Pharmacological approaches for the treatment of strabismus*

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Abstract
Strabismus is an eye movement disorder characterized by misalignment of the eyes and affects 1-5% of preschool-aged children. Lack of successful treatment in children may result in amblyopia, a lasting visual impairment. Current treatments to realign the eyes include surgery and botulinum toxin (Botox). We are working to develop two potential alternative treatments. The first uses muscle-specific immunotoxins, such as ricin-MAb35 where ricin is conjugated to a monoclonal antibody to the nicotinic acetylcholine receptor. We show that this significantly weakens the treated muscle, and our studies indicate that its muscle-weakening effects last up to 24 weeks after a single injection. The second strategy uses known myogenic growth factors, such as insulin-like growth factor (IGF), to strengthen an underlying muscle. A single injection of IGF-II resulted in significant increases in muscle force generation. Our studies thus far confirm that these agents can significantly alter muscle force generation, and we are actively pursuing additional drug strategies to improve the effect and duration of these agents. If successful, these agents may provide new alternatives to surgery for the treatment of strabismus.

Introduction
Strabismus is a common ophthalmological disorder that is manifested by a misalignment of the eyes. It affects between 1% and 5% of the population of preschool-aged children in the U.S. (1-3). This percentage increases significantly in certain subgroups. For example, 13% of prematurely born infants develop strabismus (4, 5) and neurologically impaired children are also much more likely to develop the disorder (6).

The clinical spectrum of strabismus is complex and includes several different conditions. A convergent ocular deviation is called esotropia, while a divergent deviation is called exotropia. These involve either overaction or underaction of one or more of the extraocular muscles, or a combination of both. Less commonly, vertical or torsional misalignment of the eyes may also develop. 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. The prevalence of amblyopia in adults is approximately 3%, with strabismus the cause in approximately half of these cases (7). Amblyopia is a common cause of life-long visual impairment in the affected eye. While the exact nature of the mechanisms that cause the permanence of this vision loss is under debate, there is a great deal of evidence to suggest that there is a critical period for binocular vision development in the cortex (8, 9). In contrast to visually immature children, an acquired misalignment of the eyes results in diplopia or visual confusion.

The goal in the treatment of strabismus is to realign the eyes and to re-establish binocularity over a sufficiently long period of time that central adaptive mechanisms

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can develop that will allow for long-term maintenance of alignment. The treatment of strabismus often begins with the use of eye patches, glasses, prisms or orthoptic exercises. However, many patients require incisional surgery. Unfortunately, strabismus surgery, for multiple reasons, has not proven to be the optimal treatment we might desire. Surgical success rates vary depending on the type of strabismus. Overall, however, strabismus surgery has a favorable outcome in only about half of the treated patients (10-12). This may be due, in part, to the fact that incisional surgery results in compromised muscle dynamics, altering the arc of contact of the operated muscle with the globe and decreasing the elasticity of the operated muscle due to scarring. Furthermore, surgery alters physiological properties of the muscle, including the resting tension of the agonist-antagonist pair (13) and the generated twitch tension (14). Surgery may also disrupt extracocular muscle relationships with connective tissue pulley structures in the orbit (15).

In 1973, Scott et al. first showed the feasibility of pharmacological treatment for focal dystonias and strabismus by direct intramuscular injection of botulinum toxin A (Botox) (16). Botulinum toxin A blocks the release of acetylcholine from the presynaptic terminal into the synaptic cleft, creating a temporary paralysis of the muscle. Since its introduction, Botox has been used for the treatment of both childhood and adult strabismus in large numbers of patients (17, 18). A National Library of Medicine search for strabismus and botulinum toxin produces a list of 274 articles on its use. This collective experience has highlighted several drawbacks to the routine use of Botox in strabismus treatment. First and most obvious, Botox can only be used to weaken the effect of an extraciliary muscle. Second, alignment following a single injection of Botox cannot consistently be achieved, especially in children with large initial angles of strabismus (19, 20). Several studies report rates of acceptable alignment following Botox treatment that range between 54% and 89% (21-23). Thus, significant numbers require reinsertion or other forms of treatment (22). This is due, in part, to the relatively short duration of action of Botox (24).

The short duration of effect following Botox treatment presumably does not allow sufficient time for the central nervous system to reorganize its output for coordinated binocular control. Moreover, Botox is only effective on an overacting extraocular muscle, leaving an underacting muscle untreated.

However, the use of Botox has shown that the pharmacological treatment of strabismus is possible, and has paved the way for further investigation of other agents. Remarkably, the development of new pharmacological agents for the treatment of strabismus has not been pursued until recently, despite the potential advantages of ease of administration, limitation of postoperative scarring, and preservation of normal extraocular muscle-globe mechanical dynamics. We have been developing new agents that address some of the limitations of Botox mentioned above. Our hope is that by expanding the therapeutic armamentarium available, we might make it possible to move away from incisional surgery in appropriate patients while simultaneously improving treatment success rates and patient comfort. The thrust of this research has been to develop muscle-weakening agents of greater duration than Botox, and, at the same time, to develop agents that could strengthen underacting muscles. These new agents could be used alone or simultaneously in agonist-antagonist extraocular muscle pairs to alter the rotational position of the globe, and thus treat the strabismus.

The first approach uses immunotoxins, where a potent toxin is conjugated to a monoclonal antibody to a muscle-specific cell-surface molecule. This targets the toxin directly to mature myofibers in order to weaken an overacting muscle. The second approach is to use myogenic growth factors that are known to act specifically on muscle fibers in order to strengthen the treated muscle.

A great deal of work has demonstrated that childhood strabismus must be treated as soon as possible to obtain binocular vision; this is due to the presence of a critical period in developing the visual cortex (25, 26). During this critical period, cortical changes are extremely rapid (27); however, the time course for creating stable alterations is more difficult to assess. There is some basis for estimating the time course that can be gleaned from visual deprivation studies in animal models. One of the most powerful in defining parameters for duration of effect is from Innocenti et al. (28), where central nervous system alterations from their visual deprivation paradigm were not seen after 1 month of deprivation, but required 3 months of visual deprivation. Two months of visual experience did not restore the normal visual system connectivity after 3 months of visual deprivation. Using a different visual deprivation paradigm, only animals that suffered from visual deprivation for 40 and 55 days developed a persistent strabismus (29). This gives us a window for altering muscle force of more than 3 months. Thus, we are choosing immunotoxins and methods of growth factor application that we anticipate will have a duration of effect of over 3 months.

Use of immunotoxins to weaken an underacting extraocular muscle

The use of immunotoxins in the treatment of disease is well established (30-32). Several studies have shown that ricin conjugated to antibody molecules such as CD22, CD25 or CD19 targets its toxicity to specific cancer or immune system cells (33, 34), reducing the risk to patients of systemic toxicity (35, 36). Clinical trials using immunotoxin therapy in human brain cancer patients are in progress testing either ricin A or a genetic mutant of diphtheria toxin conjugated to transferrin. These trials have proved very successful, and the patients have shown no evidence of systemic toxicity with these target-ed immunotoxins (35, 36). Thus, the strategy of linking a potent toxin to a targeting antibody or molecule is proving to be a successful approach for the direct pharmacologi-
cal treatment of a number of diseases. If ricin could be targeted to cancer cells, then it seemed reasonable that it could be targeted specifically to muscle cells.

Ricin is a cytotoxic protein composed of a ribosome-inactivating enzyme, the A chain, linked by a disulfide bond to a galactose/N-acetylglucosamine-binding lectin, the B chain. Ricin, by irreversibly inactivating ribosomes, inhibits protein synthesis and kills affected cells. The immunotoxin ricin-MAb35 consists of ricin chemically linked to a monoclonal antibody against the nicotinic acetylcholine receptor (37). The antibody specifically targets the ricin to mature myofibers that express that receptor. Intramuscular injection of this skeletal muscle-specific immunotoxin results in long-lasting weakness in limb muscle as a consequence of direct local myotoxicity. It was suggested that it could serve as a potential treatment for focal muscle dystonias (37). This molecule seemed a good candidate to test as a potential treatment for strabismus.

Muscle loss after direct ricin-MAb35 injection into extracuticular muscle

A single injection of ricin-MAb35 directly into the superior rectus muscles in adult rabbits resulted in significant myofiber loss, both acutely and over the long term (38, 39). Based on the maximally tolerated dose that had been determined for mice (37), we titrated the dose to 0.2 µg/kg (1/10th the maximally tolerated dose for rats [rMTD]). This dose resulted in significant muscle injury, yet produced only a relatively short-lived inflammatory reaction (Fig. 1). Significant muscle loss was readily apparent in the acutely injected superior rectus muscle, but was maintained over a long period of time. Even 105 days after a single injection of the ricin-MAb35 immunotoxin, there was still a 45-69% muscle loss compared to the un.injected control muscle in both total myofiber number and in total muscle cross-sectional area (39). The monoclonal antibody to the nicotinic acetylcholine receptor was effective at targeting the ricin to the mature myofibers. Thus, satellite cells that do not express the receptor were spared, allowing slow regenerative regrowth of muscle fibers. In addition, capillaries and peripheral nerves within the treated extracuticular muscle were normal (see the electron micrograph to the right in Figure 2).

The long-term effects of direct injection of ricin-MAb35 in extracuticular muscles were also examined (39). Fifty-six days after a single treatment, there was a 45% loss in myofiber number in the treated muscle compared to the contralateral control muscles. By 105 days after a single ricin-MAb35 injection, there was a 68.7% myofiber loss compared to normal. By 1 year, the muscles treated with ricin-MAb35 were reduced in myofiber number by 10%, which was not statistically different from the normal control muscle myofiber number. However, myofiber cross-sectional area and myosin heavy-chain isoform expression were still abnormal 1 year after a single injection of ricin-MAb35. To summarize, our preliminary studies demonstrated that a single injection of ricin-MAb35 into the superior rectus muscle resulted in a substantial and prolonged period of myofiber loss and subsequent regeneration. These long-lasting myotoxic effects on extracuticular muscles of rabbits are discrete, sustained and well tolerated. The toxic effects of the ricin-MAb35 appeared to be directed at muscle only and did not appear to spread to neighboring structures within the orbit. This decreases the likelihood of unwanted myotoxic effects on nearby extracuticular muscles, or of nonspecific toxicity to other orbital tissues. Ricin-MAb35 did not appear to be toxic to peripheral nerve or capillaries within the treated muscles acutely, nor at the long postinjection intervals. In addition,
it is important to note that none of the treated animals showed any evidence of systemic toxicity at any time. The muscle-specific binding property of the antibody to the nicotinic acetylcholine receptor targets the ricin directly to the mature myofibers and presumably plays an important role in the containment of the toxin after a direct muscle injection.

One additional study in the literature reports the use of an immunotoxin, saporin-MAb73, which is also specific for the acetylcholine receptor (40). This immunotoxin resulted in short-term focal muscle loss, but no long-term follow-up was reported. The potential utility of ricin-MAb35 treatment for strabismus is clear, and our published studies show that the effects of ricin-MAb35 are similar in the orbicularis oculi muscle as well (41). Thus, it may have broader use for the treatment of focal dystonias such as blepharospasm.

Loss of muscle force after direct ricin-MAb35 injection into extraocular muscle

The goal of strabismus treatment is to induce long-term, dose-related adjustments in the force generation of specific extraocular muscles, which would in turn result in permanent changes in eye alignment. Our initial studies demonstrated that direct injection of ricin-MAb35 into extraocular muscles of rabbits resulted in a long-lasting reduction in muscle mass (38, 39). This suggests that the reduction in extraocular muscle force generation should be of longer duration than that following a single injection of Botox.

A single injection of ricin-MAb35 was administered directly into the superior rectus muscles of rabbits (0.2 µg/kg, or 1/10th the mMTD, in a volume of 0.1 cc), while the contralateral superior rectus muscles received an equal volume of normal saline. Both the treated and control superior rectus muscles were tested for muscle force generation in situ (42) at 1, 6, 12, 24 and 48 weeks after treatment. At 1, 6, 12 and 24 weeks postinjection, there was a decrease in generated tension at all stimulation frequencies tested (Fig. 3) (43, 44). These decreases in force in the ricin-MAb35-treated muscles ranged between 40% and 65% of control. By 48 weeks after a single injection of ricin-MAb35, there was no difference in force generation between injected and control muscles. Reduction of force for up to 24 weeks is a critical observation, as it doubles the effective time of muscle weakening when compared with botulinum toxin, which only lasts for an average of 8-12 weeks. The duration of effect is critical if we are to alter the motive force of agonist and antagonist pairs of muscles long enough to allow binocular realignment and central nervous system remodeling of the afferent control of eye movements. These effects must last...
sufficiently long that sensory and motor adaptation can occur to create a sustained change in the rotational position of the globe. Just as important, however, is that the muscle recover. Permanent weakness could result in consecutive strabismus opposite to the initial deviation.

**Myogenic growth factors increase force generation in extraocular muscle**

As useful as a long-acting agent to weaken extraocular muscle might be in the treatment of strabismus, its effect could be augmented if simultaneous strengthening of the antagonist extraocular muscle were possible. In strabismus surgery, recession of an extraocular muscle (a weakening procedure) is often combined with resection of the antagonist (a strengthening procedure) in the same eye to effect a given change in the rotational position of the globe. Myotoxic or paralytic agents for weakening muscle are relatively easy to find. Developing an agent to increase the motive force of a muscle is a more complex undertaking, due to the large number of factors known to promote myogenic growth. There is recent evidence in the literature to suggest that increased expression of insulin-like growth factor (IGF) in muscle tissue results in myofiber hypertrophy (45, 46). Improved muscle force generation in both aged and dystrophic muscle was demonstrated after prolonged IGF exposure (47-49). Exogenously supplied nerve growth factor (NGF) prevented the effects of strabismus in a rat model (50), which the authors felt was affecting visual cortical plasticity directly. Thus, there is a precedent outside our work that supports this approach.

Work in our laboratory and others has shown that, in contrast to limb skeletal muscles (51, 52), normal adult extraocular muscles continue to express a number of growth factors and growth factor receptors (Fig. 4) (53, 54). In both the orbital and global layers, satellite cells were positive for the IGF receptor, as visualized immunohistochemically (Fig. 4). The basal lamina around the myofibers in the orbital layers was also positive for IGF receptor staining, while leg muscle controls were negative for the IGF receptor. Contrary to limb skeletal muscles, the extraocular muscles also express IGF-I and epidermal growth factor (EGF). We recently demonstrated that the extraocular muscle in mammals maintains a population of activated satellite cells (55), as well as retaining the ability to add myonuclei to normal, uninjured myofibers in the adult (56-58). If IGF treatment in aging or dystrophic muscles results in significant increases in myofiber size and strength, then the presence of IGF receptors and the increased plasticity normally present in adult extraocular muscle (56, 58) support the hypothesis that IGF treatment should have a dramatic effect on extraocular muscle myofiber size, and force generation as well.

We are testing the effects of direct injection of myogenic growth factors and/or their receptors on myofiber number, size and strength in rabbit extraocular muscle.

Our data suggest that direct injection of IGF-I or -II results in increased muscle mass, as evidenced by morphometric analysis. It is interesting that the morphological changes caused by each of these growth factors are distinct; apparently each growth factor affects different mechanisms of muscle growth (Fig. 5). While the mean myofiber cross-sectional area increased significantly after a single injection of IGF-I, no statistical difference was seen in this measure after IGF-II. However, total muscle mass was increased in the IGF-II-treated muscles (59).

*In situ* assessment of whole muscle strength 1 week after a single injection of 10 ng IGF-II into the superior rectus muscle of an adult rabbit demonstrated a significant increase in muscle force generation at all stimulation intensities (Fig. 6A). The effect of direct injection of IGF-I
was tested in an in vitro apparatus (60). There was an increase in muscle force generated by the IGF-I-treated muscles, but in no case was this increase as significant as that seen after IGF-II treatment (Fig. 6B). It will be important to demonstrate that these increases in both muscle mass and muscle force can be maintained for a sufficient duration to be effective in the treatment of strabismus. No significant difference in force is seen by 2 weeks after a single injection of IGF-II.

Other work has supported the findings of these studies, as it was recently demonstrated that an injection of IGF-I and/or cardiotropin-1 into the orbit of hatching chicks resulted in increased single twitch force in the muscles of the treated orbit (61). Thus, the injection of growth factors into extraocular muscle may prove to be a viable means of increasing extraocular muscle force generation. This represents a novel approach to the pharmacological treatment of strabismus, specifically directed at increasing the strength of an underacting extraocular muscle.

Future directions

Our future work will focus on two main areas. We will be testing a new immunotoxin, which theoretically should be more myotoxic than ricin-MAb3S. This immunotoxin consists of a fusion protein of the diphtheria A chain and the ricin A chain (RTA-DTA), and this more potent toxin will be chemically linked to the monoclonal antibody to the nicotinic acetylcholine receptor. We have named the diphtheria-ricin-antibody construct DR-ITox.

Diphtheria is a potent cytotoxic protein in its native form. It kills cells by inactivating elongation factor-2 (EF-2) by ADP phosphoribosylation and by inhibiting protein synthesis (62). The substrates for RTA and DTA are different. The fusion protein of the A chains of both these toxins was prepared by genetic manipulation and tested for its toxic characteristics compared to either chain alone using an in vitro cell system (63). By delivering the A chains of both these toxins into cells, their combined toxic effects on the cells were enhanced. The hybrid toxin retained the full enzymatic activity of both the ricin and diphtheria A chains, and their potency in inhibiting tumor cell growth in vitro was increased 100-1,000-fold (63, 64). When the RTA-DTA construct was linked to an antibody reactive to breast and ovarian cancer cells (64), the toxicity of this construct against tumor cells was greater than the RTA-immunotoxin and DTA-immunotoxin alone. The RTA-DTA-immunotoxin reduced protein synthesis to 50% of control in tumor cells starting at a concentration of \(10^{-5}\). The RTA-immunotoxin reduced protein synthesis to 50%
of control at 10². RTA alone or RTA-DTA alone did not reduce protein synthesis to these levels (63). Thus, by extension, we are expecting our RTA-DTA-immunotoxin to be at least 10 times as toxic as ricin-MAb35 in extraocular muscle.

Since recovery of both muscle mass and muscle force generation after treatment with ricin-MAb35 is dependent on muscle regeneration, a more potent toxin would have the potential to increase the amount of muscle injured after a single injection. This should allow smaller doses of the toxin-antibody construct to be injected into the muscle, which in turn would decrease possible concerns about systemic toxicity. The other attractive feature of the RTA-DTA fusion protein is that it lacks the B chains of each of those potent toxins; the B chains allow cells to internalize the molecule in a wide variety of cells. The elimination of the B chains eliminates a serious concern about possessing potentially large amounts of biotoxic agents. The A chain fusion protein would not be toxic by itself. The increased cytotoxicity of DR-Itox should, in turn, allow effective treatment of longer duration than Botox or ricin-MAb35, hopefully at lower doses. While we have not seen any evidence of systemic toxicity with ricin-MAb35, lower doses of immunotoxin would reduce the possibility of systemic toxicity. If the theoretical potential of DR-Itox proves correct, it should result in a greater loss in both muscle mass and muscle force generation. This should extend the duration of muscle regeneration, which will allow for a longer time window for sensory and motor adaptation of the brain regions that control coordinated eye movements.

Since the muscle-strengthening effects of a single injection of IGF-II were relatively short-lived, we are testing the application of IGF-I and IGF-II in timed-release pellets. In addition, we are testing other myogenic growth factors that have been reported in the literature to increase muscle mass to determine if they will also strengthen the extraocular muscles.

These approaches will hopefully result in a combination of drugs that can be tailored to a given motility issue and may reduce our dependence on incisional surgery, with its inherent adverse motor consequences. At the very least, the pharmacological treatment of strabismus will expand currently available treatment options and will hopefully improve long-term outcomes.

References


53. McLoon, L.K., Peters, E., Wirtschafter, J.D. Adult extracocular muscles express HSPG. Syndecan and growth factor receptor molecules that normally are down regulated in adult skeletal muscle. Invest Ophthalmo Vis Sci ARVO ??? 1999, 40: S408.

54. Fischer, M.D., Gorospe, J.R., Felder, E., Bogdanovich, S., Pedrosa-Domeliof, F., Ahima, R.S., Rubinstein, N.A., Hoffman,


