Autogenous Tissue-Engineered Cartilage
Evaluation as an Implant Material
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Objectives: To determine whether autogenous tissue-engineered cartilage grafts can be synthesized in predetermined shapes, to compare tissue-engineered cartilage with native cartilage with respect to histological characteristics and biomechanical properties, and to demonstrate how multiple transplantations affect tissue-engineered cartilage.

Design: Controlled, prospective animal study.

Subjects: Twenty New Zealand white rabbits, 3 weeks old.

Interventions: Autogenous chondrocytes were seeded onto biodegradable polyglycolic acid–poly-lactic acid copolymer templates in 1 of 3 shapes (cross, nasal tip graft, or auricle). Grafts and controls of sculpted cartilage were divided among 3 groups: short-term implantation (4 or 8 weeks), long-term implantation (6 or 12 months), and a reimplantation group. The gross morphological features, histological findings, and tensile strength of grafts were assessed.

Results: Production of tissue-engineered cartilage was confirmed in 30 of 31 implants. Histological evaluation demonstrated characteristic cartilaginous matrix, but with prominent vascular and fibrous tissue ingrowth. In long-term implantation grafts (n = 4), foci of osteoid were evident by 6 months. In the subset of transplanted grafts (n = 7), 5 of 7 demonstrated significant loss of cartilage viability. Tensile strength measurements demonstrated values 24% and 41% of those of controls at 4 and 8 weeks, respectively.

Conclusions: Tissue-engineered autogenous cartilage can be reliably produced, and predetermination of graft shape is possible. Histologically, grafts represent composites of mature cartilage infiltrated by vasculature and fibrous tissue, with delayed osteoid formation. Graft viability is compromised by early transplantation, and tensile strength is less than that of native cartilage. These results demonstrate the feasibility of tissue-engineered cartilage as a future graft material.


The ideal implant material for use in plastic and reconstructive surgery remains elusive. Sculpted cartilage autografts and homografts are commonly used despite their inherent disadvantages. These implants are associated with donor site morbidity, graft distortion, resorption, and limited tissue availability. Inorganic alloplastic implants are another option, but extrusion, infection, and poor biocompatibility are major considerations.

The concept of tissue-engineered cartilage has been proposed by several authors as an alternative in addressing reconstructive needs. This technology uses a synthetic, biocompatible, biodegradable, highly porous polymer mesh impregnated with isolated chondrocytes. By means of tissue culture techniques, the impregnated mesh is incubated briefly in vitro, allowing amplification of chondrocyte number and attachment to the polymeric scaffold. The mesh-chondrocyte complex is then implanted back into the subject, where the deposition of matrix substance begins. This promotes the development of mature cartilage, which adopts the morphological characteristics of the biodegradable polymer mesh.

The pioneering work by Vacanti et al. in tissue-engineered cartilage has demonstrated the ability to produce viable, mature, structurally stable cartilage in predictable shapes. Bovine chondrocytes have been harvested, seeded onto polymeric templates, and implanted into athymic mice. Use of an immune-incompetent recipient avoids host rejection of the chondrocyte xenograft.

The logical next step toward establishing the clinical utility of this technology is autogenous chondrocyte implantation with...
MATERIALS AND METHODS

STUDY DESIGN

Twenty New Zealand white rabbits were entered into the study. At 3 weeks of age, all rabbits had cartilage harvested from the right ear. Chondrocytes were isolated, seeded onto polymer templates, and incubated in vitro for 5 days. After incubation, the polymer templates were implanted on the flank of the respective donor animal. Three template shapes were evaluated within the experimental groups: a nasal tip graft, a cross, and a template molded into the shape of a human auricle. For all cross and nasal tip grafts, identical control grafts were fashioned from cartilage of the rabbit’s left ear and implanted.

Animals were entered into 1 of 3 study groups. A short-term implantation group consisted of animals whose grafts remained in vivo either 4 or 8 weeks before retrieval and analysis (n = 20). A long-term implantation group consisted of animals whose grafts were retrieved after either 6 or 12 months (n = 4). The third group involved animals whose implants were transplanted to another site after 4 weeks in vivo and then allowed to grow an additional 4 weeks before retrieval (n = 7). In many cases, multiple implants per animal were placed, depending on the template size and the yield of chondrocytes from donor cartilage.

The animal protocol was reviewed and approved by the University of Virginia Animal Care Advisory Committee. Animals were housed and cared for at the University of Virginia Health Sciences Center, Charlottesville, animal facility. Animals were marked for positive identification and were kept with their mothers until approximately 8 weeks of age.

TEMPLATE PREPARATION

A polyglycolic acid (PGA) nonwoven felt (Sherwood-Davis & Geck, Danbury, Conn) was used as the biodegradable template. This polymer is highly porous, with an interfiber distance of 100 to 200 µm (manufacturer’s data) and a density of 0.07 g/cm. The PGA felt was immersed for 2 minutes in a 5% (wt/vol) solution of poly-L-lactic acid (PLLA), with a molecular weight of 200 000 (Polysciences Inc, Warrington, Pa), dissolved in methylene chloride. The PLLA served to provide cross-linking of PGA fibers and enhance template rigidity.

For preparation of nasal tip and cross templates, the PGA/PLLA copolymer was allowed to dry and templates were cut by means of a precise x-ray film stencil. For the auricle template, wet PGA/PLLA was placed into a sculpted polymethyl methacrylate mold and allowed to dry. All templates were allowed to dry in a vacuum hood for 24 hours, allowing evaporation of all residual methylene chloride. Weights and dimensions of templates were recorded before the templates underwent ethylene oxide gas sterilization.

CARTILAGE HARVESTING

Ears were harvested from 3-week-old rabbits, soaked in povidone-iodine, dried for 5 minutes, and then serially rinsed with sterile saline to remove povidone-iodine. The overlying skin and perichondrium were meticulously removed and the cartilage was diced into pieces, none greater than 2 mm. The cartilage was placed in a sterile container of saline with 5% penicillin-streptomycin, and the exact weight of the cartilage was noted.

CHONDROCYTE ISOLATION

After cartilage harvest, chondrocyte isolation was promptly initiated. The cartilage pieces were bathed in 20 mL of 0.05% hyaluronidase (Sigma Chemical Co, St Louis, Mo) and allowed to rotate in a 37°C water bath for 30 minutes, after which the solution was aspirated and the cartilage rinsed with saline and penicillin-streptomycin. The cartilage was then incubated in the rotating water bath for 2 hours submerged in a solution of 2 mL of 0.25% trypsin (Gibco, Grand Island, NY) and 4000 U of purified bacterial collagenase (Worthington Biochemical Corp, Freehold, NJ). At this point, the chondrocytes were liberated from the cartilage and in solution.

The chondrocyte suspension was then centrifuged at 30g for 5 minutes and the supernatant discarded. The pellet was resuspended and the process repeated twice to remove residual digesting enzymes. The pellet was suspended in 1 mL of culture medium composed of Eagle minimum...
essential medium with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid solution and penicillin-streptomycin. The cell concentration of the solution was determined by means of a hemocytometer with trypan blue staining to determine the proportion of viable chondrocytes.

CHONDROCYTE SEEDING OF POLYMER TEMPLATES

Because the PGA/PLLA copolymer is largely hydrophobic, the template retards water and inhibits optimal chondrocyte seeding. It has been suggested that wetting the template with ethanol before seeding will allow better penetration of cell culture media and more uniform cell distribution. Templates were therefore soaked in 70% alcohol for 30 minutes and then washed with incomplete medium. The template was soaked in the medium for 2 hours and then washed twice more to displace ethanol from the polymer matrix. The PGA/PLLA templates were placed in 60-mm culture plates (Sarstedt Inc., Newton, NC), and then a volume of isolated chondrocyte solution was applied to the polymer template in an effort to achieve a cell concentration of 1 to 2 × 10^7/cm^3 of template material. This concentration of cells has been shown to provide reliable neocartilage growth without excessive overgrowth or distortion of the original template dimensions.15

TISSUE CULTURE

After the concentrated chondrocyte suspension was gently seeded onto the polymer mesh, 3 hours was allowed to promote cell adhesion to the mesh before it was immersed in culture medium (Eagle minimum essential medium plus HEPES buffer and 10% fetal calf serum). This was incubated in a 5% carbon dioxide incubator at 37°C for 5 days, allowing for additional cell growth and adherence. Culture medium was changed every 24 to 48 hours. With the aid of an inverted microscope, cultures were monitored for cell growth and evidence of bacterial contamination.

GRAFT IMPLANTATION AND HARVEST

After 5 days of tissue culture, grafts were implanted into the respective hosts. Rabbits were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg) intramuscularly, in addition to 1 ml of 1% lidocaine hydrochloride subcutaneously at the implantation site. On their flanks, pockets were developed beneath the panniculus carnosus for graft placement, and wounds were closed in 2 layers with chromic gut suture. Experimental and control grafts were placed on opposite sides of the animals. Grafts retrieved during transplantation and harvesting followed the same protocol.

ANALYSIS

The exact morphological characteristics and dimensions of harvested tissue-engineered grafts were compared with those of the original templates and control grafts for each animal. Specimens were photographed to record results and to assist with these comparisons. The quality of cartilage produced and retention of the morphological characteristics of the original template were compared between study groups.

Tensile strength for tissue-engineered cartilage specimens after 4- and 8-week implantation was determined and compared with that of native auricular cartilage. Freshly harvested specimens were cut into standard dumbbell shapes and kept in saline at 37°C until testing (1.5 to 3 hours). Ultimate tensile strength (UTS) was assessed by means of a tensiometer (Applied Test Systems, Butler, Pa) and 5-kg load cell, applying incremental axial loads to each specimen at a strain rate of 0.1 inch per minute to the point of specimen failure (Figure 1). Break strengths were normalized to the cross-sectional area at the specimen “neck” as determined by micrometer measurements, allowing calculation of UTS.

Chondrocyte viability, matrix production, and host tissue response were assessed via hematoxylin-eosin stains. In addition, special stains were used to confirm the production of characteristic constituent molecules within the cartilage matrix. Masson trichrome staining was performed to document collagen content, elastin von Gieson staining for elastic fibers, and aldehyde fuchsin alcian blue to indicate sulfated glycosaminoglycans.

Brous tissue. Gross morphological evaluation of specimens showed that cartilage grafts were produced in the prescribed shapes of a cross, a nasal tip graft, and an auricle (Figure 2). All control grafts of auricular cartilage were recovered; however, most lost their original sculpted shape because of folding, warping, and continued cartilage growth. This distortion of control grafts prevented useful morphological comparisons with the tissue-engineered cartilage grafts.

Histological analysis of the short-term implantation specimens with hematoxylin-eosin stains demonstrated viable chondrocytes within lacunae surrounded by newly synthesized matrix substance. Tissue-engineered cartilage specimens typically demonstrated a surrounding fibrous tissue layer as well as a unique fibrous and vascular ingrowth (Figure 3). Basophilic-staining PGA fibers were commonly found within the 4-week implantation specimens with an accompanying foreign body giant cell reaction. By 8 weeks after implantation, polymer filaments were largely absent. Foreign body giant cell reaction and the ingrowth of fibrous tissue and vasculature were not seen in the control specimens. Special histological stains were used to characterize the matrix substance of the cartilage produced. Masson trichrome staining demonstrated collagen content, and prominent staining with aldehyde fuchsin alcian blue indicated sulfated glycosaminoglycans (Figure 4). Elastin von Gieson staining was exhibited in the lacunar territorial matrix, indicating the presence of elastic fibers, although to a lesser extent than that seen in control specimens.

The UTS for specimens of tissue-engineered cartilage showed values 24% and 41% of those of native controls after 4- and 8-week implantation, respectively (Figure 5). The mean UTS of tissue-engineered specimens at 4 weeks was 0.85 ± 0.32 MPa as compared with
3.60 ± 0.79 MPa for control cartilage. Values for 8-week cartilage showed UTS of 2.40 ± 0.63 MPa and 5.70 ± 2.40 MPa for controls. Although formal flexion testing was not performed, manipulation of cartilage grafts showed that they were flexible and resilient without excessive brittleness.

Long-term implantation specimens (6 and 12 months) demonstrated thick adherent fibrous tissue capsules, with general retention of implant morphological features, although fine details, such as in the auricle graft, were lost. Histological examination of these grafts at 6 and 12 months all showed viable cartilage without residual polymer material or foreign body reaction. Interestingly, all grafts from this group showed foci of bone development along with marrow and associated hematopoietic elements (Figure 6). Long-term controls showed no evidence of bone formation. Because of bone formation within these grafts, specimens could not be cleanly cut without prior decalcification. This precluded the manufacture of dumbbell-shaped specimens for tensile strength testing.

The third group of specimens (reimplantation group) demonstrated gross characteristics similar to those in the previous groups, but with retention of original implant morphological characteristics in only 1 of 7 grafts. Histological examination showed devitalization of cartilage in 5 of 7 specimens, as evidenced by the disappearance of chondrocytes from lacunae and poorly staining matrix material (Figure 7). All transplanted controls remained viable. Because of the amount of graft distortion and loss of viability, formal mechanical testing was not pursued.

**COMMENT**

Tissue engineering shows promise as a means of developing new implant materials for the future. While previous reports have dealt primarily with xenograft chondrocyte implantation from a bovine donor to a murine recipient, our results demonstrate the ability to reliably generate cartilage from autogenous chondrocytes in the rabbit model. For up to 12 months in vivo, tissue-engineered cartilage implants have been well tolerated by the host. The cartilage produced resembles native cartilage yet possesses certain unique characteristics. 2

Many of our tissue-engineered cartilage grafts failed to retain the precise shape of the initial template. These flaws in graft morphological features are thought to result from the newly synthesized cartilage incompletely replacing the original polymer template. This can result from inadequacies in seeding the polymer template or from infiltration of noncartilaginous tissues.

Uniform chondrocyte seeding of the entire polymer mesh is a prerequisite for optimal graft development. Problems with template seeding can arise from inconsistencies in the template porosity, or may be caused...
by the inherent hydrophobicity of the PGA/PLLA co-polymer. It is known that PLLA adds stability to the PGA mesh by cross-linking fibers, but at the expense of decreased porosity of the copolymer.

Microscopy of developing tissue-engineered cartilage shows an intense foreign body giant cell response interspersed around and between areas of developing cartilage. Miyamoto et al evaluated polylactic acid impregnated with growth factors for promotion of osteogenesis and found an intense foreign body giant cell reaction (especially with higher–molecular-weight polylactic acid polymers) that retarded bone formation. We hypothesize that foreign body giant cell reaction may likewise play a role in limiting chondrogenesis. Vacanti et al used similar copolymers for tissue engineering and noted only a mild inflammatory reaction. Whether the difference in observations is caused by their use of an immunocompetent host, or differences in polymer composition, is uncertain.

The appearance of bone formation in the long-term implantation specimens (6 and 12 months) and the peculiar vascular infiltration of the cartilage grafts are likely related phenomena. Cartilage is typically avascular tissue. Chondrocytes are thought to produce an angiogenesis inhibitory factor that retards vascular ingrowth. The chondrocytes in our tissue-engineered cartilage specimens appear to have a reduced or absent production of this inhibitory substance. This may be caused by dedifferentiation of chondrocytes back to a progenitor cell such that the chondroblastic phenotype and the production of angiogenesis inhibitor are lost. Dedifferentiation of
chondrocytes is known to occur when cells are in tissue culture, although this is thought to be minimized by using 3-dimensional substrates such as our polymer template.22,23 Vacanti et al24 described the transformation of cartilage to bone in tissue-engineered specimens in which PGA mesh was seeded with bovine osteoblasts. These specimens were initially composed of hyaline cartilage but gradually developed osteoid tissue with increasing vascularity. Endochondral bone formation with bone marrow elements was achieved by 20 weeks. Additionally, the rate of morphogenesis from cartilage to bone appears to correlate with the vascularity of the implant site.7 It is known that chondrocytes and osteocytes derive from a common skeletal progenitor cell, and transition between chondroblast and osteoblast cell lines can occur.25 We hypothesize that a fraction of the chondrocyte isolates may have dedifferentiated to a common skeletal progenitor cell, allowing reexpression of an osteoblastic phenotype and production of endochondral bone. This transition of chondroblast to osteoblast cell line has not been described with other tissue-engineered cartilage models and may be unique to the rabbit auricular chondrocyte. Dedifferentiation of chondrocytes to fibroblasts is known to occur in tissue culture25 and may contribute to the fibrous tissue seen in our cartilage grafts, although this fibrovascular infiltrate is thought to be primarily a host response to the implant. The fibrovascular ingrowth into a porous material is not an unexpected process and likely contributes to many of the characteristic features of tissue-engineered cartilage.

Mechanical testing of tensile strength produced values of UTS comparable with those reported in the literature.26 At 4 and 8 weeks of development, UTS was found to be inferior to that of controls, although controls may have been poorly matched. For example, specimens harvested 4 weeks after implantation were compared with native auricular cartilage from the host animals, which were 8 weeks old at the time of graft harvest. Therefore, 4-week-old tissue-engineered cartilage was compared with cartilage that had been developing since before the birth of the animal. Further testing with more “mature” im-

plants may provide a more useful comparison, particularly since we noted a trend toward greater tensile strength from 4- to 8-week specimens. Determination of mechanical properties is also confounded by the heterogeneity of the specimens tested. As noted previously, specimens at 4 weeks still contained residual polymer fibers, as well as considerable fibrous tissue. Long-term grafts could not be tested because osteoid development prohibited cutting the cartilage into uniform test specimens. The relative scarcity of elastic fibers seen in tissue-engineered specimens may also contribute to compromised tensile strength. The diminished capacity of cultured rabbit auricular chondrocytes to synthesize elastin has been previously documented.27 While it is understood that the elastic fibers allow auricular cartilage its pliability, the contribution of these fibers to its tensile strength is not certain.

The third experimental group served to evaluate the feasibility of transplanting tissue-engineered cartilage grafts. For example, could grafts be matured at an intermediate site, retrieved, sculpted and refined, and then implanted at another site for final use? Overall, grafts tolerated reimplantation poorly. All grafts were transferred to a separate implantation site in a timely manner, with care taken to prevent desiccation or contamination of the grafts. Control grafts, on the other hand, seemed to tolerate the reimplantation. As noted previously, early specimens of tissue-engineered cartilage (4-week specimens) are composites of developing cartilage, foreign body giant cell reaction, neovascularization, and fibrous tissue. At this early stage, interruption of the microvasculature on the periphery of the implant may lead to cartilage loss. Additionally, the surrounding fibrous tissue capsule and inflammatory infiltrate may become devitalized, effectively insulating the developing cartilage from its nutrient supply. Further investigation allowing more time for cartilage maturation before reimplantation may help clarify this important aspect of tissue-engineered cartilage.

Tissue engineering has opened a new frontier in the development of implant materials for plastic and reconstructive surgery. It has many characteristics of an ideal implant and may obviate complications and limitations of other implant materials currently in use. To date, most work with tissue-engineered cartilage has been through a xenograft model. This study takes an additional step toward practical application by using an autograft model. Although our 12-month experience with implanted tissue-engineered cartilage is as long as any reported in the literature, the ultimate fate of these implants is still in question and deserves further investigation.

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REFERENCES


