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Abstract

Background: Tissue engineering has the potential to provide ear-shaped cartilaginous constructs in the future. Previous attempts to engineer human ear-shaped constructs mimicked human shape and characteristics but were done in immunocompromised animal models. Objectives: The authors design and evaluate a novel, 3-dimensional (3D) cell copolymer construct resembling a human ear that was subsequently implanted in an immunocompetent rabbit model.

Materials: Mesenchymal progenitor cells that were obtained from perichondrium and chondrium of a rabbit auricular cartilaginous site were expanded in vitro to chondrocytes and seeded onto biodegradable alginate and silk polymer ear-shaped scaffolds. After implantation in the back of 6 immunocompetent rabbits for 8 weeks, cell/scaffold constructs were harvested and analyzed in terms of size, shape, and histology.

Results: Data from this study suggest that auricular mesenchymal progenitor cells derived from rabbit perichondrium and chondrium are suitable for development of tissue-engineered human ear models with retention over time of 3D construct architecture. Gross morphology revealed that the silk alginate scaffold diminished slightly the size dimensions but maintained shape and flexibility. Histological analysis showed formation of cartilage tissue along with type II collagen and proteoglycan extracellular matrix components of the silk alginate construct.

Conclusions: This study demonstrates for the first time that it is possible to engineer an ear cartilage construct that resembles the human ear not only in shape but also in size and flexibility in a real test model. This study also confirms that the association of silk, alginate, and perichondrium and chondrium mesenchymal cells is a reliable method to produce an engineered auricular cartilage construct. Further long-term research needs to be done to confirm these observations.

Keywords
auricular tissue engineering, human ear, stem cells, anotia, biomolecules, biomaterial

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Total auricular reconstruction represents one of the greatest challenges for the plastic and reconstructive surgeon. The ability to construct a fully satisfactory, complete external ear has been an elusive goal for centuries. Because a suitable and reliable substitute for auricular cartilage has yet to be engineered, hand-carved autologous costal cartilage grafts and ear-shaped porous polyethylene implants are the current treatment modalities for auricular reconstruction. However, over the past decade, significant advances have been made in the field of regenerative medicine and tissue engineering. A variety of scaffolds and innovative approaches have been investigated as alternatives using autologous carved costal cartilage or porous polyethylene implants. Although investigators have demonstrated that neocartilage can be constituted in a predetermined shape and in complex 3-dimensional (3D) structures such as a human ear by using cell transplantation on polymer constructs, many unsolved problems still remain. The crucial issues for auricular tissue engineering consist of optimal cell culture environment, choice of polymers, behavior of chondrocytes, study of cell-polymer constructs in an acceptable immunocompetent animal model (“real test model”), and long-term structural integrity. One approach to functional tissue engineering of cartilage involves the in vitro cultivation of

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tissue constructs by using chondrogenic cells that can be selected, expanded, and implanted in ear-shaped scaffolds that provide a defined 3D structure for tissue development and are biodegrade at a controlled rate. \(^1,7,8\) Although a number of investigators have been able to produce neocartilage in predetermined shapes, no study has been done in a “real test” immunocompetent animal model. \(^1,9-11\)

In this study, we evaluated a novel, 3D cell-copolymer construct resembling an ear model, made of mesenchymal progenitor (stem) cells (MSCs) seeded in alginate and silk fibroin, which was subsequently implanted in an immunocompetent rabbit model.

**METHODS**

**Copy of the Human Auricle With 3D Computer-Aided Design**

To calculate a geometric model of the auricle construct, the following image-processing steps were necessary: (1) segmenting the image structure of the model, (2) calculating the surface model, (3) making a mirror image of the geometric model, and (4) exporting the geometric data in the stereolithography data format. After importing the computed tomography (CT) data in the DICOM file format, the isotropic image data volume was calculated by linear interpolation. \(^12\)

This was the basis for a 3D image segmentation of the auricle model, which was performed by automatic thresholding. \(^13\)

This process produced a binary image data volume, and the surface data could be approximated by geometric primitives such as triangles. After making a mirror image of these geometric data, the 3D visualization process could be initiated as well as the export of the data in the STL format.

**Fabrication of the 3D Human Ear Mold**

The production of the mold for the creation of the auricular cartilage construct was carried through fast prototyping, from a human ear image. The 3D image of the ear was sent to the 3D impression, where the initial mold, called “first positive,” was produced by laser impression, overlapping layer by layer in cuts of 1 micron (Figure 1A). In recent years, laser technology has proven to be a more reliable method for mold engraving (impression), as many traditional engraving methods have failed to meet the continually growing standards for precision, depth, and quality. This first “positive” scaffold allowed the preparation of a second scaffold, the so-called negative, in silicone (Figure 1B).

**Harvest of Cartilage, Perichondrocyte, and Chondrocyte Isolation and Monolayer Culture Expansion**

Auricular cartilage was dissected from the ears of 12-week-old New Zealand White rabbits. A square-shaped composite tissue of the whole layer of the auricle, 1 × 1 cm in size, was harvested. Each sample was placed in normal saline and processed within 6 to 12 hours postsurgery. Cartilage was washed with phosphate-buffered saline containing 2% fetal bovine serum (FBS). The sample was then minced into...
small fragments and digested with 2 mg/mL collagenase in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C for 3 hours, for MSC isolation. Deactivation of collagenase action was done by adding DMEM and FBS. The resulting cell suspension was centrifuged at 2000 rpm for 15 minutes. The supernatant was decanted and the cell pellet was washed to remove the remaining enzyme. After final centrifugation, the cell pellet was analyzed for total cell count with a hemocytometer. Cell viability was determined using the trypan blue dye exclusion test. Cells were expanded in low-glucose DMEM, containing 20% fetal bovine serum (CULTILAB, Campinas-SP, Brazil), penicillin, and streptomycin. All cultures were maintained in a 5% CO₂ incubator. Cells cultured in pellets were treated with transforming growth factor (TGF)-β (10 ng/mL) and bone morphogenetic protein 2 (BMP-2; 100 ng/mL). Documentation of perichondrocyte and chondrocyte monolayer culture at 2 days and 14 days under microscopic magnification is shown (Figure 2). The concentration of cells was 2 to 5 × 10³/cm².

**Preparation of Microporous 3D Silk-Alginate-Cell Scaffolds**

Cocoons from the *Bombyx mori* silkworm were boiled for 20 minutes in distilled water, and sericin was extracted with an aqueous solution of 0.1M NaOH at 70°C. After 1 hour, the silk-fibroin concentrates were dissolved with formic acid 85% with 0.01% (w/v) CaCl₂ at room temperature, as previously described. Mesenchymal perichondrocytes and chondrocytes were seeded at a density of 10 cells captured in 20 µL of alginate 1% (Sigma Fluka, St. Louis, MO) with 102 mM CaCl₂ and silk (Figure 3). The cell-seeded fibroin-based material was maintained in vitro at 37°C with 5% humidified CO₂ for 1 week.

**Implantation of the Constructs**

The fibroin-based silk-alginate-cell construct was able to assume an auricular shape (Figure 4A). The ear construct dimensions for the 6 rabbit models are shown in Table 1. Immunocompetent rabbits were anesthetized upon surgery. A minor skin incision was made at the dorsum of nude rabbits. One construct was implanted into the subcutaneous pocket of each rabbit (Figure 4B). The resulting in vivo constructs were harvested at 8 weeks postimplantation. All animals were treated in compliance with the “Guide for the Care and Use of Laboratory Animals” from the Association for Assessment and Accreditation of Laboratory Animal Care International.

**RESULTS**

After cell seeding, cells adhered to the scaffolds, proliferated, and produced cartilaginous matrices to fill the void spaces in the ear scaffold. Figure 5 shows the ear construct after 4 weeks of implantation. In Figure 6A, the auricular construct is shown after 8 weeks of implantation, and in Figure 6B, the auricular construct is shown after explantation. Although the ear constructs maintained their original shape, there was a reduction in size compared with the
preimplantation specimens (Table 2). Each construct was observed grossly at room temperature, without any fixation, and palpated with forceps clinically to assess mechanical rigidity. A thin, vascularized capsule was observed surrounding the construct. Immunohistochemistry analysis was performed (Figure 7). No ear scaffold implants exhibited extrusion or infection.

**DISCUSSION**

Tissue engineering using MSCs continues to be a challenging problem. Cartilage tissue engineering has an important role to play in the generation of graft material for head and neck reconstruction and specifically for auricular cartilage reconstruction. Although this work serves as a preliminary investigation for fabricating a human ear model, the results are important for the research of tissue-engineered cartilage. Successful tissue engineering is based on acquiring the appropriate cells, proliferating them, and accurately predicting their response in vivo. We have found that the MSCs acquired from perichondrium and chondrium of the rabbit ear cartilage have expressed markers for chondrogenesis. Although it has been shown in earlier studies that the yield for chondrocytes and extracellular matrix from MSCs has not been sufficient to support 3D auricular scaffolds, in our study, the rate yield was sufficient for constructing an ear 3D model. Comparing and testing the different possible sources of cells for construction of this specific auricular model, the combination of MSCs from perichondrium and chondrium seemed to be the most appropriate. A recently published study has

**Table 1. Ear Implant Dimensions in the 6 Rabbit Models Before Implantation**

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<th>Test Animal</th>
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Abbreviations: d1, pinna height; d2, pinna width.

**Figure 3.** Schematic representation of preparation of the auricular scaffold by mixture of perichondrocyte-chondrocyte cells, alginate, silk, transforming growth factor (TGF)-β3, and bone morphogenetic protein 2 (BMP-2).

**Figure 4.** (A) The auricular tissue-engineered construct. d1, pinna height; d2, pinna width. (B) Implantation of the auricular construct in the back of an immunocompetent New Zealand White rabbit.
confirmed also that MSCs are excellent candidates for tissue engineering, and when obtained from the perichondrium and chondrium, they have a high chondrogenic potential.\textsuperscript{10,18}

Biomaterials are used to guide the organization, growth, and differentiation of cells in the process of forming functional tissue and can provide both physical and chemical cues.\textsuperscript{19} They need to be tissue inductive to induce the proliferation of cells and tissue conductive to guide the migration of the cells. Alginate is a polyanionic polymer found in brown algae and can be cross-linked with bivalent cations to form stable ionically cross-linked gels. Alginate beads and hydrogels have been used to expand chondrocytes and induce stem cell differentiation.\textsuperscript{20} The use of alginate associated with fibroin-based material to reconstruct an ear cartilage microenvironment in vitro has been described.\textsuperscript{14,19} Silk fibroin has been shown to support stem cell adhesion, proliferation, and differentiation in vitro and promote tissue repair in vivo.\textsuperscript{21} The silk is mainly composed of a filament core protein, fibroin, which can be made in various formats and has been shown to support stem cell adhesion, proliferation, and differentiation in vitro and promote tissue repair in vivo. A recent study has shown that seeding fibrous silk scaffolds with MSC guides MSC morphology and orientation, demonstrating the impact of scaffold topography on the engineering of oriented tissues.\textsuperscript{22}

Biomolecules are biological materials that contribute to the structural integrity of tissue-engineered constructs and, at the same time, regulate their components. Growth factors, differentiation factors, angiogenic factors, and gene-modulated factors are the main components of the biomolecules. For in vitro culture, the addition of TGF-β3 and BMP-2 has stimulated enhanced chondrogenesis, regardless of the culture method chosen or scaffold composition; however, the degree of chondrogenesis is

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<th>Table 2. Dimensions of the Explanted Ear Constructs From the 6 Rabbit Models, 8 Weeks After Implantation</th>
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Abbreviations: d1, pinna height; d2, pinna width. The percent decrease in size measurements is shown in parentheses.
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scaffold dependent. Chondrogenic induction assays in the presence of TGF-β growth factor and BMP-2 were used in this study, as both factors have well-documented roles in chondrocyte maturation. Localization of long-term DNA label-retaining cells (LRC) is restricted to the perichondrium layer, and these putative stem/progenitor cells possess clonogenicity and chondrogenic capability in vitro. In a recently published study, no LRC were observed in the chondrium but LRC were observed in the perichondrial layer. These LRC, observed only in the perichondrial layer, seemed dormant, which is characteristic of putative stem cells. Distortion and shrinkage of ear-shaped constructs during scaffold degradation and neocartilage maturation in vivo have been reported. Scaffolds made of synthetic polymers often generate degradation products that cause an inflammatory reaction and negatively affect neocartilage formation in vivo. This is a pilot study, which is a small experiment designed to test logistics and gather information prior to a larger study, to improve the latter’s quality and efficiency. Future studies need to be conducted with a bigger sample size, in which the engineered ear construct is followed for a longer period. Although we are reporting preliminary results of fabricating an ear model in an immunocompetent animal, we believe that these observations could serve as guidance for future experiments to produce “off-the-shelf” engineered auricular cartilage constructs.

CONCLUSIONS

This study demonstrates for the first time that it is possible to engineer an ear cartilage construct that resembles the human ear not only in shape but also in size and flexibility in a real test model. This study also confirms that the association of silk, alginate, and MSC is a reliable method to produce an engineered auricular cartilage construct. Further long-term research needs to be done to confirm these observations.

Disclosures

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