Transplanted Tissue-Engineered Cartilage

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Objective: To evaluate the feasibility of transplanting sculpted autogenous tissue-engineered cartilage (TEC) with the hope that it will retain precise 3-dimensional morphologic features after transplantation. Transplanted TEC is described in terms of the gross morphologic and histologic characteristics in contrast to pretransplanted TEC.

Methods: Synthetic scaffolds of a polyglycolic acid and poly-L-lactic acid polymer, coated with chondrocytes derived from rabbit auricular cartilage in concentrations ranging from $2.7 \times 10^6$ to $6 \times 10^7$ cells/mL, were incubated in vivo on the dorsum of a rabbit for 8 weeks and then retrieved. The resultant TEC specimens were then sculpted into defined shapes and transplanted into a different location in the same rabbit, where they were allowed to incubate for another 8 weeks. The specimens were then retrieved and compared with the TEC before transplantation according to size, weight, and histomorphometric analysis.

Results: Thirteen chondrocyte-laden templates were successfully engineered to develop TEC. In each case, they were sculpted and transplanted to a different site in the same rabbit. Eight weeks after transplantation, all sculpted TEC specimens lost their original 3-dimensional morphologic features and experienced a significant decrease in mass. Histologically, the staining intensity of both hematoxylin-eosin and safranin O was dramatically reduced following transplantation. In addition, there was a reduction in chondrocyte viability. Two consistent histologic findings were a foreign-body reaction to the synthetic polymer and ongoing cellular activity directed toward the formation of bone.

Conclusions: Transplanting autogenous TEC does not allow the preservation of precise morphologic features that are needed for clinical implantation. The osteogenic progression and foreign-body reaction must also be controlled.

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harvesting, chondrocyte liberation, template preparation, seeding, and implantation have been described previously.²⁻⁵

**TRANSPLANTATION OF TEC (8-WEEK HARVEST)**

Immediately after harvesting the TEC at 8 weeks, each piece of cartilage was sculpted using a constant template of either a cross or square. The grafts were photographed, weighed, measured, and transplanted to the opposite side of the rabbit’s dorsum, where they were allowed to incubate for 8 weeks. The templates were stored for future comparisons. Cartilage remnants from the sculpting were similarly stained.

**POSTTRANSPLANTATION HARVEST**

(16-WEEK HARVEST)

The rabbits were killed 8 weeks after transplantation by the veterinary technician in the animal care facility by giving phenobarbital. The skin over the transplant site was shaved, and the underlying cartilage was meticulously removed. The specimen was photographed, weighed, measured for gross analysis, and sent for sectioning and staining with the same agents used for pretransplantation remnants.

**DATA ANALYSIS**

**Gross Morphologic Analysis of Pretransplantation and Posttransplantation Cartilage**

The morphologic characteristics of the harvested TEC grafts were compared with those of the original templates for each animal by comparing size, weight, and shape. Specimens were photographed to assist with these comparisons.

**Histologic and Histomorphometric Analysis**

The cartilage sections were stained using a standardized protocol at the Cell Science Core Facility at the University of Virginia. The cartilage was fixed in a 50:50 wt/vol solution of 10% neutral buffered formalin and decalcifying solution, embedded in paraffin, and sectioned in 4-µm increments. In one section, hematoxylin-eosin was used to stain the cartilage matrix. The second section was stained with safranin O to highlight the sulfated glycosaminoglycan in the form of proteoglycan, representing active cartilage growth. Paired slides were compared in regard to chondrocyte viability, matrix production, and overall cartilage integrity. Specifically, the objective measures were chondrocyte density, chondrocyte dropout ratio, and the ratio of cartilaginous matrix components. Quantitative data about these characteristics was collected using computerized histomorphologic analysis of pictures taken using microscopic photometry (BH-2 microscope; Olympus, Melville, NY; and Image-Pro Plus 4.5; Media Cybernetics, Silver Spring, Md).

As a measure of chondrocyte viability through transplantation, we followed the chondrocyte density and the chondrocyte dropout ratio (percentage of vacant lacunae). To measure chondrocyte density, 1 manually delineated ×20-magnified area of each hematoxylin-eosin slide was chosen, and Image-Pro Plus measured the density of the chondrocytes. Chondrocyte dropout ratios were counted manually on the hematoxylin-eosin slides, counting 4 different areas at ×40 magnification of each slide. The chondrocyte dropout ratios were averaged, and their pretransplantation and posttransplantation counterpart values were compared using a paired t test.

As a measure of the cartilage matrix production, we followed the intensity of safranin O staining. To quantify this, we relied on the standardization of the staining procedure and then used Image-Pro Plus to measure the intensity of the stain. The images were captured and normalized for background light penetration by white balancing. Image-Pro Plus sees each image as constructed of pixels, ranging in intensity from 0 to 255, where black is assigned 0 and white assigned 255. Thus, a higher-intensity number means that more light is getting through the slide and less proteoglycan is available to bind safranin O. Four different areas of each image at ×20 magnification were measured, the intensity values averaged, and the comparisons made in staining intensity between pretransplantation and posttransplantation cartilage. The mean of the intensity values obtained from each image reflects the amount of light penetration, which inversely reflects glycosaminoglycan presence; the higher the intensity value, the fewer glycosaminoglycans present.

**STATISTICAL ANALYSIS**

Measurements of chondrocyte density, chondrocyte dropout ratio, weight, and intensity of safranin O staining for both pretransplantation and posttransplantation samples (N = 13) were averaged and compared using a paired t test. Data were represented as a box plot with the median value marked by the central line in each box; the 25th and 75th percentiles are the respective borders of the boxes.

**RESULTS**

**PRETRANSPLANTATION HARVEST**

**Gross Morphologic Features**

As observed in previous studies, all chondrocyte-laden templates were successfully engineered into TEC. As in the past, cartilage harvested at 8 weeks did not fully retain the original shape of the implanted templates (Figure 1).² The purpose of this stage was to obtain bulk cartilage for sculpting (Figure 2).
Histologic Morphologic Features

Three major histologic features were consistently found in the cartilage harvested at 8 weeks. With hematoxylin-eosin staining, highly metabolically active chondrocytes surrounded by the periosteum and fibrous connective tissue were seen (Figure 3B). The second prominent characteristic was an extensive foreign-body reaction against the scaffold polymer fiber in some areas (Figure 3D). Third, both appositional growth and interstitial growth of cartilage were seen (Figure 3C). Also, both hyaline and elastic cartilage were seen after 8 weeks of incubation.

POSTTRANSPLANTATION HARVEST

Gross Morphologic Features

After 8 weeks of in vivo incubation, the sculpted TEC specimens failed to retain their preshaped morphologic characteristics. There was a significant reduction in specimen mass (Figure 4) from a mean of 0.63 g to 0.39 g, a 39% reduction (P=.003). Remodeling of the external surface was a prominent feature (Figure 5). Overall, the morphologic features were grossly similar to but not an exact replica of the original template.

Figure 3. Histologic features of bulk tissue-engineered cartilage (TEC) 8 weeks after implanting a chondrocyte-laden scaffold (hematoxylin-eosin; original magnification ×10) (A). B, Metabolically highly active chondrocytes were seen in the cartilage nest. Note that the perichondrial-like surface gives rise to the chondroblasts, which in turn evolve into the chondrocyte in this slide, representing appositional growth of the cartilage (hematoxylin-eosin; original magnification ×20). C, Isogenous groups in a cartilage nest, representing recently divided cells, were clearly visualized along with appositional growth from the periphery (hematoxylin-eosin; original magnification ×20). D, Bulk TEC demonstrated residual synthetic polymer and foreign-body reaction to this synthetic polymer residue (hematoxylin-eosin; original magnification ×20).

Figure 4. Mass comparison of pretransplantation and posttransplantation cartilage. Box borders represent quartiles; box line, median value; whiskers, range; and asterisks and circle, outliers.
Histologic Morphologic Features

A striking feature found in the histologic studies of the posttransplanted cartilage is the decrease in intensity of the safranin O–stained extracellular matrix compared with that of the pretransplanted cartilage (Figure 6). Also, a reduction in the relative amount of viable chondrocytes as judged by the loss of normal cellular integrity was clearly observed, and the body of cartilage was replaced by endochondral ossification with a clear transition from cartilage to bone (Figure 7).

QUANTITATIVE COMPARISON

Quantitative analysis of the chondrocyte dropout ratio demonstrated a statistically significant difference between pre-
transplantation and posttransplantation cartilage (60% pre-
transplantation vs 82% posttransplantation; \( P = .001 \)) (Figure 8A and Table). The intensity of the safranin O staining was also found to be statistically different between pretransplantation and posttransplantation sections of cartilage. The posttransplantation sections absorbed 8% less safranin O \( (P = .003) \) (Figure 8B, Table). Another prominent feature of the posttransplantation cartilage was the decreased density of the chondrocytes when compared with the pretransplantation cartilage (Figure 8C, Table). The pretransplantation chondrocyte density was 1190 cells/mm\(^2\), and the posttransplantation density was 934 cells/mm\(^2\), a reduction of 22% \( (P = .001) \).

COMMENT

Previous studies have shown the potential of TEC as the ideal implant for use in aesthetic, reconstructive, and orthopedic surgery.\(^7\) However, TEC continues to be characterized by implant distortion and resorption. An initial question is, “How and when does remodeling occur?” Our initial assumption was that remodeling occurs as cartilage matrix is deposited and the scaffold resorbed and that if TEC was placed in vivo after this phase, remodeling would not be as destructive. Therefore, we directed this experiment toward the study of transplanting TEC grown in vivo using a rabbit model. Specifically, is transplanted cartilage more resilient to remodeling than the original graft? This model did not demonstrate an improvement in retention of morphologic or histologic characteristics. We observed a decrease in weight and size and lost architectural integrity and chondrocyte viability. Furthermore, the chondrocytes dedifferentiated to mesenchymal precursors and osteocytes.

Although transplanting cartilage after it had finished its initial growth phase failed to alter the course of TEC, there are some things to be learned from this particular experiment. First, we are left with a better understanding of the underlying histologic mechanism of remodeling. We speculate that during the pretransplantation in vivo incubation, there was a gradual proliferation of the chondrocytes and organization into cartilage, with si-
multaneous loss of the artificial template. Connective tissue from the host rabbit surrounded the cartilage, appearing as fibrosis on hematoxylin-eosin slides, and possibly contributed to the loss in shape as contracture occurred. We assume that there was a continuous competition between the de novo organization of cartilage and a foreign-body reaction to the template. Therefore, initial chondrogenesis does not necessarily translate to an increase in size and shape. Some rabbits demonstrated graft expansion, whereas others showed a marked reduction. Morphologic change may also be attributed to the uneven distribution of chondrocyte seeding on the template and the varying number of total cells implanted. Despite attempts to distribute the chondrocytes in an even manner, there will be inherent variations in cell density on the surface of the template.

After 8 weeks of in vivo incubation, the cartilage matrix stained heavily with both hematoxylin-eosin and safranin O, representing large amounts of glycosaminoglycan and proteoglycan, and indicated that the chondrocytes were metabolically active with the production of the extracellular matrix. Each cartilage specimen showed nests of isogenous groups of chondrocytes, indicating recently divided cells and giving evidence of interstitial growth. At the same time, the cartilage nests revealed appositional growth of cartilage from an inner perichondrium, indicating cellular differentiation from chondroblasts. We did not expect to observe the chondrogenic activity of the perichondrium, and this may have contributed to remodeling. The perichondrium must have derived from mesenchymal precursor cells adjacent to the site of implantation, from implanted chondrocytes that dedifferentiated, or from latent auricular perichondrium not separated during the initial liberation. In the future, confirming the origin of the perichondrium with a tagged radioactive study may be important if one assumes that this appositional growth contributes to remodeling.

The role of the perichondrium in TEC may prove to be interesting and critical. After 8 weeks of in vivo incubation, the presence of scar tissue surrounding the specimen and infiltrating the graft was a predominant feature. However, there was no evidence of an inflammatory reaction directed against the polymer when there was perichondrium shielding the implant. Furthermore, the foreign-body reaction continued to develop even after transplantation, when there was no histologic evidence of a persistent synthetic polymer. Using a bioactive polymer rather than a synthetic scaffold may be a better delivery system considering the foreign-body reaction to the PGA/PLLA polymer and the effects of inflammatory cell infiltration.

Interestingly, each cartilage specimen not only failed to retain its sculptured shape, but it also underwent histologic ossification. Bone formation resembled the features

**Figure 7.** Tissue-engineered cartilage 8 weeks posttransplantation. Note that a large amount of cartilage has been replaced by bone formation, and the transitional zone from cartilage to bone is clearly visualized (hematoxylin-eosin; original magnification ×10).

**Figure 8.** Summary of objective histologic measures. A, The chondrocyte dropout ratio displayed a statistically significant increase in the number of chondrocytes losing viability after transplantation. B, Safranin O staining intensity, determined by light penetration of the slide. Note that the number on the y-axis represents the intensity of light penetrance. The higher value in light penetrance reflects the decreased proteoglycan production that absorbed safranin O. C, Chondrocyte density pretransplantation and posttransplantation. Data are represented as box plot in which box borders represent quartiles; box line, median value; and whiskers, range.

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typical of endochondral ossification seen in long bones. The nature of the devolution from tissue containing mostly differentiated chondrocytes into tissue containing osteocytes may be unavoidable. Even without sculpting and transplantation, some signs of cartilage degradation and conversion to bone were already seen before transplantation. We cannot explain why ossification was seen so consistently in these rabbits, but we believe that the osteocytes were not derived from the dedifferentiation of chondrocytes. Transitional areas were clearly seen where chondrocytes were being engulfed by osteoclasts in the process of depositing endochondral bone, identical to that of normal endochondral ossification. In other words, we postulate that precursor cells migrated into the TEC from surrounding connective tissue along with angiogenesis, followed by the arrival of bloodborne osteoblast and osteoclast precursors. The origin of the osteoblasts and osteoclasts is still unclear at this point.

Morphologic remodeling and loss of the sculpted shape after transplantation are all negative findings from a clinical point of view. These results may imply that transplanted TEC using a PGA/PLLA scaffold is a less feasible option as a potential implant. Future investigation should pursue a scaffold that minimizes the foreign-body reaction and subsequently reduces in situ remodeling. Because the perichondrium appears to have a significant role in TEC, both in terms of protection and neocartilage formation, its origin and role in appositional growth are worth exploring. Finally, preventing ossification of implanted TEC is important for long-term application.

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REFERENCES