Development of Large Engineered Elastic Cartilage Constructs

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Introduction:
Total external ear reconstruction with autologous cartilage still remains one of the most difficult problems in the fields of plastic and reconstructive surgery. As the absence of tissue vascularization limits the ability to stimulate self-repair or growth, numerous strategies have emerged to repair or reconstruct the auricle (or pinna) as a result of traumatic loss or congenital absence (e.g. microtia). While conventional surgical reconstruction can result in good clinical outcomes, this approach is limited by the amount of donor tissue that can be harvested. Alternatively, tissue engineering can offer the potential to grow autogenous cartilage suitable for implantation. While tissue engineered auricle cartilage constructs can be created, a substantial number of cells are required to generate sufficient quantities of tissue for reconstruction. Similarly, as routine cell expansion can elicit negative effects on chondrocyte function, we have developed an approach to generate large-sized, scaffold-free engineered auricle constructs (≥ 3 cm^2) directly from a small population of donor cells (6,500 or 13,000 isolated cells/cm^2).

Materials and Methods:
Sample Preparation: *In vitro* cartilaginous tissue was formed by isolated chondrocytes harvested from ear of young female New Zealand white rabbits (2 ± 0.5 kg; ~ 12 weeks old) (Charles River Laboratories) by enzymatic digestion. The cells were seeded in the wells of a continuous flow bioreactor at either 6,500 or 13,000 isolated cells/cm^2 and incubated at 37°C and 5% CO_2 in Ham's F-12 supplemented with 20 mM HEPES, 20% FBS and 100 μg/mL ascorbic acid in the presence or absence of 14 mM of sodium bicarbonate ($NaHCO_3$) to stimulate the synthesis of cartilaginous extracellular matrix. Constructs were maintained a bioreactor culture for 4 weeks. *Histological Evaluation:* Engineered constructs and native auricular cartilage tissue samples were fixed in 4% paraformaldehyde, paraffin-embedded, and sections (5 μm) stained with either Hematoxylin & Eosin (a general connective tissue stain), safranin-O (proteoglycan stain) or Weigert's resorcin-fuchsin (elastin/elastic fiber stain). Immunohistochemistry was also performed for the detection of collagen I and II. *Transmission Electron Microscopy (TEM):* Engineered constructs and native auricular cartilage tissue samples were fixed in Karnovisk solution, post-fixed in 1% osmium tetroxide, epoxy-embedded, and ultrathin sections (70 nm) stained were with uranyl acetate and lead citrate**.** *Biochemical Analyses*: Tissues were papain digested and aliquots of the digest were assayed for DNA (Hoechst 33258 assay), sulphated glycosaminoglycan (dimethylmethylene blue assay) and hydroxyproline contents (chloramine-T/Ehlrich's assay). *Statistical Analyses:* Comparisons between individual groups was conducted using a two-way ANOVA and Tukey's post-hoc testing. Significance was assigned with $p<0.05$.

Results:
Each of the bioreactor preparations resulted in the formation of large tissue constructs (~ 3 cm^2) (Fig. 1A). Histological evaluation of the engineered constructs revealed that the generated neotissue possessed a similar morphology to native auricular cartilage. The ECM stained positive for sulphated proteoglycans as well as displaying the presence of thin elastin fibres. Interestingly, this neocartilage also displayed an outer region that was similar in structure to the perichondrium of native elastic cartilage. This perichondrium-like region was predominantly fibrous and displayed little positive

proteoglycan staining (Fig. 1B-C). Immunohistochemical assessment of the engineered elastic cartilage constructs supported this finding. Collagen type I was almost exclusively located in the perichondium-like region. Interestingly, collagen type I was not detected in the any of the constructs grown in the absence of $NaHCO_3$. The distribution of collagen type II was more homogenous with staining throughout the construct. Tissue ultrastructure was examined by TEM. Chondrocytes in the central region of native elastic cartilage displayed single, large cytoplasmic lipid droplets surrounded by cytofilaments arranged in parallel. These elements were more readily observed in cells that were less elongated (typical chondrocyte morphology). The elastic fibers in the ECM were very thin and slightly electron dense. Similar morphologies were observed in the engineered tissues after 4 weeks of bioreactor cultivation with the cells possessing the same key elements of elastic tissue (Fig. 1D-E). ECM accumulation and cellularity were significantly affected by both $NaHCO_3$ and seeding density. The presence of $NaHCO_3$ increased cellularity, whereas both seeding density and $NaHCO_3$ resulted in increased accumulation of ECM macromolecules (Table 1).

Discussion:

These findings confirmed that the generated neocartilage was elastic in nature and that the cultured chondrocytes displayed normal differentiation after 4 weeks of culture. Constructs grown in the presence of $NaHCO_3$ with higher seeding densities displayed the greatest similarities to native elastic cartilage in terms of structure and ECM accumulation. For successful implantation, it is critical to generate engineered constructs that resemble the structure and function of native tissue. Although it may not be possible to precisely replicate native elastic cartilage *in vitro*, an important requirement for tissue-engineered cartilage is that the neotissue is phenotypically stable. These preliminary studies are promising lay the foundation for future work to improve the accumulation of specific ECM constituents and determining clinical effectiveness in an animal model.

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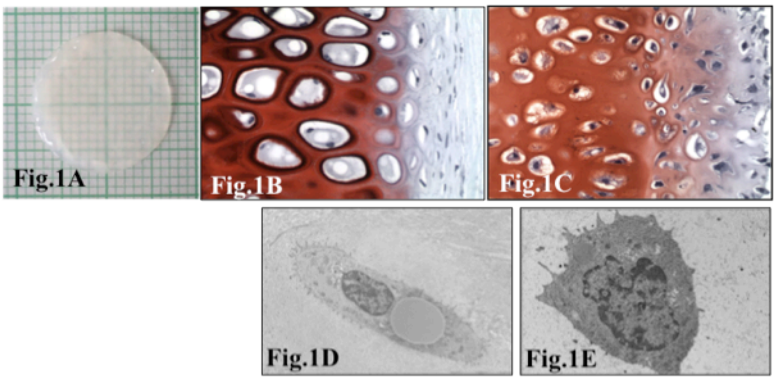

Fig. 1 – Macroscopic appearance of the tissue generated from the monolayer cell preparations after 4 weeks of bioreactor culture (Fig1A). General architectural organization of the tissue generated from the monolayer cell preparations after 4 weeks in bioreactor culture, 13,000 cells/cm^2 with $NaHCO_3$ (Fig. 1C) compared to native auricular cartilage (Fig. 1B)-Safranin-O stain. Transmission electron micrographs of cells located in the middle of the engineered elastic cartilaginous constructs and native auricular cartilage (Fig.1D- Native cartilage; 1E-Bioreactor 13,000 cells/cm^2 with $NaHCO_3$. 40X.

Table 1 – Biochemical properties of engineered elastic cartilaginous tissue. Data presented as mean ±SEM (n=6-8 samples/group).

	Seeding Density and $NaHCO_3$ Concentration			
	6,500 [cells/cm^2]		13,000 [cells/cm^2]	
	0 [mM]	14 [mM]	0 [mM]	14 [mM]
DNA [µg/mg dry wt.]	1.00 ± 0.04 ^{b}	1.1 ± 0.1	1.13 ± 0.06 ^{a,b}	1.0 ± 0.1 ^{a}
PG [µg/mg dry wt.]	1.00 ± 0.05	1.5 ± 0.5	1.3 ± 0.1 ^{a}	2.2 ± 0.9 ^{a}
Collagen [µg/mg dry wt.]	1.0 ± 0.1	1.2 ± 0.2	1.2 ± 0.2	1.4 ± 0.1
PG/DNA [µg/µg]	1.0 ± 0.1	1.2 ± 0.3 ^{b}	1.0 ± 0.1 ^{a}	2.1 ± 0.8 ^{a,b}
Collagen/DNA [µg/µg]	1.0 ± 0.1	1.3 ± 0.2 ^{b}	0.89 ± 0.09 ^{a}	1.6 ± 0.2 ^{a,b}
Collagen/PG [µg/µg]	1.0 ± 0.2 ^{a}	1.6 ± 0.6 ^{a,b}	0.76 ± 0.07	0.9 ± 0.3 ^{b}
DNA Content (mg/mg)	1.0 ± 0.05	1.72 ±0.1	1.16 ± 0.06	1.99 ± 0.3
PG Content (mg/mg)	1.0 ± 0.08	1.82 ± 0.2	1.3 ± 0.1	2.34 ±0.4
OH Content (mg/mg)	1.0 ± 0.1	2.28 ± 0.6	1.13 ± 0.1	3.2 ± 0.6

PG: Proteoglycans