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Effects of sequential injections of hepatocyte growth factor and insulin-like growth factor-I on adult rabbit extraocular muscle

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Abstract

Purpose—To determine whether hepatocyte growth factor (HGF) and insulin-like growth factor-I (IGF-I) have synergistic effects in promoting extraocular muscle fiber growth and force generation.

Methods—A superior rectus muscle of adult rabbits was treated with either a single injection of HGF or sequential injections of HGF followed 1 week later by IGF-I. One week after HGF alone and 1 week after the IGF-I injection, the superior rectus muscles from treated and control orbits were examined for alterations in force generation as well as changes in myofiber size.

Results—Injection of HGF alone did not result in changes to muscle force, specific tension, or myofiber cross-sectional area; however, it did result in a significant increase in numbers of satellite cells. Sequential injection of HGF and IGF-I resulted in significantly increased force, specific tension, and myofiber cross-sectional areas as well as increased numbers of satellite cells.

Conclusions—Preinjection with HGF augments the treatment effect of IGF-I. This is likely a result of HGF-induced activation of satellite cells. This synergistic effect should allow a reduction in IGF-I dosing required to produce a given increase in extraocular muscle force generation.

Pharmacologic treatment of strabismus is evolving. Botulinum toxin A (BTA) was developed over three decades ago¹ as a means of weakening skeletal muscle while avoiding permanent alterations in the biomechanics of the ocular rotary mechanism. BTA proved effective in some forms of strabismus, but widespread adoption of BTA has been limited, due, in part, to its relatively brief duration of action and the frequent need for reinjection. Whereas BTA only weakens extraocular muscle, growth factors can be used to strengthen them^{2–5}; however, no growth factors have been approved for clinical use. Recent studies

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using the local anesthetic bupivacaine have shown changes in eye position, but the mechanism is unclear.^{6,7} This potential to increase and decrease extraocular muscle strength pharmacologically, in a way that is easily and safely administered, bears further development and study.

Recently we showed that single, direct intramuscular injections of insulin-like growth factor (IGF) I or II into adult rabbit extraocular muscles result in significantly increased muscle force (grams) and specific tension (mN/cm²), with most effective doses at 10 µg and 25 µg.^{2,5} This increased muscle force was maintained by sustained delivery of IGF-I to the extraocular muscle,⁴ as IGF-I and its receptor are both expressed in adult extraocular muscle.^{2,8} There is some concern over the potential effect of high doses of IGF-I in extraocular muscles because recent data has shown a possible link between the IGF-I receptor in orbital fibroblasts in active thyroid eye disease and fat expansion.⁹ Thus we tested a strategy for lowering the dosage of IGF-I while maintaining equivalent force augmentation. Prior to clinical use, several important issues need to be addressed, including the dosage required for both maximal and lasting effects as well as demonstration that no local or systemic toxicity result from sustained release of these growth factors.

Hepatocyte growth factor (HGF) stimulates mitogenesis and plays an important role in tissue regeneration.¹⁰ In skeletal muscle, HGF activates quiescent satellite cells, which are the regenerative population.^{11,12} It also inhibits differentiation of the activated satellite cells.¹³ This should result in increased numbers of myogenic precursor cells in treated muscles; however, HGF alone does not result in increased myofiber growth,¹⁴ unlike IGF-I.^{15–17} Studies show that the expression of these two growth factors increases in skeletal muscle during repair, growth, and remodeling.^{18,19} The present study examined whether preinjection of HGF prior to IGF-I treatment would augment the effect of IGF-I alone by providing greater numbers of satellite cells and therefore promoting increased myofiber size and force generation. If this injection strategy is effective, less IGF-I could be injected in order to obtain the same overall muscle-strengthening effect. The potential mechanisms of action are also investigated.

Methods

Adult New Zealand rabbits were obtained from Bakkom Rabbitry (Viroqua, WI) and housed with Research Animal Resources in their AAALAC-approved facility at the University of Minnesota. All studies were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and followed the guidelines for animal research set forth by the National Institutes of Health. Rabbits were anesthetized with an intramuscular injection of ketamine and xylazine (1:1, 10mg/kg:2mg/kg, resp.). Every experimental injection was accompanied by an injection of an equal volume of saline on the contralateral side. One group of 8 rabbits received a single injection of 2.5 µg HGF in 100 µl sterile isotonic saline into one superior rectus muscle (rmHGF, R & D, Minneapolis, MN), with a similar volume of saline injected into the contralateral superior rectus muscle. The second group of 8 rabbits received a single injection of HGF as described, followed 1 week later with an injection of 5 µg IGF-I in 100 µl sterile isotonic saline (rmIGF-I, R & D) into the same superior rectus muscle treated with HGF. This timing is the point of peak satellite cell proliferation based on in vitro studies (Hebert and McLoon, unpublished studies, 2012). The contralateral superior rectus muscle received a second saline injection of a similar volume. As an additional control for the potential effect of injection alone causing the subsequent increased force measurements seen with the HGF/IGF-I treatments, another group of 6 rabbits received bilateral injections of 100 µl sterile isotonic saline followed one week later with an injection of 5 µg IGF-I in 100 µl sterile isotonic saline into one of the saline treated superior

rectus muscle. An additional 4 rabbits were injected with HGF only and 4 rabbits were injected with HGF/IGF-I as described but prepared for histological analysis only.

After one week for the HGF-only treated rabbits, or 2 weeks for the HGF/IGF-I treated rabbits or saline/IGF-I treated rabbits, the animals were deeply anesthetized with ketamine and xylazine, followed by a thoracotomy. The superior rectus muscles were removed from the sclera to the apex of the orbit and analyzed for force and specific tension generation as previously described.^{2,4,20,22} Maintained in oxygenated Krebs solution at 30° C for the duration of the experiment, the muscles were attached to force transducers in in vitro incubation chambers (Aurora Scientific, Aurora, Ontario, Canada) and stimulated by flanking platinum electrodes. After length-tension curves were generated, all further tests were performed with supramaximal stimulus intensities at optimal preload. After muscles achieved stabilization,²³ they were stimulated at frequencies of 10, 20, 40, 100, 150, and 200 Hz at a train duration of 500 ms with a 2-minute interstimulation rest and force in grams was determined. Percent differences were calculated for each stimulation frequency for each rabbit, and the mean percent difference was determined based on these values. For any given treatment, the mean values of all treated rabbits were averaged to determine the final percent difference.

The HGF only (N = 8), saline/IGF-I (N = 6), HGF/IGF-I (N = 8), and control superior rectus muscle (N = 14) were prepared for histologic examination. All superior rectus muscles were embedded in tragacanth gum, frozen in 2-methylbutane chilled to a slurry on liquid nitrogen, sectioned at 12 µm in a cryostat, and stored at -80° C until processed for immunohistochemistry. The following primary antibodies were used for immunostaining using our standard methods: fast, neonatal, and developmental myosin heavy chain (MyHC) isoforms (1:40; Vector Labs, Burlingame, CA); Pax7 (1:100; Hybridoma Bank, Iowa City, IA), and neurofilament (1:1000; Covance, Princeton, NJ). After blocking in normal serum, selected sections were incubated in primary antibody, rinsed in phosphate buffered saline, incubated with reagents in the Vectastain Elite ABC kit (Vector Lab), followed by incubation in diaminobenzidine using heavy metal intensification.⁵ Cross-sectional areas were measured for each of the three MyHC isoforms in both the orbital and global layers within sections taken from the muscle midbelly and within 200 µm of the end of myofibers in the tendon (referred hereafter as “in the tendon region”). Mean area and percent positive for expression of each isoform (see Figure 3) were calculated, with a minimum of 200 myofibers counted per layer and region. The effect on satellite cell number was assessed by analysis of Pax7-positive nuclei. Pax7 is a widely accepted marker for quiescent satellite cells.²⁴ Pax7-positive nuclei were counted per myofiber number, with a minimum of 200 fibers counted per field for each layer and region. Neurofilament-stained HGF, HGF/IGF-I and saline-treated superior rectus muscles were assessed by determining the area positive for axon bundles as a percent of total muscle area per cross-section, and orbital and global layers were compared in both the midbelly and tendon region.

Data were expressed as mean plus or minus the standard error of the mean. All data were analyzed for statistical significance using either an unpaired two-tailed *t* test (if two groups were being compared) or an analysis of variance (ANOVA) and Dunn’s multiple comparison tests aided by the Prism and Statmate software (Graphpad, San Diego, CA) for multiple group comparisons. An *F* test was used to verify that the variances were not significantly different. Additional statistical analyses included ANOVA with Bonferroni adjustment. A *P* value 0.05 was considered statistically significant.

Results

Single injections of HGF alone did not result in significantly increased force generation 1 week after treatment, whether examined in grams (Figure 1A) or specific tension (not shown). In contrast, sequential injections of HGF followed 1 week later by IGF-I did result in significantly increased force (Figure 1B) and specific tension (not shown) at all stimulation frequencies. When compared to the mean percentage change in force generation observed with escalating doses of IGF-I alone,² the combined sequential injection of HGF and IGF-I resulted in significantly increased force generation over that seen after single injections of IGF-I regardless of dose (Figure 2). Injections of only HGF resulted in forces that were significantly below all doses of IGF-I alone and even more significantly below the combined HGF/IGF injections. Preinjection with saline prior to injection with IGF-I resulted in levels similar to IGF-I alone.²

Mean myofiber cross-sectional areas were unchanged after HGF alone but were significantly greater after the sequential treatment with HGF and IGF-I compared to control levels, specifically in the orbital middle, and global middle and tendon regions (Figure 3). It should be noted that there was a great deal more heterogeneity of myofiber cross-sectional area in the HGF treated global region myofibers (Figure 3). This may have been due to concentration differences along the muscle after injection. Future studies will examine how local HGF concentration might alter these effects. The HGF-only treated superior rectus muscles had significantly more Pax7-positive nuclei per myofiber number than the untreated control muscles. This increased density of satellite cells was maintained in the HGF/IGF-I treated superior rectus muscles (Figure 4). No change in nerve density was seen in the HGF/IGF-I treated muscles at this short post-treatment interval (not shown).

The sequential treatment of superior rectus muscles with HGF and IGF-I resulted in few changes in fast MyHC isoform expression, with significant decrease only in the orbital tendon region (Figure 5A). The global layer showed significant changes in developmental and neonatal MyHC isoform composition, with significant decreases in the percentage of global layer myofibers expressing developmental MyHC isoform (Figure 5B) and a significant increase in neonatal MyHC isoform expression (Figure 5C). There was no measureable change in orbital layer fibers expressing developmental MyHC; however, the percentage of myofibers expressing neonatal MyHC did decrease significantly.

Discussion

A single injection of HGF was insufficient to cause a significant change in muscle force generation 1 week after treatment. However, when HGF was injected intramuscularly 1 week prior to an injection of IGF-I, both mean myofiber cross-sectional area and force generation significantly increased. A prior injection of HGF before an IGF-I injection also resulted in significant elevation of muscle force generation above forces normally observed with a single IGF-I injection.² Thus combined sequential treatment by HGF and IGF-I resulted in muscles significantly stronger than after any dose of IGF-I alone.² This would allow for administration of a smaller amount of IGF-I to produce the same or even enhanced muscle force.

IGF-I is well known for its ability to build muscle mass and strength on skeletal muscle in a variety of conditions¹⁷ and has been shown to increase muscle mass and force generation capacity in, for example, mouse models of muscular dystrophy,^{23,25} aging,²⁶ and muscle cachexia from cancer,²⁷ and other chronic debilitating diseases.²⁸ Several possible mechanisms may explain why injection of HGF followed by IGF-I increased force generation greater than with IGF-I alone. HGF activates quiescent satellite cells, resulting in

entry into the cell cycle and increased replication.^{11,12} As predicted from these studies, HGF injection into rabbit extraocular muscle resulted in significant increases in satellite cell number (Figure 4), which has been specifically linked to myofiber hypertrophy.^{29,30} However HGF treatment also inhibits skeletal muscle differentiation.^{13,14} IGF-I can induce satellite cell proliferation, resulting in increased number of these muscle precursor cells.³¹ However, IGF-I also significantly contributes to the development of myofiber hypertrophy, increasing protein synthesis rates³² and decreasing protein degradation pathways.³³ This can occur even in the absence of increased satellite cell number.¹⁵ Sequential up-regulation of HGF followed by IGF-I is seen in regenerating and diseased skeletal muscle^{19,34} and has even been shown to foster regeneration of infarcted pig heart.³⁵ The rationale for sequential treatment is based on these collective studies.

Changes in MyHC isoform composition were most pronounced in the global layer, which produces the majority of force needed for eye movements. It is unclear how an increased percentage of global layer myofibers positive for neonatal MyHC and a decreased percentage of fibers positive for developmental MyHC would alter muscle function in light of the complex coexpression patterns of MyHC isoforms in single fibers in the extraocular muscle³⁶; nevertheless, similar MyHC isoform changes were seen after single injections of IGF-I in our previous study² as well as in other studies that examined MyHC isoform composition after either exogenously supplied IGF-I treatment or other experimental paradigms that result in localized increases in IGF-I.^{37,38} Our previous work showed that, despite increased neonatal MyHC expression, the addition of new myonuclei into existing EOM myofibers did not correlate with neonatal MyHC fiber expression.³⁹ Although not specifically measured in the present study, alteration of MyHC isoform expression patterns would be expected to change shortening velocity⁴⁰; thus functional sequelae would be predicted from these changes.

Both IGF-I and IGF-I receptor are expressed in extraocular muscle tissue throughout life^{2,8} and are thought to play a role in maintaining the continuous myofiber remodeling that occurs in adult mammalian extraocular muscle.⁴¹ Thus the cellular machinery for rapid response to exogenously increased IGF-I is present without requiring receptor up-regulation. Recently an interrelationship was suggested between orbital tissue in Graves' ophthalmopathy and antibodies to IGF-I receptor.⁴² Expression was found to be coresident with the expression of antibodies to thyroid stimulating hormone receptor.⁴³ However, recent studies somewhat allay the concern that exogenously added IGF-I might provide a "trigger" for an autoimmune response in the orbit. When mice were immunized with IGF-I receptor, thyroid stimulating hormone receptor, or both proteins, only immunization with thyroid stimulating hormone receptor protein alone resulted in antibodies to both thyroid stimulating hormone receptor and IGF-I receptor and moderate tissue fibrosis in orbital tissues.⁴⁴ This suggests that the precipitating factor for orbital disease is development of antibodies to thyroid stimulating hormone receptor. It should be noted that all previous studies of IGF-I treatment of extraocular muscle report no changes in connective tissue density²⁻⁵; nevertheless, our findings demonstrate that equivalent or greater efficacy using lower doses of IGF-I is possible in extraocular muscle.

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FIG 1.

A, Effect one week after a single injection of HGF compared to saline treated controls on muscle force in grams. B, Effect of sequential treatment of superior rectus muscles by HGF followed one week later by a single injection of IGF-I compared to saline treated controls on muscle force in grams. The asterisk indicates significantly different from control. N = 8. Data are shown as mean plus or minus the standard error of the mean.

FIG 2.

Comparison of percentage change of means in specific tension of either HGF alone or IGF alone compared to sequential treatment with HGF and IGF. Percentage change of injected IGF-I muscles was calculated based on Anderson and colleagues.² The asterisk indicates significant difference of HGF/IGF injections compared to all doses of IGF only, HGF only and saline/IGF. The σ indicates significant difference of HGF only compared to all doses of IGF only or HGF/IGF. While not shown on the graph there were significant differences between 1 and 25μg IGF-I doses, and between 10 and 25μg IGF-I doses. N = 6–8. (ANOVA, Bonferroni: $P < 0.05$).

FIG 3.

Mean cross-sectional areas in HGF/IGF-I co-treated superior rectus muscles compared to saline treated controls determined on sections immunostained for fast MyHC. A, Control global layer muscle fibers immunostained for fast MyHC 2 weeks after sequential saline injections. B, Global layer myofibers examined one week after a sequential treatment of HGF followed one week later by IGF-I. Bar is 20 μm. C, Mean cross-sectional areas of the middle region of the superior rectus muscles treated with saline only (control), HGF only, or sequential HGF/IGF-I. D, Mean cross-sectional areas of the tendon region of the superior rectus muscles treated with saline only (control), HGF only, or sequential HGF/IGF-I. N = 4. White indicates control, light gray indicates HGF treatment, and black indicates HGF/IGF treatment. Data are shown as mean plus or minus the standard error of the mean. An asterisk indicates significantly different from saline-only control muscles.

FIG 4.

Density of Pax7+ satellite cells (arrows) in HGF/IGF-I co-treated superior rectus muscles compared to saline treated controls. A, Saline-treated control global layer superior rectus myofibers immunostained for Pax7. B, Global layer myofibers immunostained for Pax7 examined one week after a sequential HGF/IGF-I treatment. Bar is 20 microns. C, Quantification of density of Pax7+ cells per number of myofibers the middle region of the muscle. D, Quantification of density of Pax7+ cells per number of myofibers the tendon region of the muscle. White indicates control, light gray indicates HGF treatment, and black indicates HGF/IGF treatment. N = 8. Data are shown as mean plus or minus the standard error of the mean. The asterisk indicates significantly different from saline-only control muscles.

FIG 5. Quantification of percentage of myofibers expressing A, fast MyHC, B, developmental MyHC, and C, neonatal MyHC in HGF/IGF-I cotreated SR compared to saline treated controls. N = 8. Data are shown as mean plus or minus the standard error of the mean. The asterisk indicates significantly different from saline-only control muscles.