Increasing Extraocular Muscle Strength with Insulin-like Growth Factor II

Linda K. McLoon and Stephen P. Christiansen

Purpose. Botulinum toxin type A and, more recently, the immunotoxin ricin-mAb35 have been effective as means of pharmacologically weakening the extraocular muscle. However, currently there are no drug treatments to strengthen an underacting extraocular muscle. In limb muscle, treatment with insulin-like growth factor causes myofiber hypertrophy. In this study, the short-term effects of insulin-like growth factor II (IGF-II) on extraocular muscle morphometry and force generation were examined.

Methods. One superior rectus muscle in normal adult rabbits received a single injection of 10 μg IGF-II, and the contralateral muscle received an injection of saline only. One week after injection, muscle morphology and muscle force were compared between the IGF-treated and control muscles.

Results. In the treated muscle, there was no significant change in the mean cross-sectional area of myofibers compared with the control. However, there was an increase in the heterogeneity of myofiber cross-sectional area, with increases in both small and very large myofibers. Mean single-twitch force generation was 0.48 ± 0.12 mN/cm^3 compared with 0.27 ± 0.04 mN/cm^3 ($P = 0.0473$) in control samples. Mean tetanic force generation was increased significantly at all stimulation frequencies. Treatment had no effect on muscle fatigability.

Conclusions. Extraocular muscle is very responsive to direct injection of IGF-II. Although no difference was seen in mean myofiber cross-sectional area, overall there was sufficient alteration in myofiber heterogeneity to result in increased force generation. If a sustained treatment effect can be achieved with IGF-II and, potentially, other growth factors, the pharmacological treatment of strabismus could be advanced by simultaneous injection of agonist–antagonist pairs with agents that weaken and strengthen the treated extraocular muscle. (*Invest Ophthalmol Vis Sci.* 2003;44:3866–3872) DOI:10.1167/iovs.03-0223

Strabismus is a common ophthalmologic problem that affects between 2% and 5% of school-aged children in the United States. Any significant misalignment of the eyes disrupts binocular fusion. In the visually immature child, this often results in cortical suppression of the afferent input from one eye and may lead to amblyopia. Untreated amblyopia can result in visual loss that persists into adulthood. In the older child or adult, an acquired misalignment of the eyes results in diplopia or visual confusion.

Patching, glasses, prisms, or orthoptic exercises may be useful in patients with some forms of strabismus. However, many patients must undergo a surgical procedure for correction of alignment. This is most often accomplished with incisional surgery or chemodenervation by direct muscular injection of botulinum toxin A (Botox; Allergan, Irvine, CA). Both treatments, however, have their advantages and drawbacks. The critical issues in the use of botulinum toxin A in strabismus relate to the timing of administration, the duration of its effect, and the spectrum of cases in which it is most effective.[1] Incisional surgery, however, may compromise normal muscle dynamics by altering the arc of contact of the muscle with the globe, the intrinsic elasticity of the operated muscles, the resting tension on the agonist–antagonist pair,[2] and the generated twitch tension.[3] In addition, surgery unavoidably induces scarring and may disrupt muscle relationships with soft-tissue pulleys that could further alter extraocular muscle function.[4,5]

Botulinum toxin A, approved for clinical use two decades ago, has been used effectively to weaken the overacting muscle in both childhood and adult strabismus[6,7] and has shown that the concept of drug treatment of strabismus is possible. Remarkably, the development of new pharmacological agents for the treatment of strabismus has not been pursued, despite the potential advantages of ease of administration, limitation of postoperative scarring, and preservation of normal extraocular muscle–globe mechanical dynamics. Recently, however, a myotoxic immunotoxin, ricin-mAb35, has been developed, which targets its myotoxicity directly to mature myofibers.[8,9] A single injection into the superior rectus muscle of adult rabbits results in long-term muscle loss and significant weakening.[10,11]

Although drug-induced extraocular muscle weakness can now be accomplished rather predictably, a pharmacological means of strengthening extraocular muscle has yet to be realized. One approach would be to inject the target extraocular muscle with certain growth factors that are known to increase limb muscle mass.[12] Fiber hypertrophy or other motor enhancements induced by the growth factor could result in greater force output from the treated muscle. If such an effect could be demonstrated, growth factor treatment of a single extraocular muscle alone, or in combination with the treatment of the antagonist by weakening agents such as botulinum toxin A, could greatly enhance our ability to manage strabismus without the mechanical hazards of incisional procedures highlighted previously.

Insulin-like growth factor (IGF) is a myogenic growth factor that can act to both stimulate proliferation and differentiation of satellite cells.[13] Increased levels of IGF in muscle can result in significant muscle hypertrophy.[14,15] More significantly, IGF treatment improved muscle force production in both aged and dystrophic muscle.[12,16,17] In this study, we examine whether direct injection of a myogenic growth factor, specifically IGF-II, may be a viable means of increasing extraocular muscle strength.

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Supported by EY13979 and EY11375 from the National Eye Institute; grants from the Neuro-ophthalmology Research Fund, the Minnesota Medical Foundation, the Minnesota Lions and Lionesses; and an unrestricted grant to the Department of Ophthalmology from Research to Prevent Blindness, Inc.

Submitted for publication March 4, 2003; revised April 18, 2003; accepted April 24, 2003.

Disclosure: **L.K. McLoon**, None; **S.P. Christiansen**, None

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Investigative Ophthalmology & Visual Science, September 2003, Vol. 44, No. 9
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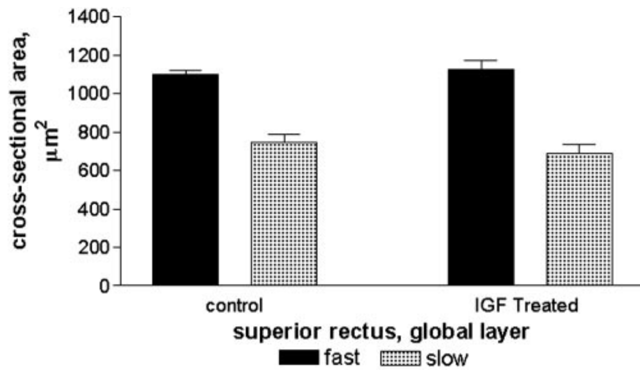


FIGURE 1. Analysis of the mean cross-sectional areas of myofibers 1 week after a single injection of IGF-II into the superior rectus muscle compared with the saline injected controls. There was no significant difference between control and IGF-II–treated mean areas.

MATERIALS AND METHODS

Adult New Zealand White rabbits were obtained from Bakkon Farms (Viroqua, WI) and housed with Research Animal Resources. All studies were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and were in compliance with National Institutes of Health guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Seven rabbits were anesthetized by an intramuscular injection of 10 mg/kg ketamine and 2 mg/kg xylazine. IGF-II, 10 μg in 100 μL sterile saline (R&D Systems, Minneapolis, MN), was injected intramuscularly into one superior rectus muscle, randomized before surgery. IGF-II was selected based on earlier observations that extraocular muscles express the IGF receptor and IGF (McLoon LK, et al. *IOVS* 1999;40:ARVO Abstract 2150)[18] and that in IGF-treated extraocular muscle a small population of highly hypertrophic myofibers develops (McLoon LK, et al. *IOVS* 1999;40:ARVO Abstract 2150). The contralateral muscle received an injection of sterile saline to serve as a control.

Rabbits were anesthetized with ketamine and xylazine 1 week after injection for measurement of in situ muscle force development of the superior rectus muscles in both orbits. After endotracheal intubation, inhalational anesthesia with isoflurane was initiated (≤5%). The rabbits were ventilated continuously and were monitored with a pulse oximeter. Core body temperature was monitored with a rectal probe and maintained within the normal range by a heating pad. The heart rate was also monitored. The animal's head was fixed in a stereotactic frame and secured with a nose clamp to prevent head movement. The superior rectus muscles of both orbits were exposed by removal of the upper eyelids and the superior bulbar conjunctiva. The insertional tendons were cut from the globe and sutured to isometric force transducers with 4-0 silk. The force transducers were mounted on isometric tension clamps with the line of force perpendicular to the animal's midline and elevated slightly above the horizontal plane of the animal's head. This allowed adjustments in the resting length of the muscles with minimal drag from the globe. Generated force in grams was recorded on a computer (LabView software; National Instruments Corp., Austin, TX). Grams are converted to milliNewtons per cubic centimeter (mN/cm^3) by using the constant, 1.056 g/cm^3. Muscle weight and the muscle length were measured to calculate muscle volume.

Bipolar platinum contact electrodes were positioned on the distal third of the muscles, near the insertional tendons, to avoid indirect stimulation of the muscles by terminal nerves or the endplate region. Both control and IGF-treated superior rectus muscles were tested simultaneously. The supramaximal stimulation intensity was determined by increasing voltage until maximal contraction was achieved using square-wave pulses of 0.4 ms duration. Isometric length–tension curves were determined by stimulation of each muscle at supramaximal intensity (≥5 V, 0.4 ms) while varying the preload (resting length) over a range of 0.15 to 10.0 g. The optimal preload was determined by incrementally increasing the resting muscle length to achieve maximum isometric twitch force, allowing 60 seconds of rest between stimuli. All further testing was performed with supramaximum stimulus intensities at optimal preload.

Tension development was measured for single, double, triple, and quadruple pulses (0.4 ms pulse duration) with 2 minutes rest between stimuli. The muscles were then stimulated at 10, 20, 40, 100, 150, and 200 Hz (250 ms train duration) with 4 minutes of rest between stimuli. Fatigability was tested by stimulating the muscles at 100 Hz (250 ms train duration), every 10 seconds for 1600 seconds, or until there was a 50% reduction in generated force. Data from treated and control muscles were pooled at each postinjection interval and compared with the paired *t*-test. $P \leq 0.05$ was considered statistically significant.

At the completion of testing, the animals were killed with an overdose of intravenous barbiturate anesthesia. The superior rectus muscles of each eye were removed, weighed, measured, and prepared for histologic examination. The superior rectus muscles from each orbit were embedded in tragacanth gum and frozen in 2-methylbutane

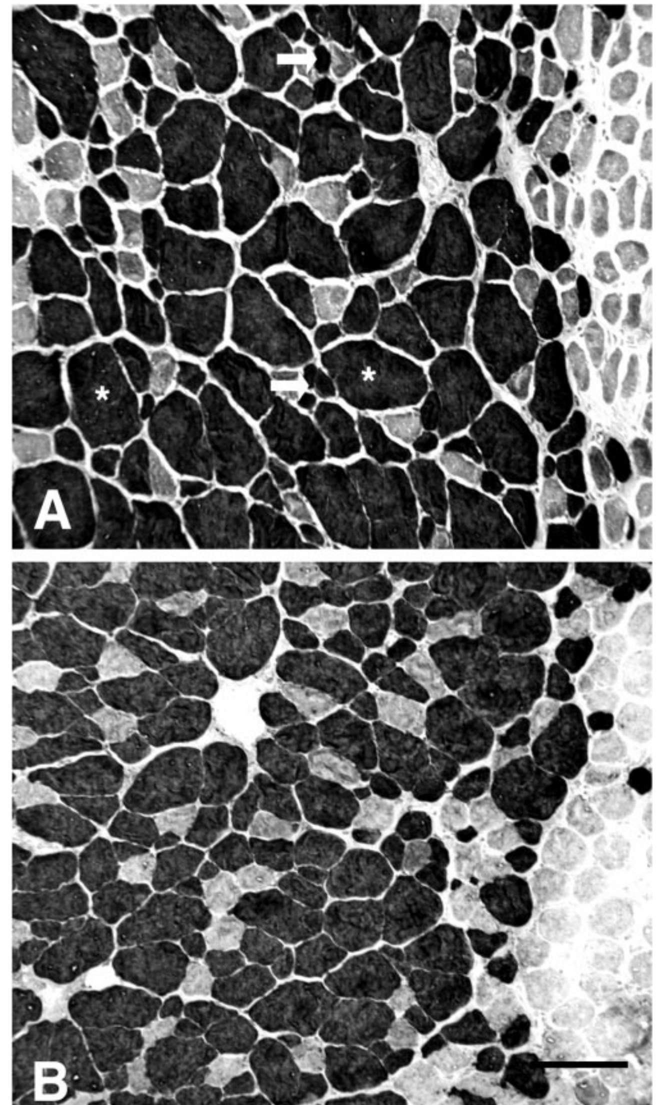


FIGURE 2. Photomicrograph of an IGF-injected superior rectus muscle (**A**) and a saline-injected control muscle (**B**) 1 week after a single injection immunostained with an antibody against the fast MHC isoform. *Arrows*: myofibers with small cross-sectional areas; (*) hypertrophic myofibers. Bar, 50 μm.

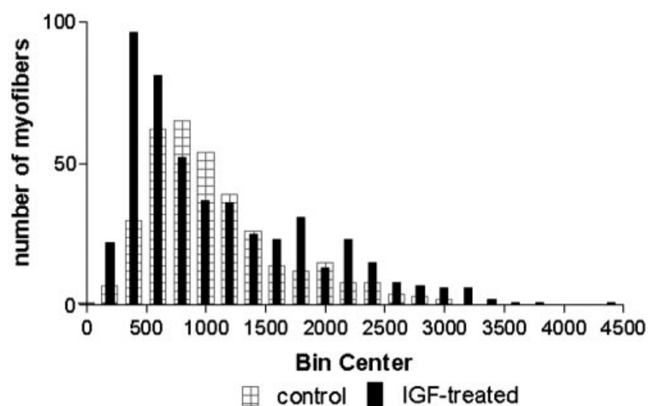


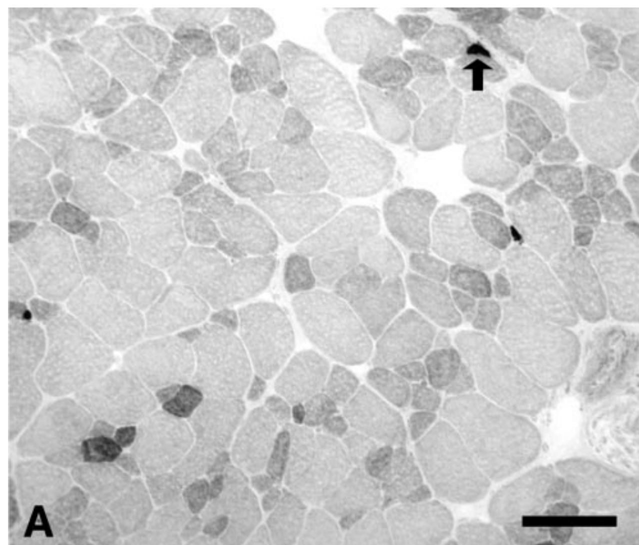
FIGURE 3. Histogram of individual myofiber cross-sectional heterogeneity for one representative superior rectus muscle pair. One muscle was injected with IGF-II, and the contralateral side was injected with saline only. There was an increase in the percentage of very small and very large myofibers in the IGF-II–injected muscle.

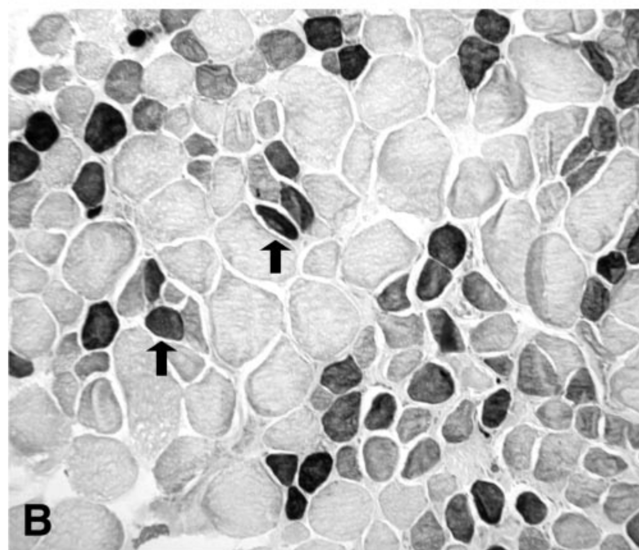
chilled to a slurry in liquid nitrogen. A second set of four rabbits that received injections of either 10 μg IGF in saline or saline alone was also prepared, to control for any changes induced by the physiological manipulations. These rabbits were killed with an overdose of barbiturate 1 week after injection, and both superior rectus muscles were removed, embedded in tragacanth gum, and frozen in methylbutane that had been chilled to a slurry in liquid nitrogen. Muscles were sectioned serially at 12 μm and processed for immunohistochemical visualization of the heavy chain isoforms of fast, slow, and neonatal myosin. For pan-fast myosin heavy chain (MHC; Novocastra, Newcastle, UK) no fixation was performed, and the sections were incubated with the antibody at a 1:40 dilution. For slow and neonatal myosin (Novocastra), the primary antibody dilution was 1:20. The sections were blocked for nonspecific binding with horse serum and avidin-biotin blocking reagents (Vector Laboratories, Burlingame, CA), and incubated for 1 hour with the primary antibody. The sections were rinsed and incubated using the avidin-biotin complex method (Vectastain Elite ABC kit; Vector Laboratories). The reacted tissue sections were processed by using the heavy metal–intensified diaminobenzidine procedure.

At least three cross-sections from each muscle were analyzed for individual myofiber cross-sectional area by manual tracing under bright-field microscopy. A minimum of three fields, or between 200 and 400 myofibers, were analyzed on each cross-section for each of the 11 rabbits studied. Mean myofiber cross-sectional areas for all myofibers were determined on a computer (Bioquant Image Analysis System; Bioquant, Nashville, TN). In addition, histograms were constructed from the cross-sectional area measurements of the individual control and IGF-treated muscles. The cross-sectional areas were divided into bins of 200-μm increments. The percentage of myofibers positive for the neonatal MHC was determined. Care was taken to analyze sections from similar regions along the muscle length, because the percentage of myofibers positive for neonatal myosin changes along the tendon-to-tendon muscle length.[19] All data were analyzed for statistical significance by an unpaired, two-tailed *t*-test or analysis of variance (ANOVA) or the Dunn multiple comparison test, on a computer (Prism and Statmate software; Graphpad, San Diego, CA, or SigmaStat 2.03; SPSS Science, Chicago, IL). An F-test was used to verify that the variances were not significantly different. Data were considered significantly different if $P \leq 0.05$.

RESULTS

No toxic effects of IGF treatment were observed in any of the animals. No obvious change in the rotational position of the globe was seen in the IGF-treated eyes. A single injection of IGF





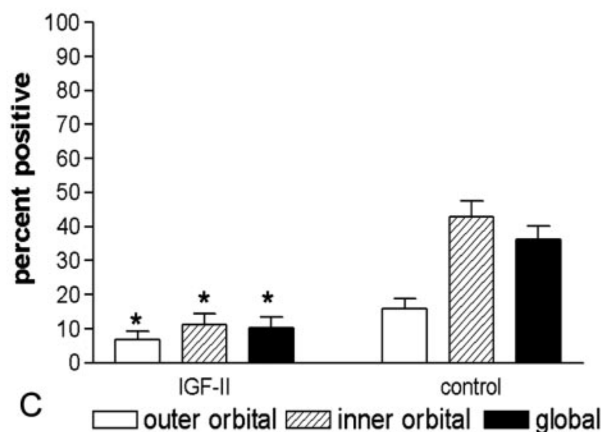


FIGURE 4. Photomicrograph of an IGF-injected superior rectus muscle (**A**) and a saline-injected control muscle (**B**) 1 week after a single injection immunostained with an antibody against the neonatal MHC isoform. *Arrows*: myofibers positive for neonatal myosin. Bar, 50 μm. (**C**) Percentage of myofibers positive for the neonatal MHC isoform per total number of myofibers counted in representative cross-sections from IGF and saline-treated superior rectus muscles. $^*P \leq 0.05$.

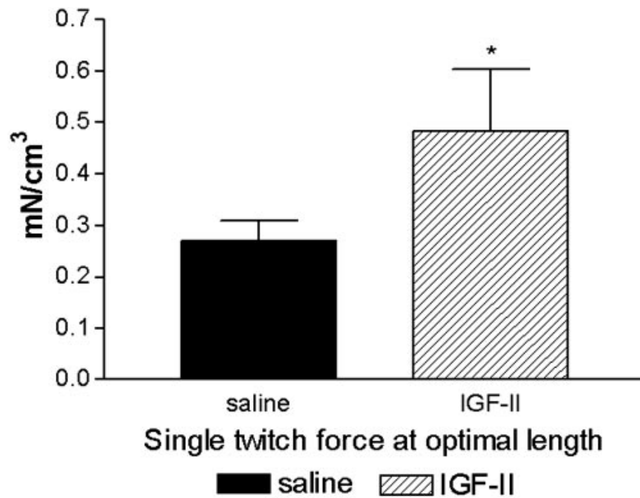


FIGURE 5. Generated force at 0.1 Hz stimulation frequency in IGF-II and saline-injected control superior rectus muscles in rabbits at 1 week ($n = 7$). Mean single twitch force generation was 0.48 ± 0.12 mN/cm^3 after IGF-II treatment compared with 0.27 ± 0.04 mN/cm^3 for the saline injected control muscles. *$P = 0.0473$.

did not result in a significant increase in mean cross-sectional area of myofibers positive for the fast or slow MHC 1 week after treatment (Figs. 1, 2). Although mean myofiber cross-sectional area did not change, in six of the seven muscle pairs examined, the total muscle wet weight of the IGF-II–treated muscle increased an average of 5.5% ($P = 0.0162$). There was also an increase in the heterogeneity in myofiber cross-sectional areas compared with control muscles, producing both very large and very small myofibers (Figs. 2, 3). In particular, there was a significant increase in the population of hypertrophic myofibers ($P = 0.05$), those with cross-sectional areas of greater than 3000 μm^2, as well as an increase in the number of myofibers with cross-sectional areas of 400 μm^2 or less. The population of hypertrophic fibers with cross-sectional areas over 3000 μm^2 represented 2% of the total myofibers in the IGF-treated muscle. There were few to none in this size range in the normal superior rectus muscle cross-sections. The population of myofibers with cross-sectional areas less than or equal to 400 μm^2 represented 24.3% of the total in the IGF-treated superior rectus muscles, compared with 9.7% of the myofibers in this size range in the saline-injected control samples.

The expression of MHC isoforms plays a role in the contractile properties of skeletal muscles. The superior rectus muscles treated with IGF were examined for alteration in expression patterns of neonatal MHC compared with saline-injected control extraocular muscles. The single injection of IGF resulted in a significant decrease in the number of myofibers expressing neonatal MHC (Fig. 4).

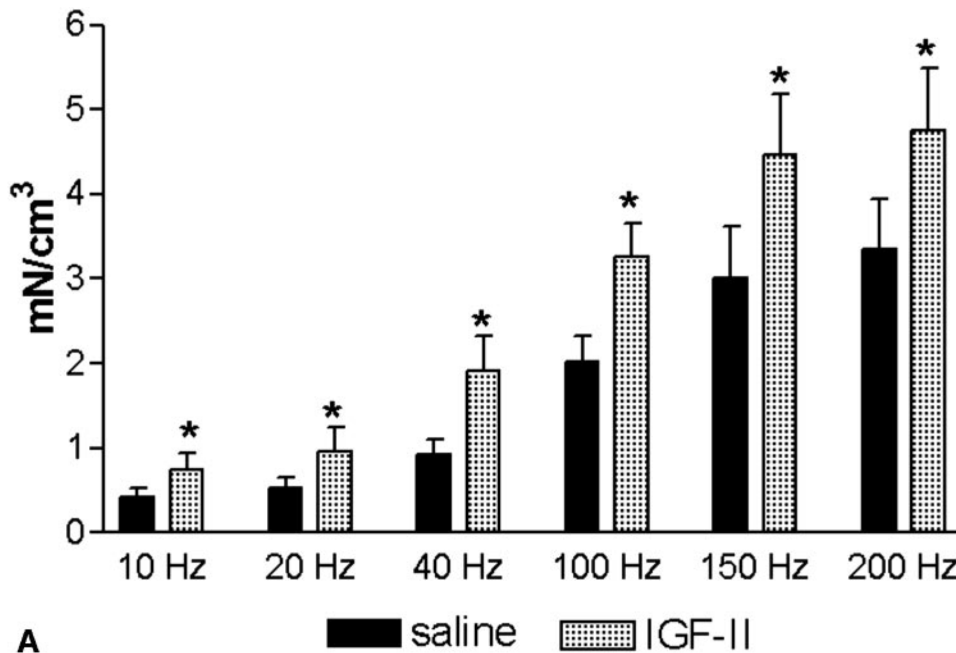
IGF-treated superior rectus muscles showed a significant increase in both single-twitch and tetanic tension development 1 week after a single injection ($P = 0.0473$; Figs. 5, 6). In the IGF-treated muscles, mean developed tension at a stimulation frequency of 10 Hz was 0.747 ± 0.2 mN/cm^3 compared with 0.422 ± 0.1 mN/cm^3 in the control ($P = 0.0230$). This represents a 56.5% increase in force generation compared with the saline-injected control. At 20 Hz, the force was increased 54.5% and at 40 Hz, by 47.7% ($P = 0.0352$). At the higher stimulation frequencies, similar increases in force generation were observed in the IGF-treated muscles. At 200 Hz the mean tetanic tension of the treated muscles was 4.752 ± 0.7 mN/cm^3 compared with 3.342 ± 0.6 mN/cm^3 in the saline-injected control ($P = 0.0156$); this represents a 70.33% increase over the saline-injected muscle. Tension development after double, triple, and quadruple pulses (0.4 ms pulse duration) was also significantly larger 1 week after IGF injection (Fig. 7). There was, however, no difference in fatigability between control and IGF-treated muscle (Fig. 8).

DISCUSSION

A single injection of IGF-II into the superior rectus muscles of rabbits resulted in a significant increase in twitch and tetanic force development compared with the saline-injected control. In contrast to limb muscle, IGF-II treatment did not increase the average cross-sectional area of extraocular myofibers, although overall total muscle wet weight increased. The heterogeneity of individual myofiber cross-sectional areas, however, was increased. In particular, a significant increase in the number of markedly hypertrophic myofibers occurred in the IGF-treated muscles. Thus, the combination of increased muscle weight and the increase in the population of significantly hypertrophic fibers suggests that significant alterations occur within the IGF-treated superior rectus muscles. Preliminary data suggest that increasing the IGF-II dose to 20 μg yields an even greater increase in force generation than that occurring after injection of 10 μg IGF-II (McLoon LK, et al. *IOVS* 2003; 44:ARVO E-Abstract 3125). These results suggest that treatment with injectable growth factors may be a viable means of strengthening underacting extraocular muscle.

The lack of a significant increase in mean myofiber cross-sectional area after a single injection of IGF-II into the superior rectus is interesting in light of the large number of studies where elevated expression of IGF-I or -II resulted in satellite cell proliferation,[20] increased myogenic differentiation,[21] or marked myofiber hypertrophy.[14,16,22] In the present study, only a single injection of IGF-II was made into the superior rectus muscle. When IGF-I was infused continuously over a 2-week period into limb muscles, the weight of the treated muscle increased by approximately 9%.[14] However, the muscle was not examined histologically, and individual myofiber cross-sectional areas were not determined. In a transgenic mice model in which IGF was overexpressed in skeletal muscle specifically using an actin promoter, myofibers were significantly hypertrophic.[22] Again, in the transgenic mouse model, the muscles were exposed to elevated levels of IGF-I over a significant period, because the actin promoter is turned on early in muscle development. In a third approach, virus-mediated expression of IGF-I or IGF-II was used to increase localized skeletal muscle expression, and again, hypertrophy of individual myofibers developed.[12,13,16] Most important, the continued upregulation of IGF was associated with an increase in force generation in the hypertrophic limb muscles.[12,17,23] Our preliminary studies have indicated that by 2 weeks after a single injection, extraocular muscle strength returns to control levels. We are currently looking at methods to increase the duration of muscle exposure to IGF. On the basis of the published literature, we surmise that the increase in extraocular muscle force generation that occurs after a single injection of IGF-II will be enhanced and extended if the IGF treatment is sustained, by continuous infusion, extended release, or treatment with a viral vector.

On the contrary, elevated expression of IGF-II in limb muscle results in a number of muscle changes distinctly different from IGF-I. One particularly interesting effect of elevated levels of IGF-II is a decrease in programmed cell death in proliferating myogenic cell lines[24] and in limb muscles of the *mdx* mouse.[25] Because there is an ongoing process of myonuclear turnover in normal extraocular muscle,[26–28] it is possible that a single injection of IGF-II results in a decrease in myonuclear apoptosis



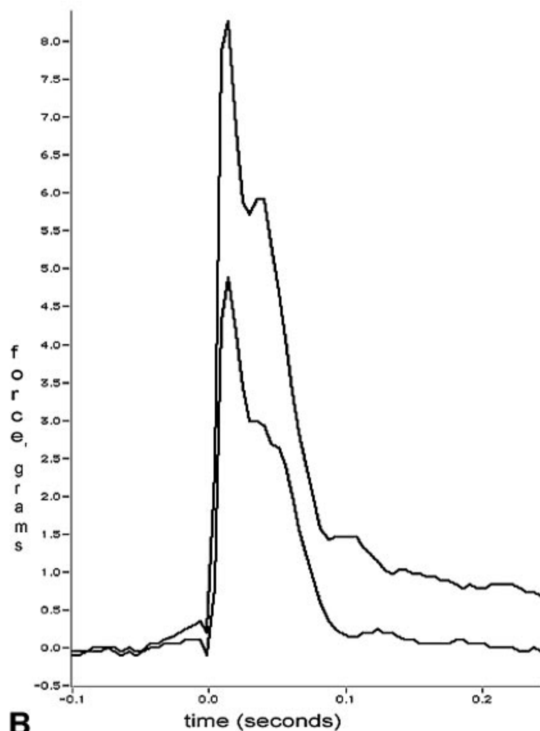


FIGURE 6. (A) Generated force with increasing stimulation frequencies of 10, 20, 40, 100, 150, and 200 Hz (250 ms train duration) with 4 minutes of rest between stimuli in IGF-II and saline-injected control superior rectus muscles in rabbits 1 week after a single injection ($n = 7$). *Significant at $P \leq 0.05$. (B) Representative force response to a single 10-Hz simulation pulse. The *top trace* is the force produced by a 10 Hz stimulation of the IGF-II injected superior rectus muscle, with the *trace* from the contralateral, saline-injected control muscle stimulated at 10 Hz superimposed on the same scale.

in the treated superior rectus muscles. This is a particularly interesting possibility. We are currently looking at the effect of IGF-II on myonuclear apoptosis in the treated superior rectus muscles.

Although mean myofiber cross-sectional area did not change, the total muscle wet weight of the IGF-II–treated muscle increased by 5.5%. The heterogeneity of myofiber cross-sectional areas also increased, with increases in the percentage of myofibers with very small and very large myofiber cross-sectional areas. The population of hypertrophic fibers, with cross-sectional areas over 3000 μm^2, represented 2% of the total myofibers in the IGF-treated muscle, with few to none in the control superior rectus muscle cross-sections. In addition to the very large myofibers, there was also an increase in the number of myofibers with cross-sectional areas less than or equal to 400 μm^2. These smaller myofibers represented 24.3% of the myofibers in the IGF-II–treated muscles, compared with 9.7% in the control muscles. This observation is interesting in light of a recent study that different types of skeletal muscles use different modes of hypertrophy.[29] In muscles with a single endplate band, hypertrophy is characterized by increased myofiber cross-sectional areas; however, in muscles with multiple endplate bands and myofibers that end intrafascicularly, no increase in myofiber cross-sectional areas occurs. Instead, these muscles respond to elevated IGF levels by increasing individual myofiber length. The extraocular muscles have mul-

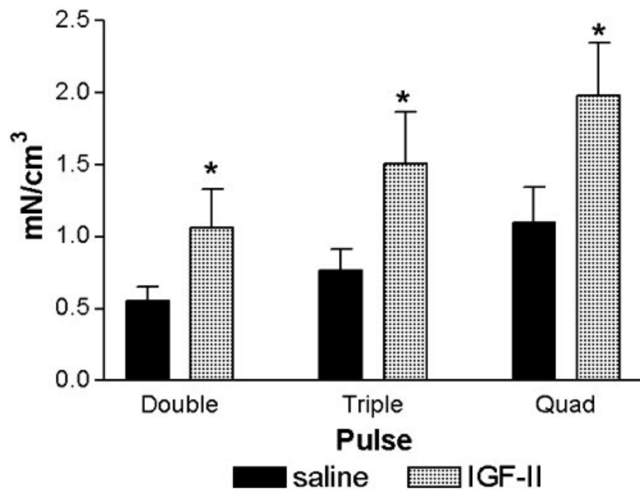


FIGURE 7. Tension development was measured for single, double, triple, and quadruple pulses (0.4 ms pulse duration) with 2 minutes rest between stimuli in IGF-II– and saline-injected control superior rectus muscles in rabbits 1 week after a single injection ($n = 7$).$^*P \leq 0.05$.

tiple endplate bands[19,30] and are composed of many short myofibers.[19,31] Thus, the observation that there were large numbers of myofibers with small cross-sectional areas is consistent with fiber lengthening as the mode of hypertrophy in response to IGF treatment in extraocular muscles. We are currently looking at myofiber length to confirm this.

Skeletal muscles are composed of heterogeneous populations of myofibers, each expressing different myosin heavy and light chain isoforms. This isoform diversity is associated with diversity in the physiological properties of single myofibers, including maximum shortening velocity and maximum power.[32,33] Myosin isoform expression is extremely adaptable, with changing patterns of myosin coexpression in single myofibers in response to different physiologic demands placed on the muscle.[34] IGF treatment has been shown to alter the phenotype of limb muscle fibers and was associated with increased muscle force.[17] The decrease we observed in the number of myofibers expressing the neonatal MHC isoform after IGF-II treatment may also be a factor in changing force generation. A more inclusive quantitative analysis of myosin

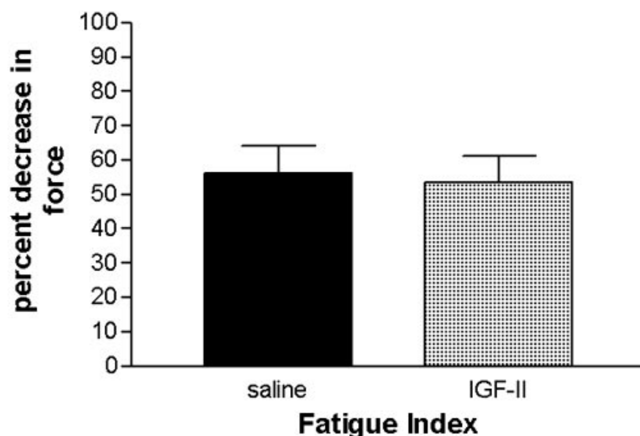


FIGURE 8. Fatigability was tested by stimulating the muscles at 100 Hz (250 ms train duration) every 10 seconds for 1600 seconds or until there was a 50% reduction in generated force. No significant difference in fatigability was noted between IGF-II– and saline-injected superior rectus muscles 1 week after a single injection ($n = 7$).

isoform changes in the IGF-treated superior rectus muscles is needed to clarify the action of IGF treatment on force generation, because isoform switching could be part of the myriad of changes that occur in the IGF-treated EOM.

Currently, the only treatment available to strengthen an underacting extraocular muscle is traditional surgery. The use of direct application of growth factors to these muscles to increase muscle strength would be a powerful addition to the treatment choices available for patients with strabismus. It is important to demonstrate that this increase in muscle force can be maintained for a sufficient duration to be effective in the long-term treatment of strabismus. However, the present study demonstrates that injection of IGF into extraocular muscle is a viable means of acutely increasing extraocular muscle strength.

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