Short communication

Elucidation of extracellular matrix mechanics from muscle fibers and fiber bundles

Gretchen A. Meyer\textsuperscript{a,b}, Richard L. Lieber\textsuperscript{a,b,*}

\textsuperscript{a} Department of Bioengineering, University of California, San Diego and Veterans Affairs Medical Center, La Jolla, CA 92093, USA
\textsuperscript{b} Department of Orthopaedic Surgery, University of California, San Diego and Veterans Affairs Affairs Medical Center, La Jolla, CA 92093, USA

\textbf{A R T I C L E I N F O}

Article history:
Accepted 27 October 2010

Keywords:
Muscle
Extracellular matrix
Connective tissue
Mechanical properties

\textbf{A B S T R A C T}

The importance of the extracellular matrix (ECM) in muscle is widely recognized, since ECM plays a central role in proper muscle development (Buck and Horwitz, 1987), tissue structural support (Purslow, 2002), and transmission of mechanical signals between fibers and tendon (Huijing, 1999). Since substrate biomechanical properties have been shown to be critical in the biology of tissue development and remodeling (Engler et al., 2006; Gilbert et al., 2010), it is likely that mechanics are critical for ECM to perform its function. Unfortunately, there are almost no data available regarding skeletal muscle ECM viscoelastic properties. This is primarily due to the impossibility of isolating and testing muscle ECM. Therefore, this note presents a new method to quantify viscoelastic ECM modulus by combining tests of single muscle fibers and fiber bundles. Our results demonstrate that ECM is a highly nonlinearly elastic material, while muscle fibers are linearly elastic.

Published by Elsevier Ltd.

1. Introduction

Extracellular matrix (ECM) is essential for the development, maintenance and regeneration of skeletal muscle (Buck and Horwitz, 1987; Purslow, 2002). ECM is involved in the macromolecular structure of muscle, arranging fibers into bundles, bundles into fascicles and integrating muscle structure with aponeurosis and tendon. Additionally, ECM is thought to play a vital role in mechanotransduction and transmitting force laterally from the fiber to the tendon and vice versa (Fomovsky et al., 2010; Huijing, 1999; Purslow and Trotter, 1994; Street, 1983). The mechanical strength and elasticity of the ECM are critical to its functional performance—it must be strong enough to sustain the loads of contraction yet elastic enough to prevent tearing under externally applied strains (Purslow, 2002). These properties change both with age and disease, where chronic alterations to the ECM appear to impair muscle function (Lieber et al., 2003; Zhou and Lu, 2010). Unfortunately, there are almost no data available regarding skeletal muscle ECM viscoelastic properties. This is primarily due to the impossibility of isolating and testing muscle ECM.

Attempts to remove muscle cells chemically from the ECM to test its properties directly have all met with some degradation or compromised mechanical properties (Borschel et al., 2004; Qin and Qin, 2009). Additionally, the geometry of the ECM structure is modified when part of its composite structure (the fiber) is removed, which likely affects the orientation of collagen fibers and thus the modulus of elasticity. This report describes a new technique for indirectly determining the mechanical properties of the ECM without digestion, by combining tests from single muscle fibers and fiber bundles and using the analytical approach of composite theory.

2. Methods

Experiments were performed on single muscle fibers and muscle fiber bundles from the 5th toe of the extensor digitorum longus (EDL) muscle in mice (129/Sv 7–9 weeks old; Taconic Farms, Germantown, NY, USA). Details of the dissection procedure and the solutions used have been described previously (Shah et al., 2004). All procedures were performed in accordance with the NIH Guide for the Use and Care of Laboratory Animals and were approved by the University of California and Department of Veteran’s Affairs Committees on the Use of Animal Subjects in Research.

Briefly, muscles were skinned overnight in a glycerinated relaxation solution and single fibers and bundles (composed of 10–20 muscle fibers) were dissected in chilled relaxation solution composed of (mM): EGTA (7.5), potassium propionate (17.0), magnesium acetate (2.0), imidazole (5.0), creatine phosphate (10.0), ATP (4.0), leupeptin (17 μg/ml) and E64 (4 μg/ml) to prevent protein degradation. One end of the specimen (either fiber, fiber group or fiber bundle) was attached via 10-0 suture to a motor arm (Newport MT-RS; Irvine, CA, USA) that controlled specimen length and the other to a force transducer (Aurora Scientific 405 A; Aurora, Ontario, Canada) that recorded force. The experimental paradigm (Fig. 1) illustrates the three types of specimens tested: (1) single fibers, (2) bundles of 10–20 fibers or (3) 10–20 single fibers dissected individually that were then secured together to approximate the size of a bundle, but these “fiber groups” contained no interfibrillar ECM. Sarcomere lengths provided objective assessments of muscle strain and myofibrillar
array quality control and were measured by transilluminating the specimen with a low power laser diode.

Elastic properties were derived from an incremental stress relaxation protocol. Specimens were stretched in ~10% strain increments at 2000%/s to impose a 0.25 μm sarcomere length change per stretch. Length was then maintained for 3 min, while the specimen was allowed to stress-relax. Elastic properties were determined from quadratic fits to fully stress-relaxed data. Elastic properties of the ECM were derived using the rule of mixtures for composites (Eq. (1)), where $E_m$ is the modulus of the ECM, $E_f$ is the fiber modulus, $E_c$ is the composite or bundle modulus and $A_m$ is the cross-sectional area fraction occupied by ECM in the bundle.

$$E_m = \frac{E_c - E_f (1 - A_m)}{A_m}$$

Data were compared across sample type by one-way ANOVA and considered significant ($\alpha$) at $p < 0.05$. Statistical power ($1 - \beta$) was calculated for differences that were not significant. Individual experimental groups were compared using Tukey’s multiple comparison test. Data are presented as mean ± SEM.

### 3. Results

The relaxed quadratic modulus, representing the amount of nonlinearity present in the stress–sarcomere length relationship of the three experimental groups revealed that fiber bundle modulus was six fold greater than the modulus of individual fibers, where elasticity is essentially linear (Fig. 2). This indicates that a source of nonlinearity present in the fiber bundles is not present in isolated fibers.

Because fiber bundles are composites of fibers and ECM, nonlinearity could arise from natural variations in the passive tension of fibers or from an intrinsic nonlinearly elastic ECM. This concept is illustrated graphically (Fig. 3), where the relaxed stress–sarcomere length relation is plotted for 10 experimentally tested fibers for two different theoretical cases. In the first case, fibers all develop passive tension at approximately the same sarcomere length and the fiber composite stress–strain relationship is linear (Fig. 3A). If this was the case for fibers in the bundle, the observed nonlinearity would have to arise from the ECM surrounding the fibers. In the second case, fiber stress–strain curves are artificially shifted such that each fiber develops passive tension at slightly different sarcomere lengths (Fig. 3B). This results in a nonlinear fiber composite stress–sarcomere length relationship even though the single fibers are linearly elastic. Thus, if fibers in a bundle were at different sarcomere lengths at a given bundle length, bundle stress–strain behavior could appear to be nonlinear whereas each of its components was actually linear. In this case, the contribution of the ECM to the nonlinearity of bundle elasticity could not be determined by subtraction from the contribution of fiber variability.

![Fig. 1. Schematic illustration of the arrangement of the three specimen types. Single fibers (curved pink lines) were isolated from the muscle and either tested individually or secured in groups. Bundles of a similar number of fibers embedded in ECM (light pink) were isolated and similarly secured. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image1)

![Fig. 2. Quadratic moduli for fibers, fiber groups and fiber bundles. Fiber bundles have a significantly higher modulus than either individual fibers or fiber groups. Fiber and fiber group moduli were not significantly different from each other ($p > 0.1$). Fibers: n = 17, fiber groups: n = 6, fiber bundles: n = 10. Bars indicate $p < 0.05$.](image2)

![Fig. 3. Illustration of the potential sources of nonlinearity in fiber bundles. (A) Experimental data from 10 fibers is plotted in green (each thin line represents an individual fiber) with composite fiber behavior shown in thick blue line. In this case, all fibers develop tension at the same sarcomere length and the composite behavior is linear. Since nonlinear bundle behavior is observed, ECM elasticity must be nonlinear (dashed line). (B) Same experimental data as in (A) are plotted but now each fiber has been shifted to develop tension at different sarcomere lengths, which yields a nonlinear composite behavior (dashed line). In this case, nonlinear bundle elasticity could be obtained with a linearly or nonlinearly elastic ECM.](image3)
and composite theory could not be used to determine ECM
elasticity.

To differentiate between these possibilities, we combined and
biomechanically tested individual isolated fibers into “groups”
without the lateral connection of ECM. In this case, the contribution
of fiber-to-fiber variability to the nonlinearity of the stress–strain
relationship was isolated. Our results demonstrated that “fiber
groups” have a quadratic modulus that is significantly lower than
fiber bundles indicating that interfibrillar variability is not the
primary contributing factor to fiber bundle nonlinearity (Fig. 2). In
fact, fiber group modulus was not significantly different from single
fiber modulus suggesting that a group of fibers behaves like an
individual fiber (p > 0.1, 1 – β = 0.9). Based on these data, we can
calculate bundle ECM modulus using Eq. (1), knowing only fiber
and bundle mechanics and fiber area fraction.

Assuming that 5% of the bundle cross sectional area is made up
of ECM (Lieber et al., 2003), and using experimentally-derived
modulus values (Fig. 2) the quadratic modulus of ECM in fiber
bundles from the mouse 5th toe EDL muscle is 692 kPa/μm². This
indicates that the elasticity of ECM in muscle is highly nonlinear.

4. Discussion

Here we describe a new method to quantify passive mechanical
properties of the muscle ECM without tissue digestion. Previous
studies used methods of subtraction, where the ECM was “pre-
ferentially” digested from muscle and its properties inferred from
subtracting the digested state from the undigested state (see
review by Fomovsky et al., 2010; Granzier and Irving, 1995).
However, this method includes the uncertainty of incomplete or
non-specific digestion. Additionally, it has not been rigorously
demonstrated that the differences observed were due to only the
removal of ECM. The current study provides a control by using
groups of fibers, which differ from bundles only in that they contain
no ECM that could interconnect muscle fibers. Such interconnec-
tions have been shown to be significant in defining the mechanical
properties of tendon (Haraldsson et al., 2008). The modulus of the
fiber groups was not significantly different from the modulus of
individual fibers indicating that the observed difference between
the elasticity of fibers and bundles is likely due to ECM.

It is possible that our fiber isolation had some effect on
mechanical properties. Fibers were dissected from intact muscles
after chemical skinning and the shear stress applied during
isolation or the skinning procedure itself could cause fibers to
behave differently in isolation than in their unperturbed state. For
instance, the sarcolemma and associated proteins could be dis-
rupted or collagen fibrils could remain attached during isolation,
afflicting the elasticity of the fiber although this possibility seems
unlikely, as hydroxyproline assays were unable to detect the
presence of collagen in samples composed only of isolated fibers
(unpublished data). Further studies will be needed to determine
the extent to which these factors affect our conclusions.

Bundle quadratic modulus was almost four fold larger than that
of fiber groups. The magnitude of this effect supports the
 conclusion that the primary contributor to bundle stiffness is the
ECM. Small perturbations to proteins on the fiber surface would
likely contribute minimally to elasticity, as would the occasional
remaining collagen fiber. Although this method was evaluated on
bundles of only 10–20 fibers it could be extended to larger scales
using fascicles and whole muscles for a more complete picture of
the material properties of the ECM.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgement

We gratefully acknowledge the National Institutes of Health
grant AR40050 and the Department of Veterans Affairs. We also
thank Dr. Sam Ward and Lucas Smith for helpful discussions.

References

matrix to the mechanical properties of the heart. J. Mol. Cell Cardiol. 48, 490–496.
Gilbert, P.M., Havenstrite, K.L., Magnusson, K.E., Sacco, A., Leonard, N.A., Kraft, P.,
Granzier, H.L., Irving, T.C., 1995. Passive tension in cardiac muscle: contribution of
Haraldsson, B.T., Aagaard, P., Qvortrup, K., Bojesen-Moller, J., Krogsgaard, M.,
Koskinen, S., Kjaer, M., Magnusson, S.P., 2008. Lateral force transmission between
Lieber, R.L., Runesson, E., Einarsson, F., Fjoden, J., 2003. Inferior mechanical proper-
ties of spastic muscle bundles due to hypertrophic but compromised extra-
Purslow, P.P., 2002. The structure and functional significance of variations in the
desmin in series-fibred muscles: variations with muscle length. J. Muscle
Cell Motil. 15, 299–308.
Qing, Q., Qin, T., 2009. [Optimal method for rat skeletal muscle decellularization].
Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 23, 836–839.
Shah, S.B., Davis, J., Weisleder, N., Costanzo, S., Webb, A.D., Ralston, E.,
Street, S.F., 1983. Lateral transmission of tension in frog myofibers: a myofibrillar
network and transverse cytoskeletal connections are possible transmitters.