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Fiber Type-specific Desmin Content in Human Single Muscle Fibers

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FIBER TYPE-SPECIFIC DESMIN CONTENT IN HUMAN SINGLE MUSCLE FIBERS

by

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GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

Heidi Ghent Snyder

This thesis has been read by each member of the following graduate committee and by a majority vote has been found to be satisfactory.

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ABSTRACT

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Contractile and cytoskeletal protein concentrations have been shown to differ on the basis of fiber type in whole muscle homogenates. The purpose of this study was to compare the content of the intermediate filament protein, desmin, between type I and type IIa single muscle fibers from a mixed muscle in human subjects. Biopsies were taken from the vastus lateralis of six recreationally active males. Approximately 150 single muscle fibers were dissected from each sample and analyzed using SDS-PAGE to determine myosin heavy chain (MHC) composition. Following identification, muscle fibers were pooled into two groups (MHC I and MHC IIa). Desmin and actin content within the pooled samples was determined via immunoblotting. On average, muscle samples were composed of 51 ± 7 % type I, 2 ± 1% type I/IIa, 27 ± 5% type IIa, 19 ± 4% type IIa/IIx and 1 ± 1% type IIx MHC single fibers. Desmin and actin contents were 40% and 34% higher in type I fibers compared to type IIa fibers, respectively ($P < 0.05$).

However the desmin to actin ratio was similar between pooled type I and IIa single muscle fibers within the vastus lateralis. These data suggest that desmin and actin content is a function of muscle fiber type. These differences in cytoskeletal protein content may have implications for differences in contractile function and eccentric damage characteristics between fiber types.

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Fiber Type-specific Desmin Content in Human Single Muscle Fibers

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Abstract

Contractile and cytoskeletal protein concentrations have been shown to differ on the basis of fiber type in whole muscle homogenates. The purpose of this study was to compare the content of the intermediate filament protein, desmin, between type I and type IIa single muscle fibers from a mixed muscle in human subjects. Biopsies were taken from the vastus lateralis of six recreationally active males. Approximately 150 single muscle fibers were dissected from each sample and analyzed using SDS-PAGE to determine myosin heavy chain (MHC) composition. Following identification, muscle fibers were pooled into two groups (MHC I and MHC IIa). Desmin and actin content within the pooled samples was determined via immunoblotting. On average, muscle samples were composed of 51 ± 7 % type I, 2 ± 1% type I/IIa, 27 ± 5% type IIa, 19 ± 4% type IIa/IIx and 1 ± 1% type IIx MHC single fibers. Desmin and actin contents were 40% and 34% higher in type I fibers compared to type IIa fibers, respectively ($P < 0.05$). However the desmin to actin ratio was similar between pooled type I and IIa single muscle fibers within the vastus lateralis. These data suggest that desmin and actin content is a function of muscle fiber type. These differences in cytoskeletal protein content may have implications for differences in contractile function and eccentric damage characteristics between fiber types.

Introduction

Contractile proteins, such as actin and myosin, are the primary proteins involved in force production (11,15) while structural proteins, such as desmin, facilitate force transmission from the sarcomere and maintain the structural integrity of the muscle fiber (14, 17, 19). The expression of these proteins assist in determining the functional properties of the muscle fiber (11, 20, 22). For example, muscle fibers may be classified based on their expression of myosin heavy chain (MHC) isoform (21). The myosin heavy chain isoform expressed within a fiber is closely related to its contractile characteristics (2). MHC type I muscle fibers have a lower peak tension and slower shortening velocity compared to fibers that express MHC type II (5).

Contractile and structural protein content differs between fiber types (4, 8, 10). Total MHC content in single human muscle fibers is greater in type IIa fibers compared with type I fibers (8). The content of cytoskeletal proteins (actin, alpha-actinin and desmin) is highest in muscle homogenates from muscle groups containing primarily type I muscle fibers (10). The exact factors that determine cytoskeletal protein content in type I and II muscle fibers are unclear, but literature (13, 23) indicates that force production and fiber type recruitment patterns may contribute. For example, in desmin knockout mice architectural disruption and degeneration occur more readily in the heart, soleus and diaphragm muscles compared to the back, biceps, and quadriceps muscles (1, 16). Work by Chopard and colleagues (10) indicate that the chronic nature of the force generation in type I antigravity muscles may increase the need for enhanced cytoskeletal reinforcement in that fiber type. Desmin content in human skeletal muscle increases in response to

muscle damage associated with eccentric contraction (13) and high intensity concentric contractions (23). Our interpretation of these data is that increased muscle fiber contractile activity may increase the need for stronger cytoskeletal structures. Alternatively, the increase in desmin content may reflect an adaptation to muscle injury associated with high contractile forces.

The 52 kDa protein desmin provides structural support to a muscle cell. It links adjacent Z discs to each other and to the sarcolemma, nuclei, costameres, and mitochondria (11). Desmin is expressed in all types of muscle tissue from a single copy gene, and is one of the earliest markers to be expressed in the development of skeletal muscle (6). Desmin proteins are thought to be important in the structural integrity of the sarcomere and in force transmission (7). If desmin is important for effectively transmitting force, then we hypothesize that muscle fibers that are frequently activated (type I), should have greater desmin content than type II muscle fibers (10). The current study tested this hypothesis by characterizing the fiber type-specific expression of desmin protein in human muscle.

Methods

This study measured desmin content of a pooled sample of single type I and IIa muscle fibers from the human vastus lateralis muscle. Six recreationally active college age males were used for this study. Their age was 22 ± 3 years, weight was 82 ± 17 kg, and height was 181 ± 6 cm. Subjects were not currently participating and had not participated in any type of formal resistance and/or endurance training for the past six

months. The university human subjects institutional review board approved this study and all subjects signed an informed consent document prior to participation.

Fiber Analysis

A muscle biopsy was obtained from the vastus lateralis from each subject. Samples were cleaned of any connective tissue and immediately frozen in liquid nitrogen. Single fibers were dissected in cold dissection buffer under a light microscope. The dissection buffer contained 25 mM hepes, 4 mM EDTA, 25 mM benzamidine, 1 μM leupeptin, 1 μM pepstatin, 0.15 μM aprotinin, and 2 mM phenyl methylsulfonyl fluoride (pH 7.4). Individual fibers were homogenized in 15 μl of a homogenization buffer containing 2% SDS in 10 mmol/l Tris-HC, and 0.1 mmol/l EDTA. Following homogenization, the sample was heated for 2 minutes at 60º C, vortexed, and centrifuged. Two μl of single fiber homogenate was used to determine MHC isoform expression. The remaining sample was stored at -80º C until fiber type was determined, after which the samples were pooled on the basis of MHC composition.

MHC Analysis

Single fibers underwent sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine MHC isoform distribution as described previously (18). Briefly, 2 μl of sample were diluted with six μl of loading buffer containing 2% SDS, 125 mM Tris-HCl, (pH 6.8) 12.5% glycerol, 5% β-mercaptoethanol, and 0.005% bromphenol blue, and five microliters of this solution was loaded onto a 3% acrylamide stacking gel with a 5% acrylamide separating gel. Gels were run at 150 volts for approximately 15 hours, after which they were silver stained. MHC composition was

determined based on protein band migration patterns (18). Fibers were labeled either type I, I/IIa, I/IIx, I/IIa/IIx, IIa, IIa/IIx, or IIx. Only type I and IIa fibers from each subject underwent immunoblotting procedures for desmin and actin.

Based on single fiber MHC composition, muscle fiber homogenates were pooled into type I and type IIa samples (12). For each subject, a 5 µl aliquot from each single fiber homogenate was collected into a common MHC I or IIa sample. Total protein content was determined in triplicate for each pooled homogenate sample (BCA Protein Assay; Pierce Biotechnology, Rockford, IL). A standard curve was established to compare total protein values within each sample. Protein concentration measured (Perkin-Elmer, Victor 3 multilabel reader) at a wavelength of 562 nm.

Desmin and actin content were determined using immunoblotting as described previously (23). Two nanograms (ng) of total protein from each single fiber sample pools were diluted in a 1:1 ratio with sample loading buffer (2% SDS, 125 mM Tris-HCl, pH 6.8, 12.5% glycerol, 5% β-mercaptoethanol, 0.005% bromphenol blue) and were loaded onto a 12% polyacrylamide gel. Each gel contained a molecular weight ladder, two desmin standards, two actin standards and single fiber homogenates. Each single fiber homogenate was run in triplicate (Figure 1). The gels were run at 200 V for 40 minutes. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane at 350 mA for 30 minutes. The PVDF membrane was blocked in a 5% milk phosphate-buffered saline with Tween (PBST) solution for 1 hour, followed by 2 10-minute rinses in PBST and 2 5-minute rinses in phosphate-buffered saline (PBS). Each blot incubated overnight (16 hours) in a 1:500 dilution of primary desmin antibody (mouse anti-human, D33,

Dako, Carpinteria, CA) and a 1:500,000 dilution of actin antibody (AC-40, Sigma). Following incubation in primary antibody, blots were rinsed with PBST and PBS, followed by a one hour incubation with a 1:3000 dilution of secondary antibody (horse anti-mouse horseradish peroxidase congegate, Vector Laboratories, Burlingame, CA). Blots were rinsed with PBST and PBS and bands were detected using a chemiluminescent solution (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Desmin and actin bands were quantified with densitometry (Un Scan It version 4.3, Silk Scientific, Orem, UT). Desmin and actin sample band intensity was calculated using the mean intensity of the desmin or actin standard bands on that gel. Desmin and actin standard curves ($P<0.001$, $r^2 = 0.99$; $P<0.001$, $r^2 = 0.94$) were created to verify that desmin and actin density values fell within a linear range of values corresponding to antibody dilutions. The amount of desmin protein mass loaded on the standard curve ranged from 0.015 to 0.002 μg, and all samples fell within this range (0.014 to 0.002 μg). The amount of actin protein mass loaded ranged from 0.52 to 1.52 μg, and all samples fell within this range (0.072 to 0.80 μg).

Statistical Analysis

Desmin and actin levels were normalized for total protein content, allowing us to compare the desmin:actin ratio of type I and type IIa fibers. Based on the unidirectional hypothesis that type I fibers will have more desmin than type II fibers, a one-tailed *t*-test was used to compare the variance of desmin, actin, and desmin:actin ratios between fiber type groups. For statistical analysis, a P value of ≤ 0.05 was used to indicate significant differences. Values are reported as mean $\pm$ SEM.

Results

A total of 812 single fibers (135±2 fibers/subject) were analyzed from muscle samples taken from the vastus lateralis. MHC content analysis identified 372 type I fibers and 246 type IIa fibers. Mean fiber type distribution for each subject was 51 ± 7 % type I and 27 ± 5 % type IIa fibers (Table 1).

Total protein concentration for each pooled single fiber homogenate was 205.9 ± 33.7 µg/ml and 257.5 ± 42.3 µg/ml for type I and IIa fibers, respectively. Actin content greater in type I versus type IIa fibers (191.9 ± 31.6 ng actin/µg total protein vs. 121.0 ± 28.8 ng actin/µg total protein, type I and type IIa fibers, respectively) ($P<0.05$) (Figure 2). Mean desmin values were also greater in type I compared to type IIa fibers (5.1 ± 0.6 ng desmin/µg total protein and 2.9 ± 0.9 ng desmin/µg total protein, type I and type IIa fibers, respectively) ($P<0.05$) (Figure 3).

The desmin to actin ratio was not significantly different between type I and IIa fiber types (type I: 0.028 ± 0.002; type IIa 0.026 ± 0.006) (Table 2).

Discussion

Muscle fibers are composed of various proteins that generate and transmit force. These proteins are integral in determining the structural and functional properties of muscle fibers (3, 11, 20, 22). In some cases, skeletal muscle proteins can possess fiber type-specific characteristics (8). In rodents, previous research indicates that more desmin is found in slow twitch muscles of the hind limb (4, 10). According to the present work, we report that desmin content greater in type I versus type IIa single fibers within the vastus lateralis in healthy, recreationally active humans.

Cytoskeletal protein content has been shown to differ between slow twitch and fast twitch whole muscle homogenates in rodents (4, 10). Chopard et al. (10) homogenized whole rat soleus and extensor digitorum longus (EDL) muscles and used immunoblotting to identify protein content. Actin content was 10% higher, alpha-actinin 50% higher, and desmin 90% higher in the soleus, as compared to the extensor digitorum longus (EDL). Balogh et al. (4) dissected and homogenized whole mice soleus and psoas muscles and found similar results. Immunoblotting results measured twice as much desmin in the slow twitch soleus muscle compared with the fast twitch psoas muscle (4). The difference in cytoskeletal protein content may be due to the differences in muscle loading and demand. Since type I fibers generate force for long periods of time while maintaining sarcomeric structure, it is logical that type I fibers would have an increased need for cytoskeletal reinforcement (3, 4, 10). However recent work in humans from Chopard and colleagues disagrees with our current findings. In biopsies obtained from the gastrocnemius and soleus of volunteers prior to and following exposure to prolonged bedrest, no differences in desmin protein content were observed at baseline or following the intervention (9). It is noteworthy that the desmin content was measured in whole muscle homogenates and not in single muscle fibers.

Recruitment patterns within a muscle likely play an influential role in determining desmin protein content. Differences in desmin protein have been reported as a function of increased muscle activity (13, 23). Since increased muscle recruitment during training has been linked to increased desmin content (23), it is possible that different levels of muscle recruitment would be a significant factor in creating different levels of desmin

expression. Fibers in a mixed muscle, particularly the vastus lateralis, may have different recruitment patterns from fibers in a single type muscle. If desmin content increases with increasing fiber recruitment, then a highly recruited muscle fiber would have more desmin than a rarely recruited muscle fiber, regardless of fiber type.

Our current findings show that type I and IIa fibers within a human mixed muscle differ in desmin content. This agrees with previous data (3, 4, 10) on studies using whole muscle homogenates. Nevertheless, the large difference in actin content we report between type I and type IIa fibers is discrepant from other research (8, 9, 10). Carroll et al. (10) examined MHC and actin content in type I and IIa fibers. They reported greater amounts of MHC in type II fibers with no difference in actin content. They utilized silver staining to quantify protein amounts as compared to the immunoblotting procedures used in the current study. Chopard et al. (11) has reported that type II muscle has 10% less acting that slow tissue as measured in whole muscle homogenates from rodents. Findings may be different due to the origin of the muscle fibers used as samples in each study. Whole muscle homogenates containing predominantly one fiber type may not accurately represent data gathered from pure single fibers.

In conclusion, these data indicate that desmin and actin content is different between type I and type IIa fibers within the vastus lateralis of human subjects. However, the desmin:actin ratio does not differ on the basis of fiber type demonstrating that the protein mass relationship between the proteins is consistent. Desmin content has been shown to be influenced by contractile activity therefore it is possible that the different

recruitment patterns of type I and IIa fibers in the vastus lateralis of normal, active individuals accounts for this difference.

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Table 1. *MHC distribution*

I	I/IIa	IIa	IIa/IIx	IIx
51±7	2±1	27±5	19±4	1±1

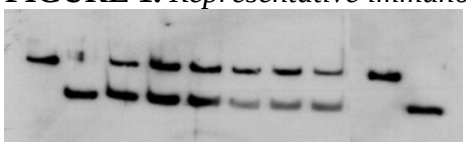
values are mean±SEM

Table 2. *Desmin to Actin ratio*

Type I	0.028±0.002
Type IIa	0.026±0.006

values are mean ± SEM

FIGURE 1. *Representative immunoblot of subject 1.*



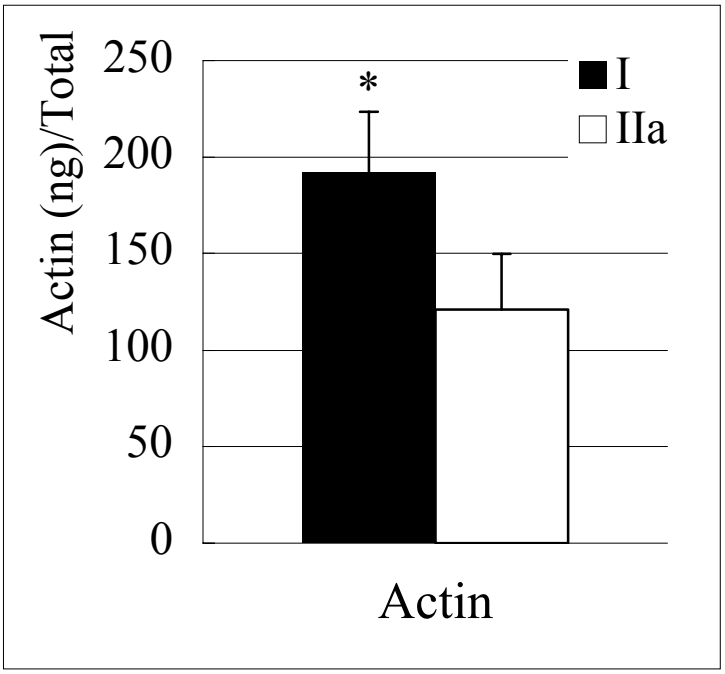


FIGURE 2. *Actin content in type I and type IIa fibers.*

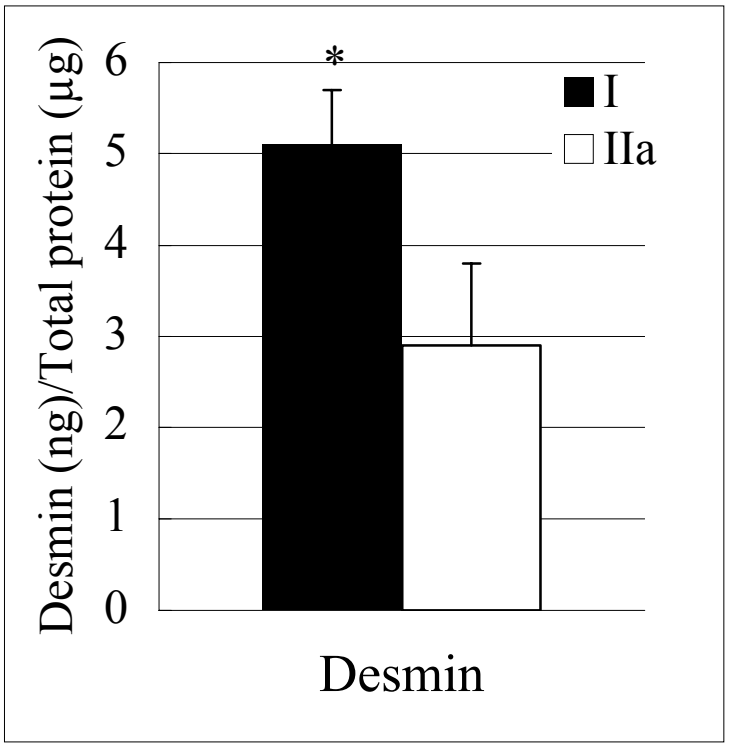


FIGURE 3. *Desmin content in type I and type IIa fibers.*

Appendix A

Prospectus

Chapter 1

Introduction

Muscle fibers are composed of proteins that allow force generation and transmission. Contractile proteins produce force, and structural proteins transmit force while maintaining the structural integrity of the fiber. Both of these proteins determine the functional properties of the muscle fiber. Muscle fibers may be categorized based on contractile protein content. The type of myosin heavy chain (MHC) expressed within a fiber defines its functional characteristics. MHC I muscle fibers have a lower peak tension and shortening velocity compared to muscle fibers with type II MHC.

Previous research has shown that contractile and structural protein content differs between fiber types. Carroll et al. (2004) examined single muscle fibers and found that MHC content is greater in type IIa fibers compared with type I fibers. Chopard et al. (2001) examined whole rat muscles and found increased levels of cytoskeletal proteins (actin, alpha-actinin and desmin) in predominantly type I fiber muscles. The differences in force and recruitment of type I and II fibers may be an influencing factor in determining cytoskeletal protein content. Since type I fibers generate force for long periods of time while maintaining architectural structure of sarcomeres, it is plausible that they would have an increased need for cytoskeletal reinforcement (Chopard, 2001).

The 52 kDa protein desmin provides structural support to a muscle cell. It links adjacent Z discs to each other and to the sarcolemma, nuclei, costameres, and mitochondria (Clark, 2002). Its three main areas of function include: 1) structural integrity of the sarcomere, 2) force transmission, and 3) possible mechanochemical

signaling (Capetanaki & Milner, 1998). Desmin is expressed in all types of muscle tissue from a single copy gene, and is one of the earliest markers to be expressed in the development of skeletal muscle (Capetanaki et al., 1984). While MHC protein content has been shown to be fiber type-specific (Carroll et al., 2004), it is unclear if cytoskeletal proteins, such as desmin, also exhibit fiber type-specific patterns.

Desmin content in human skeletal muscle increases with acute and chronic muscular activity (Feasson et al., 2002; Woolstenhulme et al., 2004;). This suggests that the cytoskeleton adapts to accommodate differences in muscle function. Furthermore, experiments using desmin knockout mice report more architectural disruption and degeneration during normal activity in muscles that are chronically recruited (type I), such as the heart, soleus and diaphragm, when compared with those that are not, such as the back, biceps, and quadriceps muscles (mixed) (Agbulut et al., 2001; Milner et al., 1996). Desmin appears to be critical to cytoskeletal integrity in frequently activated muscles.

Increased muscle fiber contractile activity may increase the need for stronger cytoskeletal structures. Frequently activated muscles such as the heart, soleus, and diaphragm are comprised primarily of type I fibers. If desmin is important for effectively transmitting force, then muscle fibers that are frequently activated (type I), should have greater desmin content than type II muscle fibers (Chopard et al., 2001). No previous studies have examined fiber type-specific characteristics related to the cytoskeleton in human skeletal muscle. Exploring fiber type characteristics as they relate to desmin

content in human skeletal muscle will provide insight into the structure-function relationships of cytoskeletal proteins in various fiber types.

Statement of Purpose

The purpose of this study is to quantify the desmin protein content difference in type I and IIa muscle fibers obtained from a mixed muscle (vastus lateralis) in human subjects.

Hypothesis

Desmin protein content will be greater in type I fibers compared to type II fibers.

Null Hypothesis

No differences will be found between desmin content in type I and type II muscle fibers.

Assumptions

1. Protein composition within a fiber varies based on fiber type-specific contractile properties and recruitment, and not based on individual differences.
2. The analysis of single fibers, taken from a small portion of the vastus lateralis, a muscle tissue, represents single fibers throughout the vastus lateralis muscle.

Definition of Terms

Recreationally active: Normal, healthy individuals who are not currently participating in a structured exercise plan. They must not be currently training and not have been training for the past 6 months.

Significance of the Study

Previous research has shown that desmin may play a role in cell signaling, force generation, and adaptation (Agbulut et al., 2001; Haubold et al., 2003; Shah et al., 2001; Shah et al., 2004). Fiber type differences in desmin content may, in part, explain differences in muscle fiber contractile properties and adaptations. An understanding of fiber type-specific protein characteristics may improve the efficacy and efficiency of interventions intended to improve whole muscle function in a variety of populations.

Chapter 2

Review of Literature

Skeletal Muscle Architecture

The unique architecture of skeletal muscle supports force production and transmission along muscle fibers to accomplish voluntary movement. Whole muscles are comprised of bundles of fibers called fascicles. Each fascicle contains multiple muscle cells or fibers. Each fiber is surrounded by a plasma membrane called the sarcolemma. Within each individual fiber are myofibrils, which are comprised of sarcomeres in series. The sarcomere is the smallest functional contractile unit, containing many myofilaments. The organization of proteins within a muscle fiber directly affects its ability to generate and transmit force. Thus, skeletal muscle structure is directly linked to its function.

Contractile, Regulatory, and Structural Proteins

Skeletal muscle is comprised of: contractile, regulatory, and structural proteins. Contractile proteins, actin and myosin, are responsible for force generation. Actin is the primary component of the thin filament, originating from the Z disc and extending towards the center of the sarcomere. Myosin, or the thick filament, contains globular heads which form crossbridges that attach to the thin filament. Regulatory proteins, tropomyosin and troponin, control the binding of myosin to actin by blocking or exposing the active binding sites. Structural proteins, such as desmin, provide and maintain an architectural scaffolding within a sarcomere to facilitate force transmission across a fiber. In the absence of desmin, skeletal muscle will not optimally transmit force between sarcomeres (Patel et al., 1997).

Differences Between Fiber Types

A myosin molecule is comprised of a pair of high molecular weight chains (MHC) attached to a globular head containing the adenosine triphosphate (ATP) and actin active sites. The MHC determines contractile properties of individual fibers. Slight variations in the MHC protein, or isoforms, produce different muscle fiber types and varied contractile characteristics. In human skeletal tissue, there are three MHC isoforms: type I, type IIa, type IIx. In addition, some fibers contain a combination of MHC isoforms and have been termed hybrid fibers.

Type I fibers generate less peak tension at a slower shortening velocity, but are more resistant to fatigue and are recruited more often than type II fibers. Type II fibers generate more tension at an increased velocity, and therefore posses greater power than type I fibers (Fitts et al., 1998). Fitts et al. reported type II soleus fibers produce 1.7 times the force of type I soleus fibers, and type II medial gastrocnemius fibers produce 8.5 times more power than type I medial gastrocnemius fibers. The differences in fiber type correspond directly with whole muscle function.

Protein Content and Fiber Type

MHC protein content varies with muscle fiber type (Carroll et al., 2004). MHC content relative to total protein is 26% greater in type IIa fibers as compared with type I fibers. There is no difference in actin between fiber types. The difference in MHC content may partially explain the functional differences in peak tension and shortening velocity between fiber types.

Cytoskeletal protein content differs between fiber types (Chopard et al., 2001).There are increased levels of cytoskeletal proteins in slow twitch (type I) whole rat muscles (Chopard et al., 2001). Actin content is 10% higher, alpha-actinin is 50% higher, and desmin is 90% higher in the soleus as compared to the extensor digitorum longus (EDL). The difference in cytoskeletal content may be due to the difference in muscle demand. Since type I fibers generate force for long periods of time while maintaining sarcomeric structure, it is likely that type I fibers would have an increased need for cytoskeletal reinforcement (Chopard et al., 2001). If cytoskeletal protein content is greater in type I fibers, then desmin content may be fiber type-specific.

Desmin Function

The 52 kDa protein desmin links adjacent Z discs to each other, the sarcolemma, nuclei, costameres, and to mitochondira (Capetanaki & Milner, 1998; Lazarides et al., 1980). Its three main areas of responsibility include: 1) structural integrity, 2) force transmission, and 3) possible mechanochemical signaling (Capetanaki & Milner, 1998; Shah et al., 2004). Desmin is present in all types of muscle tissue from a single copy gene, and is one of the earliest markers in the development of skeletal muscle (Capetanaki et al., 1984). Desmin is not required for myogenesis, but is necessary for optimal force generation. When desmin is no longer detectable immediately after eccentric exercise or not present as in myopathies, there is a significant reduction in muscle function (Agbulut et al., 2001; Balogh et al., 2002; Balogh et al., 2003; Sam et al., 2000; Shah et al., 2001).

Several studies have used desmin null mice to further determine the role of desmin within skeletal muscle. Agbulut et al. (2001) observed that muscle regeneration in desmin null mice causes many structural and functional defects. The mice had small and disorganized myofibers, decreased myofiber tension, delayed regeneration of the neuromuscular junction, and a tendency for fibers to transform to a slower phenotype. Indeed, desmin plays a critical role in proper muscle regeneration and structural integrity.

Haubold et al. (2003) measured acute and chronic endurance performance in desmin null mice, and found that during treadmill and wheel running, endurance performance was significantly impaired. In addition, the desmin null mice did not adapt to the endurance stimulus as the normal mice did; desmin null mice failed to improve running time or speed. In contrast, Balogh et al. (2003) suggested that the soleus muscles of desmin null mice have an increased resistance to fatigue. The intact soleus muscle exhibited increased resistance to fatigue during repeated short tetani, although desmin null mice generated less force. Differences between findings may be due to the differences in experimental designs *in vivo* data and *in vitro* data concerning desmin do not always support each other (Capetanaki & Milner, 1998); yet, both studies illustrate that desmin is critical for optimal muscle function.

Desmin may play a role in cellular signaling. Desmin associates with nuclei in the muscle cells (Shah et al., 2001; Shah et al., 2004). In this way, desmin creates a signaling pathway, connecting the extracellular and nuclear matrix, so "a mechanical tug on cell surface receptors can immediately change the organization of molecular assemblies in the cytoplasm and nucleus" (Maniotis et al., 1997). The contraction and relaxation of the

muscle fibers may cause desmin filaments (and/or other cytoskeletal proteins) to pull on the nuclear envelope, causing changes in gene expression (Lelievre & Bissell, 1998; Maxwell & Hendzel, 2001).

Many studies show that desmin null mice experience increased myofibrillar misalignment and a loss of force generation (Agbulut et al., 2001; Balogh et al., 2002; Balogh et al., 2003; Sam et al., 2000; Shah et al., 2001). Although desmin is not essential for sarcomerogenesis, it does regulate optimal arrangement of sarcomeres, which in turn affects overall force generation (Shah et al., 2001; Shah et al., 2002). Mice lacking desmin are able to generate muscle, but the quality of muscle produced is reduced. Desmin null muscle fibers are generally smaller, generate less force when controlled for fiber size, are less organized, and have abnormalities within mitochondria and nuclei distribution and function. These abnormalities are more pronounced in muscles that are recruited more often.

The degree to which desmin-null muscles experience functional and structural abnormalities depends on the type of muscle. In developing desmin null-mice, frequently activated tissues during normal activity, such as the heart, soleus, tongue, and diaphragm, are the most affected by the lack of desmin, displaying the most disruption in normal function (Agbulut et al., 2001; Milner et al., 1996). These muscles are comprised primarily of type I fibers. In contrast, mixed muscles such as the quadriceps, biceps and back muscles show a lesser amount of damage. There is a probable correlation between the extent of muscle use and the degree of muscle damage caused from lack of desmin (Capetanaki & Milner, 1998). If desmin is associated with frequency of recruitment, the

amount of desmin required for type I fibers may be different from the amount of desmin required for type II fibers (Chopard et al., 2001).

Desmin Adaptations to Chronic and Acute Training

Both contractile and cytoskeletal proteins adapt to chronic stimulation. In animal models using chronic low-frequency electrical stimulation, fibers adapt to the increased stimulation and shift from fast to slow MHC isoform expression (Baldi & Reiser, 1995; Brown et al., 1983; Sreter et al., 1973). In addition, there are significant changes in desmin after three weeks of chronic electrical stimulation (Baldi & Reiser, 1995). Interestingly, the changes in cytoskeletal proteins precede the transformation of contractile proteins. Absolute levels of desmin increased during the first two weeks of stimulation, after which the increase reached a plateau. Desmin increases, allowing the "force-bearing portion of the sarcomere to remain intact during increased activity" (Baldi & Reiser, 1995). The increased activity caused a need for increased intermediate filament structure. This idea supports the finding of Chopard et al. (2001) in that higher levels of desmin are associated with slower fiber types that are activated more frequently.

High-tension overload in animals causes dramatic acute changes in desmin levels within skeletal muscle. Desmin is lost in rat tissue as early as 6 hours after a single bout of 30 eccentric contractions, with maximal loss occurring at 12 hours (Barash et al., 2002). Desmin levels recover by 72 hours after eccentric contraction, and continue to increase through 168 hours. Peters et al. (2003) found similar results and reported desmin to decrease acutely in rats after a single bout of eccentric contractions, followed by an increase in desmin during the recovery period. Lieber et al. (1996) was the first to report

desmin loss after eccentric exercise. The study specifically examined desmin loss in regards to fiber type. A larger percentage of desmin was lost from fast twitch fibers as compared with slow twitch. These findings correspond with an earlier study that there is more muscle damage to fast twitch fibers after eccentric exercise (Friden & Lieber, 1992). The muscle damage consisted of disrupted Z- and A-bands, damaged intermediate filament system, and misaligned myofibrils. The increased muscle damage may correspond with the increased percentage loss of desmin.

Human studies have not been able to replicate the rapid loss of desmin after eccentric exercise seen in animals. However, desmin content does change within humans. Feasson et al. (2002) had human subjects run downhill for 30 minutes and measured desmin content before, immediately after, 1 day after, and 14 days after eccentric exercise. There was no loss in desmin immunostaining immediately following eccentric exercise, nor was there a significant increase in desmin one day after exercise. But by 14 days after eccentric exercise, desmin levels were significantly increased. Desmin appears to be involved in the remodeling response after eccentric contractions.

Yu et al. (2002) also reported changes in desmin in human skeletal muscle after eccentric exercise. Some of the muscle samples taken after a bout of eccentric exercise displayed varying degrees of desmin staining which corresponded to fiber type. Type I fibers had a weaker desmin staining than type II fibers. This finding is contrary to the findings of Lieber et al. (1996) and Friden & Lieber (1992) who found that type II fibers had more muscle damage than type I fibers. This may be due to the differences between animal and human models of eccentric exercise-induced injury.

In our lab, we have observed changes in desmin content within normal eccentric and concentric overloads. By the end of eight weeks of bicycle sprint training (high tension concentric contractions), desmin content increased by 80%, and the desmin to actin ratio increased by 66% (Woolstenhulme et al., 2004). It appears that desmin increased to reinforce the cytoskeleton, facilitating the increased force output in response to the training overload.

In a previous experiment in our lab, subjects participated in 12 weeks of either resistance or endurance training. Resistance training consisted of 3 sets of 8-10 reps at 75% of 1RM for 3 lower body exercises. Endurance training consisted of training at 70% of $VO2_{max}$ 3 times a week, with exercise time increasing from 30 minutes to 90 minutes per session. Resistance training caused a 125% increase in desmin content while endurance training had no change effect on desmin content. Actin content did not change in either group. The high-tension stimulus seems to cause changes in the cytoskeleton as demonstrated by the increase in desmin content.

As illustrated within our lab's studies, the level of tension of the stimulus appears to be a key factor in determining cytoskeletal remodeling. The role of eccentric contractions in cytoskeletal remodeling may also be important as resistance training causes a greater increase in desmin content when compared with sprint training. Overall, it is clear that cytoskeletal adaptations occur in response to normal exercise training.

Desmin is essential for optimal force generation. Desmin appears to be more critical to frequently activated muscles . In addition, desmin changes with many different

types of stimuli. In summary, cytoskeletal adaptations appear to be part of the normal adaptive process in both human and animal models.

Within human and animal studies, there is a relationship between the frequency of muscle activation and desmin content. In desmin knockout models, frequent activation leads to increased sarcomere damage. In normal human models, high-tension loads lead cytoskeletal remodeling as shown by an increase in desmin content.

Desmin content is muscle-specific in rats (Chopard et al., 2001). Within human single fibers, MHC content is fiber type-specific (Carroll et al., 2004). It is possible that additional proteins are expressed in a fiber type-specific pattern within human single fibers. Based on the literature reviewed, it is likely that the cytoskeletal protein desmin will possess fiber type-specific characteristics in human skeletal muscle. To date, there is no human data examining fiber type-specific issues related to desmin content in human muscle, and no human or animal data examining desmin content within a mixed muscle, such as the vastus lateralis. We propose to measure desmin content in human type I and IIa fibers within the vastus lateralis.

Chapter 3

Methods

This study is designed to measure desmin content in pooled type I and IIa muscle fibers. Muscle fibers will be dissected, typed, pooled and measured for total protein (Pierce Protein Assay) and desmin protein content. Fibers will be characterized based on MHC composition. Fibers expressing MHC I and IIa isoforms will undergo analyses for desmin protein content.

Subjects

Six recreationally active college age males will be recruited for this study. Subjects will not be currently participating and will not have participated in any type of formal resistance and/or endurance training for the past six months. Approval for the use of human subjects will be obtained from the university human subjects institutional review board. All subjects will understand the risks and benefits of this study prior to participation, and will sign an informed consent document.

Muscle Samples

A muscle biopsy from the vastus lateralis will be obtained from each subject. Prior to administering the biopsy, the area around the biopsy site will be shaved and cleaned with an antiseptic solution (Betadine). Two cc's of anesthetic (1% lidocaine with epinephrine) will be administered at the biopsy site. After five minutes, a small incision (1 cm) will be made through which a 5 mm biopsy needle will be inserted. Once the needle is inserted, suction is applied, the muscle sample is cut, and the needle is withdrawn. Immediately after the needle is withdrawn, direct pressure will be applied

with sterile gauze pads for about 10 minutes to prevent excess bleeding. The incision will be closed with a bandage. Muscle samples will be cleaned of any connective tissue and immediately frozen in liquid nitrogen.

Fiber Analysis

Single fibers will be dissected on ice under a light microscope in a dissection buffer containing 25 mM hepes , 4 mM EDTA, 25 mM benzamidine, 1 μM leupeptin, 1 μM pepstatin, 0.15 μM aprotinin, and 2 mM phenyl methylsulfonyl fluoride (pH 7.4) (Carroll et al., 2004). Individual fibers will be placed into 15 μl of a homogenization buffer containing 2% SDS in 10 mmol/l Tris-HC, and 0.1 mmol/l EDTA (Carroll et al., 2004). Fibers will be centrifuged, then heated for 2 minutes at 60º C, vortexed, and centrifuged again. A portion of the sample will be used to determine total protein content and MHC. The remaining sample will be stored at -80º C until fiber type is determined, after which the samples will be pooled.

MHC Analysis

Single fibers will undergo sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine MHC isoform distribution. Samples will be diluted with a buffer containing 2% SDS, 125 mM Tris-HCl, (pH 6.8) 12.5% glycerol, 5% β-mercaptoethanol, and 0.005% bromphenol blue. Approximately 2.5 μl of sample will be loaded on a 3% acrylamide stacking gel and a 5% acrylamide separating gel. Gels will be run at 150 volts for approximately 15 hours, after which they will be silver stained. MHC composition will then be determined based on the intensity of band staining, quantified using demsitometry. Fibers will be labeled either type I, I/IIa, I/IIx,

I/IIa/IIx, IIa, IIa/IIx, or IIx. Type I and IIa fibers from each subject will undergo immunoblotting procedures for desmin and actin.

Fiber Pooling

Based on single fiber MHC analysis, fibers will be pooled into type I and type IIa fiber groups (Daugaard et al., 2000). A 5 µl aliquot of the homogenate from each fiber will be collected into a common MHC I or IIa pool for each subject. Fiber pools will be stored at -80º C until total protein assay and electrophoresis.

Total Protein Assay

Total protein content will be determined for the pooled homogenate (Pierce Protein Assay). Each sample will be run in triplicate. A standard curve will be established to compare total protein values within each sample. Samples will be measured using a microplate reader at a wavelength of 560 nm.

Immunoblotting

Desmin and actin content will be determined using immunoblotting. Sample pools will be diluted in a 1:1 ratio with sample buffer (2% SDS, 125 mM Tris-HCl, (pH 6.8) 12.5% glycerol, 5% β-mercaptoethanol, and 0.005% bromphenol blue). Two ng of total protein will be loaded onto a 12% polyacrylamide gel. Lane 1 will contain 5 µl of a molecular weight ladder, lane 2 will contain 0.02 µg of desmin standard, and lane 3 will contain 0.4 µg of actin standard. Lanes 4-12 will contain pooled fiber samples. Each sample will be run in triplicate. The gels will run at 200 V for 40 minutes. The proteins will then be transferred to a polyvinylidene difluoride membrane (PVDF) at 350 mA for 30 minutes. The PVDF membrane will rock in a 5% milk PBST solution for 1 hour,

followed by 2 10-minute rinses of PBST and 2 5-minute rinses of PBS. Blots will then incubate overnight (16 hours) in the primary antibody (mouse anti-human) containing 1:500 anti-desmin and 1:500,000 anti-actin dilutions. The next morning, the blots will be rinsed with PBST and PBS as previously described, followed by one hour of incubation with the secondary antibody (horse anti-mouse horseradish peroxidase). Blots will be rinsed again with PBST and PBS and taken to a dark room for developing. The autoradiography procedure entails exposing each blot to a chemiluminescent solution (ECL, Amersham) for one minute, after which blots will be exposed to X-ray film for 5 minutes and sent through a developer.

Desmin and actin bands will be quantified with densitometry (Un Scan It computer software, Silk Scientific Corporation, Orem, UT). Desmin and actin standard curves will be created to compare single fiber desmin and actin values to a known standard. The band intensity will be normalized for each protein on the same blot so that percent change in density can be calculated.

Statistical Analysis

A *t* test will be used to compare the variance of desmin content between fiber type groups. The independent variable will be fiber type groups (type I and type IIa), and the dependent variable will be desmin content. For statistical analysis, a p value of < 0.05 will be used to indicate significant differences.

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Appendix B

Additional Results

Fiber type distribution (%)

subject	I	I/IIa	IIa	IIa/IIx	IIx
1	27	0	39	32	2
2	71	5	13	9	2
3	47	2	33	17	2
4	57	0	23	20	0
5	39	1	30	25	4
6	32	2	46	19	2
mean	51	2	27	19	1
sd	18	2	11	10	1
se	7	1	5	4	0

Desmin/Actin Ratio

subject	I	IIa
1	0.030	0.047
2	0.026	0.015
3	0.033	0.035
4	0.015	0.008
5	0.032	0.024
6	0.031	0.031
mean	0.028	0.027
sd	0.007	0.014
se	0.003	0.006