Published in final edited form as:

J Voice. 2003 June ; 17(2): 245–254.

Staining of Human Thyroarytenoid Muscle with Myosin Antibodies Reveals Some Unique Extrafusal Fibers, but no Muscle Spindles

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Summary

This study describes the myosin composition of extrafusal and intrafusal muscle fibers found in the human thyroarytenoid (TA) and sternohyoid (control) muscles. We sought to determine the presence of muscle spindles in the TA muscle, and to identify unusual extrafusal fiber types, using the commonly accepted approach of tissue staining with myosin isoform specific antibodies. Extrafusal fibers are organized into motor units, which subsequently produce muscle movement, whereas intrafusal fibers compose muscle spindles, the primary stretch receptor that provides afferent (feed back) information to the nervous system for regulation of motor unit length and tonicity. Immunohistochemical identification of muscle spindles was confirmed in sternohyoid, but not in TA samples; however, some extrafusal fibers contained tonic myosin. These results indicate that human TA muscle functions similar to some mammalian extraocular muscle, performing unloaded (non-weight bearing) contractions without afferent information from native muscle spindles.

Keywords

Muscle spindles; Human thyroarytenoid muscle; Myosin heavy chain; Intrafusal fibers

INTRODUCTION

The thyroarytenoid muscle (TA muscle) is closely associated with the vocal folds. In humans, a portion of the muscle is continuous with underlying fold connective tissue. The TA muscle was first described as compartmentalized into two parts by Sonesson,¹ a medial “vocalis” compartment associated with fine vocal fold movements and the remainder more closely resembling a typical muscle belly designated as the “muscularis.” Recently, there has been interest in further description of intrinsic laryngeal muscles because it is recognized that without a more thorough understanding, advanced clinical procedures such as laryngeal muscle rehabilitation following injury will be unsuccessful. A recent finding regarding the TA muscle is that its superior medial quadrant is abundant in muscle spindles.² These results

were obtained by identifying spindles anatomically after staining with hematoxylin and eosin.

Muscle spindles provide afferent information to the central nervous system regarding muscle stretch. They play essential roles in reflex activity, load compensation, motor control, and kinesthesia of skeletal muscle. Mammalian muscle spindles are composed of nerves associated with the gamma (γ)–fusimotor system, intrafusal fibers (the contractile elements organized in highly adapted muscle fiber cells), and a connective tissue encapsulation. Much is known about the characteristics of intrafusal fibers, especially with regard to anatomy, innervation, myosin isoform expression, and physiologic activity. Anatomically, there are three subclassifications of intrafusal fibers, namely, nuclear bag 1, nuclear bag 2, and chain. Spindle fibers have striated contractile elements located at the poles. Nuclear bag fibers have an expanded equatorial area, which contains no contractile elements and multiple cell nuclei. Chain fibers differ in that they are of a significantly smaller diameter and have cell nuclei distributed in the middle third of the fiber with no equatorial bag area. Spindles usually contain two to three nuclear bag fibers and four to six nuclear chain fibers, although these relative numbers vary according to species, muscle type, and locomotor function. The relative ratios of bag and chain fibers appear to be more highly variable in human muscle.³ Myosin expression is characteristic of fiber type. Bag fibers may be distinguished histochemically or immunohistochemically because bag 1 fibers contain the tonic isoform of myosin heavy chain (MHC) and almost no neonatal MHC, whereas bag 2 fibers contain an abundance of neonatal MHC.⁴

Spindles have highly adapted motor and sensory innervations. Motor activity is controlled by fusimotor nerve activity. These nerve fibers are anatomically and functionally subdivided into two groups, the γ and β fusimotor nerve systems.⁵ The γ nerves have been well defined anatomically. γ 1 fusimotor neurons have a plate motor ending attached to spindle muscle fibers, whereas γ 2 fusimotor neurons have trail motor endings.⁶ Neurophysiologic recordings further characterize γ and β fusimotor neurons as preferentially active during static or dynamic spindle function.^{7–9} The mechanical properties of the contractile elements and activation of motor innervation strongly influence afferent information and firing properties of sensory nerve terminals of bag and chain fibers. All spindle fibers have sensory terminals with annulospiral endings originating from a population of Ia afferent axons. Chain fibers have additional innervation from another population of smaller diameter afferents. Neurophysiologic recordings distinguish the Ia group as “primary spindle afferents” and the latter as “secondary spindle afferents”.¹⁰ Intrafusal fibers, those skeletal fibers that compose the muscle spindle, can be easily distinguished from extrafusal fibers, those fibers that make up the motor units of the muscle by size. In human muscle, extrafusal fibers increase in size generally along the continuum of fiber type, from type I to type IIA to type IIX, although there is variation in fiber diameter within a fiber type class. Intrafusal fibers are smaller than extrafusal fibers, so much so that the diameter of an entire muscle spindle may approximate the size of extrafusal fiber (Figure 3).

Given the wide-ranging information available to characterize mammalian muscle spindles, the goal of our study was to provide further characterization of muscle spindles and other fiber types previously identified in human TA muscle by hematoxylin and eosin staining. As spindle fibers can be routinely identified and classified by differential staining for neonatal and tonic MHC isoform expression, we systematically sampled sections from whole human TA muscle preparations by staining immunohistochemically using MHC specific antibodies.

MATERIALS AND METHODS

Tissue sampling

Tissues from the thyroarytenoid and sternohyoid muscles were excised from patients undergoing total laryngectomy at the University of Pittsburgh Medical Center. The research protocol and informed consent process for human subjects was followed as approved by the University of Pittsburgh Internal Review Board (IRB #991280). The patient population consisted of 8 males and 4 females, the majority being Caucasian. The age range of the population was 56 to 86 years. Proper laryngeal function was determined prior to obtaining the muscle biopsy, and only normal functioning larynges were sampled.

Tissue processing

Anatomically, the TA has a vocalis and muscularis portion. Initially, sampling of the TA muscle compartments was random, based on the availability of the muscle upon resection. Ten TA samples from nine patients (two samples obtained from one patient) were sampled in this manner for preliminary analysis of the MHC isoform content. For more complete analysis, three additional patients provided complete TA muscles for dissection into the two compartments. A whole vocalis and muscularis portion from one side of the larynx was collected from one of the three patients. Whole TA muscles, without separation into vocalis and muscularis portions, were sampled from both left and right sides of the larynx in the additional two patients. Therefore, the whole of the TA muscle, from origin to insertion, was frozen for complete sectioning and staining analysis.

The muscle biopsies were processed for cryosectioning by snap freezing in 2-methylbutane cooled by dry ice. Sternohyoid was prepared and frozen along side of the laryngeal muscle to provide a control for the staining procedures. The muscle blocks were serially sectioned, cross-sectional to the fibers, at 10 μ m thickness. Slides were stored at -70°C until staining procedures were carried out.

Additional control muscle was obtained from rat limb muscle. Soleus and peroneus muscle was prepared and frozen together separate from the human muscle. The rat limb muscle contained multiple muscle spindles and provided confirmation of the tonic and neonatal antibody reactivity.

Immunological staining

Immunohistochemistry was completed by the indirect immunoperoxidase method, using either a peroxidase conjugated secondary antibody or a biotin-labeled secondary antibody visualized by extravidin peroxidase. The following MHC-specific antibodies were used: Type I (BA-F8), IIA (SC-71), II (MY-32) (all fast isoforms of MHC), neonatal (rabbit polyclonal),¹¹ α -cardiac (Sera-Lab cloneMAS366, Leicestershire, England), IIB (BF-F3), and tonic (rabbit polyclonal).¹¹ Antibodies against neural cell adhesion molecule (Sigma cloneN-CAM-OB11), natural killer cells (HNK-1) (Sigma cloneVC1.1, St. Louis, MO), and desmin (Chemicon cloneMAB1698, Chemicon International, Temecula, CA) were also used to determine reinnervation or degeneration in any of the biopsies. Previous use of these antibodies and references of their use can be found in Brandon et al.¹² When analyzing the whole muscle from origin to insertion, immunohistochemistry for the set of antibodies was completed once every 30 sections, to allow complete representation of the muscle fiber population throughout the whole of the thyroarytenoid.

Hematoxylin and eosin staining was done for each sample to examine the general morphology of the tissue, and as a comparison to the previously published results of Sanders et al.² Additionally, the presence of centralized nuclei can be observed as an indicator of

regenerating fibers, and as a comparison for any desmin, HNK-1, or N-CAM antibody staining.

Data Analysis

The fixed sections were viewed using an Olympus BX40 light microscope with a MTI CCD72 digital video camera interfaced to a computer (Olympus Corporation, Melville, NY). The tissue image was projected on to the monitor through image analysis software (Image Pro-Plus/Media Cybernetics Corporation, Bethesda, MD). This software allowed for image capture, saving, and printing for direct comparison of serial sections.

RESULTS

Fiber type characteristics of the sternohyoid muscle

Staining of the sternohyoid was used as a control muscle with typical human fiber types to compare the results of laryngeal muscle staining. This muscle showed antibody reactivity positively identifying type I, type IIA, and IIX fibers. Neonatal isoforms were rare, but could be identified in fibers, which also expressed IIA MHC. Coexpression also occurred in fibers with type I and the fast isoforms, type IIA, or IIX (Results not shown).

General fiber type characteristics of the thyroarytenoid muscle

Thyroarytenoid (TA) muscles were analyzed by histochemical and immunohistochemical methods to characterize the fiber type composition. The TA is composed of the typical slow and fast fiber types (Figure 1). All of the samples contained type I (Figure 1A), type IIA (Figure 1C), and some contained type IIX (Figure 1B). As no anti-IIX MHC antibody currently exists, the general type II antibody staining is compared with the anti-IIA antibody. The additional fast fibers staining with the general type II antibody were therefore classified as type IIX. Large mammals, including humans, do not possess type IIB fibers or IIB MHC. Type II fibers were more abundant than type I in the TA muscle. Coexpression was observed among various fiber types in the TA. Evidence was found for fibers coexpressing type I and IIX (Figure 1, fiber indicated by ↓). The neonatal isoform was also present in the TA, coexpressing with both slow and fast isoforms. Fibers coexpressing type I, IIX, and neonatal MHC protein (Figure 1, fibers indicated by •), and neonatal and type I (Figure 1, fiber indicated by *) could be identified. Additionally, a few rare fibers staining positively for both α -cardiac and type I were found (results not shown). There was no positive staining with N-CAM, HNK-1, or desmin antibodies.

Tonic fibers and muscle spindles in sternohyoid, TA, and rat limb muscle

The presence of muscle spindles in control muscle sectioned with the TA provided a positive control for reactivity with the tonic and neonatal antibodies (Figure 2). The bag and chain fibers could be distinguished by differences in fiber diameter and differential reactivity for MHC specific antibodies. Bag fibers could also be distinguished due to differential staining for neonatal and tonic MHC antibodies (Figure 2D and 2F). As spindles in our sternohyoid muscles were rather complex and their MHC antibody staining somewhat variable, as is characteristic from other reports on human muscle spindles, we went on to stain spindles in the rat peroneus muscle to demonstrate specificity of our tonic and neonatal antibodies. The spindle in Figure 3 contained one bag 2 fiber, expressing neonatal MHC isoform, one bag 1 fiber expressing tonic MHC isoform, and two chain fibers. These findings are typical of small mammals and are also illustrated in our previous experiments in cat muscle using the same antibodies.¹³

Previous reports indicate the presence of an abundance of muscle spindles in the TA compartments, especially in the superior medial quadrant of the vocalis portion. The tonic

antibody did not result in positive staining of fibers resembling intrafusal fibers, indicating a lack of muscle spindles throughout the muscle. Neonatal isoforms are also present in muscle spindle fibers, yet no reactivity was associated with anatomic areas, which may appear to look anatomically like spindles (Figure 4). Therefore, these structures could easily be excluded as spindles due to the lack of appropriate MHC expression. Further, they also lacked the connective tissue capsule, which typically surrounds muscle spindles, as seen by hematoxylin and eosin staining. We could, however, identify small, isolated numbers of tonic positive fibers in the central portion of the TA muscle belly (Figure 5). These fibers were not associated with muscle spindles or spindle-like structures. When analyzed methodically throughout the whole of the thyroarytenoid muscle, extrafusal tonic fibers were a rare finding. Most of these fibers were of relatively small diameter.

Discussion

Our findings for MyHC isoforms that are present in the TA muscle compartments are consistent with previous reports. Typically, the TA muscle has a majority of type II fast fibers, and less type I or slow fibers.¹⁴ Extrafusal fibers containing unusual MHC isoforms have been reported previously in specialized cranial muscles,^{15–18} so it is not surprising to find limited populations of fibers expressing neonatal or tonic isoforms. We further characterized for the first time human sternohyoid muscle fiber types with the additional finding that this muscle is rich in spindles. Unlike spindles that are anatomically much simpler in smaller mammals, as demonstrated in rat hind limb staining, spindles from human sternohyoid muscle are anatomically complex and their bag and chain fibers have much more variable myosin expressions. Other craniofacial muscles involved in maintenance of posture have similar characteristics. For example, human masseter muscle spindles have a relatively high number of intrafusal fibers, with some as many as 36.¹⁹ Further, although afferent and motor innervation is similar to other mammals, human spindles appear to be more highly variable in its different muscle groups.²⁰

The lack of muscle spindles in the TA is an interesting finding in light of the position of Sanders et al,² who find abundant spindle populations in the superior vocalis subcompartment. On repeated trials, we could not reproduce these results, even though we were certain that our myosin antibodies could positively identify intrafusal fibers in rat and human control muscles samples stained alongside human TA muscles. From 10 μ m serial sections along the entire muscle length, we routinely stained with H&E and with antibodies every 20–30 sections finding spindles in the sternohyoid muscle, but never the TA muscle. Small groups of fibers that resembled spindles were found along the region where the muscle and the vocal fold ligament merge (as in Figure 4). Besides appearing spindle-like, these groups of fibers did not meet the criteria for truly being muscle spindles. These fibers were not reactive to the tonic and neonatal antibody, and H&E staining showed no surrounding capsule. There are a number of reports in the otolaryngology literature, which come to opposite conclusions to our present findings. Bowden et al²¹ and Keene²² were among the first to report muscle spindles in human intrinsic laryngeal muscles using nerve terminal and connective tissue stains. Indeed strong claims were made that anatomically “it is clear that Y. Neuromuscular spindles are present in all the laryngeal muscles of man in considerable numbers, and also that these spindles exhibit the basic pattern described by Sherrington in 1894.”²² Although recent contemporary reports using the same connective tissue stains uphold these findings,² such findings are taken out of context with our present knowledge on muscle spindles. Neurological recordings of fusimotor neurons demonstrate that γ -discharge patterns are different than α -motor neuron patterns,²³ and further, intrafusal spindle fibers have evolved unique MHC expression patterns to coincide with γ -fusimotor activity requirements.¹¹ Further confirmatory data is necessary if we are to uphold claims that intrinsic laryngeal muscles possess muscle spindles such as (1) presence of tonic

and neonatal myosin in their intrafusal fibers, (2) demonstration of nerve terminals that resemble those previously described for afferent and efferent axons, and (3) presence of a connective tissue sheath positively identified as a spindle encapsulation.

Even the connective tissue sheath of spindles has been investigated for unique ultrastructural characteristics. Recent findings demonstrate that fibronectin, tenascin, and laminin have distributions in the connective tissue and periaxial space, which enable the positive identification of a spindle without further information regarding other structures in the receptor. It is likely that these proteins act as connective tissue framework in which intrafusal fibers are stabilized so afferent and efferent activation can progress uninterrupted and the surrounding muscle performs work.²⁴ Such staining would be of help in resolving the discrepancies between our findings and other previous reports, which positively identify spindles from connective tissue stains.

One ongoing controversy is whether nonweight bearing muscles have a physiologic need for muscle spindles. Muscle spindles as first described by Professor Sherrington (1906) were muscle receptors necessary for both reflex activity and the maintenance of posture, tonicity, and coordinated musculoskeletal movements.²⁵ A good example of the lack of consensus regarding non-weight bearing muscles is that of the extraocular muscles. For these muscles, there are no changes in load and no anti-gravity function. Further, these muscles are thought to have no stretch or myotatic reflex.²⁶ Yet, spindles are present in sheep and human extraocular muscle,²⁷ but not in other mammals, such as cat.²⁸ Beyond the muscle spindle as the prototypical stretch receptor in mammalian muscle, specialized cranial muscles have evolved “spindle like” structures whose physiologic functioning is not yet clearly understood. As an example, in cat extraocular muscles, afferent sensory nerves have unusual endings on muscle fibers, including palisade endings, compact endings with dense terminal arborizations, and complex endings with many terminal varicosities over 140 μm in length.²⁹ Added to this information are previous findings that physiologic recordings from extraocular muscle afferents have responses resembling well-described muscle spindle responses.³⁰ It is hypothesized that these receptors are used to produce microsaccadic movements in visual fixation, a highly adapted function of these muscles.²⁹ Therefore, it is quite plausible that human laryngeal muscles have also evolved specialized movements, especially the TA muscle during vocalization, requiring “spindle-like” receptors and afferents.

It is very likely that human laryngeal muscles will prove to be anatomically similar to some extraocular muscles that contain no muscle spindles, but instead utilize specialized “spindle-like” receptors and afferents. For human TA muscle determining the type and location of these receptors will be an important step in the ongoing process of studying the intricacies of human voice production. It is hypothesized that such specialized structures provide essential feedback regarding vocal fold length and probably vocal fold position. Given the obvious and essential coordination between antagonist and agonistic muscles during the complex laryngeal motor activities, it is reasonable to suppose that “spindle-like” receptors and afferents, if found in the TA, would assist in the complex kinesthetic activity found in sound production and modification. The speed of which vocal fold vibration occurs is beyond what any neural receptor can detect, but the global position and tension or stretch on the TA is most likely recorded as afferent information by highly adapted kinesthetic receptors. Like the TA, other individual intrinsic laryngeal muscles must be examined in similar fashion to determine the presence of spindle or spindle-like structures. The presence or absence of these structures is not necessarily intuitive, and work is underway to determine their presence in the other major muscle groups involved in vocal fold positioning and tension: posterior crico-arytenoid and inter-arytenoid.

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FIGURE 1.

Antibody staining of the TA muscle. (A) anti-type I (BA-F8), (B) anti-general type II (MY-32), (C) anti-type IIA (SC-71), (D) anti-neonatal. ● represents two fibers coexpressing types I, II and neonatal. ↓ represents a fiber coexpressing types I and IIX. * represents a fiber coexpressing types I and neonatal.

FIGURE 2.

Muscle spindle found in control muscle of the neck. Antibody staining done with the following antibodies: (A) anti-type I, (B) anti-type II, (C) anti-IIA, (D) anti-neonatal, (E) anti-alpha cardiac, (F) anti-tonic. ← represents a bag 1 fiber without neonatal MHC.

FIGURE 3.

Muscle spindle from rat limb muscle. Immunohistochemistry completed with the following antibodies: (A) anti-type I, (B) anti-II, (C) anti-neonatal, (D) anti-tonic. Arrow (←) indicates bag 1 fiber staining with the tonic antibody. * indicates Bag 2 and chain fibers staining with neonatal antibody.

FIGURE 4.

High and low power views of vocalis portion of TA muscle. (A) H&E staining showing low power view of vocalis adjacent to the vocal fold (bottom). (B) through (F) Small groups of fibers in vocalis area nearest to vocal fold: (b) H&E stain, (C) anti-type I, (D) anti-type II, (E) anti-neonatal, and (F) anti-tonic.

FIGURE 5.
High power view of small tonic fibers found in human thyroarytenoid muscle (400×).
Staining completed with anti-tonic antibody.