Age-Related Changes in Muscle Fiber Regeneration in the Human Thyroarytenoid Muscle

Leslie T. Malmgren, PhD; David B. Lovice, MD; Matthew R. Kaufman, MD

Background: Muscle fiber regeneration is essential to maintain normal muscle fiber populations and muscle mass by continuous replacement of fibers lost to acute muscle injury or overuse. However, the extent of ongoing muscle fiber regeneration in the laryngeal muscles is unknown.

Objective: The present study provides statistically unbiased, quantitative estimates of the content of regenerating fibers in the human thyroarytenoid muscle over the adult lifespan.

Design: In the adult, only regenerating muscle fibers express the developmental myosin isoform. Therefore, regenerating fibers were identified using immunohistochemical techniques. The content of regenerating muscle fibers in the entire muscle volume was then estimated using stereological techniques. Through the use of a computer-automated sampling protocol, stereological data were collected from sets of isotropic uniform random cryostat sections. Overprojection error was minimized by using a confocal laser-scanning microscope to image thin optical sections for use as sample fields.

Subjects: Eight autopsy cases, subjects ranging in age from 19 to 81 years.

Results: The summed length of fibers expressing developmental myosin increased significantly (P = .02) with age when compared with the overall muscle fiber length.

Conclusions: This finding indicates that muscle fibers maintain the capability for spontaneous regeneration, and that the proportion of regenerating fibers increases as the thyroarytenoid muscle ages. This increase is possibly a compensatory response to an age-related increase in muscle fiber injury or death.

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The number of Americans older than 65 years will double in the next century, and the number older than 85 years is expected to triple during that period. In response to this trend, there will be an increasing demand for improved methods of preventing and treating age-related dysfunctions. Age-related laryngeal dysfunction compromises the protection of the airway and voice quality, and atrophy of the laryngeal muscles contributes to the diminished performance of these motor systems in the elderly. However, the pathogenetic mechanisms are largely unknown, and the design of more effective methods for the treatment or prevention of age-related laryngeal dysfunction will require an understanding of the underlying aging process.

It has been demonstrated that there is a preferential 27% age-related loss of type 1 (slow-twitch) fibers in the human thyroarytenoid (TA) muscle, in contrast to the selective type 2 (fast-twitch) fiber loss typical of aging limb muscles. This is consistent with other differences between the laryngeal muscles and limb muscles, and it demonstrates a corresponding distinction in the characteristics of the aging process in the laryngeal motor units.

The present study further examines the mechanisms underlying age-related changes in muscle fiber populations in the human TA muscle. Muscle fiber regeneration is essential to maintain muscle mass and strength by compensating for muscle fiber injury or cell death, and studies on the limb muscles have suggested that a diminished capacity for muscle fibers to regenerate may contribute to age-related losses in muscle fiber numbers and a decline in strength. There is evidence for an age-related increase in a cycle of denervation and reinnervation in the laryngeal motor neurons, but the contribution of muscle fiber regeneration to...
METHODS

Thyroarytenoid muscles were obtained from male and female postmortem subjects (aged 19-81 years; N=8). None of the individuals studied had a history of neuromuscular disease or cancer. The TA muscles were dissected from the larynx within 48 hours of death and subsequently frozen and stored at -80°C. Either the right or left TA muscle was randomly selected for study. The weight and volume of the muscle were recorded, and the specimens were coded so that the patient's age and identity remained unknown to the investigators performing the staining and data collection.

IMMUNOHISTOCHEMICAL ANALYSIS

Cryostat sections were cut (10 µm) and fixed in 4% paraformaldehyde and 0.2% picric acid for 60 seconds. The immunocytochemical imaging of the developmental myosin heavy-chain isoform provided a reliable indication of regenerating muscle fibers because only regenerating muscle fibers express developmental myosin in the adult.14 The primary antibody used was mouse monoclonal RNMy2/9D2 (Novocastra Laboratories Ltd, Newcastle upon Tyne, England); the secondary antibody was biotinylated goat anti-mouse IgG H+L (Vector Laboratories, Burlingame, Calif). The label was ExtrAvidin fluorescein isothiocyanate (Sigma Chemical Co, St Louis, Mo). The excitation maximum was 554 nm, and peak fluorescence was 520 nm. Concanavalin A labeled with tetramethylrhodamine isothiocyanate (excitation maximum, 554 nm; emission maximum, 576 nm) (Molecular Probes Inc, Eugene, Ore) was used to visualize muscle fiber surfaces to allow accurate determination of fiber diameters. The sections were then mounted in Vectashield (Vector Laboratories) to reduce fading and stored at 4°C. Negative controls omitted the primary antibody.

RESULTS

Immunocytochemical techniques for the detection of the developmental myosin heavy-chain isoform provided a reliable and sensitive marker for muscle fiber regeneration in the human TA muscle (Figure 1). The regenerating fibers were found either alone or in groups (Figure 1), and their mean ± SD length was significantly smaller (20.8±6.3 µm; P=.002) than other muscle fibers in the TA (33±5.0 µm). However, there was not a significant age-related change in the diameters of regenerating fibers.

The results indicate that muscle fiber regeneration is ongoing in the TA muscle throughout the adult lifespan. However, the quantitative stereological data demonstrate age-related changes in the content of regenerating fibers. The stereological results include absolute length (L), surface (S), and volume (V) data for the content of regenerating fibers in the TA muscle as well as relative data referenced either to the entire muscle volume or to data for nonregenerating fibers (Table). The absolute data show a trend toward an age-related increase in the total length of regenerating fibers and a significant age-related increase in the total surface of regenerating fibers despite the fact that the older TA muscles were slightly smaller than those of the younger group. The relative stereological data references either the muscle volume or nonregenerating fiber dimensions to provide content estimates that are independent of age-related differences in the muscle volume. The results for variables referenced to the muscle volume were similar to those for the absolute dimensions with a trend toward an age-related increase in the length density (LV regenerating fibers, muscle) as well as the surface density (SV regenerating fibers, muscle). Since references to the muscle volume can be confounded by age-related changes in the volume fraction of the extracellular space and connective tissue, analyses were also carried out using the content of nonregenerating fibers as a reference. These data demonstrated a significant age-related increase in both the regenerating fiber...
488-nm laser line with a triple primary dichroic and a 560-nm secondary dichroic that included a 530-nm discriminating bandpass filter in channel 1 and a 610 long-pass barrier filter in channel 2 to improve separation of the fluorescein isothiocyanate and TRITC fluorochromes.

The stereological data concerning the dimensions, length, surface, and volume of regenerating muscle fibers were collected using custom software that implemented an automated isotropic uniform random sampling protocol using computer interfaced x-, y-, and z-axis stepping motors. The sample fields were distributed in a regular lattice pattern that was randomly positioned with respect to the tissue, and were defined by a graphic overlay that included an unbiased counting frame.\(^2\) Estimates of regenerating and normal muscle fiber volume fractions were made using test point counting:\(^3\)

\[
\hat{V}_{Y,ref} = \frac{\sum_{i=1}^{n} P_i}{\sum_{i=1}^{n} P_{ref}}
\]

where \(P_i\) is the number of lattice points hitting phase \(Y\) and \(P_{ref}\) is the number of points hitting the reference space. Total fiber volumes (cubic centimeters) were obtained by multiplying muscle fiber volume fractions by the muscle volume.

Surface densities of normal and regenerating fibers were estimated using counts of line intersections:\(^4\)

\[
\hat{S}_{Y,ref} = \frac{2 \cdot \sum_{i=1}^{n} l_i}{l/p \cdot \sum_{i=1}^{n} P_i}
\]

where \(l_i\) is the number of line intersections, \(l/p\) is the length of test line per sample lattice point, and \(P_i\) is the number of points hitting the reference space on each sample field. Total surface areas (centimeters squared) were obtained by multiplying surface densities by the muscle volume.

Estimates of regenerating and normal muscle fiber length densities were made using muscle fiber profile counting:\(^5\)

\[
L_{Y,ref} = 2 \cdot \frac{\sum_{i=1}^{n} Q_i}{af \cdot \sum_{i=1}^{n} P_i}
\]

where \(Q_i\) is the number of profiles counted,\(^6\) \(af\) is the area of an unbiased sampling frame,\(^7\) and \(P_i\) is the number of frame-associated points hitting the reference space. The unbiased counting frame graphic established by Gundersen\(^8\) was used to eliminate bias due to the “edge effect.” Briefly, this counting frame is formed by 2 contiguous acceptance lines and 2 exclusionary lines that are fully extended in opposite directions into a surrounding guard area. According to this sampling technique, fiber profiles that are cut by the exclusionary lines are not counted, and those that are included entirely within the frame or cut only by the acceptance lines are counted. Total fiber lengths (centimeters) were obtained by multiplying length densities by the muscle volume. Length fractions \((L_{Y,ref})\) were calculated as ratios of the regenerating and normal fiber lengths.

**STATISTICAL ANALYSIS**

Separate variance (Welch) \(t\) tests were used to test the equality of group means for stereological descriptors from the “young” (aged 19-59 years) and the “old” (aged 60-81 years) group using BMDF Software (BMDF Statistical Software; SPSS Inc, Chicago, Ill). A matched pairs \(t\) test was used to compare the diameters of regenerating muscle fibers to other fibers over the entire age range. The linear regression plots of the regenerating muscle fiber length fraction against age report \(P\) value for the test of the null hypothesis that the population correlation is zero. The Pearson product-moment correlation \(r\) was computed from all pairs of values.

The results demonstrate that regenerating muscle fibers compose only a very small proportion of the total muscle fiber population at any one time. However, when the contribution of this infrequent process is summed over the lifespan, it is evident that regeneration contributes in a major way to muscle fiber population dynamics. For example, if it can be assumed that regeneration is completed in 21 to 28 days,\(^9\) the results suggest that regeneration replaces almost twice the original population by age 70 years.

In spite of the importance of ongoing muscle fiber regeneration to the maintenance of muscle fiber populations, muscle mass and strength, the technical challenge of obtaining quantitative estimates of this rare process has discouraged investigations in this area. The efficiency of stereological techniques\(^10\) has made it feasible to sample this rare event in the entire volume of the TA muscle in 8 subjects, ranging in age from 19 to 81 years. In addition, the high z-axis resolution of the confocal laser–scanning microscope made it possible to eliminate substantial overprojection error\(^11\) by collecting the stereological data from extremely thin optical sections.

Muscle fiber necrosis may involve either the entire muscle fiber or only an injured segment, followed by either segmental (continuous) or complete (discontinuous) regeneration, respectively.\(^12\) Consequently, the contribution of muscle fiber regeneration to the maintenance of muscle mass and strength is primarily a function of the total regenerated muscle fiber length rather than the number of regenerated muscle fibers. The present stereological estimates of the length density and length fraction of regenerating fibers in the human TA muscle include both continuous and discontinuous regeneration. Therefore, they provide statistically unbiased, quantitative, 3-dimensional estimates of the total contribution of regeneration to maintenance of the muscle fiber content in the entire volume of the human TA muscle. Furthermore, these estimates can be combined with quantitative, 3-dimensional data from other stereological studies to contribute to multivariate quantitative mod-

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**COMMENT**

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els of age-related changes in the system dynamics of cell populations and tissue components in the human TA muscle.

The results demonstrate a significant age-related increase in the length fraction of regenerating muscle fibers in the human TA muscle. However, it is unlikely that this process is due to an increase in the regenerative capacity of the TA. In limb muscles there is an age-related decrease in the capacity for regeneration of muscle mass and maximum force. In addition, there is an age-related decrease in the rate of regeneration in some skeletal muscles, which would increase the number of fibers in the process of regeneration at any particular time.

An age-related increase in the frequency of muscle fiber injury and cell death probably also contributes to the observed increase in the content of regenerating muscle fibers. It has been demonstrated that extensor digitorum longus muscles of old mice are more susceptible to injury caused by eccentric contractions than muscles of young or adult mice and that free radical accumulation contributes to a secondary injury 3 days after the first injury. Although it is well known that eccentric contractions contribute to muscle fiber injury or cell death in other skeletal muscles, the likely role of eccentric contractions as a mechanism underlying use-related or age-related laryngeal dysfunction has received little attention. Exercise-induced injury to muscle fibers also results

Figure 1. Confocal laser–scanning micrographs of human thyroarytenoid muscle. Concanavalin A (red fluorochrome) images the extracellular matrix and surface of unstained muscle fibers. Top, Small regenerating muscle fibers express the developmental myosin heavy-chain isoform (green fluorochrome) in isolated regenerating fiber (arrow); bottom, cluster of regenerating fibers (arrow). Bars indicate 50 µm.

Figure 2. The relationship between the length fraction of regenerating muscle fibers and age. Solid line represents linear regression (P = 0.02; r = 0.779; y = −1.6e−03 + 9.96e−05x); dashed line, the 95% confidence interval.

<table>
<thead>
<tr>
<th>Stereological Descriptor</th>
<th>Young (Aged 19-59 y; n = 4)</th>
<th>Old (Aged 60-81 y; n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle volume, cm³</td>
<td>1.15 ± 0.17, 0.35</td>
<td>0.91 ± 0.18, 0.35</td>
</tr>
<tr>
<td>Total fiber length, cm</td>
<td>26.4 ± 10, 21</td>
<td>140 ± 38, 76†</td>
</tr>
<tr>
<td>Total fiber surface, cm²</td>
<td>0.238 ± 0.09, 0.17</td>
<td>1.11 ± 0.28, 0.58‡</td>
</tr>
<tr>
<td>Total fiber volume, cm³</td>
<td>0.0001 ± 0.00004, 0.00007</td>
<td>0.0006 ± 0.00002, 0.0005</td>
</tr>
<tr>
<td>LV regenerating fibers, muscle, cm²/cm³</td>
<td>21.9 ± 7.4, 15</td>
<td>175 ± 60, 129†</td>
</tr>
<tr>
<td>SV regenerating fibers, muscle, cm²/cm³</td>
<td>0.215 ± 0.09, 0.18</td>
<td>1.38 ± 0.45, 0.91†</td>
</tr>
<tr>
<td>VL regenerating fibers, all fibers, cm²/cm³</td>
<td>0.0001 ± 0.00004, 0.00007</td>
<td>0.0008 ± 0.00004, 0.0007</td>
</tr>
<tr>
<td>SS regenerating fibers, all fibers, cm²/cm³</td>
<td>0.0001 ± 0.00004, 0.00007</td>
<td>0.0061 ± 0.0013, 0.0026‡</td>
</tr>
<tr>
<td>VS regenerating fibers, all fibers, cm³/cm³</td>
<td>0.0003 ± 0.0001, 0.0002</td>
<td>0.0033 ± 0.0011, 0.002‡</td>
</tr>
<tr>
<td>SV regenerating fibers, all fibers, cm³/cm³</td>
<td>0.0003 ± 0.0001, 0.0002</td>
<td>0.0024 ± 0.0010, 0.0021</td>
</tr>
<tr>
<td>Diameter, µm</td>
<td>20.7 ± 4.6, 8.0</td>
<td>20.8 ± 3.0, 6.1</td>
</tr>
</tbody>
</table>

* Absolute dimensions (length [L], surface [S], and volume [V]) are for the mean content of regenerating fibers in the entire muscle. Stereological descriptors for muscle fiber categories are referenced either with respect to unit volume for the entire muscle (eg, LV) or as a fraction of the corresponding dimension for all muscle fibers (LV, SS, VS). The values are mean ± SEM, SD.
† Trend (P < .10) toward difference from the young group.
‡ Significant difference (P < .05) from the young group.
in apoptosis of myofibers and satellite cells, and it has been suggested that eccentric contractions and/or oxidative stress might trigger this program of cell suicide. A recent stereological study has demonstrated that apoptosis plays a role in the age-related remodeling of muscle fiber and satellite cell populations in the human TA muscle. This muscle fiber injury in the human TA would contribute to the reported age-related muscle fiber loss in this muscle if there is an age-related increase in the frequency of muscle fiber death relative to the frequency of muscle fiber regeneration. The current finding of an age-related increase in the content of regenerating muscle fibers in the TA is consistent with the hypothesis that muscle fiber regeneration plays an important role in opposing this age-related increase in muscle fiber injury. However, the demonstrated net age-related loss of type 1 fibers in the human TA muscle suggests that muscle fiber regeneration is not sufficient to compensate for the age-related increase in type 1 fiber injury.

Each cycle of muscle fiber death and regeneration depends on successful reinnervation for functional recovery. Therefore the observed age-related increase in the frequency of regenerating muscle fibers in the human TA places an increased demand on the success of reinnervation with increasing age. However, there is evidence that the viability of the laryngeal motor innervation is progressively impaired with increasing age. Myelinated nerve fibers in the rat recurrent laryngeal nerve show an age-related increase in a degeneration/regeneration cycle. In addition, stereological studies on the human TA muscle have demonstrated an age-related increase in muscle fiber–type grouping and muscle fiber–type transitions, which indicate a process of partial denervation followed by reinnervation. Studies in limb muscles report an age-related decrease in the capacity for reinnervation of regenerated muscle fibers. If there is also an age-related decrease in the capacity for reinnervation in the TA muscle, the demonstrated cycles of muscle fiber injury and regeneration would place an increased demand on an age-impaired reinnervation capacity and thereby contribute to muscle fiber denervation and atrophy.

Vocal fold atrophy and bowing is one of the most common causes of hoarseness in the elderly. The present study has demonstrated a substantial age-related increase in the contribution of muscle fiber regeneration to the maintenance of the muscle fiber content in the human TA. However, the observed age-related increase in the rate of regeneration is not sufficient to prevent an overall age-related loss of muscle fibers. Therefore, new clinical techniques based on manipulation of the regenerative capacity of this muscle may increase the rate of muscle fiber regeneration sufficiently to compensate for the age-related increase in muscle fiber injury as an alternative to surgical intervention. In addition, a better understanding of the mechanisms underlying muscle fiber injury in the human TA is likely to provide additional clinical targets for pharmacological intervention or gene therapy. It may be possible to identify use-related mechanisms, such as concentric contractions, that might negatively contribute to a loss of muscle fibers in the elderly and may suggest preventive strategies or treatment techniques.

**CONCLUSIONS**

The results demonstrate that muscle fiber regeneration plays a major role in maintaining the strength and mass of the TA muscle throughout the human lifespan. In addition, the content of regenerating fibers increases significantly in the elderly. However, this finding cannot be interpreted as an age-related increase in the regenerative capacity. It is more likely that the age-related increase in muscle fiber regeneration is a compensatory response to an increase in muscle fiber injury or cell death and/or a decrease in the rate of regeneration.

**REFERENCES**


