A Quantitative Assessment of Extraocular Muscle Growth in Peripheral Nerve Autografts

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In previous studies, we have documented new growth of extraocular muscle fibers within axon-depleted motor neurons of denervated muscle. This study was designed to quantitate the regenerative growth of extraocular muscle within autologous peripheral sensory nerve transplants and to determine whether acutely denervated extraocular muscle affects this growth. Fifteen anesthetized beagles were subjected to an intracranial lesion of the left third cranial nerve. Segments of the infraorbital sensory nerve were removed from the nose and implanted between the lateral rectus and inferior oblique muscles in both orbits. At 2, 4 and 8 weeks postoperatively, five dogs were killed and the nerve grafts were removed. Muscle fibers were counted at four levels along each nerve segment. Fiber number increased significantly at each successive postoperative interval in implanted nerve segments on both the denervated and non-denervated side. At 8 weeks, fiber number was significantly greater on the denervated side. These results indicate that autologous peripheral sensory nerve is capable of supporting regenerative growth of extraocular muscle and that denervation has a significant positive influence on muscle fiber growth. Invest Ophthalm Vis Sci 31:766-770, 1990

In a previous study we unintentionally produced abundant new growth of extraocular muscle (eom). The study was designed to reinnervate paretic eom by secondary muscular neurotization, but in addition to the expected nerve growth we found new muscle fibers which grew preferentially within axon-depleted intramuscular nerve fascicles. These observations suggested that degenerating nerve may be a particularly good support matrix for muscle fiber growth, and that entirely new neuromuscular units may be induced to grow outside the normal confines of eom if appropriate growth-stimulating characteristics can be established.

Multiple factors may be responsible for nerve fascicle support of eom growth. These include surface characteristics of existing basal lamina, trophic factors that stimulate myogenesis, and adhesive macromolecules that enhance myoblast adhesion and migration. Identification of these factors may permit the development of a biodegradable support matrix for the growth or regeneration of eom. The ability to grow eom autografts in the orbit could allow replacement of paretic muscles by functional muscle grafts and lengthening of maximally recessed or over-resected muscles. Other investigators have used synthetic materials and autologous or heterologous noncontractile tissue grafts to lengthen extraocular muscle for similar purposes. While the dynamic characteristics of regenerated extraocular muscle grafts are unknown, they have the theoretical advantage over these materials of more normal contractility and compliance. Reproducible growth of extraocular muscle within a nonmuscle matrix, in vivo, may also provide a good model for studying muscle growth and repair.

This study was designed to evaluate nonmuscular peripheral nerve segments as a support matrix for eom growth and to quantify the contribution of an acutely denervated muscle to fiber growth in the nerve-segment graft. The ultrastructural characteristics of this growth have been described in another paper.

Materials and Methods

Fifteen adult, standard-sized, female beagles were each subjected to a unilateral lesion of the left third cranial nerve under general anesthesia (pentobarbital 28 mg/kg). A long (3.5–4.5 mm) intracranial segment of the third nerve was removed to prevent regrowth. Two segments of the infraorbital sensory nerve were excised from the left side of the nose 2 cm from the infraorbital foramen. Each was approximately 1.5
cm long and 0.3 cm in diameter. Bilateral lateral orbitotomies were performed to expose the lateral rectus and the inferior oblique muscles. In each orbit, the lateral rectus muscle was severed 1 cm from its insertion, and the insertional stump was removed. One cut edge of the infraorbital nerve segment was sewn to the cut edge of the lateral rectus muscle. The other end of the nerve segment was sewn into the belly of the centrally denervated inferior oblique muscle (Fig. 1). The anastamosis was performed using two 7-0 Polypropylene sutures on each end of the nerve implant. The orbitotomies were then closed in layers. Methylprednisolone Na succinate, 60 mg, was injected subconjunctivally in both eyes. The dogs recovered under the supervision of animal care personnel. All procedures were performed in compliance with the ARVO Resolution on the Use of Animals in Research. Surgery was performed aseptically in an NIH-approved facility.

After postoperative survival times of 2, 4 and 8 weeks, five dogs were killed at each interval with an overdose of pentobarbital and euthanasia solution. The specimens (inferior oblique muscle–nerve segment–lateral rectus muscle complex) were removed, en bloc, from both orbits. Each specimen was quick-frozen in isopentane chilled in liquid nitrogen. Twelve micron transverse sections were made at four different levels along the length of each nerve segment using a Bright cryostat at −20°C. Each level was 330 μm apart, level 1 being closest to the lateral rectus muscle and level 4 closest to the inferior oblique muscle. Sections were stained with modified Gomori trichrome and examined by light microscopy. All muscle fibers within the using a computerized digitizing program. Mean number of fibers and standard errors of the mean were calculated at each level, for each time interval. An analysis of variance was done as outlined by Winer (Case I) to determine whether the effects of independent variables on muscle fiber count were significant. The independent variables were treatment (denervated vs. nondener- vated), section level and time interval.

Results
Most of the specimens from both denervated and nondenervated sides of each dog showed muscle fiber growth from one end of the implanted nerve segment to the other. All muscle fiber growth was parallel to the long axis of the nerve. No fibers were seen traversing the epineurium of the nerve (Figs. 2, 3). Statistical analysis showed no significant difference between the mean number of fibers at different section levels in the nerve segments at each time interval. Therefore, the data from all section levels at each time interval were pooled and are presented in Table 1. Some specimens contained no muscle fibers in the nerve segment, explaining the high standard errors of the mean.

The mean number of fibers increased significantly with each successive postoperative interval in nerve segments sewn to both denervated and nondenervated muscles. At 8 weeks postoperatively, there were significantly more fibers in nerve segments on the denervated side (P < 0.01). In fact, at 8 weeks, by light microscopy, these nerve segments were virtually filled with mature muscle fibers.

Discussion
In earlier studies directed at reinnervating paretic eom by means of secondary muscular neurotization,
we have documented substantial growth of muscle fibers within the axon-depleted intramuscular nerve fascicles of acutely denervated eom.\textsuperscript{1,2} This unexpected discovery suggested to us that there were features of axon-depleted nerve fascicles that were conducive to muscle fiber growth. Because of the potential applications of eom graft growth, we designed this study to quantitate muscle fiber growth in nerve fascicles of an autogenous free segment of nonmuscular peripheral nerve and to assess the contribution of an acutely denervated muscle to the growth process.

As evidenced by light microscopy, substantial muscle fiber growth was observed throughout the length of implanted peripheral nerve segments on both the denervated and nondenervated sides at all postoperative intervals. There were more fibers seen at each successive interval. The analysis of variance showed that denervation had a significant positive influence on muscle fiber growth at the 8-week interval. This indicates that the presence of an acutely denervated muscle does act as a stimulus to growth of eom fibers. This may be the result of diffusible growth or trophic factors that are able to infiltrate the nerve segment and reach the growing fibers\textsuperscript{9} and of satellite cell proliferation that is known to occur in denervated muscle.\textsuperscript{10} The identification of such factors may be beneficial to growth of eom fibers in therapeutic situations.

Perhaps of equal importance to the finding that denervated eom is a stimulus to fiber growth is the finding that substantial growth occurs even in the absence of a denervation stimulus. This implies that eom may be induced to grow in circumstances of either chronic denervation or no denervation at all. Because eom regeneration would be useful clinically in either setting, this is a welcome finding.

No difference in muscle fiber number was seen at different section levels of the nerve segment between the lateral rectus and the inferior oblique. We analyzed this variable in an attempt to learn the direction of muscle fiber growth. For example, if fibers grew from the healthy lateral rectus toward the denervated inferior oblique, then a significant increase in fiber number might be seen in the sections closer to the lateral rectus, especially at the early time intervals.
Fig. 3. Longitudinal section through an implanted nerve segment demonstrating striated muscle fibers (arrows). Modified Gomori Trichrome, X400.

We offer two possible explanations for why this was not seen. First, muscle fiber growth into the nerve segment may proceed from both the healthy lateral rectus and the denervated inferior oblique. Extraocular muscle is known to be relatively resistant to the degenerative effects of denervation compared to limb muscle.\textsuperscript{11,12} In addition, nerve extract is known to prevent denervation atrophy.\textsuperscript{13} Thus, it is possible that the muscle fibers in denervated eom remain viable enough to regenerate into the nerve segment, which then provides a hospitable environment for growth, at least up to 8 weeks. Second, growth may indeed proceed from the LR to the IO, but the small size and immaturity of fiber growth may have prohibited their detection by light microscopy in the early stages. At the time of identification by light microscopy, growth had proceeded all the way through the nerve segment. Each of these mechanisms could be investigated in future experiments.

We propose that the peripheral nerve segment may provide both a conducive scaffolding for orderly growth of muscle fibers and growth factors to facilitate fiber infiltration of the graft. Further studies on the relative importance of these properties may be helpful in investigating the development of synthetic biodegradable materials as a support matrix for the purpose of eom graft growth.

Table 1. Mean fiber counts within nerve segments anastomosed to denervated and nondenervated muscles

<table>
<thead>
<tr>
<th>Post-op interval (weeks)</th>
<th>Denervation</th>
<th>No denervation</th>
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<tbody>
<tr>
<td>2</td>
<td>41.05 ± 23.98 (a)</td>
<td>46.6 ± 22.26 (a)</td>
</tr>
<tr>
<td>4</td>
<td>198.9 ± 109.38 (b)</td>
<td>73.15 ± 36.98 (b)</td>
</tr>
<tr>
<td>8</td>
<td>490.2 ± 59.91 (c)</td>
<td>259.2 ± 123.83 (d)</td>
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Means with different letters following in parentheses are significantly different ($P < 0.05$).

Table 1. Mean fiber counts within nerve segments anastomosed to denervated and nondenervated muscles

Key words: extraocular muscle, denervation, regeneration, peripheral nerve autograft, basement membrane

References