Extraocular Muscle Regeneration in Freeze-Treated Extraocular Muscle Autografts

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New growth of extraocular muscle has been demonstrated in degenerating peripheral nerve autografts implanted between two extraocular muscles. This suggests that extraocular muscle may be lengthened for therapeutic purposes if a suitable matrix can be found to support this new growth. Investigators of peripheral nerve regeneration have found that the basal lamina of freeze-killed skeletal muscle remains intact and supports axonal regeneration. This study was designed to investigate the feasibility of inducing regenerative growth of extraocular muscle in freeze-treated extraocular muscle autografts. In six beagles the inferior oblique muscle was removed from both orbits, frozen in liquid nitrogen, and allowed to thaw at room temperature. The freeze–thaw cycle was repeated. The freeze-treated grafts were then sewn in an end-to-end fashion between the cut end of the lateral rectus and the globe. At both 4 and 8 weeks postoperatively, three dogs were killed, and the grafts were removed from both orbits. These were prepared for light and electron microscopic examination. This revealed robust growth of mature-appearing, innervated muscle fibers in the proximal graft that could be differentiated by adenosine triphosphatase histochemistry. Rare, immature fibers were seen in the distal graft. These results demonstrate that freeze-treated extraocular muscle autografts support regenerative growth of extraocular muscle.


Extraocular muscle is capable of regeneration outside its normal confines. This has been demonstrated in recent studies in which new muscle fiber growth occurred in peripheral nerve autografts sewn between normal and denervated extraocular muscles.1,2 This new growth may have been stimulated by trophic factors that enhance muscle regeneration and cell adhesion3–6 and by neural basal laminae that are structurally similar to that found in skeletal muscle.7

This regenerative growth raises the possibility that functional extraocular muscle autografts can be grown in the orbit. The new muscle could be used to expand transposition techniques in the therapy of paralytic strabismus, to replace paretic extraocular muscle as a free graft, to lengthen muscles that previously have been maximally recessed or resected, or as a model for muscle fiber growth and repair. To be clinically useful, however, the new muscle fibers must grow in sufficient number and diameter to generate a functional force and be of sufficient length to allow surgical manipulation. Furthermore, the new muscle growth must be supported by a matrix that is expendable, that stimulates regeneration, and that allows contraction.

As a supporting matrix for muscle growth, peripheral nerve autografts fail in two respects. First, nerve grafts can be harvested only from small nerves such as the infraorbital sensory nerve or the sural nerve, the removal of which does not cause major morbidity. As a consequence, the diameter of the graft and the fibers it contains are relatively small.1 Ideally, the graft diameter would be approximately the same as the muscle to which it is sewn. Second, the implanted nerve limits graft contractility. Because of these limitations, an alternative graft matrix must be sought.

In this study, we investigated freeze-treated extraocular muscle as a potential supporting matrix for extraocular muscle regeneration. An earlier study using fresh extraocular muscle transplants showed almost complete replacement of the muscle with fibrous connective tissue by 4 weeks postoperatively.8 In contrast, freeze-treated muscle has been successfully used as a supporting matrix for nerve regeneration9–11 and for nonocular skeletal muscle regeneration.12 Extraocular muscle is a unique type of skeletal muscle and little is known about its capacity for regeneration. This study was designed to assess the feasibility of inducing regenerative growth of functional extraocular muscle in the empty tubular basement membranes of devitalized, freeze-treated extraocular muscle autografts.
Materials and Methods

Six adult female beagles were anesthetized with intravenous pentobarbital (28 mg/kg) and artificially ventilated. A left lateral orbitotomy was done, and the left inferior oblique muscle (LIO) was removed from origin to insertion. The muscle was fixed to a sterile tongue depressor, immersed in liquid nitrogen for 7 sec, and then allowed to thaw at room temperature. The freeze–thaw cycle was repeated once. The left lateral rectus muscle (LLR) was transected 1 cm from its insertion. One end of the freeze-treated LIO was sewn to the cut edge of the LLR with 7-0 polypropylene. The free end of the LIO graft was then sewn to the globe, maintaining the entire LLR–LIO complex at slight tension in primary position (Fig. 1). The orbitotomy wound was repaired in layers, and methylprednisolone sodium succinate 60 mg was injected subconjunctivally. The procedure was repeated in the right orbit. In an additional dog, a freeze-treated IO muscle was implanted subcutaneously in the scalp as a control. The dogs were revived under the supervision of animal-care personnel. All procedures were performed in compliance with the ARVO Resolution on the Use of Animals in Research. The surgery was done aseptically in a National Institutes of Health-approved facility.

Three dogs were killed at 4 and 8 weeks postoperatively with an overdose of intravenous pentobarbital followed by euthanasia solution. The LR-IO complexes were removed from both orbits in each dog. Specimens from one dog at each interval were fixed in 10% buffered formalin and embedded in paraffin. Thirty-micron longitudinal or 7-μm transverse sections were stained with Masson’s trichrome for general morphology. Specimens from the second dog at each interval were frozen in isopentane and chilled in liquid nitrogen. Fifteen-micron longitudinal sections were cut from one specimen on a cryostat and stained with combined silver-acetylcholinesterase (AChE) stain. Twelve-micron cross-sections were cut from the midbelly of the graft from the second orbit and stained for adenosine triphosphatase (ATPase) after preincubation at pH 4.6. Small segments from the midbelly of the implanted muscle were removed from both orbits of the third dog at each interval. These were fixed in 3.5% glutaraldehyde in 0.1 M phosphate buffer for 4 hr at 4°C. These selected sections were washed in cold phosphate buffer overnight and then prepared for electron microscopy. The subcutaneously implanted graft was removed after 4 weeks, fixed in 10% buffered formalin, and embedded in paraffin. Thirty-micron longitudinal sections were stained with Masson’s trichrome.

Results

Gross Examination

At necropsy, the grafts implanted in both orbits appeared well attached to the host LR and the insertion site on the globe even at 4 weeks (Fig. 2). The grafts were adherent to the underlying sclera, and they were more pale, fibrotic, and irregular than the LR to which they were attached.

Light Microscopy

Longitudinal sections stained with trichrome revealed numerous muscle fibers crossing the anastomosis site from the LR into the graft. The maximum extent of muscle fiber growth into the grafts ranged from 8–12 mm from the anastomosis site. Examination of serial cross-sections stained with trichrome revealed mature-appearing muscle fibers organized into typical fascicles in the proximal graft. In distal sections, rare immature muscle fibers and myotubes were seen separated by abundant connective tissue.

Longitudinal sections stained with the combined silver-AChE stain showed nerve fibers crossing the anastomosis site and terminating at neuromuscular junctions on graft muscle fibers 8 weeks postoperatively (Fig. 3). Four weeks postoperatively, rare endplates were seen in the graft without terminal nerves.

Cross-sections stained for ATPase showed little fiber differentiation 4 weeks postoperatively. However, at 8 weeks, both fast and slow fiber types with...
extremely variable fiber diameters could be differentiated (Fig. 4). The smallest diameter fibers were seen at the periphery of the graft, and the largest diameter fibers were central. Although this is suggestive of orbital and global fiber layers, no clearly distinguishable layer could be identified. No fiber-type grouping was seen.

Sections through the subcutaneously implanted, freeze-treated muscle revealed only dense fibrosis. No muscle tissue was seen.

Electron Microscopy

At both 4 and 8 weeks postoperatively, transverse and longitudinal sections from the midbelly of the grafts, examined by electron microscopy, demonstrated mature and immature fibers with typical sarcomere banding patterns (Fig. 5). Several fibers with central nuclei were seen. All fibers were surrounded by abundant collagen.

Discussion

In previous studies, we showed that extraocular muscle is capable of new growth outside its normal confines, supported by preexisting or autogenously implanted neural tissue.1,2,14 The results of this study suggest that freeze-treated extraocular muscle is also capable of supporting new extraocular muscle growth.
Fig. 4. Cross-section through the proximal graft at 8 weeks showing differentiated fiber types with variable fiber diameters. ATPase pH 4.6. Bar = 50 μm.

Fig. 5. Longitudinal section through the mid-belly of the graft showing regenerating extraocular muscle fibers with typical sarcomere banding 8 weeks postoperatively. Bar = 4 μm.
Survival of a free-muscle graft, such as this, is dependent on a source of muscle precursor cells, an intact basal lamina, vascularization, and innervation. The most likely source of muscle precursor cells in this model is the population of satellite cells that exists under the basal lamina of the transected host LR muscle. These activated satellite cells presumably migrate across the anastomosis site and along the empty basal lamina of the freeze-treated graft. They then fuse to form myotubes and eventually myofibers.

The graft itself probably does not contribute significantly to the pool of muscle precursor cells that repopulate it. Tissue cultures of freeze-killed muscles and cell-marker studies of freeze-treated limb muscle grafts by other investigators reveal no viable cells and no detectable contribution to the regenerated graft. This implies that the freeze–thaw technique we used renders the graft itself nonviable. The lack of identifiable muscle tissue in the subcutaneously implanted, freeze-killed extraocular muscle supports this.

Intact basal lamina in the graft is extremely important to muscle regeneration. Basal lamina is a remarkably resilient material that has been shown to survive freeze–thaw treatment, denervation atrophy, and ischemic necrosis. It appears to function as a scaffold, supporting satellite cell adhesion, division, and myotube growth. As a consequence, the orientation of regenerating myofibers is usually parallel with the orientation of the existing basal lamina. The importance of intact basement membranes to muscle regeneration was demonstrated in a recent study in which new limb-muscle fiber growth was seen in autogenously transplanted peripheral nerve grafts. Regenerating myofibers were seen growing both alongside and within crenated, empty, neural basal lamina tubes. The close proximity of all growing myofibers to a basal lamina suggests a strong affinity of muscle precursor cells for basement membrane.

Adhesive macromolecules present in basement membranes, such as laminin and fibronectin, may promote migration and adhesion of muscle precursor cells. Laminin binds both to muscle and to matrix components such as type IV collagen. Thus, laminin may play a role in stabilizing the abundant collagen matrix seen in our preparations and in anchoring satellite cells to the graft's basement membrane.

The functional characteristics of the regenerating graft are unknown. If functional regeneration is to occur, the graft must be innervated by the motor nerves of the host LR. We showed that, by 8 weeks, nerve had crossed the graft–host junction and formed neuromuscular junctions with the regenerating myofibers. Fewer motor end-plates were seen in the graft than in the host LR, even at 8 weeks. This observation is typical of regenerating free muscle grafts. The physiologic consequences of the decreased density of motor end-plates is unknown. Even when grafts have been successfully reinnervated, the maximum tetanic tension per motor unit is reduced. Therefore, even though our grafts are reinnervated, functional deficits may persist. Electrophysiologic studies will be necessary to determine the functional potential of the grafts.

The timing of reinnervation of the implanted grafts coincides with the appearance of fiber differentiation by ATPase histochemistry. Regenerating myofibers remain histochemically homogeneous until reinnervation; at this time, fast and slow fibers can be differentiated.

This study demonstrated that freeze-treated extraocular muscle grafts support regenerative growth of extraocular muscle. The use of these grafts, in the surgical treatment of strabismus, may be limited by fibrosis of the graft, adhesion to the sclera, and limited muscle growth in the distal graft. Further studies with other graft materials, growth factors, and extracellular matrix components will be necessary to maximize extraocular muscle regeneration.

Key words: extraocular muscle, regeneration, basal lamina, myogenesis, freeze-killed

References