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Insights into the role of elastin in vocal fold health and disease

Jaime Moore, B.A. and

Department of Surgery and Communication Disorders and Sciences, 5107 Wisconsin Institutes for Medical Research, University of Wisconsin Madison, 1111 Highland Ave., Madison, WI 53705-2275

Susan Thibeault, Ph.D., CCC-SLP

Department of Surgery, 5107 Wisconsin Institutes for Medical Research, University of Wisconsin Madison, 1111 Highland Ave., Madison, WI 53705-2275, thibeault@surgery.wisc.edu, Phone: (608) 263-0121, Fax: (608)262 -0992

Abstract

Elastic fibers are large, complex and surprisingly poorly understood extracellular matrix (ECM) macromolecules. The elastin fiber, generated from a single human gene - elastin (ELN), is a self assembling integral protein that endows critical mechanic proprieties to elastic tissues and organs such as the skin, lungs, and arteries. The biology of elastic fibers is complex because they have multiple components, a tightly regulated developmental deposition, a multi-step hierarchical assembly and unique biomechanical functions. Elastin is present in vocal folds, where it plays a pivotal role in the quality of phonation. This review article provides an overview of the genesis of elastin and its wide- ranging structure and function. Specific distribution within the vocal fold lamina propria across the lifespan in normal and pathological states and its contribution to vocal fold biomechanics will be examined. Elastin and elastin-derived molecules are increasingly investigated for their application in tissue engineering. The properties of various elastin- based materials will be discussed and their current and future applications evaluated. A new level of understanding of the biomechanical properties of vocal fold elastin composites and their molecular basis should lead to new strategies for elastic fiber repair and regeneration in aging and disease.

Keywords

Elastin; vocal fold; fibroblasts; biomechanics

INTRODUCTION

Elastin fiber, comprised of the elastin protein and fibrillar components, is the extracellular matrix (ECM) protein that is responsible for tissue elastic recoil. It is an essential protein found in the vocal fold in the lamina propria where it appears as thin fibers approximating 9% of this tissue type relative to tissue total protein.¹ In comparison, elastin constitutes 2–4, 30 and 70% of total tissue protein in skin, lung and large arteries, respectively. This implies that the vocal fold lamina propria (VFLP) experiences greater amounts of mechanical strain relative to skin but less amounts when compared to that of the lungs and arteries.¹

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Correspondence to: Susan Thibeault.

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Unfortunately, there is a paucity in the literature in regard to how elastin fibers are formed and organized in the vocal fold lamina propria, throughout the lifespan in normal and diseased tissue. Changes in the formation, structure and organization of this fiber likely affects elasticity, thereby altering propagation of the mucosal wave and vocal fold vibration.^{2,3} Elastin allows tissue to stretch under load and recoiling to its original configuration after the load is released. It has the ability to extend and contract in repetitive motion when hydrated. This review paper serves to provide a comprehensive synopsis on elastin fibers; how and when they are made and broken down and their distribution in vocal fold lamina propria, vocal fold pathologies and other tissue types. We will discuss avenues that are being investigated to minimize elastin loss and to augment naturally occurring elastin with chemically modified elastin fibers in tissue engineering applications.

TROPOELASTIN/ELN GENE

The elastin protein is an insoluble cross-linked polymer assembled from monomers of the protein tropoelastin.⁴ A single copy gene, the elastin gene, or ELN encodes it, which is one of the biggest in the human genome. It is located on the long arm of chromosome 7, at position q11.23 and consists of 34 exons.⁵ The gene contains exonic sequences which code for the elastin protein scattered between intronic sequences.⁶ Its initial product is tropoelastin, the $\approx$ 70 kDa soluble antecedent to elastin. There are at least eleven human tropoelastin exons characterized resulting from developmentally regulated alternative splicing of exons 22, 23, 24, 26A, 30, 32, and 33.⁷ This large amount of splicing is thought to adapt the protein's function in various tissues.⁸ The amino acid makeup of tropoelastin is similar to that of insoluble elastin, except for the lack of cross-linking and an increase in lysine residues.⁹ Therefore, elastin arises through lysine-mediated cross-linking of tropoelastin, established through the enzyme lysyl oxidase.¹⁰ (see Figure 1) Once cross-linked by lysyl oxidase (*in vivo*) or other cross-linkers (*in vitro*), the elastin protein becomes insoluble and is considered resistant to proteolytic cleavage.¹¹ Abnormalities in the ELN can result in disorders such as Supravalvular Aortic Stenosis (SVAS) and Williams Syndrome (WS). In a study done by Watts et al., (2008), researchers measured acoustic and perceptual characteristics of vocal quality in individuals with SVAS and WS. The findings indicated that these individuals demonstrated instability in pitch as well as perceived hoarseness compared to controls, supporting the theory that ELN abnormalities can negatively influence vocal fold biomechanics.¹²

The only class of proteolytic enzymes responsible for breakdown of the elastin protein, as well as, the metabolism of elastic fibers are known as elastases.¹³ These enzymes are categorized into four groups of proteases: serine, aspartic, cysteine and metalloproteinases.¹⁴ Of particular interest are the matrix metalloproteinases (MMPs), which degrade proteins in the ECM. Another class of enzymes exists to regulate matrix degradation by these MMPs known as tissue inhibitors of MMPs (TIMPs). In normal ECM function there is a balance among all the proteins, MMPs and TIMPs.¹⁵ Assemblages of MMPs or a change in the normal homeostatic function can cause the degrading of elastic fiber such as that seen in aging or disease.¹⁶

GENESIS AND STRUCTURE OF ELASTIN

Elastin is laid down during development. Late fetal and early neonatal periods are when elastogenesis predominantly takes place. Varying cell types with tissue-specific induction of elastin expression such as smooth muscle cells, fibroblasts, endothelial cells, chondroblasts, and mesothelial cells produce and secrete elastin during development.^{17,18} After puberty, elastin is typically not produced by cells of any type. Not only is it no longer produced, but becomes less functional in aged tissue.¹⁹ Under pathological conditions, such as

hypertension, vascular cells can synthesize elastin as part of the reaction to increased mechanical stress.^{20,21} It has been demonstrated however that these newly produced elastic fibers are never as effective as those produced during development and, in fact, might even contribute to the loss of wall resilience in hypertension.²² Elastin expression has been measured when fibroblasts are exposed to vibration. In Titze et al., (2004), mRNA levels of elastin showed no significant differences when laryngeal fibroblasts were exposed to 100 Hz vibratory strain compared to controls.²³ In Kutty and Webb (2010), analysis of mRNA expression of elastin in normal human dermal fibroblasts when exposed to vibratory stimulation at 100 Hz showed no significant changes from the static controls as well.²⁴ These studies further suggest the differences in elastin structure and function within various tissues that undergo different levels of mechanical stress.

In healthy tissues, elastin is exceptionally strong and does not significantly degrade; it is approximated to have a half-life of 70 years.²⁵ The major component of an insoluble elastic fiber, elastin is located in the fiber's amorphous center and confers a high capacity for stretch and recoil (elasticity). Elastin is surrounded by a mesh of 10 –12 nm microfibrils comprised of a heterogeneous mix of microfibrillar proteins. This fibrillar component emerges first in the extracellular space of developing tissue and is thought to function as a framework upon which tropoelastin molecules are laid down.²⁶ Tropoelastin molecules are exuded into the extracellular space and become exceedingly cross-linked to each other, creating a system of fibers and sheets. These cross-links are developed between lysine residues.²⁶ These molecules are then thought to be “chaperoned” by an elastin-binding protein to the microfibrillar scaffold site of fiber formation.²⁷ This cross-linked molecular arrangement (Figure 2) is what yields structural integrity, proper function and elastic quality of elastin,²⁸ critical for repetitive stretch and relaxation cycles characteristic of vocal fold vibration.²⁷

Elastin is a protein of 72 kDa which functions as a perfect coil. To confer such biologic function, elastin is highly hydrophobic composed primarily of amino acids such as alanine, valine, leucine, and glycine. One-third of the amino acids in elastin are glycine. Glycine is distributed randomly throughout the elastin molecule and this random distribution makes elastin hydrophobic. This hydrophobic quality allows elastin molecules to slide over one another, or stretch, to maintain structural integrity and provide recoil.⁶

FUNCTION OF ELASTIN

Elastin is responsible for resiliency in elastic fibers. As a fibrous protein, it is fundamental to the shape and form of tissue and designed to manage stress.²⁹ Elastic fibers give vertebrate tissues the ability to distend and recoil, an essential quality for normal homeostatic function. Tissues that undergo constant variations in stretch and recoil contain the highest levels of elastin,²⁸ in addition to the fact that the structure of elastic fiber matrices differ depending on tissue function and location. For example, in arteries, elastic fibers form concentric rings, whereas in the lungs elastic fibers appear more like latticework through the entire organ.²⁷ Elastin is most often intertwined with inelastic collagen fibrils to regulate stretching, which prevents tissue damage and allows the elastic fiber to endure billions of expansion and recoil cycles without breaking down.²⁶

ELASTIN IN VOCAL FOLDS

Elastin plays a pivotal role in vocal fold vibration. The vocal fold lamina propria consists of fibroblasts that secrete the matrix constituents proteoglycans, glycoproteins, collagen, and elastin.² Elastin is present in three different forms within the lamina propria -- oxytalan, elaunin and elastic fibers. The elastin fibers are often referred to as “mature” elastic fibers as they are more elastic than oxytalan and elaunin. However, oxytalan and elaunin are not

immature forms of elastic fibers since they are present in many adult tissues.²⁹ The superficial layer of the lamina propria (SLLP) contains considerable amounts of elastin in the forms of oxytalan and elaunin, whereas the intermediate and deep layers of the lamina propria contain larger amounts of the mature elastic fibers.³ Previous quantitative analysis of elastin distribution within the lamina propria has demonstrated higher concentrations in the deeper layers bordering the thyroarytenoid muscle with decreasing amounts of elastin toward the epithelial layer.¹ This supports Hirano's description of the elastic conus, which contains the deep layer of the lamina propria and is connected to the vocalis muscle, or the thyroarytenoid. The elastic conus, compared to the more superficial layers of the lamina propria, is composed of more dense fibrous tissue, including elastin.³⁰

Changes in elastin density correlated with age and gender have been relatively inconclusive. In a study by Kahane, (1983), twelve larynges (six male, six female; one each from the third through eighth decades) were examined to obtain histologic data on changes in laryngeal tissues as a result of aging. Tissue sections were stained with Iron-Gallein Elastic Stain to look specifically at collagen and elastic fibers. Within the elastic conus, elastic fibers in males displayed a greater than usual waviness, more separation between fibers, and more fragmentation of fibers with age. In females, the fibers became more compact.³¹ In work done by Hirano and colleagues, a decrease in elastin density was also shown within the superficial and intermediate layers of the lamina propria after age 40, more so in males than females.³² In more recent years, studies have shown divergent results. In a study by Hammond et al., (1998), elastin fiber density in the vocal fold lamina propria was not found to be significantly different between males and females at any time point, but it varied greatly between different age groups.³³ Shortly after birth, the LP exists as a hypercellular monolayer which contains trace amounts of elastin fibers. Throughout development, the amount of elastin increases as the three-layered structure of the lamina propria starts to take shape.³⁴ According to Hammond et al., (1998), the amount of elastin in the vocal folds increases with age. Verhoeff's elastic tissue stained vocal folds revealed minimal detection of elastin in infants, with thin and coiled fibers; middle-aged adults had moderate levels of elastin with thicker fibers than infants; geriatric vocal folds had the most abundant level of elastin with the thickest fiber size.³³ As we age, oxidative cross-linking takes place which increases elastin content, but decreases the properties of elasticity.^{35,36} In a study by Chen and Thibeault (2008), quantitative real-time PCR was used to measure elastin mRNA levels in human vocal fold fibroblasts (hVFFs) from three donors aged 21, 59, and 79 years. The mRNA from the 79-year old donor increased to 238% and 201% of the 21- and 59-year olds, respectively.³⁷ This increase in elastin gene expression as age increases parallels the finding in the Hammond, 1998 study. Therefore, loss of elasticity in the aged vocal fold is likely related to homeostatic changes of the ECM also present in multiple body tissues. In Ding & Gray (2001), the use of a reverse-transcriptase polymerase chain reaction (RT-PCR) measured mRNA levels of tropoelastin, elastase, lysyl oxidase and TIMPs in rats, which revealed age-associated changes in tissues of the vocal fold, skin and lungs, similar to changes that occur in these respective human tissues. In contrast to the increase of mRNA expression in elastin in the previously mentioned study, Ding & Gray found a decrease of mRNA expression for tropoelastin in aged rats compared to that of neonatal levels.³⁸ The increase in oxidative cross-linking that occurs during aging may explain the increase in elastin mRNA expression seen along with a decrease in mRNA expression of tropoelastin. Further studies identifying how these changes in mRNA expression of elastin and tropoelastin affect aging in the vocal folds are warranted.

The location, amount and type of elastin in the vocal folds aid in determining its biomechanical properties.² However, it is not currently known how the concentration of elastin throughout the vocal folds endures the tensile stress caused by the thyroarytenoid and cricothyroid muscles during phonation. Chan et al., 2007 observed how collagen and elastin

contribute to this tensile stress within the vocal fold cover and vocal ligament for males and females. For males there was a significantly higher level of elastin in the cover than in the ligament. On average, the elastic modulus of the male cover was about twice that of the female at high-tensile strain (35–40%), whereas the male ligament was 3–5 times stiffer than the female in the same range. The ligament was stiffer than the cover for male, but the opposite was observed for female. These findings suggest that collagen and elastin could contribute differentially to elasticity of the cover and the ligament.³⁹ Continued research in understanding elastin's contribution to the viscoelasticity could lead to new information regarding surgical procedures and tissue engineering of the vocal fold lamina propria. Specifically, how elastin quantity and distribution may affect tissue constructs to reduce dysphonia.³⁹

Relative amounts of elastin in the vocal folds have been studied in vocal fold scarring. Vocal fold scar can result from trauma, inflammatory response to infection, surgical treatment of a growth or tumor, radiation, or damage to the tissues in the airway.⁴⁰ Scarred tissue has been shown to display disperse networks of elastic fiber; a possible contributory cause for the decrease in viscoelasticity that has been reported in animal investigations. In a study done by Thibeault et al., (2002), scarred vocal fold tissue from rabbits was observed post operatively at two months. Elastin concentration was less dense with shorter and more compact fibers. After six months, the density of elastin was similar to that of normal vocal folds but the fibers were still disordered.^{41,42} Similar results were found in Rousseau et al., (2003). Scarred canine larynges were examined post-operatively at 2 and 6 months, with tissue samples exhibiting decreased levels of elastin as well as tangled and disorganized elastic fibers.⁴³ Since histological variances in human scarred vocal folds are likely different from that of animal models, more human studies are needed. One such study investigated the changes of the ECM proteins in scarred human vocal folds, including elastin. Hirano et al., (2009) compared previous animal studies to their current investigation of ECM proteins measured in human scarred vocal folds obtained via cordectomy. Nine of the ten cases in the study showed disorganized elastin structure or complete absence of the protein.⁴⁴ These results are comparable to the previous findings of decreased levels or lack of elastin in scarred vocal folds of animals (Rousseau et al., 2003; Thibeault et al., 2002).^{43,42} (see figure 3a & 3b) A study by Dikkers & Nikkels (1999) also revealed disorganized elastic fiber arrangement in individuals with benign vocal fold lesions, like polyps, nodules and Reinke's edema.⁴⁵ All of these findings are of particular importance since modified states of elastin fibers could possibly impede production of new elastic fibers.⁴⁶

In a recent study, Yamashita et al., (2010) utilized fluorescent immunohistochemistry to analyze quantitative changes in ECM proteins, including elastin, in the vocal fold lamina propria of mice pre-injury and at several time points post-injury. In contrast to previous investigation of scarred vocal folds, Yamashita and colleagues found variable measures of elastin at the different time points, but no significant changes compared to the pre-injured mice.⁴⁷ These contrasting findings warrant further investigation of elastin and its relationship to vocal fold pathologies, which may shed light on possible treatment of scarred vocal folds.

ELASTIN AND BIOMATERIALS

Incorporation of elastin into biomaterials is especially significant when its elasticity or biological effects can be exploited. This interest has been fueled by the remarkable properties of this structural protein such as elasticity, self-assembly, long term stability and biological activity. Due to its heavily cross-linked and insoluble nature, purified elastin has proven difficult to manipulate as a biomaterial for tissue engineering.⁴⁸ Additionally, previous attempts at isolating elastin from natural tissues exhibits batch variation along with

possible disease transmission.⁴⁹ As a result, recent biomaterial designs have applied the use of synthetic biodegradable elastomers as three-dimensional scaffolds.⁵⁰ To date, recombinant tropoelastin, solubilized elastin and elastin-based peptides are mainly used in the assembly of these biomaterials which can be electrospun into fibrous scaffolds or cast into hydrogels, mimicking the properties of native elastin.⁵¹ These constructs have significant clinical implications with electrospinning of recombinant elastin fiber networks offering possible insight to the generation of arterial substitutes and hydrogels used as implants resembling the ECM of living tissues.⁵² It is beyond the scope of this review to present all biomaterials that have incorporated elastin derivatives, subsequently those relevant to vocal fold tissue engineering will be presented in addition to Table 1 which presents a summary of types of elastin used in regeneration research.

Using *E-coli*, Woodhouse et al. has developed, expressed, produced and purified a family of recombinant human elastin polypeptides. These elastin-like polypeptides (ELPs) mimic the alternating domain structure of native tropoelastin.^{53,54,55} ELPs are biopolymers with the pentapeptide repeat of amino acids valine-proline-glycine-Xaa-glycine. The smallest peptide contains one cross-linking domain flanked by two hydrophobic domains, and is roughly 10 kDa in size. The largest EP (31 kDa,) contains 5 hydrophobic domains, and 4 cross-linking domains. The elastin polypeptides have been demonstrated to behave similarly to elastin *in vitro*.^{54,55} Currently, ELPs combined with HA hydrogels that have demonstrated improved wound healing in the vocal folds⁵⁶ are being studied in anticipation of an increase in elastin expression. Future *in vivo* vocal fold work is expected from this group.

Recently, Grieshaber et al., (2009) have developed an elastin-mimetic hybrid polymer (EMHP) which resembles the structure of tropoelastin with the elastic quality of mature elastin. In this study, Grieshaber and colleagues covalently cross-linked the EMHP with hexamethylene diisocyanate (HMDI), creating an elastomeric gel (xEMHP) similar to a commercial polyurethane elastomer (Tecoflex SG80A). *In vitro*, primary porcine vocal fold fibroblasts were able to grow throughout three days of culture and assumed normal cell morphology in the presence of xEMHP.⁵⁷ This xEMHP has been specifically developed for eventual *in vivo* testing for vocal fold disease.

CONCLUSIONS

Elastin plays an important role in the biomechanical function of vital body tissues and organs such as the heart, lungs, skin and the vocal folds. Fundamental to voice production, is its role as a stretch and recoil protein in vibratory function of the vocal fold lamina propria. Elastin production changes throughout the lifespan -- ceasing at puberty with decreasing function as one ages. Dysfunctional elastin organization has been implicated in multiple vocal fold diseases including benign lesions and vocal fold scarring. Future development of biomaterials that include elastin-like entities will be beneficial in treating and improving the biomechanical properties of the vocal fold.

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Figure 1.

Schematic of stretching and recoil network of elastin molecules. Elastin molecules are joined via lysyl oxidase cross linking. Each molecule can expand and contract as a random coil thereby the entire elastin molecule can then stretch and recoil as one entity. Adapted from Alberts et al.²⁶

Figure 2. Schematic representation of the classical model of elastogenesis. A. Tropoelastin molecules are released into the extracellular space where cross-linking takes place via lysyl oxidase. B. The cross-linked elastin molecules are "chaperoned" to the microfibrillar scaffold site where fiber formation occurs. C. Elastin, together with the microfibrils creates the elastic fiber. Figure reproduced from Mithieux & Weiss.²⁷

3a

3b

Figure 3. Elastin Van Gieson stain illustrating elastin fibers (black) in **A.** Normal rabbit vocal fold/ Fibers run parallel to the free edge of the vocal fold and parallel to collagen fibers (pink fibers), and **B.** Compact and disorganized elastin fibers (black) in a coronal section of a scarred rabbit vocal fold (60 days post injury). 40× magnification for both images. Figure from Thibeault et al., 2002.³⁸

Table One

Various form of naturally occurring and synthetic elastin utilized in tissue engineering.

	Derivation	Characteristics of Biomaterials	Applications
Purified elastin	Pure elastin in fibrous forms containing its natural crosslinks	<ul style="list-style-type: none"> No immunological reaction to contaminants Can be molded into different shapes and scaffolds 	Aorta and artery
Hydrolyzed elastin	Elastin fibers degraded with elastases	<ul style="list-style-type: none"> Ease of solubility making handling and analysis straightforward Typically used with other preparations ie. collagen May exert biological effect on a wide variety of cell types 	Spinal cord discs Tympanic membrane Skin substitutes
Tropoelastin	Human tropoelastin expressed in a recombinant bacterial system and highly purified	<ul style="list-style-type: none"> No crosslinks like elastin Recombinant tropoelastin can be crosslinked Different isoforms of tropoelastin can be made 	Small diameter vascular grafts Skin substitutes
Tropoelastin fragments	Fragments expressed in <i>E. coli</i>	<ul style="list-style-type: none"> Better elastic moduli than purified elastin Inhibit thrombosis, platelet adhesion and activation 	Coat synthetic materials Hydrogels
Elastin – like polypeptides	Chemical synthesis utilizing various polymers and crosslinker systems	<ul style="list-style-type: none"> Can be produced in high quantities Material characteristics can vary to needs 	Synthetic tubing Drug deliver Hydrogels