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Biochemical Properties of Tissue-Engineered Cartilage

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Abstract

Objective—Microtia is treated with rib cartilage sculpting and staged procedures; though aesthetically pleasing, these constructs lack native ear flexibility. Tissue-engineered (TE) elastic cartilage may bridge this gap; however, TE cartilage implants lead to hypertrophic changes with calcification and loss of flexibility. Retaining flexibility in TE cartilage must focus on increased elastin, maintained collagen II, decreased collagen X, with prevention of calcification. This study compares biochemical properties of human cartilage to TE cartilage from umbilical cord mesenchymal stem cells (UCMSCs). Our goal is to establish a baseline for clinically useful TE cartilage.

Methods—Discarded cartilage from conchal bowl, microtic ears, pre-auricular tags, rib, and TE cartilage were evaluated for collagen I, II, X, calcium, glycosaminoglycans, elastin, fibrillin I and III. Human UCMSCs were chondroinduced on 2-D surfaces and 3-D D, L-lactide-co-glycolic acid (PLGA) fibers.

Results—Cartilage samples demonstrated similar staining for collagens I, II, X, elastin, fibrillin I, and III, but differed from rib. TE pellets and PLGA-supported cartilage were similar to auricular samples in elastin and fibrillin I staining. TE samples exclusively stained for fibrillin III. Only microtic samples demonstrated calcium staining.

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Conclusions—TE cartilage expressed similar levels of elastin, fibrillin I, collagens I and X when compared to native cartilage. Microtic cartilage demonstrated elevated calcium, suggesting this abnormal tissue may not be a viable cell source for TE cartilage. TE cartilage appears to recapitulate the embryonic development of fibrillin III, which is not expressed in adult tissue, possibly providing a strategy to control TE elastic cartilage phenotype.

Keywords

Mesenchymal Stem Cells; Chondrogenesis; Microtia; Nanofibers; Tissue Engineering; Fibrillin; Elastin

Introduction

Microtia is a deformity of the external auricle, which presents in .843 to 4.34 cases in 10,000 live births [1]. Because microtia is a noticeable physical deformity, it can have a detrimental impact on a child's psychosocial well-being and development. Children with microtia become self-aware of the malformation at the ages 5 to 6 and have been shown to be at a higher risk for interpersonal difficulties, depression, and aggression/hostility[2]. Treatments for microtia utilize endogenous costal cartilage grafts or synthetic implants. In costal cartilage implantation, techniques typically performed are ones popularized by Brent, Nagata, and Firmin[3]. These surgical techniques involve rib cartilage harvest and sculpting, with implantation, followed by staged surgeries to create the semblance of an external ear [4]. Risks associated with harvest include pneumothorax, chest wall retrusion, and postoperative thoracic scoliosis; risks associated with the implant site include infection, extrusion, and loss of the graft[5]. While the reconstructed ear may be aesthetically pleasing, [6] costal cartilage (a hyaline cartilage) does not have the same deformability as native auricular tissue. Alternatives to autologous cartilage include synthetic implants, such as Medpor®, but have higher risks of infection and extrusion, while still lacking the deformability of native auricular tissue [7]. Solutions to these limitations noted in both autologous and synthetic ear reconstruction may be found in tissue-engineered (TE) cartilage. The potential advantages of TE cartilage constructs include an unlimited supply of engineered cartilage, fewer surgeries, and the avoidance of attendant complications. However, to date, the major limitation to TE cartilage is that it tends to calcify, and become inflexible in a predictable fashion after implantation [8]. From a clinical standpoint, TE elastic cartilage must maintain its elastic phenotype, have characteristics that allow ear flexibility, and yet, must be rigid enough to withstand the deforming forces of the healing soft tissue envelope.

In previously published work, our laboratory has utilized human umbilical cord mesenchymal stem cells (hUCMSCs) as a cell source for TE cartilage [9]. Nanofiber-supported hUCMSC chondrogenesis promoted increased glycosaminoglycans (GAG) and improved collagen II to I ratio (differentiation index) compared to standard pellet formation, indicating an elastic cartilage phenotype [10]. However, we also noted increased expression of collagen X, and decreased expression of elastin mRNA, both of which suggest the development of a hypertrophic cartilage phenotype. Because hypertrophic cartilage tends to be less flexible, and may indicate a tendency to calcify after implantation, we wanted to further evaluate our tissue-engineered cartilage in comparisons to normal auricular cartilage (conchal bowl), pre-auricular cartilage remnants, microtia samples, and hyaline cartilage from rib, which is the current gold standard for cartilage source during external ear reconstruction.

In order to create and maintain flexible TE elastic cartilage beyond our current capabilities, we need to maintain control over elastic fiber deposition, fibrillin production, eliminate calcium deposition, and diminish collagen X production. Elastic fibers are formed when

tropoelastin binds to fibrillin I in the ECM and becomes cross-linked[11]. As such, maintaining fibrillin I content in tissue engineered cartilage provides the appropriate template for elastin deposition and cross-linking, thereby maintaining tissue engineered cartilage flexibility. In addition to fibrillin I, two other types of fibrillin have been identified. Fibrillin II is expressed with fibrillin I prenatally, and is only minutely expressed in mature tissues [12]. Fibrillin III is expressed prenatally and no longer present following birth[13]. To our knowledge fibrillin expression in TE cartilage has not been characterized and may eventually provide insight into maintaining elastic cartilage phenotype following implantation.

Elastic cartilage flexibility also decreases as calcium deposition in the matrix increases[14]. Calcium cross-links to binding domains in fibrillin, making the fibrillin template inflexible; therefore, minimizing calcium matrix deposition is another strategy to maintain cartilage flexibility. A last set of strategies to prevent phenotype change from elastic cartilage to hypertrophic cartilage is to control the presence of certain collagens, namely, to maintain a differentiation index (collagen II/I ratio) above 1 (with greater collagen II); a ratio below 1 is an indication of fibrocartilage development, and to control the collagen X content of the cartilage. Increased collagen X is an initial indication that hypertrophic cartilage is developing.

Lastly, MSC source may play a role in maintaining an elastic cartilage phenotype. To date, abdominal adipocytes have proven to be the most reliable source for adult tissue-engineering[15]. In infants and children, minimal sources of fat exist for harvesting, making this a less than ideal cell source. Other discarded tissue sources–including cartilage itself--may be useful to generate TE elastic cartilage, including pre-auricular cartilage-skin remnants, microtic cartilage harvested at the time of staged ear reconstruction, and rib cartilage. In order to determine whether a cell source is a legitimate alternative for TE cartilage, the biochemical properties of these sources must be determined.

The goal of this study is to accurately define the expression of various extracellular matrix (ECM) components in auricular cartilage, including collagen I, II, X, calcium; glycosaminoglycans (GAGS), elastin, fibrillin I and II. We wish to compare our tissue engineered pellets and nanofiber--supported TE cartilage to these tissues to determine a baseline of understanding how to create and maintain elastic cartilage phenotype in TE cartilage.

Materials/Methods

Discarded human cartilage samples were collected with IRB approval (IRB # 10-1580 and 10-1299) and included conchal bowl, microtia, pre-auricular skin and cartilage remnants, and rib cartilage. Samples were fixed in 10% formalin solution up to 48 hours and stored in 70% ethanol before biochemical characterization.

Tissue engineered cartilage was generated from hUCMSCs harvested using an explant technique. Passage 2 cells were chondroinduced as previously described [9]. Briefly, 4×10^5 isolated hUCMSCs were placed in 0.3 mL of chondrogenic media in a 2 mL conical tube and grown for 21 days, after which the pellet was fixed in 10% formalin and frozen. PLGA nanofibers were electrospun as mats as previously described[16]. 4×10^5 hUCMSCs were seeded on the nanomats and subjected to chondrogenesis for 21 days, after which, scaffolds were fixed in 10% formalin solution up to 48 hours and stored in 70% ethanol before biochemical characterization.

Antibodies and Immunohistochemistry

Mouse monoclonal anti-Collagen I, II and X antibodies were purchased from Abcam (Cambridge, MA). Rabbit polyclonal anti-Fibrillin I was obtained from Sigma-Aldrich (St. Louis, MO); goat polyclonal anti-Fibrillin III was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Chondrocyte cultures and human samples were fixed in neutral-buffered formalin for 24 hours and stored in 70% ethanol for staining. Samples were paraffin embedded; 4 μm sections were cut with a microtome. IHC was performed in a Bond Autostainer (Leica Microsystems Inc. Norwell, MA). Slides were de-waxed in Bond Dewax solution (AR9222) and hydrated in Bond Wash solution (AR9590). Antigen retrieval for collagen I and X and fibrillin I antibodies was performed for 15 min at 37° C in Pepsin (Invitrogen; Camarillo, CA). Collagen II antigen retrieval was performed using the Bond Enzyme Pretreatment kit (AR9551); no antigen retrieval was necessary for fibrillin III. Detection of the collagen I, II, X and fibrillin I antibodies was performed using the Bond Polymer Refine Detection System (DS9800). For fibrillin III the secondary antibody and polymer from the kit were replaced by Goat IgG and the HRP polymer from Biocare (Concord, CA). Stained slides were dehydrated and prepared with a coverslip. Positive and negative controls were included for each antibody.

Histological Stains

Elastin was detected using the Verhoeff Elastic Stain kit (KTVEL); calcium was detected with the Alizarin Red Stain kit (STARE; American MasterTech; Lodi, CA).

Digital imaging and image analysis

Stained slides were digitally imaged at 20× magnification using the Aperio ScanScope XT (Aperio Technologies, Vista, CA). Digital images were stored and analyzed within the Aperio Spectrum Database. Expression levels of biomarkers were measured using the Aperio Color Deconvolution V9 algorithm with minor modifications. Optical densities and intensity thresholds were modified based on levels in positive and negative controls. The percent area with strong (+3), medium (+2) and weak (+1) positive signals, as well as total positive signal, were used to compare biomarker levels.

Results

Cartilage from conchal bowl, preauricular remnants, microtic, and TE samples (pellets grown on 2-D surfaces, and PLGA-supported cartilage) stained in similar distributions for collagen I and X; however, costal cartilage stained less intensely for both (Figure 1). Conchal bowl and preauricular cartilage remnants stained similarly for collagen II; microtic cartilage stained less positively; both costal and TE cartilage stained lightly for collagen II. Alizarin red stain was negative in all samples, with the exception of microtia samples, where calcium was noted in the extracellular matrix. All of the human cartilage samples demonstrated similar percentage of cells positive for collagen II (Table 1); the TE pellet (45%) and PLGA cartilage (63%) stained less intensely for collagen II. Collagen X was expressed similarly in native tissues (>95%) but were below 91% in both TE pellets and PLGA-supported cartilage.

All native tissues demonstrated similar extracellular glycosaminoglycan by alcian blue staining (Figure 2); the pellet and PLGA samples stained less intensely, but were uniformly positive. Native auricular cartilage (conchal bowl, preauricular tags, microtia sample) stained similarly for elastin, with less intense staining noted in TE cartilage and none in costal cartilage. Fibrillin I stains were similar in all stained tissue samples, with the

exception of costal cartilage, where staining was lighter. All native cartilage types stained negative for fibrillin III; however, both TE cartilage samples were positive for this immature fibrillin.

Elastin staining in cartilage from the conchal bowl, microtia, and preauricular cartilage were similar to the TE pellet, but higher than the PLGA-supported cartilage and rib cartilage (Table 2). Fibrillin I stain quantification was similar in the native auricular samples (> 96% positive), lower in the TE cartilage samples (90%) and lowest in rib cartilage (41%). Fibrillin III stain was minimally present in conchal bowl, preauricular, and microtic cartilage, and absent in rib cartilage. Both the TE pellets (55%) and PLGA-supported cartilage (92%) demonstrated extensive fibrillin III staining.

Discussion

TE elastic cartilage is a potential solution for current limitations in microtia reconstruction. This cartilage will overcome the rigidity and lack of deformability of the reconstructed ear seen in both costal cartilage and synthetic implant strategies, eliminate the surgical risks associated with rib harvest, and result in a non-immunogenic construct. However, before clinical utility can be established, there must be demonstration that this cartilage is phenotypically similar to native auricular cartilage, and will maintain an elastic cartilage phenotype when implanted. Because conchal cartilage is the true normal in our set of comparisons, initial comparisons need to be established between TE and conchal cartilage. Both of the TE cartilage samples (2-D and PLGA supported) expressed similar levels of collagen I, X, and fibrillin I compared to conchal bowl cartilage. However, some notable differences also existed, including lower levels of elastin and collagen II in the PLGA nanofiber-supported cartilage. Both of these findings will eventually need to be remedied in our TE cartilage prior to implantation.

An interesting finding in TE cartilage is the presence of fibrillin III (to the best of our knowledge, ours is the first report of this immature fibrillin in TE cartilage). Lower levels of elastin in the TE PLGA-supported cartilage may in part be explained by the immaturity of TE cartilage, demonstrated by the presence of fibrillin III. The TE cartilage pellet exhibited 55% fibrillin III positivity, while PLGA-supported TE cartilage exhibited higher positivity (92%), indicating that the TE cartilage is more primitive, and may have similarities to pre-natal cartilage. In the human embryo, fibrillin III is thought to be instrumental in the initial formation of microfibril elastic cores; fibrillin III then disappears during maturation of the embryo, and fibrillin I becomes the primary extracellular matrix protein for elastic microfibrils[17]. In order for this fibrillin III to convert to fibrillin I, TE cartilage may need to be housed in vitro for longer time periods, or may need to be reassessed following implantation. Further studies are required to determine if fibrillin III expression in TE cartilage changes over time. While 40 weeks (analogous to human gestation) is probably not needed for maturation of *in vitro*-generated TE cartilage, time periods greater than the current 21 days will likely be required[13].

Further indication of the immaturity of the TE cartilage can be seen in the relative distribution of collagen I and II in both the nanofiber construct and pellet. In mature cartilage, collagen I is normally expressed by chondrocytes in a thin layer covering the surface, whereas collagen II is expressed more interiorly. However, seen in the staining of the both the nanofiber-supported and pellet TE cartilage, collagen I seems to not only be expressed by chondrocytes in the periphery, but also throughout the entirety of the construct. The collagen II-producing chondrocytes are found in a central location, as would be expected in native cartilage. The wide distribution of collagen I is a possible indication of TE cartilage immaturity. This hypothesis is further strengthened because the staining pattern

of the PLGA and pellet constructs seems to more closely resemble the preauricular cartilage remnants (a more immature cartilage) than the conchal bowl staining.

A second goal of this work was to assess potential cell sources for the purpose of engineering elastic cartilage. The most readily available sources, in descending order, are preauricular cartilage remnants (generally excised and discarded, and often done at an early age), microtic cartilage (easily accessible during surgery for microtia repair and discarded), rib cartilage (potentially discarded after multiple procedures, including microtia repair), and normal ears (rarely discarded). Histological staining of these native cartilage sources reinforces the known differences between costal (hyaline cartilage) and auricular cartilage (elastic cartilage)[18]. Elastic cartilage [19] is populated with larger chondrocytes, higher expression of ECM components, specifically elastin and collagen II, and faster proliferation *in vitro* [20]. The use of rib cartilage as a stem cell source for auricular chondrocytes would be expected to lead to insufficient elastic generation, and secondarily to an incorrect collagen II to I ratio. This is supported by work with bovine chondrocytes harvested from several anatomical locations, including costal cartilage. These cells were seeded on poly (l-lactide-ε-caprolactone) scaffolds, and implanted for 40 weeks in nude mice. At study completion, the costal chondrocyte scaffold was rigid, exhibiting calcified nodules[21], and did not appear to be suitable for generating elastic cartilage.

Conchal, preauricular, and microtia cartilage samples all exhibited similar staining for elastin, fibrillin I and III, as well as collagen I and II. Microtic cartilage was the only cartilage sample, however, that stained positive for calcium, suggesting abnormalities in this cartilage that make unsuitable as a stem cell source for TE cartilage. Among these samples, the best source for TE cartilage may be preauricular cartilage remnants (given the similarity in staining patterns to conchal cartilage, a relatively high incidence among patients (which may be as high as 1% of the population)[22], and the fact that they are surgically excised relatively early in life and discarded). Preauricular cartilage also expressed a higher fibrillin III level among the endogenous cartilage tested, suggesting that it may represent an immature auricular cartilage, which could be advantageous for TE cartilage. However, the amount of cartilage present within the preauricular remnants is variable (sometimes absent altogether) which presents a challenge as a cartilage cell source.

The umbilical cord as a discarded source of MSCs capable of undergoing chondrogenesis appears to be an ideal source of stem cells for TE cartilage but there continue to be biochemical hurdles that must be overcome in order to replicate normal auricular cartilage including maintaining Collagen II/I ratio above one, controlling elastin and fibrillin content, minimizing collagen X content and preventing calcium deposition.

Summary

TE cartilage using hUCMSCs on PLGA nanofiber-supported scaffolds results in cartilage that is similar to conchal bowl in staining for collagen I, X, and fibrillin I. However, elastin and collagen II expression levels are lower and the presence of fibrillin III suggests immaturity. TE cartilage needs to have higher levels of elastin and collagen II, lower levels of fibrillin III and be less deformable in order to justify use in microtia repair. An on-going, and unanswered question regarding TE cartilage, is how to find a balance between the needs for construct flexibility and a sufficient rigidity to withstand the deforming forces of the overlying skin envelope. Though this study sets the stage for creating a flexible elastic cartilage phenotype, significant further studies will be required to determine efficacy *in vivo*.

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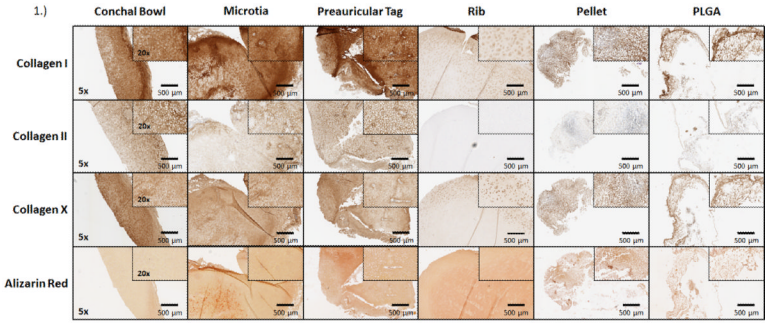

Figure 1.

Histochemical analysis of cartilage. Images are representative of collagen I, II, X, and alizarin red staining for calcium of frozen adult conchal bowl, pediatric microtia, pediatric preauricular cartilage remnants, and adult costal cartilage. Similar distributions of collagens I, II, and X were seen in all samples, with the exception of rib cartilage (a hyaline cartilage). Calcium was present in only the microtia samples. The TE pellet and PLGA –supported cartilage demonstrated similar distribution of Collagen I, X, and calcium in the extracellular matrix as native auricular cartilage. Collagen II distribution was less than native auricular cartilage samples, but greater than rib.

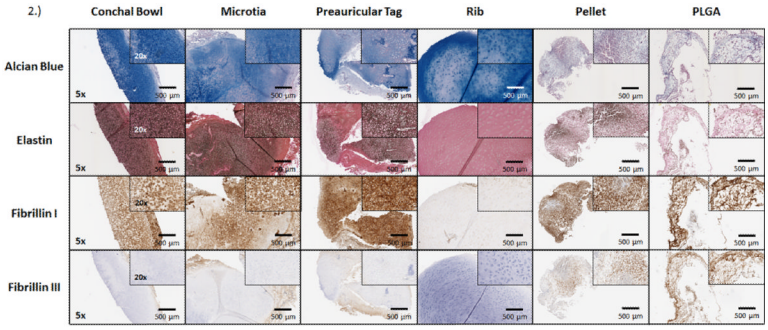

Figure 2.

Histochemical analysis of cartilage. Images are representative of alcian blue, elastin, fibrillin I, and III staining of frozen adult conchal bowl, pediatric microtia, pediatric preauricular cartilage remnants, and adult costal cartilage. All samples demonstrated glycosaminoglycans in the extracellular matrix. The TE and auricular cartilage showed similar elastin and fibrillin I distributions, with rib minimally demonstrating these proteins. Fibrillin III was positively expressed in TE samples, while not present in endogenous tissues.

Table 1

Semi-quantitative image analysis of collagens I, II, X, and calcium deposition. Total positivity of stains was analyzed using Spectrum software. Positivity was designated as strong, medium, or weak, depending on the intensity/darkness of each pixel. Similar distributions were seen in the TE (both pellet and PLGA-supported cartilage) and auricular-derived cartilage for collagens I and X, with marginal presence in rib samples. The microtia samples had a significantly higher presence of calcium, unlike the other samples. Collagen II was most intense in auricular-derived cartilage, lower in the TE cartilage, and nearly absent in rib samples.

Stain	Tissue	% Positive	%Strong+	%Medium+	% Weak+
Collagen I	Conchal bowl	99	66	29	4
	Microtia	99	63	28	8
	Preauricular Tag	100	84	14	2
	Rib	93	0	21	72
	Pellet	91	32	39	20
	PLGA	92	26	42	24
Collagen II	Conchal bowl	97	23	50	23
	Microtia	90	9	45	36
	Preauricular Tag	96	26	49	22
	Rib	3	0	1	2
	Pellet	45	0	12	33
	PLGA	63	3	21	39
Collagen X	Conchal bowl	100	53	41	6
	Microtia	99	14	62	23
	Preauricular Tag	99	17	57	25
	Rib	95	6	19	69
	Pellet	91	21	40	31
	PLGA	90	17	38	34
Alizarin Red	Conchal bowl	0	0	0	0
	Microtia	**4.14**	**0.55**	**1.18**	**2.40**
	Preauricular Tag	0.28	0	0.01	0.27
	Rib	0.85	0.02	0.11	0.72
	Pellet	0.01	0	0	0.01
	PLGA	0.02	0	0	0.02

Table 2

Semi-quantitative image analysis of elastin, fibrillin I, and III. Total positivity of the stain was analyzed using Spectrum software. Positivity was then quantitated as strong, medium, or weak, depending on intensity/darkness of each pixel. Increased elastin and fibrillin I were seen in all samples, with the exception of rib cartilage. Fibrillin III was present in the TE samples (PLGA-supported greater than pellet), with absent staining in endogenous tissues.

Stain	Tissue	% Positive	%Strong+	%Medium+	% Weak+
Elastin	Conchal bowl	49	1	18	29
	Microtia	47	1	15	31
	Preauricular Tag	38	1	11	26
	Rib	10	2	3	5
	Pellet	48	2	14	32
	PLGA	30	1	5	25
Fibrillin I	Conchal bowl	97	26	46	24
	Microtia	96	22	47	27
	Preauricular Tag	99	48	41	10
	Rib	41	1	12	27
	Pellet	89	29	39	22
	PLGA	90	23	42	24
Fibrillin III	Conchal bowl	1	0	0	1
	Microtia	5	0	1	4
	Preauricular Tag	7	0	0	7
	Rib	0	0	0	0
	Pellet	**55**	**6**	**20**	**29**
	PLGA	**92**	**11**	**50**	**30**