
ABSTRACT: The mechanical properties of isolated single muscle fiber segments were measured in muscle cells obtained from patients undergoing surgery for correction of flexion contractures secondary to static perinatal encephalopathy (cerebral palsy). "Normal" muscle cells from patients with intact neuromuscular function were also mechanically tested. Fiber segments taken from subjects with spasticity developed passive tension at significantly shorter sarcomere lengths ($1.84 \pm 0.05 \mu\text{m}$, $n = 15$) than fibers taken from normal subjects ($2.20 \pm 0.04 \mu\text{m}$, $n = 35$). Elastic modulus of the stress-strain relationship in fibers from patients with spasticity (55.00 ± 6.61 kPa) was almost double that measured in normal fibers (28.25 ± 3.31 kPa). The fact that these muscle cells from patients with spasticity have a shorter resting sarcomere length and increased modulus compared with normal muscle cells suggests dramatic remodeling of intracellular or extracellular muscle structural components such as titin and collagen. Such changes in muscles of patients with spasticity may have implications for therapy.

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SPASTIC MUSCLE CELLS ARE SHORTER AND STIFFER THAN NORMAL CELLS

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The treatment of patients with spasticity represents a tremendous clinical challenge. These patients suffer from upper motor neuron lesions due to diverse disorders such as static perinatal encephalopathy (cerebral palsy), stroke, degenerative diseases, head injury, and spinal cord injury.^{3,4,8} They present with increased tone, clonus, joint contractures, muscle weakness, and loss of dexterity. Patient management options range from conservative treatment consisting of splinting, physiotherapy, and electrical stimulation³⁰ to surgical intervention consisting of releases, nerve blocks, tendon transfers, and muscle-tendon unit lengthening.^{9,25}

It is not clear why muscles become spastic, but there are data suggesting that spastic muscles are not normal. For example, based on histochemical analysis of biopsies from children with cerebral palsy,

Rose et al. documented increased variability in muscle fiber size and a fiber type distribution biased toward type I fibers, suggesting an altered use pattern and altered myosin heavy chain expression.²¹ Additionally, recent experiments demonstrated that reflex stiffness of ankle extensors from patients with a spastic hemiparesis was normal, but that the muscle itself was intrinsically stiffer, suggesting a change in intramuscular or extracellular supporting structures.^{22,23} Finally, Tardieu and colleagues have suggested that muscle fiber length in children with cerebral palsy is shorter than normal, which would tend to increase passive stiffness at large joint angles.²⁷ The literature is not without controversy in that Mirbagheri et al. have shown increased intrinsic stiffness as well as increased reflex stiffness in the plantarflexors from patients with spinal cord injury and spasticity.¹⁷ Thus, there is no clear consensus regarding whether muscles or muscle cells from patients with spasticity have normal properties. This is due in part to the fact that there remains a paucity of objective data regarding the mechanical, physiological, or biochemical properties of spastic muscle. Such information is important for developing rational treatment programs for these patients.

We recently reported dramatic architectural changes in the flexor carpi ulnaris (FCU) muscles of

See accompanying editorial on page 131.

Abbreviations: ANOVA, analysis of variance; ATP, adenosine triphosphate; DTT, dithiothreitol; FCU, flexor carpi ulnaris; EGTA, ethylene-glycol tetraacetic acid; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

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patients with wrist flexion contractures secondary to cerebral palsy. Surprisingly, these greatly shortened FCU muscles had extremely long sarcomere lengths.¹² Based on a biomechanical analysis of FCU sarcomere length and wrist joint kinematics, we concluded that spastic muscle fibers had a normal fiber length even though the spastic muscles were shorter than normal. This provided the explanation for the long sarcomere lengths—in order for shorter muscles (less fibers in series) to span from origin to insertion, the fibers themselves were greatly stretched. These greatly stretched fibers with extremely long sarcomere lengths raise significant questions regarding the properties of the spastic muscle cells themselves: Are fibers from the muscles of patients with spasticity (i.e., “spastic fibers”) much *less* stiff than normal so that, even at very long sarcomere lengths, their passive tension is relatively normal, or are spastic fibers *more* stiff than normal, in which case long sarcomere lengths would present a tremendous passive mechanical resistance? Although there is evidence for increased whole-muscle stiffness in spasticity secondary to multiple sclerosis²³ and cerebral palsy,²⁷ there are no data available that describe the elastic properties of human muscle at the cellular level. Therefore, the purpose of this study was to define the elastic properties of human muscle cells based on mechanical measurements from segments of single cells obtained from both normal subjects and patients with spasticity.

MATERIALS AND METHODS

Patient Characteristics. All patients included in this study provided informed consent for muscle biopsies that were obtained secondary to planned surgical procedures. All procedures were performed with the full approval of the human ethics committee at Göteborg University as well as the committee on the use of human subjects in research at the University of California, San Diego and VA Medical Centers. Biopsies were obtained from human subjects in two experimental groups: those with normally innervated muscles and no upper motor neuron lesion (“normal”) and those with spasticity (“spastic”). Biopsies from normal patients were obtained from muscles that were exposed incidental to another procedure; for example, FCU biopsy during repair of an ulnar fracture. Spastic muscle fibers were obtained from patients with cerebral palsy in conjunction with elective tendon transfer procedures of the upper extremity (Table 1). In total, 41 biopsies from normal subjects and 15 biopsies from subjects with spasticity were obtained.

Table 1. Experimental subject characteristics.

Patient classification	Age (years)	Muscle studied
Cerebral palsy	7.8 ± 1.3	Flexor carpi ulnaris (n=6) Pronator teres (n=2) Brachioradialis (n=2) Digital flexor (n=1) Thumb adductor (n=1)
Normal controls	37.4 ± 4.1	Flexor carpi ulnaris (n=1) Pronator teres (n=1) Brachioradialis (n=2) Digital flexor (n=9) Digital extensor (n=9) Thumb extensor (n=6) Wrist extensor (n=6) Other (n=7)

Sample Preparation. After excision, biopsies were immediately placed in a muscle-relaxing solution composed of (in millimoles per liter): ethylene-glycol tetraacetic acid (EGTA), 7.5; potassium propionate, 170; magnesium acetate, 2; imidazole, 5; creatine phosphate, 10; adenosine triphosphate (ATP), 4; leupeptin, a protease inhibitor, 17 mg/ml; and E64, a protease inhibitor, 4 mg/ml. This solution prevented depolarization across any site of disrupted membrane and proteolytic degradation, which would destroy the specimen. Special care was taken at the time of biopsy to hold only a small region of one of side of the specimen, which was then dropped into relaxing solution without additional handling. Biopsy size was typically a cylinder with dimensions 10–15 mm in length × 2 mm in diameter and consisted of ~1500 fibers, at least half of which were completely intact. Muscle fibers were either immediately dissected from the fresh biopsy (9 of 41 normal and 2 of 15 spastic) or, in most cases, placed into a storage solution composed of relaxing solution mixed with 50% glycerol and stored at 0°C. Fibers stored in this manner have been shown to have stable active mechanical properties for up to 3 months,^{18,31} but the fibers in this study were not stored for longer than 21 days. Stored fibers showed no signs of deterioration such as alterations in translucency or structural abnormalities.

Single muscle fiber segments were dissected from biopsies in chilled relaxing solution under 40× magnification (Leica MZ8, Heerbrugg, Switzerland) with epi-illumination (Model DCR II, Fostec, Auburn, NY) using microsurgical forceps (S&T P-00019, Neuhausen, Switzerland). Fibers were selected from the biopsy to obtain a segment free of obvious defects that maintained a normal translucent appearance, but were not selected with any other intentional bias.

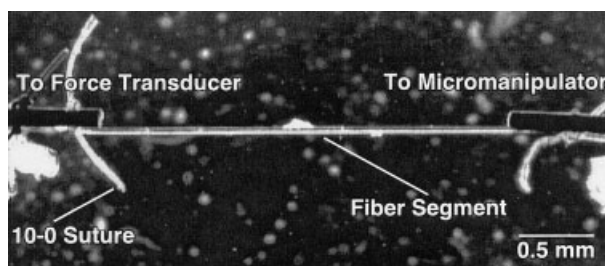


FIGURE 1. Single skeletal muscle cell segment secured to 125- μm titanium wires on either side, which are mounted to a force transducer and micromanipulator.

No information regarding the location of the fiber segment within the biopsy nor within the muscle of origin was obtained.

Mechanical Testing Protocol. The testing protocol was designed to measure the muscle fiber's elastic properties apart from any velocity-dependent properties.⁵ The dissected single fiber segment was secured on either side to 125- μm titanium wires using 10-0 silk suture loops. One wire was secured to an ultrasensitive force transducer (Model 405, sensitivity 10 V/g, Aurora Scientific, Ontario, Canada) and the other was secured to a micromanipulator (Fig. 1). The fiber was transilluminated by a 7-mW He-Ne laser to permit sarcomere length measurement by laser diffraction.¹³ The appearance of a clear diffraction pattern with well-defined diffraction orders of nearly equal intensity indicated that the biopsy and dissection procedures did not disrupt the cellular myofibrillar lattice. Sarcomere lengths were calculated based on the distances between both the \pm first- and \pm second-order diffraction lines to provide two sarcomere lengths at each deformation. Resolution of this method is approximately 5 nm.¹ The system was calibrated with 1.66- μm , 2.50- μm , and 3.33- μm plastic blazed diffraction gratings prior to experimentation (Diffraction Gratings, Inc., Nashville, TN). At the conclusion of the experiment, the gratings were again placed at the level of the fiber in the chamber and the absolute value of the percent error between predicted and measured diffraction spacing was calculated. Mean absolute error for the 60 experiments reported here was $5.11 \pm 2.84\%$ (mean \pm standard deviation).

The mounted fiber segment was examined via a dissecting microscope (Model MZ95, Leica, Heerbrugg, Switzerland) under 20 \times magnification, which permitted measuring of fiber diameter to within 5 μm and the setting of slack length. The fiber segment was set to "slack length," which was defined as the length where: (1) the diffraction pat-

tern became oriented along the fiber axis; (2) tension remained within ± 1 standard deviation of the noise level of the transducer (10 mV, equivalent to 0.5 mg of tension); and (3) the fiber did not have a curved appearance in any plane. In practice, this was easily reproducible. Repeated setting of slack sarcomere length on a pilot study of normal fibers revealed a coefficient of variation of less than 1% (mean \pm SD: $0.67 \pm 0.16\%$; $n = 5$ repeated measures on three different muscle fibers), indicating reliability of the method.

Mounted fibers were then lengthened in 250- μm increments after which stress-relaxation was permitted for 2 min and both sarcomere length and tension were again recorded. This amount of time has been shown to permit tension to fall to within $\sim 10\%$ of baseline values⁵ so that tension levels reflected elastic as opposed to viscoelastic mechanical properties. Segments were elongated until mechanical failure. The sarcomere length recorded prior to the lengthening that resulted in failure was defined as peak sarcomere length. Fibers were discarded if they did not produce a clear diffraction pattern, if any irregularities appeared along their length, or if they were severed or slipped at either suture attachment point during the test.

After mechanical testing, fibers were transferred into microfuge tubes and suspended in 10 μl of sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer consisting of dithiothreitol (DTT; 100 mmol/L), SDS (2%), Tris-base (80 mmol/L) at pH 6.8, glycerol (10%), and bromophenol blue (0.012% w/v). Samples were boiled (2 min) and stored at -80°C for up to 3 weeks prior to loading onto gels to determine myosin heavy chain composition as previously described.^{14,26}

Data Analysis. Muscle fiber area and force were used to calculate fiber stress for each mechanical experiment performed. Sarcomere length-stress relationships were usually linear for normal fibers and usually nonlinear (resembling exponential or sigmoidal functions) for spastic fibers. Fiber modulus was calculated as the slope of the fiber's stress-strain curve according to:

$$E_f = \frac{\Delta\sigma_f}{\Delta\varepsilon_f} = \frac{(\sigma_{\max} - \sigma_{\min})}{\left(\frac{\text{SL}_{\max} - \text{SL}_{\min}}{\text{SL}_{\min}}\right)}$$

where SL_{\min} and SL_{\max} represent the minimum and maximum limits of the linear region of the

sarcomere length–stress relationship (see later), σ_{\min} and σ_{\max} represent the calculated stresses at these respective sarcomere lengths, $\Delta\sigma_f$ represents the change in fiber stress, $\Delta\epsilon_f$ represents the change in fiber strain over this linear region, and E_f represents fiber elastic modulus. Linear regression was applied to stress–strain curves over the entire deformation range when the curve was linear, or to a restricted range when the curve was nonlinear. In all cases, the restricted range (i.e., definition of SL_{\min} and SL_{\max}) was chosen, which had a linear coefficient of determination (r^2) of ~ 0.9 and a length of at least $1 \mu\text{m}$. In cases where more than one linear region could be defined, the modulus was measured over the first portion of the fiber deformation. In practice, such decisions were limited to a few spastic fibers with sarcomere length–stress relationships that had a sigmoidal shape (e.g., see Fig. 2C). Tangent modulus was measured over a sarcomere length range of $2.54 \pm 0.22 \mu\text{m}$ for normal fibers and $1.57 \pm 0.28 \mu\text{m}$ for spastic fibers, representing a substantial portion of the entire relationship (see examples in Results section), so that local variations in these curves did not affect the results presented.

Values for parameters measured were screened for normality to justify the use of parametric statistics. Then, mean values were compared between groups by one-way analysis of variance (ANOVA). Post hoc comparisons were not necessary as the study was composed of only two groups. Data are expressed as mean \pm SEM except where previously noted.

RESULTS

A total of 38 normal muscle fibers, from 8 different muscle groups, were successfully tested as well as 15 muscle fibers from 5 different muscle groups with spasticity (Table 1). For the normal muscle fibers tested, the majority (22 of 38, or 58%; Fig. 2A) of sarcomere length–stress graphs were highly linear ($r^2 = 0.966 \pm 0.005$) and modulus was unambiguously obtained by linear regression over the entire range of sarcomere lengths tested. The next most common graph shape for normal fibers was exponential (11 of 38, or 29%; Fig. 2B) and modulus was calculated as a tangent modulus as described earlier. In contrast, for spastic fibers, the predominant shapes of the sarcomere length–stress graphs were sigmoidal (6 of 15, or 40%; Fig. 2C) or exponential (6 of 15, or 40%; Fig. 2B). Only 20% (3 of 15) of the graphs from spastic fibers were linear (Fig. 2A). As a result of the shape differences between experimen-

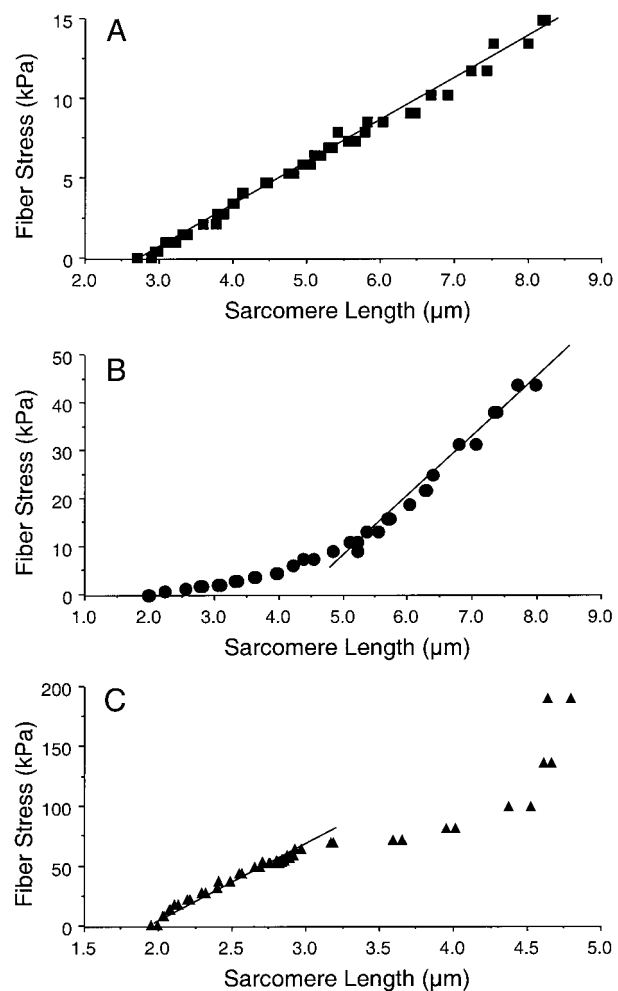


FIGURE 2. Examples of the types of sarcomere length–stress curves that were obtained for this study. **(A)** A linear curve from a normal human flexor carpi ulnaris muscle. **(B)** An exponential curve from a spastic pronator teres muscle. **(C)** A sigmoidal curve from a spastic flexor carpi ulnaris muscle.

tal groups, tangent modulus was calculated over a significantly longer sarcomere length range for normal fibers compared with spastic fibers ($P < 0.02$). The absolute sarcomere length range for calculation of modulus for normal fibers was from sarcomere lengths $2.90 \pm 0.13 \mu\text{m}$ to $5.44 \pm 0.25 \mu\text{m}$, whereas for spastic fibers it was 2.55 ± 0.35 to $4.12 \pm 0.53 \mu\text{m}$, demonstrating that spastic fiber data systematically represent shorter sarcomere lengths. This has the effect of slightly biasing the spastic data toward lower stress values.

The most dramatic effect observed between groups was that fiber segments taken from subjects with spasticity developed passive tension at significantly shorter sarcomere lengths than fibers from normal subjects. Normal fibers had a resting sarcomere length of $2.20 \pm 0.04 \mu\text{m}$ (Fig. 3A), whereas

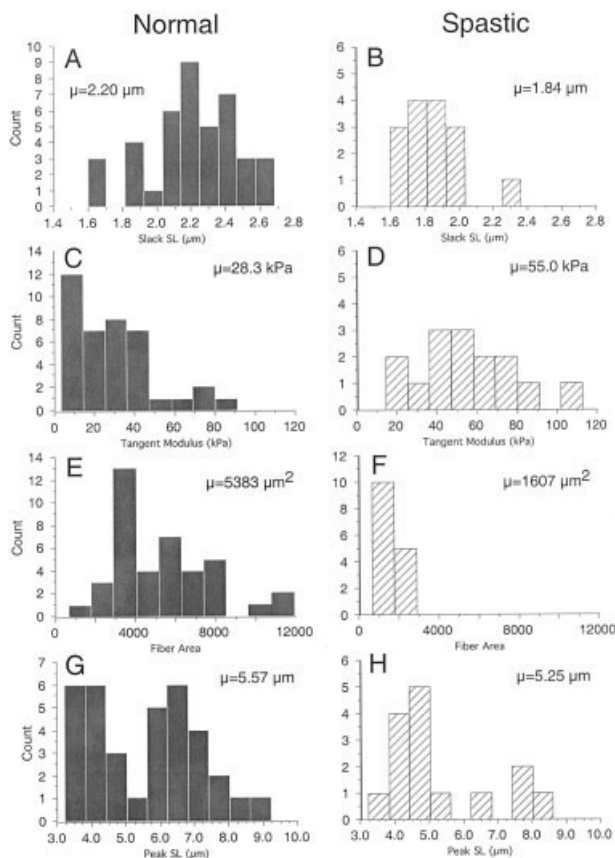


FIGURE 3. Histograms of important parameters measured in this study from normal muscle fibers (left panels, solid bars) and muscle fibers obtained from patients with spasticity (right panels, hatched bars). (A,B) Resting sarcomere length. (C,D) Muscle fiber tangent elastic modulus. (E,F) Muscle fiber cross-sectional area. (G,H) Sarcomere length at fiber failure ($n = 32\text{--}38$ for the normal group and $n = 13\text{--}15$ for the spastic group).

spastic fibers were about 20% shorter with an average resting sarcomere length of only $1.84 \pm 0.05 \mu\text{m}$ (Fig. 3B; $P < 0.001$). The distribution of these sarcomere lengths was roughly normal, suggesting that subpopulations of different fibers did not exist with respect to resting length.

The tangent modulus of the sarcomere length–stress relationship in spastic fibers ($55.00 \pm 6.61 \text{ kPa}$; Fig. 3D) was almost double that measured in normal fibers ($28.25 \pm 3.31 \text{ kPa}$; $P < 0.001$; Fig. 3C). Although the distribution of tangent modulus was nearly normal for spastic fibers, normal fibers showed a tendency toward skewing to smaller values. There was no significant correlation between fiber size and tangent modulus for either the normal ($P > 0.2$) or spastic fibers ($P > 0.2$) in spite of the fact that the spastic fibers ($1606 \pm 156 \mu\text{m}^2$; Fig. 3F) were less than one third the size of normal fibers ($5383 \pm 392 \mu\text{m}^2$; $P < 0.001$; Fig. 3E). These results indicate that the mechanical properties measured actually reflect

the cellular material properties and were not an artifact of swelling, which is known to occur in skinned muscle cells.^{6,16}

The failure properties between normal and spastic fibers were elucidated based on interpretation of two parameters. First, peak sarcomere length, that is, the sarcomere length at which muscle fibers failed, was not significantly different between groups ($5.57 \pm 0.26 \mu\text{m}$ for normal, $5.25 \pm 0.40 \mu\text{m}$ for spastic; $P > 0.5$). Interestingly, the distribution of peak sarcomere length appeared to be bimodal for both groups, suggesting either two different failure mechanisms or distinct subpopulations of muscle cells tested (Fig. 3G,H). Second, failure stress for spastic fibers ($73.88 \pm 16.04 \text{ kPa}$) was approximately three times greater than failure for normal muscle cells ($26.15 \pm 2.43 \text{ kPa}$; $P < 0.0001$) and, approximately, normally distributed.

Myosin heavy chain content was obtained for 9 of 15 of the spastic fibers and 12 of 25 of the normal fibers. As has been observed by others, no type 2B myosin heavy chain was expressed in human muscle cells.^{24,29} Both spastic and normal fibers expressed all three adult myosin heavy chain isoforms (types 1, 2A, and 2X; Fig. 4). Co-expression of types 2A and 2X was observed for both normal and spastic fibers, whereas co-expression of types 1 and 2A was observed only in normal fibers. However, because the number of fibers sampled was small, we would not claim that these expression combinations represent spastic muscle fibers in general.

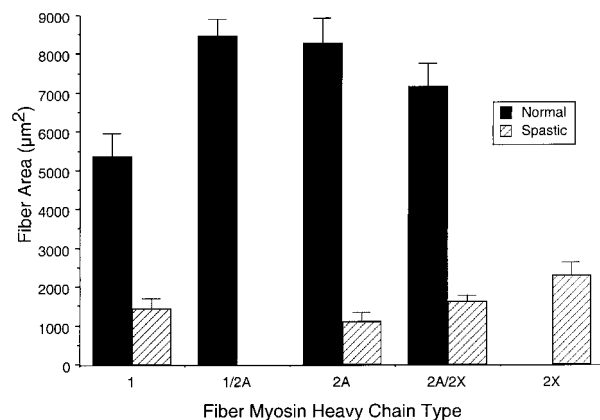


FIGURE 4. Myosin heavy chain content of single muscle cells from normal (filled bars) or spastic (hatched bars) experimental groups. Myosin heavy chain type is shown on the abscissa whereas fiber size is plotted on the ordinate. Note that several fibers coexpressed two isoforms. When two isoforms are listed, the first isoform listed is the predominant isoform ($n = 12$ for normal group and $n = 9$ for spastic group).

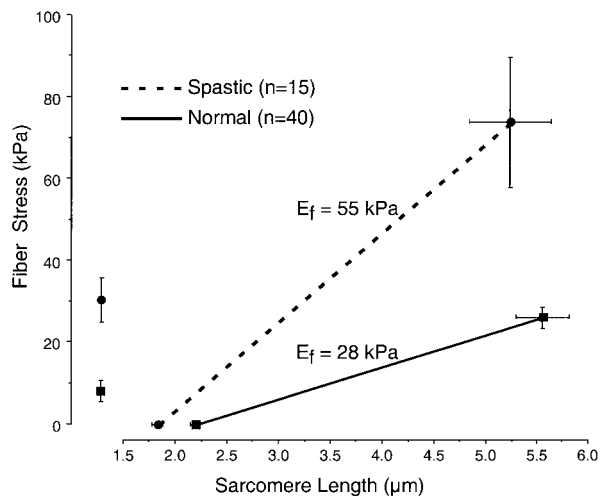


FIGURE 5. Graphic summary of differences between normal fibers and fibers from patients with spasticity based on the current experimental data. Normal fibers have a resting sarcomere length of 2.2 μm , an ultimate stress of 26 kPa, and an elastic modulus of 28 kPa (squares and solid line), whereas fibers from patients with spasticity have a resting sarcomere length of 1.8 μm , an ultimate stress of 74 kPa, and a modulus of 55 kPa (circles and dotted line). Isolated symbols to left of figure reveal *in vivo* stress calculated at a sarcomere length of 3.5 μm . ($n = 35$ for normal group and $n = 15$ for spastic group). Abbreviation: E_f , fiber elastic modulus.

DISCUSSION

We found that muscle cells obtained from patients with spasticity had a decreased resting sarcomere length and nearly double the elastic modulus compared with normal muscle cells, as shown schematically in summary form in Figure 5. The structural basis for these observations is unknown, but the results clearly suggest that those muscle structures involved in passive load bearing and setting of resting sarcomere length are altered in cells obtained from patients with spasticity.

The most obvious candidate structural protein for the observed effects is the giant intramuscular titin molecule (also called connectin) that connects the myosin filament to the Z-disk.⁷ Variations in titin isoforms have been shown to relate to variations in muscle stiffness, especially between cardiac and skeletal muscle.¹⁰ To our knowledge, these data represent the first report of a change in resting sarcomere length in skeletal muscle under conditions of altered use. Whether the observed changes can be attributed to variations in titin isoform, titin concentration, or other structural components remains to be determined.

A second source of passive elasticity in muscle is the extracellular matrix protein, collagen. Even though single cell segments were tested, significant

extracellular matrix proteins are present in this preparation. The most abundant extracellular structural protein is collagen. Skeletal muscle is endowed with a densely arranged collagen network around fibers and bundles of fibers that appear to play a significant role in force transmission between muscle fibers and the external tendon.^{20,28} In frog muscle, the passive elasticity of the titin intracellular filament network dominates this extracellular component.¹⁵ Analogous data are not yet available for mammalian muscle. However, based on our recent intraoperative measurements in the FCU muscle of patients with spasticity,¹² we argue against significant load bearing in spastic muscle by extracellular components. The argument proceeds as follows: sarcomere length with the wrist fully flexed in spastic FCUs measures $3.48 \pm 0.44 \mu\text{m}$ ($n = 6$).¹² Average cellular stress from the 15 spastic fibers measured in this study at a sarcomere length of $\sim 3.5 \mu\text{m}$ was determined from the raw data to be $30.1 \pm 5.2 \text{ kPa}$ (Fig. 5). Given the physiological cross-sectional area of the FCU of 3.42 cm^2 ,¹¹ an FCU from a patient with spasticity would be predicted to bear a load of $\sim 10 \text{ N}$ (i.e., $30.1 \text{ kPa} \times 3.42 \text{ cm}^2$). This force level is consistent with recent preliminary intraoperative force measurements (Fridén and Lieber, unpublished observations) suggesting that intracellular load-bearing is sufficient to account for the passive mechanical forces in whole FCU muscle. Detailed biomechanical analyses are pending.

A challenging aspect of this study is attempting to understand the nature of the significant structural and mechanical changes observed. Clearly, there is precedent in the literature for disuse-induced atrophy of skeletal muscle² and even overuse-induced atrophy of skeletal muscle, when cells are electrically stimulated chronically.¹⁹ Thus, the fact that muscle cells from patients with spasticity are severely atrophic (Fig. 3E,F) does not provide support for either an increased- or decreased-use model. However, almost without exception, when shifts in use patterns occur, the myosin heavy chain profile of the muscle cells shifts as well—chronic disuse leading toward a shift toward faster isoforms and chronic increased-use leading toward a shift toward slower isoforms. Based on the limited analysis of the fibers studied here, we cannot provide strong support for either altered-use level. Although we did observe smaller slow muscle fibers compared with fast fibers for normal muscle cells (Fig. 4, filled bars), no such pattern was observed for spastic muscle fibers (Fig. 4, hatched bars). It appears that the spastic adaptive response does not follow the simple adaptive response patterns demonstrated for normal muscles

under the control of an intact nervous system. The upper motor neuron lesion seems to leave the spastic muscle in a “confused” state in which the normal matching between fiber size and type is lost. Therefore, at this point, it is inappropriate to classify spasticity as either an increased- or decreased-use model. Finally, with respect to surgical intervention, these mechanical data demonstrate that a surgical release or Z-lengthening procedure applied to a spastic skeletal muscle will result in a muscle that would shorten considerably and even to lengths shorter than normal. The usual assumption that tendon lengthening will permit the muscle in spastic patients to retract to “normal” muscle lengths must therefore be challenged based on the results of the current study. If the spastic muscle is to be transferred, the surgeon must appreciate that greater forces will be required to stretch the muscle to a sarcomere length comparable to that of a normal transferred muscle in the same joint configuration. Studies are now underway to determine the extent of force required to produce physiological sarcomere lengths as well as the nature of the muscular adaptation observed after tendon transfer or surgical lengthening.

Several cautionary points must be made with regard to interpretation of these data. First and foremost, the spastic data were obtained primarily from FCU muscles whereas the normal data were obtained across a wider range of muscles (Table 1). Thus, the spastic fibers disproportionately represent the FCU. The small-sample statistical analysis did not show a significant difference between the spastic FCU and other spastic muscles ($P > 0.4$) nor between the normal FCU and other normal muscles ($P > 0.5$). Additionally, the spastic patients were much younger (~8 years old) compared with normal subjects (~37 years old; Table 1). One might be concerned that the effects reported here represent an age effect rather than a consequence of the spasticity. This concern can be addressed if we are able to provide age- and muscle-matched control specimens to make a direct comparison of the same muscles from same-age individuals. Finally, the experimental data reported here are from upper extremity muscles, whereas significant clinical endeavor has been devoted to treating spasticity in the lower extremity. Based on our lack of data from lower extremity muscles, extrapolation of these results to the lower extremity must be made with caution.

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