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Gene shifting: a novel therapy for mitochondrial myopathy

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Mutations in mitochondrial DNA (mtDNA) are the most frequent causes of mitochondrial myopathy in adults. In the majority of cases mutant and wild-type mtDNAs coexist, a condition referred to as mtDNA heteroplasmy; however, the relative frequency of each species varies widely in different cells and tissues. Nearly complete segregation of mutant and wild-type mtDNAs has been observed in the skeletal muscle of many patients. In such patients mutant mtDNAs predominate in mature myofibers but are rare or undetectable in skeletal muscle satellite cells cultured *in vitro*. This pattern is thought to result from positive selection for the mutant mtDNA in post-mitotic myofibers and loss of the mutant by genetic drift in satellite cells. Satellite cells are dormant myoblasts that can be stimulated to re-enter the cell cycle and fuse with existing myofibers in response to signals for muscle growth or repair. We tested whether we could normalize the mtDNA genotype in mature myofibers in a patient with mitochondrial myopathy by enhancing the incorporation of satellite cells through regeneration following injury or muscle hypertrophy, induced by either eccentric or concentric resistance exercise training. We show a remarkable increase in the ratio of wild-type to mutant mtDNAs, in the proportion of muscle fibers with normal respiratory chain activity and in muscle fiber cross-sectional area after a short period of concentric exercise training. These data show that it is possible to reverse the molecular events that led to expression of metabolic myopathy and demonstrate the effectiveness of this form of 'gene shifting' therapy.

INTRODUCTION

The mitochondrial encephalomyopathies are a heterogeneous group of multisystem disorders with a wide spectrum of clinical phenotypes and prominent involvement of the central nervous system and skeletal and cardiac muscle (1,2). Impaired oxidative phosphorylation in these disorders commonly results from a mutation in mitochondrial DNA (mtDNA); either a large-scale deletion or a point mutation in a tRNA, rRNA or protein coding gene. In the majority of cases, mtDNAs carrying a pathogenic mutation coexist with normal wild-type mtDNAs, a condition known as mtDNA heteroplasmy. The expression of a pathological phenotype depends primarily on the relative proportions of mutant versus wild-type mtDNA copies per cell (3,4). When this ratio exceeds a critical threshold, oxidative energy metabolism is impaired and clinical disease ensues.

Mitotic segregation of mutant mtDNAs during development, growth and normal cellular turnover of mitochondria can lead to different degrees of mtDNA heteroplasmy among tissues within an individual and this is to some degree responsible for the selective tissue involvement in these disorders (2). Recently, two cases were reported in which dramatic tissue-specific variation in the degree of mtDNA heteroplasmy was observed (5,6). Both patients had a point mutation in $tRNA^{leu(CUN)}$, which was the predominant mtDNA species (>90% of total mtDNAs) in skeletal muscle. The mutant mtDNA could not, however, be detected in other tissues examined such as red blood cells, platelets, fibroblasts or, most importantly, skeletal muscle satellite cells. This nearly complete segregation apparently resulted from the accumulation of mutant mtDNAs with time in post-mitotic skeletal muscle fibers and the loss of the mutant mtDNAs, probably by random genetic drift, in mitotic cell populations (5,6). A similar distribution of mutant and wild-type mtDNAs has been observed in the muscles of patients with Kearns–Sayre syndrome, a disorder associated with large-scale mtDNA deletions (7,8), and several other patients with mtDNA point mutations (9,10) or small deletions (11).

These studies raised the intriguing possibility of normalizing the skeletal muscle mtDNA genotype in these and similar patients by stimulating the proliferation and incorporation of satellite cells into existing myofibers. This concept was tested in muscle fibers regenerating from the traumatic muscle fiber injury induced by the biopsy procedure (12) and in muscle fibers regenerating after chemically induced muscle fiber necrosis (13). In both studies, mutant mtDNAs were rare or undetectable in regenerating muscle fibers and cytochrome c oxidase (COX) activity was restored, demonstrating the soundness of the approach.

While these results are encouraging, a practical method to implement this potential therapy remains to be found. We have

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proposed two non-invasive methods to induce satellite cell incorporation into existing myofibers in patients with skewed skeletal muscle heteroplasmy: (i) producing subtle muscle fiber injury leading to segmental necrosis and regeneration through eccentric exercise (lengthening contractions); (ii) inducing muscle fiber hypertrophy through concentric exercise (shortening contractions) (12). In this study we tested these approaches in the patient we have previously reported with a G12315A $tRNA^{leu(CUN)}$ point mutation (5,12).

RESULTS

Maximal voluntary contraction (MVC)

Before training began, maximal left elbow flexion produced a force output of 12.35 ± 0.4 kg, whereas right elbow flexion produced 13.94 ± 0.78 kg of maximal force (50% of the predicted MVC for a healthy male of this age). Eight days of eccentric exercise training with a 12 kg weight led to a slight decrease in strength in the left arm (11.20 ± 0.99 kg). Following the 9 day rest period, MVC increased to 14.14 ± 1.10 kg (14% greater than baseline) and the weight used for training was subsequently increased. After 8 days of eccentric exercise in the second training period, MVC decreased to 9.37 ± 0.20 kg. At 6 days post-training, MVC was not significantly different from baseline at 11.80 ± 0.80 kg. Concentric exercise training in the right arm also led to a decrease in MVC to 10.82 ± 0.35 kg. This recovered 6 days post-exercise to 13.40 ± 0.92 kg.

Creatine kinase (CK) activity

The CK level was abnormally elevated at 697 U/l before training began (laboratory normal range is 5–140 U/l). With exercise training, CK levels increased further to 1703 U/l at day 2, 1504 U/l at day 7 and 1592 U/l at day 9. Nine days of rest resulted in a lowering of CK to 650 U/l. After 2 days of training during the second training period, CK rose to 1036 U/l. One day post-training CK was 1964 U/l, and 1284 U/l by 2 days post-training. CK on the day of biopsy was 707 U/l. As eccentric and concentric training was performed concurrently, it is not possible to attribute changes in CK levels to one protocol or the other.

Muscle circumference

At baseline, left middle arm circumference 8 cm above the elbow joint was 25.0 cm. Five days of eccentric training resulted in a 2.5 cm increase in left middle arm circumference. This conspicuous swelling was accompanied by bruising at day 8 over the triceps area which lasted 7 days. At day 19 arm circumference was decreased to 26.0 cm. Two days of training in the second training period led to a further 0.5 cm increase and by day 27 arm circumference had increased to 27.0 cm. Mild bruising was observed again at day 25. This increase in arm circumference was maintained at 2 days post-training.

Pre-training right middle arm circumference was slightly greater than the left arm at 26.5 cm. The right arm was not exercised for the first 5 days due to severe pain (presumably caused by the eccentric exercise), which prevented the patient from raising his arm above shoulder level. Bruising was also observed in this arm at day 5 and lasted 7 days; this may have resulted from the strain placed on this arm acting to stabilize the effects of lowering the weight in the left arm. Post-training circumference measurements of this right arm undergoing concentric exercise were made in the second training period. Two days of concentric training led to a 1.0 cm increase and by day 27, arm circumference was 28 cm.

mtDNA genotype and muscle phenotype

The relative proportions of mutant and wild-type mtDNAs were measured in the left (eccentric exercise) and right (concentric exercise) arms pre- and post-training (Table 1). Wild-type mtDNAs accounted for ~18% of total mtDNAs in the left arm and 12% in the right arm before training. After training there was a small increase (8%) in the proportion of wild-type mtDNAs in the left arm and a substantial increase (33.4%) in the right arm. This was accompanied by a corresponding decrease in the proportion of COX-negative muscle fibers in the left (19.5%) and right (43.5%) arms (Table 2 and Fig. 1) and a substantial increase in muscle fiber diameter, which is especially evident in the right arm (Fig. 1D). In COX-negative fibers in the right arm, mean fiber area increased from 2542 ± 837 (n = 71) to 3629 ± 1316 μm^2 (n = 29), an increase of 43%. However, COX-positive fibers increased from 1539 ± 694 (n = 79) to 8194 ± 2648 μm^2 (n = 121), a remarkable 432% increase.

To investigate whether the exercise protocol induced muscle damage that was severe enough to induce muscle fiber necrosis and regeneration, cryostat sections were examined for evidence of anti-neural cell adhesion molecule (NCAM) immunoreactivity, a marker of muscle fiber regeneration. A few scattered NCAM-positive fibers were observed in all muscle sections, but no significant differences were seen pre- versus post-training (data not shown).

DISCUSSION

In this study we used strenuous resistance exercise training to test whether endogenous satellite cell-mediated transfer of wild-type mtDNA could normalize the mtDNA genotype in the skeletal muscle of a patient with a tRNA point mutation. We envisioned two possible mechanisms for promoting such 'gene shifting' from satellite cells to existing myofibers: inducing a cycle of necrosis and regeneration by promoting microscopic muscle fiber injury or encouraging the incorporation of satellite cells by a program of strength training.

To induce muscle injury we used a program of eccentric resistance exercise, in which muscles are lengthened during force generation, an action that is unusually destructive to muscle fibers. The extent of damage induced by high force stretching of muscle fibers depends on the intensity and duration of exercise (14) and ranges from overstretching of the sarcomeres to actual tearing of the fiber and muscle necrosis (14–17). Such damage induces the release of mitogenic factors that stimulate proliferation and migration of satellite cells to the site of injury, where they repair and regenerate the damaged myofiber segment (15,18). One bout of strenuous eccentric exercise is known to result in damage which manifests as an increase in muscle proteins (such as CK) in the blood, prolonged losses in muscle strength and range of motion, swelling and delayed onset muscle soreness (14).

Table 1. Training protocol

Training day	Eccentric contraction				Concentric contraction			
	Session/day	Set/session	Repeats/set	Initial MVC (%)	Session/day	Set/session	Repeats/set	Initial MVC (%)
1	2	3	10	100	R	R	R	R
2	2	3	10	100	R	R	R	R
3	2	3	10	100	R	R	R	R
4	R	R	R	R	R	R	R	R
5	2	3	10	100	1	3	10	75
6	2	3	10	100	R	R	R	R
7	2	3	10	100	1	3	10	75
8	2	3	10	100	R	R	R	R
9	2	3	10	100	1	3	10	75
10–18 rest period								
19	2	3	10	110	1	3	10	75
20	2	3	10	110	1	3	10	75
21	2	3	10	110	1	3	10	75
22	R	R	R	R	R	R	R	R
23	2	3	10	110	1	3	10	75
24	2	3	10	110	1	3	10	75
25	2	3	10	110	1	3	10	75
26	2	3	10	110	1	3	10	75
27	2	3	10	110	1	3	10	75

R, rest.

Table 2. Changes in the proportions of COX-negative fibers and mutant and wild-type mtDNA after resistance exercise training

	Eccentric left arm			Concentric right arm		
	Baseline	Post-training	Mean difference	Baseline	Post-training	Mean difference
COX-negative fibers (%)						
Sample 1	40.0	26.0	–19.5	66.0	13.0	–43.5
Sample 2	31.0	6.0		56.0	22.0	
Wild-type mtDNA (%)						
Sample 1	19.3	27.6	+8.0	11.8	48.4	+33.4
Sample 2	16.7	24.1		13.1	43.2	

The samples refer to different blocks analyzed from the same biopsy sample.

All of these indirect markers of eccentric exercise-induced muscle damage were observed in our patient. CK values in blood rose, muscle strength decreased, arm circumference increased and muscle soreness was evident and reported by the patient. Although the CK response to exercise-induced injury shows large inter-subject variability (19), peak values after one bout of high force eccentric exercise of the elbow flexors generally range from 1000 to 10 000 U/l and values of 6000 U/l and higher have been reported 2 days post-exercise (19,20). The relative increase in CK is generally associated with the severity of muscle damage (19). After 2 days of eccentric exercise, CK activity in the blood of our patient increased to 1703 U/l. Considering the elevated baseline CK, this value is lower than one would expect after four strenuous bouts of eccentric exercise.

We are not aware of data on the exact relationship between the CK response and the extent of direct skeletal muscle damage, so it is difficult to establish the severity of direct muscle fiber damage from blood CK levels alone. Furthermore, one bout of eccentric exercise is known to have a long-lasting adaptive effect making the muscle more resistant to damage with subsequent intense exercise in normal subjects (20), which could account for the stabilization of CK activity in our patient with repetitive eccentric exercise.

We did not find any direct evidence for muscle fiber regeneration in response to the eccentric exercise paradigm performed by our patient. A few scattered NCAM-positive fibers were observed pre- and post-training in both the left and right biceps muscles. Activation and proliferation of satellite cells

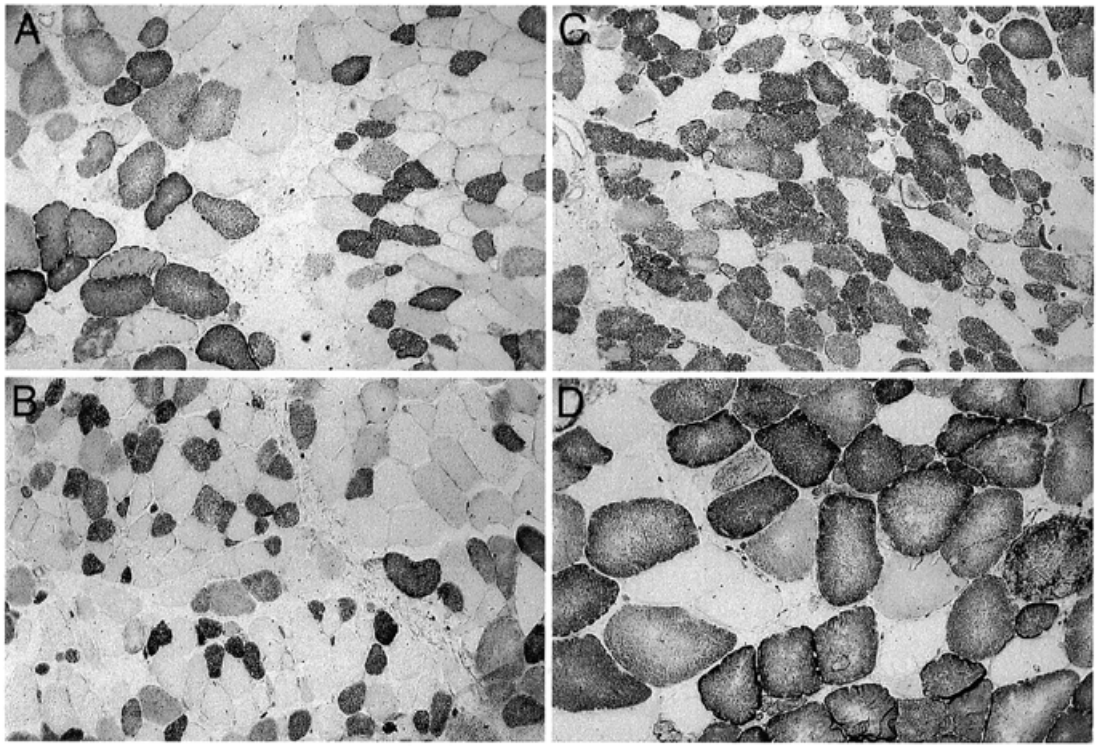

Figure 1. COX activity in patient muscle before and after resistance exercise training. (Left) Biceps muscle before (**A**) and after (**B**) eccentric exercise training. (Right) Biceps muscle before (**C**) and after (**D**) concentric exercise training. Micrographs are all shown at the same original magnification (×60).

after an acute bout of prolonged eccentric exercise in animals is known to occur within 24 h of injury (15,21) and is sustained until repair of the myofiber is complete, usually ~3 weeks in humans (20). Thus, the fact that the proportion of regenerating fibers was not increased by eccentric exercise was surprising to us, suggesting that the exercise intensity, although maximal for the patient, was not traumatic enough to induce frank muscle necrosis and regeneration. The damage induced by eccentric exercise is thought to be mechanical (17) and it may be that the absolute force that could be generated by our patient was insufficient to produce significant damage. It is also possible that the muscle fibers sustained minor tears that were promptly repaired by activated satellite cells without expression of the molecular markers associated with regenerating fiber segments. The eccentric exercise protocol produced a small change in mitochondrial genotype and a decrease in the proportion of COX-negative fibers, which is, however, difficult to evaluate in light of the heterogeneity in different muscle biopsy samples from this arm.

Concentric exercise training results in gains in muscle strength, but unlike eccentric exercise, does not cause significant muscle fiber damage. Intense tension overload provided by a concentric contraction (shortening of active muscle) provides the stimulus for muscle fiber hypertrophy and is primarily responsible for muscle growth associated with strength training (22). This is associated with the fusion of satellite cells into growing muscle fiber (15,18,22–24) to maintain a constant nuclear:cytoplasmic ratio (23–25).

Analysis of the muscle biopsy from the concentrically trained arm showed a large decrease in the proportion of COX-negative fibers (43.5%), increase in wild-type mtDNA (33.4%) and a clear increase in fiber diameter, which was especially evident in COX-positive muscle fibers (Fig. 1D). We suggest that this marked improvement resulted from the fusion of satellite cells devoid of the mtDNA mutation into existing myofibers in response to the overload resistance training. Satellite cells have a rather sparse cytoplasm with few mitochondria relative to mature myofibers, so we did not expect to observe a substantial increase in the proportion of wild-type mtDNAs due to hypertrophy alone. The fact that it occurred suggests that mtDNA replication in the myofiber during the training period, accompanying the muscle hypertrophy, occurred preferentially from templates originating in satellite cell mitochondria.

Although we did not observe a significant increase in muscle strength in the concentrically trained arm of the patient, this may simply reflect the length of the training protocol. We chose to perform the training over a period of 3 weeks in order to test for the presence of regenerating fibers in the biopsy sample due to the eccentric exercise training. The patient was not capable of initially exercising both arms, so eccentric training was started first and concentric training was only performed for a total of 11 days. Strength training studies typically involve training programs that last 8–20 weeks. Although we cannot rule out muscle pain and soreness as contributing factors, it is likely that the short training interval in this study was inadequate to observe early increases in muscle strength, which are associated with neural adaptation (26).

In conclusion, we have been able to demonstrate a dramatic increase in the proportion of wild-type mtDNAs and corresponding decrease in the proportion of COX-negative fibers in the biceps brachii muscle of a patient with a mitochondrial

myopathy after a brief period of concentric exercise training. Surprisingly, only a minor shift in mtDNA genotype was observed after eccentric training, despite expectations that this form of exercise would induce muscle necrosis and regeneration. Concentric exercise training appears to be a more potent stimulator of satellite cell activation without the effects of inducing serious muscle damage. This form of 'gene shifting' therapy should be useful in reversing the inexorable accumulation of mutant mtDNAs that occurs in muscle fibers in many patients with mitochondrial myopathy. This would be predicted to greatly increase muscle strength and exercise tolerance in these patients.

MATERIALS AND METHODS

Patient description

The patient was a right-handed 59 year old Italian-Canadian man presenting with a phenotype similar to Kearns–Sayre Syndrome due to a G12315A mutation in $tRNA^{leu(CUN)}$ (5). His primary symptoms included proximal muscle weakness and exercise intolerance. Previous quantitative analysis in this patient showed that mutant mtDNA constituted >90% of total mtDNA in two independent muscle biopsies.

Training

The training regimen, outlined in Table 1, was designed to recruit and exercise the biceps brachii muscle. A muscle biopsy of the biceps in both the left and right arm was obtained 7 days following the last day of training. This study was reviewed and approved by the Ethics Committee of the Montreal Neurological Institute and Hospital and written informed consent was obtained from the patient.

The left arm underwent eccentric strengthening by doing elbow extension while in a supine position with the elbow joint angle at a starting position 90° from full extension (corresponding to 180°). A weight equal to the patients maximal voluntary force was placed in the hand and the arm was slowly lowered to reach a fully extended position so that the contraction lasted 10–15 s. Initially the researcher aided this lowering phase as the patient was not able to do it independently. The weight was immediately lifted to the starting position by the researcher to avoid a concentric phase and the exercise was repeated to complete the set as detailed in Table 1. A 3–5 min rest period was given between sets. The two exercise sessions per day were performed at least 3 h apart. Exercise training was performed in two training periods of 9 consecutive days interspersed with 9 days of rest.

The right arm underwent concentric elbow flexion using the protocol outlined in Table 1. In a supine position, with the arm fully extended, the patient lifted a weight corresponding to 75% of MVC to reach a position 90° from start. The weight was lowered by the researcher and the exercise was repeated according to protocol. The training regimen for concentric exercise consisted of 3 days in the first training period as opposed to 9 for the eccentric arm, due to the patient's difficulty in tolerating such a sudden increase in muscle activity. Once he became more accustomed to the eccentric exercise, additional exercise in the form of concentric work was introduced and the training regimen followed that of the eccentric arm.

Measurements of muscle function and damage

MVC. Maximal voluntary isometric force generation of the elbow flexors was measured with an electronic strain gauge connected to a Macintosh Data Acquisition System (GW Instruments) while the subject was lying supine on a strength testing table (Tufts Quantitative Neuromuscular exam). The arm was at rest on the table with the elbow joint angle positioned at 90°. The subject was asked to pull with maximal force for 5 s and, after a 10 s rest period, asked to repeat the maximal force generation a second time. This test was repeated with an interval of 10–15 min. The maximal force generation was recorded on each occasion (n = 4 measurements). Initial muscle force generation was measured on two separate occasions at baseline to determine the weight used for exercise training. Testing was repeated on days 7, 19 and 27 and at 6 days post-training for the eccentrically trained arm, and on day 27 and at 6 days post-training for the concentrically trained arm. There is an ~10% measurement error associated with this testing procedure.

CK activity. Approximately 5 ml of blood was collected from the antecubidal fossa of the right arm and analyzed for resting serum CK activity at baseline, on days 2, 7, 9, 19, 21 and 27 during the training period, then at 1 and 6 days post-training.

Arm circumference. Arm circumference was used to indicate muscle damage due to inflammation. It was assessed by a tape measure at a site 8 cm above the elbow joint on the upper arm. Measurements were obtained at baseline, on days 5, 19, 21 and 27 during the training and at days post-training.

mtDNA, histochemical and morphological analyses. Muscle biopsies were obtained from the left and right biceps muscle pre- and post-training. The biopsy sites were chosen to be distinctly different from previous sites so that regenerated fibers from previous biopsies would not confound the analysis. The biopsy specimen is routinely separated into two parts and mounted separately for histological and cytochemical analysis on cryostat sections. Both blocks were analyzed to determine the proportion of COX-negative muscle fibers and the relative proportions of mutant and wild-type mtDNA. COX activity and DNA analysis were carried out as described previously (5). Cryostat sections were analyzed for anti-NCAM immunoreactivity (a marker of muscle fiber regeneration) as described by Shoubridge *et al*. (12). Muscle fiber cross-sectional areas were estimated from the minimum diameter of individual muscle fibers measured on a camera lucida drawing.

ABBREVIATIONS

CK, creatine kinase; COX, cytochrome c oxidase; MVC, maximum voluntary contraction; NCAM, neural cell adhesion molecule.

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