultured for 2 weeks (B, D) or precultured for 4 weeks (C, E) into Hyaff-11 meshes, before (A–C) or after (D–F) 2 additional weeks of implantation in nude mice. Arrows (B) indicate undegraded polymer fibers. Scale bar = 100 μm (left panels) or 40 μm (right panels).

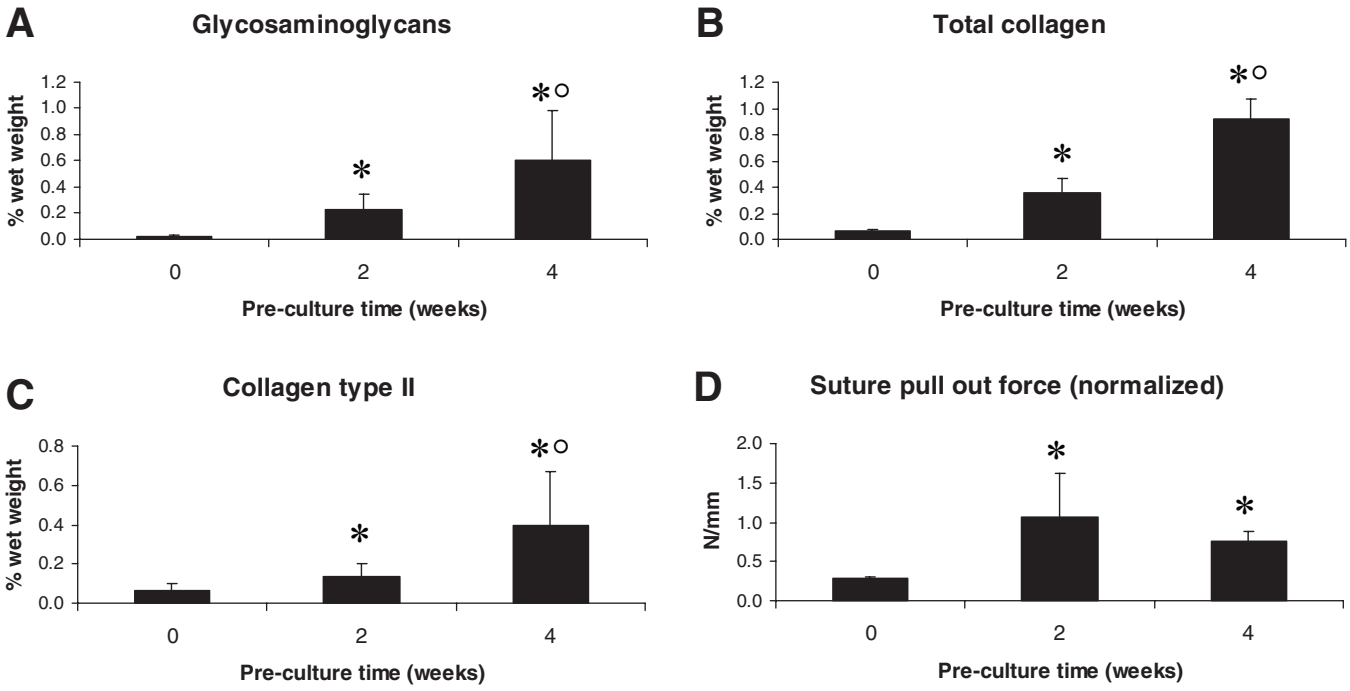

FIGURE 4. Properties of engineered nasal cartilage grafts after in vitro preculture. Wet weight fractions of GAG (A), total collagen (B), collagen type II (C), and suture pullout force, normalized to specimen thickness (D) in constructs generated by nasal chondrocytes, freshly seeded or precultured for 2 or 4 weeks into Hyaff-11 meshes. *Statistically significant difference from the freshly seeded group. °Statistically significant difference from the 2-week preculture group.

Wet weight fractions of GAG, total collagen, and type II collagen were negligible in freshly seeded scaffolds and significantly increased following preculture, reaching levels respectively 5.5-, 8.1-, and 19.5-fold lower than in the native cartilage specimens (respectively 3.3 ± 1.4, 7.8 ± 2.1, and 7.4 ± 2.0).

The suture pull out force normalized to the specimen's thickness (Fig. 4D) was 3.6-fold higher in 2-week precultured constructs than in freshly seeded scaffolds, did not further increase with longer preculture time and reached levels 4.3-fold lower than those measured in native nasal cartilage (4.5 ± 1.9 N/mm). The suture pullout strength of the cell-free scaffolds was below the sensitivity of the test.

Engineered Tissues Following In Vivo Implantation

After 2 weeks' implantation, all constructs maintained the original size and shape of the graft and displayed a smooth, shiny surface. Tissues generated by freshly seeded scaffolds were weakly stained for Safranin-O (Fig. 3D). Constructs precultured for 2 or 4 weeks yielded tissues with stronger staining intensity for GAG, but predominantly in the inner region (Fig. 3E, F), where cells had a more chondrocytic morphology and were at lower density. No vascularization or mineralization was observed in any of the explants. Constructs derived by implantation of cell-free scaffolds had a very soft consistency and displayed histologically a large amount of vascular ingrowth, without any sign of cartilaginous matrix (data not shown).

The wet weight fractions of GAG and total collagen (Fig. 5A, B) were similar in explants that were grafted immediately after cell seeding or following 2 weeks of preculture, and significantly higher if grafts were precultured for 4 weeks, reaching levels respectively 4.5- and 4.0-fold lower than in native nasal cartilage. The wet weight fractions of collagen type II following in vivo implantation (Fig. 5C) were significantly higher if constructs were precultured for 2 or 4 weeks prior to implantation than if constructs were implanted directly after seeding, and reached levels 13.1-fold lower than in native cartilage.

The modulus of elasticity in tension following 2 weeks of in vivo implantation (Fig. 5D) was not significantly different if constructs were grafted immediately after cell seeding or following 2 or 4 weeks of preculture. The level reached was 2.7-fold lower than in native nasal cartilage (6.4 ± 2.4 N/mm^2). Instead, the modulus of elasticity in bending following 2 weeks of in vivo implantation was significantly higher (rsp. 1.8/2.3-fold) if constructs were grafted after 2 or 4 weeks of preculture than immediately after cell seeding (Fig. 5E). The level reached was 7.4-fold lower than in native nasal cartilage (6.8 ± 3.8 N/mm^2). The mechanical properties of the implanted cell-free scaffolds were below the detection level of the biomechanical test setup.

DISCUSSION

With the ultimate goal of the clinical use of engineered cartilage for nasal reconstruction, in this study we demon-

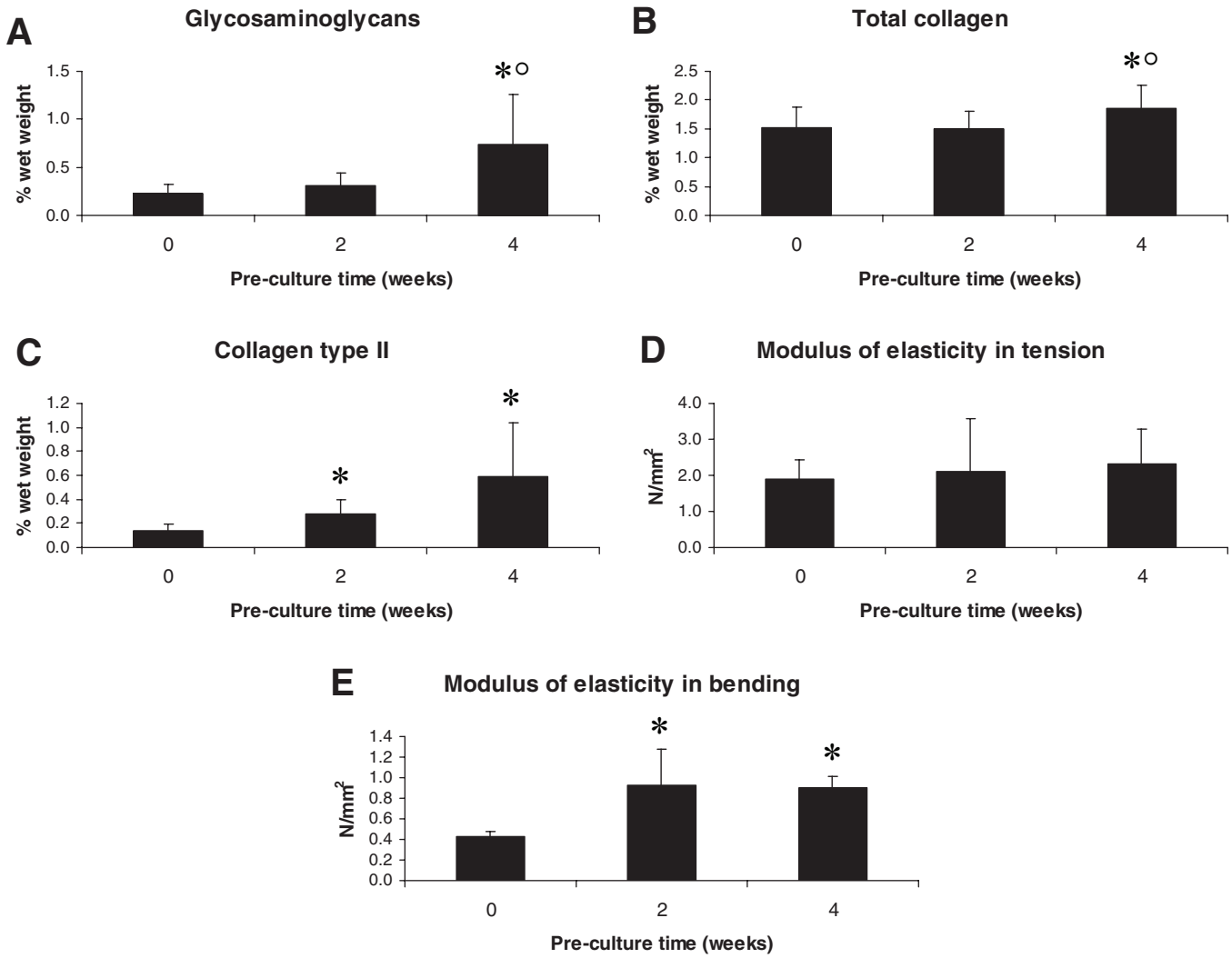

FIGURE 5. Properties of engineered nasal cartilage grafts after 2 weeks of in vivo implantation. Wet weight fractions of GAG (A), total collagen (B), collagen type II (C), and modulus of elasticity in tension (D) or in bending (E) in grafts explanted after 2 weeks of implantation in nude mice. Constructs were generated by nasal chondrocytes, freshly seeded or precultured for 2 or 4 weeks into Hyaff-11 meshes prior to implantation. *Statistically significant difference from the freshly seeded group. °Statistically significant difference from the 2-week preculture group.

strated that precultivation of human nasal chondrocytes into Hyaff-11 nonwoven meshes for 2 weeks yields engineered grafts with 1) significantly higher suture retention strength, a prerequisite for reliable implantation, and 2) significantly higher bending stiffness after 2 weeks' implantation, a prerequisite for safe removal of external fixation. Moreover, precultivated engineered tissues were reproducibly approaching histologic and biochemical properties of native nasal cartilage, which would be required to prevent fibrous tissue and vascular ingrowth and thus to support long-term stability of the graft.

Human nasal chondrocytes have been previously reported to have the capacity to generate hyaline-like cartilaginous tissues after monolayer expansion, to a higher extent than articular[16] and rib[4] chondrocytes. In addition, unlike articular chondrocytes,[17] the age of the donor and quality of the donor tissue did not effect the cell chondrogenic capacity following monolayer expansion,[7] indicating that tissue engineering of human septal cartilage is likely possible over a wide age range.[7] Our results further emphasize the reproducibility of engineering cartilage tissues starting from human nasal chondrocytes.

Despite previous extensive characterization of human engineered nasal cartilage, to the best of our knowledge, the present study is the first to use an experimental design based on clinically relevant parameters and related to issues which need to be addressed toward a clinical implementation of the procedure. In particular, the size and shape of the graft (ie, 1.5 × 0.5 cm beam) would be compatible with the use in a variety of reconstructive surgeries of the nose, including treatment of defects at the nasal septum or alar. The extent of cell expansion (ie, about 10 doublings), considering a previously deter-

mined yield of about 3.5E3 cells/mg of nasal cartilage tissue,[4] would be in the range of that required to have a sufficient number of cells starting from a biopsy of a few milligrams. Unlike subcutaneous implantation used by previous studies,[7,9] the in vivo model was selected to more closely resemble the environment of nasal cartilage, namely, a pocket between muscle fascia and subcutaneous tissue, and in vivo implantation was for a shorter period (2 weeks), which is clinically critical as the typical time before external fixation is removed. Most importantly, the biomechanical properties assessed were those specifically required for a graft to resist contraction by scar tissue formation and by exposure to local or external forces in the recipient bed, as opposed to previously characterized properties in compression or indentation tests.[7,9]

Rotter et al observed only minor differences in the in vivo formation of engineered nasal cartilage when PLA/PGA scaffolds were implanted immediately after seeding or following preculture for 3 weeks.[7] The discrepancy in comparison to our results could be explained by a number of different factors, including the scaffold used, specific growth factors inducing chondrocyte redifferentiation, the time point of explantation, and the different mechanical properties assessed. In this regard, however, it should be pointed out that the experimental design of our study did not include different times of implantation and thus does not allow to derive a conclusion on whether in vitro preculture effectively enhances cartilage tissue development in vivo or simply anticipates the time to reach a defined level of quality.

The scaffold used for this study is a nonwoven mesh composed of a benzylic ester of hyaluronic acid, a molecule naturally present in all soft tissues and playing an essential role in the maintenance of the normal extracellular matrix structure.[18] The resulting material, commercially known as Hyaff-11 and available in different forms (eg, sponges, meshes), has been extensively used for studies on cartilage tissue engineering[10,18–20] and is already in clinical use for the repair of articular cartilage defects.[12] The result that a preculture of 2 weeks improved the suture retention strength of engineered grafts is clearly related to the fact that the mesh used has intrinsically negligible mechanical properties. This observation allows to conclude that the mechanical properties reached by the grafts were merely due to the deposition and/or functional organization of new extracellular matrix and therefore are not likely to reduce with time due to scaffold degradation. Interestingly, the modulus of elasticity in tension was not modulated by precultivation time, and reached levels closest to native tissue as compared with all other parameters, suggesting that tensile properties of engineered cartilage are not a critical read-out in quality assessment. A longer implantation time was not the objective of the present study, although previous works[7,9] reported that engineered nasal cartilage tissues improved biochemical and biomechanical similarity to native tissue with time of implantation. The lack of a direct correlation between the collagen and GAG content and the measured mechanical properties of the tissues suggests the importance of a functional organization of those molecules and prompts for future investigations on the assessment of the level of collagen cross-linking.

CONCLUSION

The results of the present study demonstrate the possibility to develop in vitro a nasal cartilage graft with clinically relevant size and biomechanical properties. Furthermore, our data indicate a possible advantage in the preculturing of engineered human nasal cartilage grafts for 2 weeks prior to implantation, and together with other promising reports on the topic prompt for the clinical test of precultivated grafts in nasal reconstructive surgery.

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Discussions

DR. ANDREA FRILLING: I would like to thank the Association for inviting me to discuss this paper, and I appreciate the possibility to have seen the paper prior to the meeting for insight.

The group from Basel must be strongly congratulated for a technically very demanding and sophisticated study. The unique approach presented here, namely, the in vitro cultivation and in vivo implantation, underlines the clinical importance of this preclinical approach. The main goal of your study was to prove if precultured engineered cartilage tissues are of superior quality and long-term function when compared with the non-precultivated construct and, in particular, since studies performed in the past on this topic have generated controversial results.

So, in your model, you used 3 groups. The loaded scaffolds were alternatively implanted into nude mice directly or after seeding for 2 or 4 weeks of preincubation. Harvesting of the constructs was performed after 2 weeks. My first question would be: could you imagine that the non-precultured construct harvested after 4 weeks, that means not after 2 weeks as in your protocol, but after a longer time, could provide better results than they did after being implanted for 2 weeks only? Or the other way around: what is the rationale behind the recommendation that in the clinical settings the fixation of the nasal cartilage should be removed after 2 weeks?

And the second question. Can nasal chondrocytes be used for the augmentation of the nasal cartilage or also in distant localization such as hip or knee ankle? Thank you very much for your answers.

DR. JIAN FARHADI: Thank you very much for your comments and asking these relevant questions.

With regard to the first question, in this study we decided to focus the experimental setup to address a specific clinical goal, namely, the reconstruction of alar lobule defects. Considering that in this context traditional surgery using autologous grafts allows removal of external fixation and internal splints after 2 weeks, the envisioned tissue engineering approach should match the same criteria: thus, the engineered cartilage graft should achieve acceptable biomechanical stability 2 weeks after implantation.

Regarding your second question, indeed as compared with articular chondrocytes, human nasal chondrocytes are more easily accessible, can be harvested with lower morbidity, and allow more reproducible engineering of good quality cartilage tissues. Thus, we are also considering the possibility to use them to generate grafts for articular cartilage repair. So far, preliminary studies indicate a general compatibility of phenotype of engineered nasal cartilage with articular native cartilage. We are currently investigating whether engineered nasal cartilage is capable to respond to dynamic loading similarly to articular cartilage.

DR. ERKKI TUKIAINEN: Thank you. This was an excellent paper. Can I ask you: could you make cartilage in any forms, like for bronchus, trachea, or larynx?

DR. JIAN FARHADI: Thank you very much. We have not yet tried to engineer complex 3-dimensional structures like the trachea. In principle, that should be possible, although I anticipate that, for proper seeding and nutrition of cells within complex scaffold structures, specifically designed bioreactors would have to be introduced.

DR. HELMUT FRIESS: This was a very nice presentation and you presented very interesting and promising data on tissue engineering. To fill up your meshes with chondrocytes, you have first to isolate chondrocytes from the cartilage of donors. So my question is: how many chondrocytes do you need to fill up one mesh? For example, for 1-cm^2 mesh, how much donor cartilage do you have to use to get enough chondrocytes isolated? The background of my question is whether you are really engineering new cartilage material by first isolating chondrocytes from donor cartilage, and putting it later on a mesh? What is the expansion that you can reach?

DR. JIAN FARHADI: By taking a 4-mm-diameter biopsy of cartilage from the nasal septum and expanding the chondrocytes for 2 passages, which takes about 2 weeks, we are in principle able to engineer a cartilage graft of 16 cm^2 in 2-mm thickness. We achieve the possibility to extensively expand human nasal chondrocytes while maintaining their postexpansion capacity to form a cartilaginous tissue by using a specific cocktail of growth factors during cell growth, namely, transforming growth factor beta1, fibroblast growth factor-2, and platelet-derived growth factor-bb (see paper by Tay et al. *Tissue Eng*. 2004;10:762–770).