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Conditions for seeding and promoting neo-auricular cartilage formation in a fibrous collagen scaffold

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ABSTRACT

Background: Carved autologous costal cartilage and porous polyethylene implants (Medpor) are the most common approaches for total ear reconstruction, but these approaches may have inconsistent cosmetic outcomes, a high risk of extrusion, or other surgical complications. Engineering ear cartilage to emulate native auricular tissue is an appealing approach, but often the cell-seeded scaffolds are susceptible to shrinkage and architectural changes when placed *in vivo*. The aim of this study was to assess the most favorable conditions for *in vitro* pre-culture of cell-seeded type I collagen scaffolds prior to *in vivo* implantation.

Methods: Sheep auricular chondrocytes were seeded into this type I collagen scaffold. The cell-seeded constructs were cultured in either static or dynamic conditions for two days or two weeks and then implanted into nude mice for another six weeks. The harvested constructs were evaluated histologically, immunohistochemically, and biochemically.

Results: Robust neo-cartilage formation was found in these collagen scaffolds seeded with auricular chondrocytes, which was comparable to native cartilage morphologically, histologically, and biochemically. Culture under dynamic conditions prior to implantation improved the neo-cartilage formation histologically and biochemically.

Conclusion: Dynamic culture of this cell-seeded fibrous collagen material could permit predictable engineered auricular cartilage and a promising approach for external ear reconstruction.

1. Introduction

Reconstruction of the external ear due to congenital malformation or following traumatic injury is a challenging clinical problem in the field of plastic and reconstructive surgery. Since Gillies first carried out auricular reconstruction using autologous costal cartilage nearly a century ago, this approach has become the predominant surgical technique for partial and total reconstruction of the external ear (Brent, 1992). Although reconstruction with carved autologous tissue often has favorable results, the cosmetic result is highly dependent on the sculpting abilities of the surgeon and implantation techniques. This technique has several major drawbacks including significant morbidity to the donor rib site (Ohara et al., 1997), the potential for operative complications (Lindig et al., 1997; Walton et al., 2002), and these costal cartilage grafts are very stiff and inflexible. Costal cartilage grafts also are subject to absorption (Berghaus and Toplak, 1986) and progressive calcification (Mori et al., 2002) over time.

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[1] Potential conflict of interest: Christopher M. Bowley is an employee of Kensey Nash Corporation that supplied the collagen scaffold for this study.

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An alternative strategy has been the implantation of alloplastic materials to simulate the human ear. Numerous alloplastic scaffold materials have been tried as framework substitutes for ear reconstruction, most of which have failed (Watson et al., 1995; Kunda et al., 2006). Currently, porous polyethylene (Medpor®, Porex Surgical, Newnan, GA) is being used with clinical success to reconstruct and augment many areas of the craniofacial skeleton. Similar to carved costal cartilage grafts, however, porous polyethylene is very stiff and inflexible, and blunt force trauma to a Medpor auricular implant can cause soft tissue breakdown leading to skin perforation, and possible infection resulting in implant removal (Sevin et al., 2000). Placing the Medpor implant under a temporoparietal fascial flap (Romo et al., 2006) has eliminated some complications. However, reconstruction of the external auricle with an entirely synthetic rigid framework is not an optimal solution for all conditions.

Engineered auricular cartilage may provide a solution for auricular reconstruction that behaves more like native ear cartilage (Langer and Vacanti, 1993). For more than two decades, numerous groups have demonstrated that cartilage-like tissue can be generated *in vitro* and *in vivo* using chondrocytes or mesenchymal stem cells and a variety of biodegradable scaffold materials (Xu et al., 2004; Xu et al., 2005; Wang et al., 2005; Ibusuki et al., 2009; Fan et al., 2010; Wang et al., 2011; Tay et al., 2012). In 1997, Cao et al. (Cao et al., 1997) reported a human ear-shaped tissue engineered construct using bovine articular chondrocytes and a polyglycolic acid-polylactic acid scaffold. Several reports in the ensuing years have sought to improve engineered auricular tissue, but progress stalled because scaffolds could not maintain a satisfactory ear shape over longer time periods, even in immunodeficient mice models.

Although numerous scaffolds support neocartilage formation in small animal models, no scaffold material has been developed that permits satisfactory cartilage generation under native inflammatory conditions in the subcutaneous environment of an immunocompetent large animal. Biodegradable scaffold materials including fibrin glue, alginate, and porous polymer scaffolds, when combined with chondrocytes have been useful in generating new cartilage matrix in small animals (Tsai et al., 2011; Lee et al., 2011; Lu et al., 2011; Wang. et al., 2012). Scaffolds have been manufactured from a combination of materials such as polyglycolic acid (PGA), poly-L-lactic acid (PLLA), and polycaprolactone (PCL) to make human ear-shaped constructs (Kim et al., 1994; Vacanti et al., 1992; Cao et al., 1997; Shieh et al., 2004; Kusuhara et al., 2009; Isogai et al., 2004, 2005; Haisch et al., 2002; Liu et al., 2010), and the mechanical and degradation requirements of synthetic materials can be modified and the polymers can be combined in various ratios to meet specific biological and biomechanical properties. For example, ear-shaped constructs containing polymers with slower degradation rates, such as PCL, were better preserved at the end of the studies because the shape of the auricle was maintained mainly by the persisting scaffold material (Shieh et al., 2004; Kusuhara et al., 2009; Isogai et al., 2005; Isogai et al., 2004, 2005). However, the degradation products of the synthetic materials often cause chronic inflammation that can negatively affect neocartilage formation (Fujihara et al., 2010; Hsu et al., 2006; Lotz et al., 2009; Rotter, et al., 2005). Furthermore, many of these scaffold materials degrade rapidly before adequate extracellular matrix has formed so that the construct is unable to withstand wound contraction forces imposed by the overlying skin, especially in large animal models.

Natural biological materials are promising candidate scaffolds for cartilage engineering. Collagen, a major component of extracellular matrix (ECM), is natural protein that is abundant, biocompatible, and may eliminate the negative aspects of the degrading synthetic polymers on the neocartilage (Parenteau-Bareil et al., 2010; Glowacki et al., 2008). Although an immune response can be mounted to collagen-based products, advances in collagen purification and processing have rendered them relatively innocuous biologically (Lynn et al., 2004). Scaffolds made of collagen originating from diverse animal tissues are commercially available and have been actively used in research and clinical applications. For example, numerous biocompatible commercial products made from collagen have been used clinically for meniscus regeneration and osteochondral defect repair (Steadman and Rodkey, 2005; Gigante et al., 2011). An initial report from our laboratory has shown that a wire-reinforced scaffold using collagen was capable of permitting cartilage formation, and the wire aided in maintaining the original dimensions of an ear-shaped construct in nude mice (Zhou et al., 2011). However, chondrocyte-seeded constructs without auxiliary support often shrink and change form and dimension upon immediate *in vivo* implantation (Silverman et al., 1999; Xu et al., 2004). These dimensional changes could be disadvantageous for reconstructing an external auricle where size and shape are critical outcomes. One possible solution is to culture the cell-seeded constructs to initiate extracellular matrix formation prior to implantation with the goal of being able to predict the size and architecture of the final tissue engineered product. As part of a step-wise approach to engineer auricular cartilage, the optimal conditions for seeding cells and *in vitro* culture prior to *in vivo* implantation using a commercially available collagen scaffold currently in clinical use have not been determined. The source of animal cells chosen for this early preclinical study was sheep, which is a suitable model for obtaining autologous cells for implantation in future large animal studies. The aim of this study was to assess the most favorable conditions for *in vitro* pre-culture of cell-seeded type I collagen scaffolds prior to *in vivo* implantation.

2. Materials and methods

2.1. Scaffolds

The collagen scaffolds used in this study were manufactured from purified collagen extracts from bovine dermis, which is a source of primarily type I collagen (provided by Kensey Nash Corporation, Exton, PA, USA) with pore sizes ranging from 1.39 to 139.09 μm (16.34 ± 16.69 μm). Eighty discs measuring 5 mm diameter and 2 mm thick were punched from a sheet of fibrous collagen (Fig. 1). All scaffolds were sterilized with cold ethylene oxide gas prior to use.

2.2. Chondrocyte isolation and expansion

All actions were approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital. Ear cartilage was harvested from four, 3 to 6-month-old sheep donors. Under sterile conditions, the skin and perichondrium were carefully dissected and removed. One full-thickness piece of cartilage measuring 1 cm × 1 cm was collected from each sample and set aside for controls. The remainder of the cartilage was minced into 1 mm^3 fragments and digested in 0.1% type II collagenase (Worthington Biochemical, Lakewood, NJ, USA) for 16–18 h at 37 °C. After digestion, the isolated chondrocytes were washed twice with PBS with antibiotics (Xu et al., 2004). Cell number and viability were assessed using the trypan blue dye exclusion test. Cells were plated in roller bottles (Corning, Inc., Corning, NY, USA) at an initial density of 6×10^3 cells/cm^2, and cultured in Ham's F-12 medium with L-glutamine supplemented with 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO), 50 μg/ml ascorbic acid, 100 U/ml penicillin, and 0.1 mM non-essential amino acids (chondrocyte