Influence of Growth Factors on Tissue-Engineered Pediatric Elastic Cartilage

Carlos A. Arevalo-Silva, MD; Yilin Cao, MD, PhD; Martin Vacanti, MD; Yulai Weng, DD; Charles A. Vacanti, MD; Roland D. Eavey, MD

Objective: To investigate the influence of growth factors on tissue-engineered pediatric human elastic cartilage relative to potential clinical application.

Design: Controlled study.

Subjects: Eleven children ranging in age from 5 to 15 years provided auricular elastic cartilage specimens measuring approximately $1 \times 1 \times 0.2$ cm and weighing approximately 100 mg.

Interventions: Three million chondrocytes were plated into 4 groups of Ham F-12 culture medium: group 1, Ham F-12 culture medium only; no growth factors (control group); group 2, Ham F-12 culture medium and basic fibroblast growth factor; group 3, Ham F-12 culture medium and transforming growth factor $\beta$; and group 4, Ham F-12 culture medium and a combination of both growth factors. At 3 weeks, the cells were harvested and mixed with a copolymer gel of polyethylene glycol and polypropylene oxide (Pluronic F-127). The cell solution was injected subcutaneously into athymic mice. The constructs were harvested at up to 22 weeks of in vivo culture and histologically analyzed.

Results: The average number of cells generated in vitro was as follows: group 1, 12 million; group 2, 40 million; group 3, 7 million; and group 4, 35 million. Group 2 in vivo gross specimens were the largest and heaviest. Histologically, the control group and the basic fibroblast growth factor group (groups 1 and 2) exhibited characteristics compatible with normal auricular cartilage; groups 3 and 4 demonstrated cellular disorganization and moderate to severe fibrous tissue infiltration.

Conclusions: Basic fibroblast growth factor demonstrates the greatest positive influence on the in vitro and in vivo growth of engineered pediatric human auricular cartilage. The results suggest that basic fibroblast growth factor has the potential for clinical application in which a goal will be to generate a large volume of tissue-engineered cartilage from a small donor specimen in a short period of time and of a quality similar to native human elastic cartilage.

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ARTICLE IS vital for reconstructive surgery and is used to repair a variety of defects within the specialty of otorhinolaryngology. The major cartilage source for microtia reconstruction is rib fibrocartilage. Although ribs are a dependable donor cartilage source, there are disadvantages to their use. The amount of cartilage is limited early in life; intraoperative sculpting is required; the patient experiences discomfort and displays a chest scar; and the reconstructed ear feels quite firm.1-3

Tissue engineering, which combines biologic principles with fundamental engineering and polymer chemistry, enables the generation of new tissue replacements in animal models.4 Previous animal studies have demonstrated promising results using freshly harvested cells to generate a variety of cartilage structures.5-8 The future concept of tissue engineering for humans should require the harvest of relatively few cells from a small biopsy specimen. The cells would be allowed to multiply in vitro and would be added to a preshaped scaffold to generate new tissue. Although many human pediatric cells can be generated using current culture techniques,9 months of in vitro culture may be required to expand the cells to a sufficient number to create a full-sized auricle, which may result in aged cells with diminished properties and a tissue of suboptimal quality.10,11

In this study, we employed 2 of the most commonly used growth factors for chondrocyte culture, hypothesizing a rapid growth effect on the tissue-engineered car-

From the Department of Anesthesiology, Center for Tissue Engineering (Drs Arevalo-Silva, Cao, M. Vacanti, Weng, and C. A. Vacanti), and the Department of Pathology (Dr M. Vacanti), University of Massachusetts Medical Center, Worcester; the Department of Otolaryngology, Massachusetts Eye and Ear Infirmary, Boston (Drs Arevalo-Silva and Eavey); and the Department of Otology and Laryngology, Harvard Medical School, Boston (Drs Arevalo-Silva and Eavey).
SUBJECTS, MATERIALS, AND METHODS

CHONDROCYTE ISOLATION

Excess pediatric elastic cartilage from ear surgery was obtained from 11 children (age range, 5-15 years) by one of us (R.D.E.) at the Massachusetts Eye and Ear Infirmary, Boston, with informed consent. Perichondrium was removed under sterile conditions. The isolated cartilage was minced into small fragments; washed in phosphate-buffered saline solution containing 100-mg/L penicillin, 100-mg/L streptomycin, and 0.25-mg/L amphotericin B (Gibco, Grand Island, NY); and digested with 0.3% collagenase II (Worthington Biochemical Corp, Freehold, NJ) at 37°C for 8 to 12 hours. The digested cartilage suspension was filtered using a sterile 250-mm polypropylene mesh filter (Spectra/ Mesh 146-426; Spectrum Medical Industries Inc, Laguna Hills, Calif) and centrifuged at 6000 rpm. The resulting pellet of cells was washed twice with phosphate-buffered saline and then resuspended in Ham F-12 medium. The number of cells was calculated using a hemocytometer, and the viability of the cells was determined using trypan blue vital dye (Sigma-Aldrich, Irvine, Calif). Chondrocyte suspensions with cell viability in excess of 85% were used.

CHONDROCYTE IN VITRO CULTURE

The chondrocytes were plated onto 225-cm² cell culture flasks (Costar, Cambridge, Mass) at 2400 cells per square centimeter. Four groups of cells from each patient were plated at the same concentration. Group 1 was nourished with Ham F-12 and levoglutamine, 50-mg/L l-ascorbic acid, 100-mg/L penicillin, 100-mg/L streptomycin, 0.25-mg/L amphotericin B, and 10% fetal bovine serum (Sigma-Aldrich Corp, St Louis, Mo). Group 2 was nourished with the same Ham F-12 culture medium supplemented with 10-ng/mL b-FGF (R&D Systems Inc, Minneapolis, Minn). Group 3 was nourished with Ham F-12 culture medium supplemented with 1-ng/mL TGF-β (R&D Systems Inc). Group 4 was nourished with Ham F-12 culture medium and a combination of the 2 growth factors: 10-ng/mL b-FGF and 1-ng/mL TGF-β. The cells were maintained in vitro at 37°C and 5% carbon dioxide for 3 weeks. The culture medium was changed twice a week. The in vitro growth rate was estimated by photographic records of each group. After 3 weeks of in vitro culture, the cells were harvested using 0.25% trypsin/EDTA (Sigma-Aldrich Corp) and counted using a hemocytometer. Cell viability was determined using trypan blue vital dye.

CHONDROCYTE POLYMER SUSPENSION

After quantification, the cells were suspended in a 30% wt/vol solution of a copolymer gel of polyethylene glycol and polypropylene oxide (Pluronic F-127; BASF, Mount Olive, NJ) at 4°C and constituted in Ham F-12 medium at a cellular concentration of 6 x 10⁶ cells per milliliter. Aliquots containing 100 µL of the above-mentioned mixture were prepared.

SURGICAL IMPLANTATION

Following the animal facility guidelines of the University of Massachusetts Medical Center, Worcester, equal aliquots of the chondrocytes suspended in Pluronic F-127 were injected into the dorsal subdermal space of the athymic mice (nu/nu) (Taconic Inc, Boston), which were under general anesthesia.

HARVEST OF THE SPECIMENS

The specimens were harvested after 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 weeks of in vivo implantation. After anesthetic overdose, the constructs were carefully dissected from the surrounding soft tissue. The specimens were examined grossly, weighed, and measured.

HISTOLOGICAL EXAMINATION

Each specimen was fixed in 10% phosphate-buffered formalin (Fisher Scientific, Fair Lawn, NJ) for histological analysis. The specimens were embedded in paraffin and sectioned. Using standard histochemical techniques, slide sections were stained with hematoxylin-eosin, safranin O, Masson trichrome blue, and Verhoeff solution.

RESULTS

IN VITRO RESULTS

The chondrocytes grown in monolayer cultures in the 4 different media varied in number, size, and shape. The chondrocytes cultured with Ham F-12 culture medium (group 1) were polygonal at 1 week, with very few cells still unattached to the flask bottom. At the beginning of 2 weeks, cell multiplication became evident. The cells appeared to be of uniform size, polygonal, and distributed as islets of growth. At 3 weeks, a few cells became elon-
IN VIVO (XENOGRAFT) RESULTS

Specimens generated from the 4 experimental groups of cells were harvested after 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 weeks of in vivo culture. The gross appearance of the specimens was different among the groups. The largest specimens were from group 2, and the smallest were from group 3, with those from groups 1 and 4 being intermediate. The specimens were roughly spherical and ranged in size as follows: group 1, 3.5 to 5.5 mm; group 2, 8.7 to 9.5 mm; group 3, 3.0 to 4.0 mm; and group 4, 3.5 to 4.5 mm. The average weight of the specimens was as follows: group 1, 28 mg (range, 24-36 mg); group 2, 99 mg (range, 97-105 mg); group 3, 16 mg (range, 14-18 mg); and group 4, 40 mg (range, 35-42 mg). Histological evaluation demonstrated the differences among the 4 groups.

Group 1 generated lobules of cartilage with a basophilic matrix within evenly spaced lacunae. The lacunae varied from round to oval, with slight pleomorphism. The results of safranin O staining were strongly positive, correlating with proteoglycans production. Trichrome blue staining showed a well-defined perichondrium (Figure 2A). Verhoeff staining revealed elastic fibers that were homogeneously distributed.

The specimens in group 2 demonstrated a variable matrix with heterochromacia indicative of maturity. The cartilage was very cellular with round to oval lacunae containing single chondrocytes with single round to oval nuclei, with mild variation. The specimens were strongly positive for proteoglycans. The perichondrium was thin and well defined (Figure 2B). The elastic fibers stained evenly positive (Figure 3).

The specimens from group 3 had a small ratio of round to oval lacunae, containing single chondrocytes. There was a predominant presence of fibrous tissue surrounding the cartilage. Moreover, trichrome blue staining revealed fibrous tissue in the engineered cartilage and a thick perichondrium. The proteoglycans production was strong, as evidenced by safranin O staining. The overall quality of cartilage was poor, containing fibrous tissue and fibrocartilage (Figure 2C). There was moderately positive staining of the elastic fibers.

In group 4, the specimens stained basophilic, with areas of mild heterochromacia. The tissue was very cellular, with some grouping of cells. There was moderate variation in the size and shape of the lacunae, which occasionally contained bichondrocytes. There was also mild to moderate variation in the size and shape of the nuclei. Staining results for proteoglycans production were strongly positive. The perichondrium was well defined, with rare ingress of fibrous tissue into the cartilage (Figure 2D). There was moderate to strong staining of the elastic fibers.

Safranin O staining of the younger specimens (<6 weeks) demonstrated a hyperchromic and homogeneous matrix with abundant rounded cells and scarce evidence of multiple layers. The average total number of cells was $3.5 \times 10^7$ (Figure 1). Cell viability was more than 98%; the average cell doublings was about 5 times the original number.

![Figure 1](image1.png)

*Figure 1.* Average number of chondrocytes after 3 weeks of in vitro culture. b-FGF indicates basic fibroblast growth factor; TGF-β, transforming growth factor β.
staining for the production of proteoglycans. The older specimens (>6 weeks) demonstrated a hypochromic and heterogeneous matrix and proportionately fewer cells of shapes from round to oval, with a strong detection of proteoglycans as a bright-red stain in all the specimens.

**COMMENT**

Tissue engineering has evolved as a science that may reduce the problem of scarcity for tissue reconstruction and organ replacement. Although advances in this new field have been encouraging, the clinical applications may not be realized for several years. One major obstacle has been the generation of a sufficient number of healthy young cells in a short period of time.

This report compares the effect of 2 growth factors used in the culture of pediatric auricular chondrocytes. The in vitro findings demonstrate that b-FGF alone results in the generation of more tissue, and tissue of a higher quality, than other combinations of growth factors. For example, the number of in vitro cell divisions in conventional medium alone or in supplemental TGF-β was significantly lower than the number in b-FGF alone or in the 2 growth factors combined. Furthermore, the in vivo specimens cultured with b-FGF were notably the largest and heaviest. Most importantly, histological evaluation of specimens nourished with b-FGF demonstrated cartilage that was superior to cartilage from specimens that were nourished with TGF-β alone; specimens from the TGF-β group demonstrated a consistent infiltration of fibrous tissue into the elastic cartilage.

The specimens formed from cells nourished only with Ham F-12 medium were histologically of very good

**Figure 2.** A, Group 1 (control group). The specimen has a well-defined perichondrial layer and no fibrous ingrowth. B, Group 2. The perichondrial layer and the appearance of the cartilage are similar to those of the group 1 specimen. C, Group 3. The specimen is the smallest and shows fibrous ingrowth. D, Group 4. There is visible fibrous tissue ingrowth. All specimens were cultured for 10 weeks in vivo (Masson trichrome, original magnification ×6). Arrows indicate the perichondrium [A through D]; arrowheads, fibrous tissue [C and D].

**Figure 3.** All specimens were positive for elastic fibers, which stain black, similar to native cartilage. This group 2 specimen was cultured in vivo for 10 weeks (Verhoeff, original magnification ×40).
quality; however, the in vivo cell number was significantly less than that of specimens from the b-FGF group, suggesting a persistent effect of this growth factor. Also, even though the absolute number of in vitro cells obtained with the combination of growth factors compared with b-FGF alone was not substantially different, the amount of in vivo tissue obtained from the combination group was significantly smaller and of poorer quality, with moderate fibrous tissue extending into the cartilage. Transforming growth factor β can inhibit cell proliferation, which could be the reason for suboptimal neocartilage development.

It was previously demonstrated that a large volume of bovine chondrocytes could be induced to consistently create an auricular construct in a nude mouse model.3 However, for human clinical application, several challenges remained. For example, human pediatric elastic cartilage had to demonstrate properties necessary for tissue engineering efforts.3 A corollary then arose as to whether a limited number of chondrocytes from a small piece of human cartilage might be expanded to create a full-sized auricle. The results of this study demonstrate that in vitro chondrocyte enhancement by a growth factor produces a large volume of healthy-appearing cartilage in a reasonable period of time. Challenges remain,3 but the concept of auricular tissue engineering appears to be closer to reality.

**CONCLUSIONS**

The results demonstrated that b-FGF enhanced the development of engineered human pediatric elastic cartilage in a xenograft model. This growth factor generated a significant amount of engineered cartilage of high quality from a small number of pediatric auricular cells in an acceptable period of time.

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