Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis

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Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. J Appl Physiol 104: 1736–1742, 2008. First published April 24, 2008; doi:10.1152/japplphysiol.01215.2007.—A present debate in muscle biology is whether myonuclear addition is required during skeletal muscle hypertrophy. We utilized K-means cluster analysis to classify 66 humans after 16 wk of knee extensor resistance training as extreme (Xtr, n = 17), modest (Mod, n = 32), or nonresponders (Non, n = 17) based on myofiber hypertrophy, which averaged 58, 28, and 0%, respectively (Bamman MM, Petrella JK, Kim JS, Mayhew DL, Cross JM. J Appl Physiol 102: 2233–2239, 2007). We hypothesized that robust hypertrophy seen in Xtr was driven by superior satellite cell (SC) activation and myonuclear addition. Vastus lateralis biopsies were obtained at baseline and week 16. SCs were identified immuno histochemically by surface expression of neural cell adhesion molecule. At baseline, myofiber size did not differ among clusters; however, the SC population was greater in Xtr (P < 0.01) than both Mod and Non, suggesting superior basal myogenic potential. SC number increased robustly during training in Xtr only (117%; P < 0.001). Myonuclear addition occurred in Mod (9%; P < 0.05) and was most effectively accomplished in Xtr (26%; P < 0.001). After training, Xtr had more myonuclei per fiber than Non (23%; P < 0.05) and tended to have more than Mod (19%; P = 0.056). Both Xtr and Mod expanded the myonuclear domain to meet (Mod) or exceed (Xtr) 2,000 μm² per nucleus, possibly driving demand for myonuclear addition to support myofiber expansion. These findings strongly suggest myonuclear addition via SC recruitment may be required to achieve substantial myofiber hypertrophy in humans. Individuals with a greater basal presence of SCs demonstrated, with training, a remarkable ability to expand the SC pool, incorporate new nuclei, and achieve robust growth.

strength training; skeletal muscle; neural cell adhesion molecule; myonucleus; myogenesis

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METHODS

Subjects. Sixty-six adults were recruited from the Birmingham, Alabama metropolitan area into two age groups. Inclusion into the study was based on age ranges of 60–75 yr for the older adults and 20–35 yr for the younger group. All participants were screened by health history questionnaire, and older subjects also passed a physical examination and graded exercise stress test. Subjects were not obese (body mass index <30 kg/m²) and free of any musculoskeletal or other disorders that might have affected their ability to complete testing and/or resistance training. Subjects had not experienced any leg resistance training in the previous 5 yr before initiating the study protocol. None of the subjects were being treated with exogenous testosterone or other pharmacological interventions known to influence muscle mass. The project was approved by the Institutional Review Boards of both the University of Alabama at Birmingham, Birmingham, Alabama (UAB), and the Birmingham Veterans Affairs Medical Center, Birmingham, Alabama. Written informed consent was obtained from each volunteer before participation.

Resistance training results based on the independent variables of age and sex have been previously published on 49 (20) and 52 (25) of these 66 subjects, and our laboratory introduced the concept of K-means cluster analysis with a 2-cluster model on n = 52 subjects in this earlier work (25). After expanding the sample to 66 subjects, we clustered subjects, independent of age and sex, into three response clusters based on changes in vastus lateralis mean myofiber cross-sectional area (MFA; μm²): Xtr (n = 17), Mod (n = 32), and Non (n = 17). We recently published detailed characteristics of each response cluster including training program adherence, intensity, and volume (none of which differed by cluster) (4), along with cluster differences in myogenic gene expression (4, 19). In our experience, the cluster model has provided an extremely valuable tool for examining potential mechanisms underlying human intervention responsiveness in an unbiased manner.

Resistance training. Progressive resistance training of the knee extensors occurred 3 days/wk for 16 wk. Subjects warmed up on a cycle ergometer or treadmill for ~5 min or until warm (light sweat) before each training session. Training consisted of three exercises that included knee extension, leg press, and squats. Each exercise was performed for 3 sets of 8–12 repetitions using resistance exercise stations or plate loaded stations (barbell squats and linear 45° leg press). Rest between sets was standardized at 90 s. Initial training loads were based on 80% of baseline one-repetition maximum (1 RM) strength and progressed to maintain 8- to 12-RM loads.

Muscle biopsy and tissue preparation procedures. Percutaneous needle muscle biopsies were performed under local anesthesia (1% lidocaine) by a surgeon in the Pittman General Clinical Research Center at UAB. Muscle samples were collected from the vastus lateralis muscle with established techniques using a 5-mm Bergstrom biopsy needle under suction (11). Biopsies were taken from the left leg at baseline and 24 h after the final training session. At the bedside, visible connective and adipose tissues were removed with the aid of a dissecting microscope, and portions used for immunohistochemistry were mounted cross-sectionally on cork in optimum cutting temperature mounting medium mixed with tragacanth gum, and frozen in liquid nitrogen-cooled isopentane. All samples were stored at −80°C.

Myonuclear and myofiber type quantification. Myonuclear and myofiber type quantification was performed post hoc to localize the effect(s). Statistical significance measures ANOVA. For each ANOVA model with a significant main effect, post hoc comparisons were performed using Tukey’s honestly significant difference tests, and results were considered statistically significant at P < 0.05 for all tests.

RESULTS

Phenotypic and resistance training program characteristics of the three response clusters were reported previously (4). Briefly, the clusters did not differ in pretraining type I or type II muscle fiber distributions. Quantitation of muscle stem cells and myonuclei. Muscle-specific stem (i.e., satellite) cells were identified by cell surface expression of neural cell adhesion molecule (NCAM) using established techniques (16, 17, 25). Briefly, NCAM+ satellite cells were identified immunohistochemically on 6-μm sections in series with those used for myofiber typing and sizing. Sections were fixed for 45 min at room temperature in 3% neutral-buffered formalin and blocked with 5% goat serum in PBS for 30 min at room temperature. Sections were incubated for 1 h at 37°C with anti-NCAM/CD56 mouse monoclonal antibody (Becton-Dickinson Biosciences, 1:200 in 1% goat serum) followed by 30-min incubation at room temperature in biotinylated goat anti-mouse secondary antibody (BA-9200 Vector Laboratories, 1:200 in 1% goat serum). Vectastain ABC reagent was applied for 1 h at room temperature, and NCAM+ cells were revealed by diaminobenzidine substrate (Elite Pk-6102, Vector Laboratories). Sections were rinsed briefly with PBS followed by 5 min in double-distilled water (ddH2O). Nuclei were counterstained with Mayer’s hematoxylin for 5 min followed by 5 min rinses in warm running tap water and ddH2O. The protocol results in myonuclei stained blue and in NCAM+ satellite cells, identified as any nucleus localized to the membrane of a myofiber, stained brown or with a brown rim. Slides were mounted with Aqua-Mount (Lerner Laboratories) and stored at 4°C.

High-resolution (48-bit TIFF) bright-field images were captured at ×20 magnification and image analysis was performed using Image-Pro Plus 5.0 software (Media Cybernetics). Myonuclei, NCAM+ cells, and fibers were counted by the same analyst blinded to the time point and response cluster assignment of the sample. The total number of nuclei, NCAM+ satellite cells, and myofibers were counted and myonuclei per fiber, myonuclear domain (fiber area per nucleus), NCAM+ satellite cells per 100 fibers, and relative number of satellite cells (NCAM+ satellite cells per total nuclei) were determined from 364 ± 6 cross-sectional myofibers before training and 341 ± 8 myofibers after training.

Serum samples. Insulin-like growth factor I (IGF-I), IGF binding protein-3 (IGFBP3), and IGFBP1 serum concentrations were determined in fasted morning serum samples collected before training and 24 h after the final exercise bout of the 16 wk resistance training program. Ten millimeters of blood were withdrawn from an antecubital vein, and serum was aliquotted (500 μL) and frozen at −80°C. Samples between subjects for a given hormone or binding protein were assayed in random order. All analyses were conducted by the UAB General Clinical Research Center Hormone/Substrate Core Laboratory. IGF-I, IGFBP3, and IGFBP1 were assessed by immuno-radiometric assays (Diagnostic Systems Laboratories, Webster, TX). The interassay coefficient of variation (CV), average intra-assay CV, and assay sensitivity for each hormone/binding protein were as follows: IGF-I, 9.43%, 3.48%, and 4.89 ng/mL; IGFBP3, 2.95%, 3.30%, and 200 ng/mL; and IGFBP1, 9.36%, 9.51%, and 0.42 ng/mL.

Statistical analysis. All statistical analyses were performed using STATISTICA 6.1 (StatSoft, Tulsa, OK). Data are reported as means ± SD. K-means clustering of Xtr, Mod, and Non responders based on change in MFA has been previously reported (4). Between-groups differences in baseline variables were tested by univariate ANOVA based on the independent between-groups variable (response cluster). All variables measured before and after resistance training were analyzed using a three (cluster) × two (time point) repeated-measures ANOVA. For each ANOVA model with a significant main effect or interaction, Tukey’s honestly significant difference tests were performed post hoc to localize the effect(s). Statistical significance was accepted at P < 0.05 for all tests.
II myofiber size or myofiber type distribution; no differences were found among the clusters in average training intensity, training volume, or program adherence; and the IIX-to-IIa myofiber type shift typical of resistance training was induced equally among all three clusters (4). Despite these similarities, the propensity for myofiber hypertrophy was vastly different (Fig. 1A). K-means clustering on changes in mean myofiber size obviously precludes group × time repeated measures analysis on this variable; thus the magnitudes of hypertrophy within clusters are shown for descriptive purposes (Fig. 1A). However, it is noteworthy that before training, univariate ANOVA confirmed no differences in MFA between response clusters (P = 0.153). In separate analyses, we confirmed no pretraining age differences in the number of NCAM+ satellite cells per 100 myofibers (P = 0.29, young = 10.8 ± 0.7; old = 12.0 ± 1.0), the number of myonuclei per fiber (P = 0.74, young = 2.4 ± 0.06; old = 2.4 ± 0.1), or the number of NCAM+ satellite cells relative to total nuclei (P = 0.46, young = 4.4 ± 0.3%; old = 4.8 ± 0.3%).

**Myonuclei per fiber.** There was a significant effect of cluster on the number of myonuclei per fiber at baseline with Mod having slightly fewer myonuclei per fiber than Non (P < 0.05) but similar myonuclei counts to Xtr (P = 0.32) (Fig. 1B). A cluster × training interaction (P < 0.001) was noted as the number of myonuclei per fiber increased 9% in Mod (P < 0.05) and was most effectively accomplished in Xtr (26%; P < 0.001), whereas no changes were found for Non (P = 0.877). In the trained state, the Xtr cluster had significantly more nuclei per fiber than Non (P < 0.05) and tended to have more myonuclei per fiber than Mod (P = 0.056).

**Myonuclear domain.** As shown in Fig. 1C, all three response clusters had a similar myonuclear domain at baseline (P = 0.948). A cluster × training interaction was found (P < 0.05), because the myonuclear domain expanded in both Xtr (P < 0.001) and Mod (P < 0.01) but did not change in Non (P = 0.877). A noteworthy finding was that Mod reached (2,002 ± 64 μm²) and Xtr exceeded (2,247 ± 90 μm²) the theoretical myonuclear domain threshold of ~2,000 μm² per nucleus (25) after resistance training. In Non, the myonuclear domain after resistance training was unchanged (1,866 ± 68 μm²) and remained below 2,000 μm².

**Population of muscle satellite cells.** At baseline, there was a significant effect of cluster on the number of NCAM+ satellite cells per 100 myofibers (P < 0.01, Fig. 2A). Post hoc analysis showed that Xtr had significantly more satellite cells per 100 fibers than both Mod (P < 0.01) and Non (P < 0.01) before training. There was a significant cluster × training interaction because only Xtr increased the number of satellite cells (117%; P < 0.001). After training, Xtr had over twofold more satellite cells than Mod (P < 0.001) and nearly threefold more than Non (P < 0.001). We also found significant differences between clusters at baseline for the number of satellite cells relative to total nuclei, with Xtr having more relative satellite cells than either Mod (P < 0.05) or Non (P < 0.001) (Fig. 2B). There was a cluster × training interaction on the relative

![Graph A: Mean fiber area by response cluster before and after 16 wk of resistance training.](http://jap.physiology.org/)

![Graph B: Myonuclear number (satellite per fiber).](http://jap.physiology.org/)

![Graph C: Myofiber area (μm²) per myonucleus.](http://jap.physiology.org/)
number of satellite cells ($P < 0.01$) because Xtr increased this index from 5.7 to 9.3% ($P < 0.001$), whereas no changes were found for Mod ($P = 0.316$) and Non ($P = 0.979$). Figure 3 displays representative immunohistological images from one Xtr and one Non subject before and after training.

Circulating IGF-I system. No baseline differences were noted among clusters in serum IGF-I ($P = 0.26$) or its binding proteins IGFBP3 ($P = 0.88$) and IGFBP1 ($P = 0.09$), and group means fell within the normal ranges. Furthermore, none of these factors changed pre to posttraining ($P = 0.22–0.74$). Overall, serum IGF-I averaged 279 ± 14 ng/ml across the three clusters. Overall averages for IGFBP3 and IGFBP1 were 4,155 ± 89 and 35 ± 3 ng/ml, respectively. There was a tendency ($P = 0.09$) for Xtr (21 ± 3 ng/ml) to circulate less IGFBP1 than Non (43 ± 7 ng/ml).

**DISCUSSION**

In this relatively large cohort of 66 human subjects, the three-cluster model provides compelling evidence to support the concept that extreme myofiber hypertrophy is facilitated by satellite cell proliferation and the addition of new myonuclei to existing myofibers. Four major findings of this novel work serve to advance our understanding of mechanisms driving load-mediated myofiber hypertrophy in humans. First, the availability of satellite cells in untrained muscle appears to be an important determinant of hypertrophic potential, as shown by a greater population of these cells before training in Xtr. Second, achieving extreme hypertrophy was coupled with a more than twofold expansion of the available satellite cell pool, while modest growth was not. Third, findings in all three clusters strongly support our laboratory’s prior suggestion (25) that initial expansion of the myonuclear domain (growth via net protein synthesis) toward a threshold may drive the demand for satellite cell recruitment and myonuclear addition to facilitate continued growth. Fourth, the present findings in the Xtr cluster indicate that our laboratory’s previously proposed myonuclear domain “ceiling” of ~2,000 μm² per nucleus (25) was underestimated. Each of these key findings is discussed below.

Availability of satellite cells in untrained muscle. Each of the three clusters had similar mean fiber areas as well as myonuclear domains at baseline. However, the Xtr cluster at baseline exhibited a significantly larger satellite cell pool as evidenced by the number of NCAM+ cells per fiber as well as the number of NCAM+ cells relative to total nuclei. The number of satellite cells in adult muscle is thought to be tightly regulated and thus held fairly constant in steady-state conditions (reviewed in Ref. 9). However, our findings strongly suggest one’s myogenic potential may at least partially be predetermined by the availability of satellite cells before training. In fact, the number of satellite cells present at baseline moderately predicted the magnitude of myofiber growth ($r = 0.403, P = 0.001$). Previous studies have suggested that the microenvironment and its host of circulating and anabolic factors that bathe the satellite cell largely determine myogenic potential (5, 8, 25). IGF-I is a potent endocrine, as well as skeletal muscle autocrine/paracrine, growth factor that is known to substantially influence satellite cell function and muscle growth rates (1, 2, 14). Here, we show that the circulating IGF-I system (IGF-I, IGFBP3, and IGFBP1) did not differ by cluster. Furthermore, we have previously reported that the response clusters did not differ before training in serum or muscle myostatin protein levels (19) or in muscle expression of mRNAs encoding IGF-I isoforms [IGF-IEx and IGF-IEc (MGI)] or of mRNAs encoding other proteins thought to be important during myogenesis (myogenic differentiation factor D, myogenin, cyclin D1, myostatin, p27kip, p21cip, activin receptor IIB) (4, 19). This is certainly not a complete picture of the muscle microenvironment, but we have yet to identify a circulating or local tissue factor that differentiates the response clusters before training. It is as yet unclear why the Xtr subjects possessed a larger satellite cell pool before training, but the results strongly suggest that this conferred a decided growth advantage on stimulation by resistance training.

Ability to expand the available satellite cell pool. During the process of myofiber hypertrophy, Xtr subjects experienced a remarkable 117% expansion of the available satellite cell pool.
On the other hand, the number of satellite cells was unaltered in Mod subjects \( (P = 0.19) \), who also experienced hypertrophy but roughly only 50% of the growth seen in Xtr. This may indicate a differential demand for myonuclear incorporation between Xtr and Mod responders: clearly, Xtr incorporated more nuclei than Mod during the hypertrophy process. If, however, demand was the only driving force determining the expansion rate of the satellite cell pool, the doubling of satellite cells in Xtr would not be expected. In other words, the rate of proliferation would be expected to keep pace with the rate of myonuclear addition but not to exceed it. This may have been the case in Mod but not in Xtr. On activation via a regeneration or growth stimulus, it is well known that some proliferating satellite cells return to a quiescent state to replenish the available pool, whereas others progress down the differentiation and fusion pathways \( (9) \). The fact that proliferation far surpassed myonuclear addition in Xtr suggests an alternative fate for many of these cells, one that exceeded self-renewal leading to a “stockpile” of precursor cells, perhaps in anticipation of future mechanical stressors. As opposed to being driven solely by a demand for nuclear addition, we suggest the muscles of Xtr were simply the most mechanosensitive, leading to superior mitogenic signaling responses to each loading bout beginning early in the program. This is supported by the robust, acute increases in MGF \( (4) \) and cyclin D1 \( (19) \) transcripts previously found in Xtr. Certainly there is evidence in humans that satellite cells proliferate in response to mechanical stress even when hypertrophy is not imminent \( (22) \), suggesting that forces other than eventual myonuclear addition drive expansion of the pool.

Although we reported an expansion of the satellite cell pool among Non in our first attempt at cluster analysis \( (25) \), this analysis with fewer subjects was based on percent change in fiber size and only a two-cluster model, using a theoretical cut point of 27% hypertrophy over baseline, as suggested by Kadi et al. \( (17) \). This initial clustering was an attempt to verify the theoretical ceiling of the myonuclear domain needed to stimulate the addition of nuclei as suggested by Kadi and colleagues. However, in the new 3-cluster analysis with 66 subjects, in which response clusters were defined by absolute change in mean myofiber size, one could argue that a portion of the subjects classed as Non \( (i.e., \text{less than 27\% hypertrophy}) \) were actually misclassified in Petrella et al. \( (25) \) because we have now identified a truly nonresponsive cohort with zero hypertrophy.

Initial expansion of the myonuclear domain. A hot debate in muscle biology is whether myofiber hypertrophy can occur in the absence of myonuclear addition \( (23) \). In the present study, the rate of myonuclear incorporation lagged behind the rate of hypertrophy, because the myonuclear domain expanded significantly in both Mod and Xtr despite significant myonuclear addition. These data provide strong evidence that at least some initial myofiber expansion is possible before the recruitment of nuclei in adult myofibers of average size at baseline. If the opposite were the case, addition of nuclei preceding hypertrophy, one would expect reduction or maintenance of the myonuclear domain pre- to posttraining. Expansion of the cytoplasmic region associated with each nucleus in Xtr and Mod indicates upregulation of the muscle protein synthesis machinery within existing myonuclear domains. Although the precise upstream mechanotransduction mechanisms are as yet unclear, it is generally accepted that the phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway is sensitive to mechanical load and drives translation initiation.
during hypertrophy. We speculate that, at the outset of the training program, the capacity of each myonucleus to direct transcription and/or translation to facilitate the synthesis of new proteins extended beyond its domain. In support of this, our laboratory previously found myofiber hypertrophy in the absence of myonuclear addition in a group of older women with small myonuclear domains prior to training (25). Based on our present and prior findings, we suspect that the failure to hypertrophy in Non was driven as much by attenuated protein synthesis as the lack of satellite cell recruitment and myonuclear addition. Participants clustered as Non clearly showed blunted activation of the mitogenic machinery (4, 19) and, because ligand binding to the type I IGF receptor also activates the PI3K-Akt-mTOR signaling cascade in mature myofibers, failure to increase IGF-IIEc (MGF) transcript levels (4) could have conceivably limited the protein synthesis response in Non. It is possible that the resultant lack of myonuclear domain expansion among Non subjects diminished the demand for satellite cell proliferation. However, as discussed above, the demand for nuclear addition is unlikely to be the only force driving satellite cell proliferation. It seems more likely that the failure to respond among Non subjects may have resulted from an overall blunted mechanosensitivity (i.e., less responsive mechanotransduction machinery). This would limit load-mediated activation of processes governing both protein synthesis and satellite cell recruitment.

**Myonuclear domain ceiling.** Our laboratory has previously suggested a ceiling to the myonuclear domain of ~2,000 \( \mu \)m\(^2\) per nucleus (25), speculating that myonuclear addition may be required in order for myofiber growth to progress beyond some degree of initial protein accretion that results in the attainment of this ceiling. The results found among Mod subjects support this theoretical ceiling, because myofibers in Mod expanded to a domain of 2,002 \( \mu \)m\(^2\) per nucleus during hypertrophy. By contrast, Xtr stretched the domain to nearly 2,250 \( \mu \)m\(^2\) per nucleus during remarkable hypertrophy. This exceptional cohort of Xtr subjects cannot be readily identified in typical human resistance training studies, which tend to be limited in sample size. The degree of hypertrophy attained in Mod typified the average growth rate reported in the resistance training literature; thus, without the Xtr cohort, the data would support our previous ceiling suggestion. These new findings indicate the myonuclear domain is not constrained to the ~2,000-\( \mu \)m\(^2\) limit. Still, domains of less than 2,000 \( \mu \)m\(^2\) per nucleus have been documented in elite power-lifters with extremely large myofibers (i.e., >10,000 \( \mu \)m\(^2\)), and the number of myonuclei per fiber correlated quite well with fiber size in these athletes (10). We captured histological results after only 16 wk of resistance training, which was likely during a rapid hypertrophy phase in both Xtr and Mod. It is attractive to speculate that with continued, long-term training (much like the elite lifters) during which rates of growth are known to slow substantially, myonuclear addition may eventually “correct” the domain size of Xtr back into the expected range. Certainly the robust expansion of the satellite cell pool in Xtr would appear to have provided sufficient nuclear donors for such an effect.

**Summary.** This novel application of K-means cluster analysis has yielded a number of valuable conclusions and directions for future study. Xtr individuals who realized robust hypertrophy in response to 16 wk of resistance training, regardless of age or sex, exhibited robust myogenic potential before training as evidenced by a large pool of muscle satellite cells. Additionally, during resistance training, these individuals were most adept at expanding the satellite cell pool and incorporating new myonuclei. Expansion of the myonuclear domain may drive myonuclear addition by placing strain on existing nuclei to produce adequate gene products for the growing myofiber; however, the excessive increase in satellite cells seen among extreme responders suggests the induction of satellite cell proliferation is driven by factors beyond simply a demand for nuclear addition.

More than one-fourth of our cohort (26%) did not experience measurable hypertrophy. The complete lack of growth was associated with no domain expansion (i.e., protein accretion) and no detectable satellite cell activation. We therefore suspect failure or impaired sensitivity of a common, upstream mechanotransduction process that initiates both protein synthesis and satellite cell activation. Transmembrane protein complexes associated with dystrophin and the integrins have been considered in this regard (4). Whether these subjects are truly non-responders or actually delayed responders requires further study. The possibility certainly exists that this cohort would experience hypertrophy with additional weeks of training. Studies with prolonged recovery periods between bouts (2 days/wk loading) have often resulted in more robust hypertrophy of myofibers than 3 days/wk training for both men (15, 21) and women (12, 13), particularly in older adults. It therefore stands to reason that some subjects who failed to experience hypertrophy in this study may better respond to extended recovery time between bouts and/or additional weeks of training, and both factors should be considered in future evaluations of the mechanisms driving resistance training-mediated hypertrophy among humans.

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