Role of Exogenous and Endogenous Trophic Factors in the Regulation of Extraocular Muscle Strength during Development

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Purpose. Weak eye muscles can cause ophthalmic disorders, and in particular, strabismus. Exogenous trophic factors such as cardiotrophin (CT)-1 and insulin-like growth factor (IGF) have been shown to increase the contractile force of adult heart and skeletal muscles, respectively. In the current study, the effects of endogenous and exogenous trophic factors on extraocular muscle strength and mass were examined in the developing chicken.

Methods. Superior rectus and superior oblique muscles of hatching chicks were treated in vivo either to increase levels of trophic factors CT-1, IGF-I, glial cell line-derived neurotrophic factor (GDNF), or brain-derived neurotrophic factor (BDNF), or to decrease their levels with neutralizing antibodies and binding proteins. Forty-eight hours after factors were injected into the orbit, the contractile force of dissected muscles was measured in vitro and the morphology of muscle fibers was compared between control and treated muscles.

Results. Treatment with CT-1 or IGF-I significantly increased the mean single-twitch force generation and these trophic factors increased muscle fiber diameters when compared to control muscles. A cocktail of antibodies and binding proteins, directed against endogenous IGF-I, GDNF, and CT-1, significantly decreased mean single-twitch force. This cocktail slightly, but significantly, reduced muscle fiber diameters within treated extraocular muscles.

Conclusions. Endogenous trophic factors regulate and/or maintain extraocular muscle force through a rapid mechanism that appears to involve changes in muscle mass and specifically enlargement of muscle fiber diameters. CT-1 and IGF-I may be considered promising candidate trophic factors for therapeutic strengthening of eye muscles in the developing extraocular muscle system. (Invest Ophthalmol Vis Sci. 2004;45: 3538–3545) DOI:10.1167/iovs.04-0393

Strabismus, a misalignment of the visual axes, affects 5% of the population. In children, this disorder can result in severe central visual deficiencies such as amblyopia. In adults with a mature visual system, misalignment of the eyes results in diplopia. Current treatments for strabismus are intended to weaken the relatively stronger antagonist muscle by surgical muscle resection or botulinum toxin injection. However, both treatments can have unwanted side effects.2–7 Currently, there is no treatment for strabismus that strengthens the weaker eye muscle. Therefore, it is important to explore new treatment options for strabismus.

Trophic factors were discovered as cell survival- and differentiation-promoting agents that belong to different families: neurotrophins, the ciliary neurotrophic factor/leukemia inhibitory factor (CNTF/LIF) family, hepatocyte growth factor/scatter factor family, insulin-like growth factor (IGF) family, and the glial cell line-derived neurotrophic factor (GDNF) family.8 Viral expression of IGF-I promotes an average increase of 15% in skeletal muscle mass and a 14% increase in muscle strength in young and adult mice.9 Overexpression of IGF-I has also been shown to cause muscle hypertrophy in transgenic mouse lines.10 In two disease models of experimental animals, amyotrophic lateral sclerosis and Duchenne-type muscular dystrophy, IGF-I significantly delays the onset and progression of both diseases.11–13 Most recently, IGF-II injected into the extraocular muscles of adult rabbits was shown to increase the muscles’ contractile force.14 CT-1, a member of the family of cytokines that includes interleukins, can induce cardiac myocyte hypertrophy and promotes the survival of cultured motoneurons.15–17 These reports suggest that trophic factors may regulate muscle strength.

Several trophic factors are known to be expressed in muscles and affect muscles and their innervation. For example, IGFs are produced by satellite cells and both IGF-I and IGF-II signal through the IGF-I receptors. These receptors are abundantly expressed in skeletal muscle.18 GDNF, a distant member of the transforming growth factor-β family, is a target-derived neurotrophic factor that is expressed in Schwann cells, human skeletal muscle, and chick extraocular muscle.19,20 GDNF is also the most potent motoneuron survival factor known to date.21 Overexpression of GDNF in neonatal mice under a musclespecific (myogenin) promoter increases the number of motor axons innervating the neuromuscular junctions.22 Twitch-tension measurements in myogenin-GDNF transgenic mice demonstrate that the maximum muscle tension was elicited by activating only 66% of the number of motor axons needed to elicit maximum tension in wild-type control animals.23 BDNF, a member of the neurotrophin family, is present in skeletal muscle, including eye muscles of chick, and can prevent motoneuron death in vivo.24–26 Administration of exogenous BDNF can retard motor dysfunction in a natural motor neuron disease model and diminish denervation muscle atrophy and motor axon loss.26–27 These reports indicate that trophic factor ligands and receptors are present in muscle.

Based on their localization of expression and effects on muscle documented in previous studies, we decided to examine the effects on extraocular muscle of the following trophic factors: IGF-I, GDNF, cardiotrophin (CT)-1, and BDNF. We examined whether exogenous trophic factors can increase developing muscle strength and thus may be potential candidates for the treatment of strabismus. We also determined whether endogenous trophic factors are important in the regulation or maintenance of force in developing extraocular muscle. To test these hypotheses, we examined the effects of...
either increased or decreased levels of trophic factors on extraocular muscle force and morphology in the developing chicken. The oculomotor system is evolutionarily conserved. Although birds have smaller muscle fibers than mammals, detailed analyses have documented histologic similarities between mammalian and avian eye muscles. Furthermore, chickens have the most discretely organized oculomotor nuclei among vertebrates.

**MATERIALS AND METHODS**

**Materials**

Fertilized White Leghorn chicken eggs were obtained from local suppliers and were kept in humidified incubators at 37.0°C to 37.5°C. Embryonic day (E)20 to hatching chicks were used for our studies, and the ages of chick embryos were verified at the time of death according to the method of Hamburger and Hamilton. Experimental procedures were conducted in compliance with the Policy on the Use of Animals in Neuroscience Research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local animal care committee. GDNF, IGF-I, and CT-1 were purchased from R&D Systems (Minneapolis, MN). Insulin-like growth factor binding protein (IGFBP)-4 was purchased from GroPeP (Adelaide, South Australia, Australia).

**Iodination and Injection of Trophic Factors and Antibodies**

Iodination of trophic factors and antibodies was performed as described. The proteins GDNF, CT-1, IGF-I, and BDNF were iodinated to specific activities of 90 to 94 cpm/pg GDNF, 25 to 62 cpm/pg CT-1; 102 to 107 cpm/pg IGF-I; and 92 to 141 cpm/pg BDNF. Incorporation was typically 70% to 95% for all proteins. Radiolabeled proteins were stable within 4 weeks after iodination, as determined by trichloroacetic acid precipitation analysis. Five to 10 μL of trophic factor spiked with radiolabeled protein was injected with microfine disposable syringes (BD Biosciences, Franklin Lakes, NJ) unilaterally through the conjunctiva of the upper eyelid into the retrobulbar space of the orbit of chick embryos at E20 to E21.

**Retention of Trophic Factor Protein in Extraocular Muscle**

One hundred twenty nanograms of radiolabeled IGF-I or GDNF was injected into the orbit, targeting the superior oblique and superior rectus muscle of E20 chick embryos. The embryos were killed, extraocular muscles were dissected, and radioactivity was measured 1, 8, 20, 24, and 48 hours after injection (Fig. 1). The radioactivity present within individual muscles was measured in a gamma counter (65% counting efficiency; 1470 Wallac Wizard; PerkinElmer, Wellesley, MA) to verify successful injections and to estimate effective concentrations of the injected peptide in the extraocular muscles. Immediately after dissection, the muscles were placed in freshly oxygenated Krebs buffer and gamma counted for 1 minute each. Care was taken that they did not dry out during transfer and measurement in the gamma counter vial. Because the amount of hot and cold trophic factors in the injection volume was known, we could calculate the total amount of trophic factor that remained in the extraocular muscle at the time of killing by measuring the amount of radioactivity in each muscle. Muscles with successful injections were pinned down on one side in a chamber perfused with oxygenated Krebs-Ringer buffer at 37.5°C. The other end of each muscle was tied with 60 silk sutures to a 10g force transducer (World Precision Instruments, Sarasota, FL) with a resonance frequency of 300 Hz and was secured on a micromanipulator (Narashige, East Meadow, NY). The force transducer was positioned with the line of force perpendicular to the extraocular muscle. Two fine platinum wire electromyographic (EMG) electrodes were positioned on either side of the muscle. The muscles were stretched to the optimal length (Lo) by the micromanipulator to examine the contractile properties of the muscles. The Lo was obtained by systematically lengthening the muscle with a micromanipulator while inducing single-twitch contractions. Lo was determined as the length of the muscle that allowed maximum twitch generation. Single-twitch contraction of muscles was measured with a data-acquisition system (Digidata 1322A; Axon Instruments, Union City, CA). The muscles were stimulated with single pulses (30–60 ms duration) and increasing voltages until they reached a plateau in their contractile force (Fig. 2A), typically at 100 to 150 V and 30 to 60 ms. Relatively long stimulus durations (50–60 ms) were used, as in previous studies, because shorter pulse durations (<10 ms) failed to elicit maximum muscle contractions in vitro. Statistically significant differences were assessed by an unpaired t test with a confidence level of P < 0.05. Muscle cross-sectional area was calculated by using a constant for muscle density of 1.056 g/cm3, and determining muscle wet weight and muscle length. This is the standard method to determine cross-sectional area, although extraocular muscle may contain larger extracellular space than skeletal muscle.

**Gain-of-Function Study**

The orbits of E20 to E21 chick embryos were injected with 250 ng of the individual trophic factors (BDNF, GDNF, CT-1, and IGF-I) or 5 μg IGF-I spiked with the respective radioiodinated trophic factor. In another group, the orbit was injected with a trophic “cocktail,” a combination of trophic factors (BDNF, 175 ng; GDNF, 50 ng; IGF-I, 120 ng; and CT-1, 70 ng). For control experiments, muscles were either not injected or were injected with phosphate-buffered saline (PBS). All injections included 0.25 mg/mL bovine serum albumin (BSA) as a carrier. Forty-eight hours later, chicks were killed, and extraocular muscles were dissected. As described earlier, radioactivity present within individual muscles was measured in a gamma counter to verify successful injections and to estimate effective concentrations of the injected peptide in the extraocular muscles. Immediately after dissection, the muscles were placed in freshly oxygenated Krebs buffer and gamma counted for 1 minute each. Care was taken that they did not dry out during transfer and measurement in the gamma counter vial. Because the amount of hot and cold trophic factors in the injection volume was known, we could calculate the total amount of trophic factor that remained in the extraocular muscle at the time of killing by measuring the amount of radioactivity in each muscle. Muscles with successful injections were pinned down on one side in a chamber perfused with oxygenated Krebs-Ringer buffer at 37.5°C. The other end of each muscle was tied with 60 silk sutures to a 10g force transducer (World Precision Instruments, Sarasota, FL) with a resonance frequency of 300 Hz and was secured on a micromanipulator (Narashige, East Meadow, NY). The force transducer was positioned with the line of force perpendicular to the extraocular muscle. Two fine platinum wire electromyographic (EMG) electrodes were positioned on either side of the muscle. The muscles were stretched to the optimal length (Lo) by the micromanipulator to examine the contractile properties of the muscles. The Lo was obtained by systematically lengthening the muscle with a micromanipulator while inducing single-twitch contractions. Lo was determined as the length of the muscle that allowed maximum twitch generation. Single-twitch contraction of muscles was measured with a data-acquisition system (Digidata 1322A; Axon Instruments, Union City, CA). The muscles were stimulated with single pulses (30–60 ms duration) and increasing voltages until they reached a plateau in their contractile force (Fig. 2A), typically at 100 to 150 V and 30 to 60 ms. Relatively long stimulus durations (50–60 ms) were used, as in previous studies, because shorter pulse durations (<10 ms) failed to elicit maximum muscle contractions in vitro. Statistically significant differences were assessed by an unpaired t test with a confidence level of P < 0.05. Muscle cross-sectional area was calculated by using a constant for muscle density of 1.056 g/cm3, and determining muscle wet weight and muscle length. This is the standard method to determine cross-sectional area, although extraocular muscle may contain larger extracellular space than skeletal muscle.

**Loss-of-Function Study**

Individual antibodies and proteins as well as cocktails of antibodies and blocking proteins to trophic factors (2.5 μg GDNF antibody, 2.5 μg CT-1 antibody, 1.7 μg IGFBP-4), spiked with 2 to 10 ng of the respective radiolabeled protein, were injected...
into the orbits of E21 chick embryos. The anti-GDNF antibody neutralizes GDNF bioactivity, and the concentration we used in our study has been shown to be effective in neutralizing endogenous GDNF in chick embryos. The anti-CT-1 antibody neutralizes the biological activity of CT-1 (R&D Systems). IGFBPs bind endogenous IGF-I and -II, thus reducing the bioavailability of these endogenous trophic factors. We used a dose of IGFBP-4 that has been shown to bind endogenous IGF-I effectively in extraocular muscles of chick embryos. Antibodies were spiked with radiolabeled protein as described above to verify successful injections and to estimate effective concentrations of injected proteins in muscles 48 hours later. The extraocular muscles were dissected, and the maximum contractile force was measured as described earlier.

**Morphologic Experiments**

A cocktail of trophic factors (175 ng BDNF, 50 ng GDNF, 120 ng IGF-I, and 70 ng CT-1), CT-1 individually (250 ng), IGF-I individually (5 μg), or a combination of neutralizing antibodies or binding proteins to trophic factors (2.5 μg GDNF antibody, 2.5 μg CT-1 antibody, 1.7 μg IGFBP-4) was injected together with a trace amount of respective 125I-labeled protein (2–10 ng) into the orbit of E20 chick embryos. Control muscles were either not injected or were injected with PBS. Forty-eight hours later, the chickens were killed by decapitation, and their eye muscles were dissected and radioactivities measured. The muscles were postfixed in 4% paraformaldehyde, dehydrated in a graded ethanol series, and embedded in paraffin (Paraplast Plus; Oxford Labware, St. Louis, MO). Muscles were positioned to obtain 10 μm cross sections. Every 20th section of the 10-μm muscle sections was collected on silane-coated slides. Sections were deparaffinized, stained with thionin, dehydrated, and coverslipped with di-N-butyl phthalate-xylene (DPX; BDH Laboratory Supplies, Poole, UK). The muscles were examined under a microscope (Nikon, Tokyo, Japan) with a drawing tube to determine the total number of muscle fibers per cross section through the belly (largest cross section) and the average diameter of the muscle fibers. The total number of muscle fibers was counted under the microscope with a 40× objective. For the measurements of the average diameter of the muscle fibers, three to eight muscles were examined from each treatment group, and 60 muscle fibers were randomly chosen from each muscle. The diameter of each fiber was measured under the microscope with a 100× objective and oil immersion. The average diameter of all 60 muscle fibers was calculated. The data for all muscle fibers were plotted on a histogram to compare the relative frequencies of diameters of the muscle fibers in the injected and control muscles. Statistically significant differences between groups were assessed by the Wilcoxon signed rank sum test.

**RESULTS**

**Contractile Force of Normal Extraocular Muscle**

To determine the contractile force of normal chick extraocular muscles, the superior rectus and superior oblique muscles of hatching chicks were dissected and contractile force measured in vitro (Fig. 2C). Control injections of PBS (also containing BSA) into the extraocular muscles did not cause a significant increase in contractile force compared with muscles that were not injected (Fig. 2F), demonstrating that the injection itself does not increase muscle contractile force. Therefore, data from control animals receiving no injections were combined with controls with PBS injection. Occasionally (among 10% of all muscles examined), extraocular muscles were weaker than 0.5 mN of contractile force. These were considered to be virtually nonresponsive “dead” muscles (probably due to damage during dissection or handling) and therefore were not included in the data for analysis. Muscle force was plotted between 0.5 and 5 mN. The mean maximum contrac-
Table 1. Comparison of Concentrations of Endogenous and Exogenous Trophic Factors in Extraocular Muscles or Other Tissues, Based on Published Reports

<table>
<thead>
<tr>
<th>Protein (Dose)</th>
<th>Endogenous Factor</th>
<th>Exogenous Factor*</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDNF (250 ng)</td>
<td>1 pg/mg</td>
<td>14 pg/mg</td>
<td>14-fold</td>
</tr>
<tr>
<td>CT-1 (250 ng)</td>
<td>10.75–12.9 pg/mg</td>
<td>25 pg/mg</td>
<td>2-fold</td>
</tr>
<tr>
<td>IGF-I (5 μg)</td>
<td>46.2–150 pg/mg</td>
<td>140 pg/mg</td>
<td>1–3-fold</td>
</tr>
</tbody>
</table>

* 48 Hours after injection.

tile force of normal superior rectus muscles was 1.81 ± 0.23 and 1.43 ± 0.17 (SEM) mN for normal superior oblique muscles. Although the twitch contraction force of the superior rectus was greater than that of the superior oblique, the superior rectus also had a larger cross-sectional area (superior rectus, 0.018 cm²; superior oblique, 0.014 cm²), resulting in a similar contractile force per cross-sectional area between the superior rectus and oblique. The contractile force per cross-sectional area for the superior oblique was 99.45 ± 12.64 and 100.70 ± 16.20 mN/cm². Because the mean maximum contractile force differed slightly between the superior rectus and oblique muscles, we normalized the data to 100% and combined the data from the two groups for subsequent comparison with treated muscles. The normal contractile force of muscles from hatching chicks is lower than the contractile force of adult pigeon extraocular muscles shown in previous studies (571 mN/cm²), possibly due to the difference in stimulus parameters and settings (in vitro versus in situ) and/or the ages of animals between the two studies.

Effect of a Change in pH in the Krebs Buffer on Contractile Force

It has been reported that pH can acutely affect contractile force of skeletal muscles. We therefore examined how minor changes in pH influence the contractile force of the extraocular muscle. When muscles were superfused with pH 6.4 or 7.4 Krebs buffer, the contractile force differed by less than 2% (data not shown).

Effect of Exogenous Factors on Extraocular Muscle Strength

One previous study has shown that IGF-II can increase extraocular muscle strength in adult rabbits, however, it is not known whether trophic factors can increase extraocular muscle strength in a developing system. To examine the contractile properties of developing extraocular muscle, we dissected 163 muscles. Although the twitch contraction force of the superior rectus was slightly increased compared with normal controls, the twitch contractile force of the IGF-I treated muscle retained close to 4% of the radioactivity present in extraocular muscle at 1 hour (Fig. 1). Spiking injected trophic factors with radiolabeled proteins provided a sensitive method to determine which muscles received a sufficient dose of trophic factors. We injected the trophic factors into the orbit where they were taken up by the extraocular muscles. This appears to be a reliable method of administering exogenous trophic factors that is comparable to direct injections into the extraocular muscles, since the resultant change in force with direct muscle injection of IGF-II (+78%) is virtually identical with our orbit injections of IGF-I (+81%, described later). Considering the amounts of endogenous CT-1 and GDNF present in muscle or plasma determined in previous studies, we injected 250 ng of these trophic factors into the orbit to increase their levels. The concentrations of CT-1 and GDNF remaining in extraocular muscles after 48 hours were calculated to be, on average, 14 and 23 pg/mg wet weight, which is significantly higher than known endogenous levels of these trophic factors in skeletal muscle or plasma (Table 1). Because levels of endogenous IGF-I in muscle tissues are known to be unusually high (Table 1), we injected 5 μg of IGF-I into the orbit to increase levels of total IGF-I significantly. The concentration of exogenous IGF-I remaining in the muscle after 48 hours was 140 pg/mg which is about twice the estimated amount of endogenous IGF-I in muscle tissues.

We examined whether individual trophic factors or a cocktail of trophic factors can increase the normal extraocular muscle strength in hatching chicks compared with that of controls. Injection of CT-1 (250 ng) resulted in a similar pattern of increase in the contractile force of both the superior rectus and oblique muscles. The twitch contractile force after CT-1 treatment was 3.06 ± 0.52 mN for the superior rectus and 1.46 ± 0.26 mN for the superior oblique, which constitutes a significantly increased contractile force (by 51%, P < 0.05; Figs. 2D, 2G). CT-1 increased extraocular muscle strength with a positive dose-response curve (Fig. 3A). The cross-sectional area of the CT-1-treated superior rectus was 0.021 cm² and for the superior oblique, 0.012 cm². The cross-sectional area for the superior rectus was slightly increased compared with the normal control (0.018 cm²), indicating that increases in muscle strength may be in part due to hypertrophy as discussed later.

Injection of 5 μg of IGF-I into the orbit resulted in concentrations of IGF-I about twice the endogenous levels. The contractile force of the IGF-I–treated superior rectus was 2.74 ± 0.27 and 2.94 ± 0.029 mN for the superior oblique. When compared with the control muscles, the contractile force increased by 81% (Figs. 2E, 2H). When a lower dose of IGF-I (250 ng) was injected into the orbit (which increased total amounts by only 3% above endogenous levels), there was no increase in contractile force (data not shown). The cross-sectional area was 0.020 cm² for the superior rectus and 0.012 cm² for the superior oblique. The cross-sectional area for the superior rectus was slightly increased compared with normal controls, again indicating that increases in muscle strength may be due in part to hypertrophy, as discussed later. Neither the injection of GDNF (250 ng) individually, nor a cocktail of a lower dose of trophic factors (50–175 ng each of CT-1, GDNF, IGF-I, and BDNF) significantly increased the contractile force of the extraocular muscles (Fig. 2B).
Effect of a Cocktail of Neutralizing Antibodies to Trophic Factors on Extraocular Muscle Strength

To determine whether a neutralization of endogenous trophic factors decreases the contractile force of developing eye muscles, we injected individual antibodies or a cocktail of function-blocking antibodies or binding proteins (2.5 μg anti-CT-1 antibody, 2.5 μg anti-GDNF antibody, and 1.7 μg anti-IGF-binding protein 4 (IGFBP-4) into the orbit. When levels of available endogenous trophic factors were reduced with a cocktail of antibodies, the contractile force of extraocular muscles significantly decreased by 34% (P < 0.05; Fig. 4). However, the injection of CT-1 antibody alone (2.5 μg) did not decrease the contractile force (Fig. 4B). This indicates that a combination of endogenous trophic factors is needed to regulate or maintain extraocular muscle strength.

Effect of CT-1 and IGF-I on Extraocular Muscle Fiber Diameters

The morphology of extraocular muscles from normal and trophin-treated animals was examined in paraffin-embedded sections (Fig. 5). The mean diameter of normal extraocular muscle fibers of the hatching chick was measured to be 9.35 ± 0.44 μm (SEM), not significantly different between the superior rectus and oblique muscles. Because CT-1 or IGF-I treatment increased the contractile force of extraocular muscles, we determined whether CT-1 or IGF-I alone, or a cocktail of
trophic factors would change the extraocular muscle fiber diameter. Injection of either CT-1 (250 ng) or IGF-I (5 μg) significantly increased the mean muscle fiber diameter to 12.24 ± 0.13 and 13.50 ± 0.45 μm, respectively, and significantly shifted the percentage histogram of muscle fiber diameters to larger diameters (Figs. 6A, 6B). When a cocktail of antibodies (2.5 μg GDNF antibody, 2.5 μg CT-1 antibody, 1.7 μg IGFBP-4) was injected into the orbit, there was no significant change in mean muscle fiber diameter, but a trend toward a smaller mean (8.46 ± 0.30 μm, SEM). The more sensitive histogram analysis of muscle fiber diameters showed a small, but significant reduction of muscle fiber diameters (shift to the left, Fig. 6C). The number of muscle fibers per muscle section showed no significant difference between control muscles and muscles treated with trophic factor (data not shown). Taken together, these results demonstrate that changes in contractile force of extraocular muscles correlate with changes in muscle mass, and specifically muscle fiber diameter. The trophic factors examined in this study appeared to regulate extraocular muscle strength rapidly (within 48 hours), by altering muscle fiber diameters, but changes in extraocular muscle strength may also be caused by other parameters, as discussed later.

**DISCUSSION**

The strength of extraocular muscles is particularly important in the context of strabismus and other ocular motility disorders. The two major current treatments for strabismus include surgical muscle resection and botulinum toxin injections with the goal of weakening the stronger eye muscle of the muscle pair to allow balance and alignment of the eyes. Short-term effects of botulinum toxin on the lateral rectus muscle of the cat have been shown to cause a dramatic decrease in maximum twitch and tetanic tension. Similar to botulinum toxin, injection of ricin-mAb35, a myotoxic immunotoxin, into the superior rectus of adult rabbits results in a significant weakening of the muscle. Another study has reported an increase in adult extraocular muscle strength with IGF-II. However, it was not known whether trophic factors can regulate extraocular muscle strength during development, whether endogenous trophic factors are important in maintaining muscle strength, and whether different trophic factors can act synergistically to regulate extraocular muscle strength. It is important to determine how trophic factors may regulate extraocular muscle strength during development, because strabismus in childhood has severe clinical consequences.

**Advantages of Using Hatchling Chicks as a Model System**

Important advantages of using the hatchling chicken as a model system are that they have relatively large eyes and eye muscles, and there is a significant amount of data on the development of the oculomotor system for this species. Previously, the chicken has been widely used as a model system in visual science, especially in myopia research. Because the oculomotor system is highly conserved among vertebrate species, it is likely that the same trophic factors regulate muscle innervation and strength in different species. Trophic factors are also evolutionarily conserved, with nearly identical sequence homology between species at the amino acid level. Therefore, the same basic trophic mechanisms may regulate eye muscle strength for chicken and humans.

**Effect of Trophic Factors on the Strength of Developing Extraocular Muscle**

It is well established that trophic factors regulate several parameters of adult skeletal muscle, such as tension, force, inner...

**FIGURE 6.** (A–C) Histograms show the percentages of myofiber cross-sectional diameters of extraocular muscles in hatchling chicks as a function of fiber sizes. (A) CT-1 significantly increased the average muscle fiber diameter of extraocular muscles. The shift to the right (larger diameters) after CT-1 was statistically significant. (B) IGF-I significantly increased the average muscle fiber diameter of extraocular muscles. The shift to the right (larger diameters) after IGF-I was statistically significant. (C) A cocktail of neutralizing antibodies against endogenous trophic factors (2.5 μg GDNF antibody, 2.5 μg CT-1 antibody, 1.7 μg IGFBP-4) slightly changed the sizes of muscle fiber diameters in extraocular muscles toward the smaller fiber sizes. This shift was statistically significant (Wilcoxon signed rank sum test).
viation, and mass.9,22 IGF-I and CT-1 can induce skeletal muscle hypertrophy, and IGF-I can increase skeletal muscle strength.13,15,52 The subcutaneous injection of CT-1 in wobbler mice motor neuron disease has been shown to prevent deterioration in paw position and walking-pattern abnormalities, indicating that CT-1 exerts myotrophic as well as neurotrophic effects in a mouse model of spontaneous motor neuron disease.8,4 A recent study demonstrated also that the application of exogenous IGF-II increases extraocular muscle strength in adult rabbits.14 In our study, additional trophic factors, CT-1 and IGF-I, increased extraocular muscle strength during development and several endogenous trophic factors, probably including CT-1 and IGF-I, were important in maintaining muscle strength. Application of exogenous CT-1 or IGF-I into the muscle increased muscle strength in developing extraocular muscles rapidly (within 48 hours). Although GDNF or a low-dose cocktail of trophic factors did not increase the contractile force of normal eye muscles, in strabismic muscles there may be a lower amount of endogenous trophic factors, and it is possible that in those muscles, increased levels of such exogenous factors are effective in improving trophic factors that may have beneficial effects in regulating extraocular muscle strength in both the developing and adult oculomotor system. Furthermore, it is important to design and test chronically elevated levels of trophic factors in extraocular muscles, such as slow-release compounds or viral vector delivery.

Effect of Endogenous Trophic Factors on Extraocular Muscle Strength in Chicks

Previous studies have not examined whether lowering endogenous trophic factor levels changes muscle strength. We showed that the application of antibodies and binding proteins to GDNF, CT-1, and IGF-I resulted in a significant decrease in extraocular muscle contractile force. Lowering the levels of endogenous trophic factors in the extraocular muscle resulted in a weaker contractile force, suggesting that in the chick oculomotor system, a combination of endogenous GDNF, CT-1, or IGF-I has an important physiological role in maintaining muscle strength. However, the application of CT-1 antibody alone did not decrease the extraocular muscle strength, indicating that regulation of muscle strength is complex. Apparently, several trophic factors contribute to maintaining a “normal” muscle force, and loss of just one such factor can be compensated for by other ones. Increased levels of one factor may be sufficient, however, to increase muscle force beyond this “normal” level. Several endogenous trophic factors may act in a redundant or synergistic fashion to regulate and maintain muscle strength.

Does Regulation of Contractile Force Correlate with Changes in Muscle Mass?

We showed that the exogenous trophic factors CT-1 and IGF-I significantly and rapidly increased muscle fiber diameters of extraocular muscles (Figs. 6A, 6B). The measurements of muscle fiber diameter confirmed the results of cross-sectional area measurements that indicated that muscle mass was increased with CT-1 or IGF-I treatment. This was expected, since an increase in muscle strength is typically correlated with muscle-fiber hypertrophy.9,14,15,22 Injection of IGF-I into adult mammalian extraocular muscles also resulted in an increase in mean muscle fiber diameter (Linda McLoon, University of Minnesota, personal communication, March 2004). Our histogram analysis of frequencies of hatching chick extraocular muscle fiber diameters showed a bell-shaped curve, with similar frequencies among the very small- and the very large-diameter fibers (Figs. 6A, 6B). This result differs from a similar analysis in adult rabbit extraocular muscle where the smaller diameter fibers were much more frequent than the larger muscle fibers.14 The reason for these differences is not clear, but may relate to age differences rather than species differences.22 Lowering levels of endogenous trophic factors, including CT-1 and IGF-I, slightly reduced muscle fiber diameters (Fig. 6C). These data indicate that the mechanism by which the trophic factors CT-1 and IGF-I increase extraocular muscle strength probably involves cross-sectional muscle fiber hypertrophy.

In contrast, a recent study by McLoon and Christiansen14 indicates that increases in muscle fiber diameter may not be the only way by which increases in extraocular muscle strength can be achieved. IGF-II increased the contractile force of adult rabbit extraocular muscle, but it did not change the mean myofiber cross-sectional area, although muscle wet weight was increased in IGF-II-treated extraocular muscles. The authors considered the possibility that instead of increasing myofiber cross-sectional areas, there may be an increase in myofiber length. In muscles with multiple endplate bands (such as those of extraocular muscles) and myofibers that end intrasarcially, hypertrophy may occur through an increase in myofiber cross-sectional area or instead there may be an increase in myofiber length.14,56 In addition, expression of myosin heavy chain isoforms plays a role in contractile properties of skeletal muscle,14 and isoform switching of muscle fibers may lead to a change in force generation. Further studies are needed to determine the precise mechanisms by which trophic factors increase the contractile force of muscles.

In conclusion, several endogenous trophic factors appear to regulate and maintain the strength of developing extraocular muscle, and both CT-1 and IGFs may be considered promising candidates for the future treatment of strabismus. If levels of endogenous trophic factors are actually lower in the weaker muscles of strabismic patients, chronic application of exogenous trophic factors or increased levels of endogenous trophic factors may be useful in the treatment of strabismus.

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References


