JOURNAL OF SURGICAL RESEARCH **75,** 135–147 (1998)
ARTICLE NO. JR985271

Myosin Heavy Chain Expression in Skeletal Muscle Autografts under Neural or Aneural Conditions

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Submitted for publication August 26, 1997

Background. **Our purpose was to investigate (1) the heterogeneity of satellite cells derived from adult fast-twitch and slow-twitch skeletal muscles, (2) the influence of innervation on muscle regeneration, and (3) the differences between developmental myoblasts and satellite cells with regard to myosin heavy chain (MHC) expression.**

Materials and methods. **Autografts under neural (*nerve-intact graft;* brief denervation interval) or aneural (*aneural graft;* prolonged denervation interval) conditions of the fast-twitch extensor digitorum longus (EDL) muscle or the slow-twitch soleus muscle were performed in adult rat hindlimbs. MHC expression during skeletal muscle regeneration was determined sequentially using immunocytochemistry.**

Results. **After grafting, most muscle fibers in the EDL and soleus underwent ischemic degeneration and regeneration; at the periphery of each muscle, a few adult fibers survived. All regenerating fibers initially expressed embryonic/fetal (developmental) MHC alone, and subsequently both developmental and fast MHC. During the first week, no expression of slow MHC was observed in regenerating fibers in either the EDL or the soleus. In nerve-intact grafts, regenerating fibers expressed slow MHC as early as the second week; under aneural conditions, no regenerating fibers expressed slow MHC even 4 weeks after grafting. On the other hand, some persisting fibers in aneural grafts could maintain expression of slow MHC 4 weeks after grafting; other fibers underwent MHC transformation induced by denervation. No significant difference in MHC expression during regeneration was observed for slow compared with fast muscles, under either neural or aneural condition.**

Conclusions. **These data suggest that regenerating adult skeletal muscle fibers, derived only from satellite cells, cannot express slow MHC without motor innervation, and that persisting muscle fibers, derived from both myoblasts in fetal development and satellite cells, may be intrinsically distinct from regenerating fibers. Satellite cells derived from slow and from fast muscles may be a single, homogenous population and may be the same population as fetal (secondary) myoblasts with regard to MHC expression.**

Key Words: **myosin; satellite cell; myoblast; rat; muscle; regeneration; autograft; myosin heavy chain.**

INTRODUCTION

Clinically, embolic disease, operating tourniquet use, or microneurovascular muscle transplantation can result in prolonged muscle ischemia with muscle cell necrosis and subsequent muscle fiber regeneration [1–4]. Muscle fiber degeneration and regeneration may also be seen following direct muscle trauma, denervation, tenotomy, and immobilizaiton [5–8]. Muscle regeneration is therefore an important mechanism of muscle recovery after a wide array of clinically significant muscle injuries.

In adult muscle, fiber regeneration depends on the proliferation and fusion of satellite cells, sometimes referred to as adult myoblasts [9]. These mononucleate cells are located between the plasma membrane and the basal lamina of muscle fibers, and are considered to be the reserve of myogenic precursor cells in adult muscle. In uninjured, adult muscle, satellite cells are mitotically quiescent. In response to fiber degeneration, these cells proliferate and fuse to form new muscle fibers. In fetal development, skeletal myogenesis begins in the somite where multipotential mesodermal cells commit to the myogenic lineage. After migrating into the limbs, myoblasts withdraw from the cell cycle, align, and fuse to form multinucleated myotubes. It has been shown that, in fetal development, there are two, distinct myoblast populations, embryonic (primary) myoblasts and fetal (secondary) myoblasts, both of which are distinguished from satellite cells [9]. However, it is unclear whether, during *in vivo* regeneration of adult muscle fibers, satellite cells express MHC in a manner similar to that observed for primary or for

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0022-4804/98 $25.00

secondary myoblasts during prenatal development. It is also unclear if satellite cells in adult skeletal muscle represent one, or more than one, population of myogenic precursors.

The purpose of this study was to investigate (1) the heterogeneity of satellite cells derived from adult fast-twitch and slow-twitch skeletal muscles, (2) the effect of innervation on muscle regeneration, and (3) differences between developmental myoblasts and satellite cells with regard to myosin heavy chain (MHC). Sequential changes in MHC expression during *in vivo* muscle regeneration were determined in fast-twitch and slow-twitch rat hindlimb muscles induced to regenerate under conditions of very brief denervation (*nerve-intact graft*) or of prolonged denervation (*aneural graft*). MHC isoform expression was determined immunocytochemically using specific antibodies for distinct MHC isoforms. Whole muscle autografts were employed, because, unlike cultured fibers, any stage of the regeneration process, from degenerating fibers to regenerating and almost mature fibers, can be observed in the same section. Moreover, persisting fibers, derived from both myoblasts in fetal development (primary and secondary myoblasts) and satellite cells, can be observed and compared with regenerating fibers, which are derived only from satellite cells.

MATERIALS AND METHODS

Animal model and surgery. Four-month-old, adult Sprague-Dawley rats were anesthetized by intraperitoneal administration of pentobarbital sodium (50 mg/kg). The fast-twitch extensor digitrum longus muscle (EDL) and slow-twitch soleus muscle were utilized as donor muscles for orthotopic autografts under neural or aneural conditions, referred to in this paper as *nerve-intact grafts* or *aneural grafts*, respectively. Both tendons and nutrient vessels of nerve-intact graft donor muscles were transected with great care not to damage the motor nerve, and subsequently both tendons were repaired with 7-0 nylon. This procedure has been noted to induce ischemic necrosis of the majority of muscle fibers in the graft, and also to result in transient muscle fiber denervation due to ischemic necrosis of the terminal, intramuscular branches of the motor nerve [10]. For aneural grafts, in addition to the tendons and nurtient vessels, the motor nerve of the donor muscle was severed, and this was followed by transection of the sciatic nerve so as to denervate all surrounding muscles in the lower leg to eliminate any possibility of reinnervation by neural or muscular neurotization. Both tendons then were repaired as in the case of nerve-intact graft.

In both nerve-intact grafts and aneural grafts, donor muscle circulation is completely obstructed, leading to ischemic necrosis and muscle fiber degeneration. Both the EDL and soleus are completely surrounded by vascularized muscles and circulation is reestablished from the recipient bed. The surgically treated EDL and soleus grafts, as well as the EDL and soleus in the contralateral leg, were removed at various time intervals (4, 7, 14, 21, and 28 days) after grafting, and were frozen in isopentane cooled with liquid nitrogen.

To compare MHC expression in myoblasts derived from regenerating adult muscle fibers with that in primary and secondary myoblasts, hindlimb muscles of Sprague-Dawley rat embryos and neonates were harvested. Gestation Day 15, 17, and 19 embryos and Postbirth Day 1, 7, 14, and 28 neonates were sacrificed under general anesthesia and hindlimbs were frozen in isopentane cooled with liquid nitrogen.

All animal surgery was performed under aseptic conditions with animals in a deep plane of general anesthesia. All animal care, housing, and operative procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals; the experimental protocol was approved by the University Committee on the Use and Care of Animals.

MHC antibodies. Three types of monoclonal antibodies specific for MHC were utilized in this study: NCL-MHCd, NCL-MHCf, and NCL-MHCs. NCL-MHCd, created against native myosin extracted from the hindlimb muscle of 7-day-old rats, also is specific for embryonic and fetal (neonatal) MHC. NCL-MHCf and NCL-MHCs, respectively, were raised in mice against native myosin from rabbit psoas and soleus muscles [11]. NCL-MHCf reacts with fast MHC (both MHC2a and MHC2b) and, possibly, with fetal (neonatal) MHC [12]. NCL-MHCs is specific for slow MHC. NCL-MHCd, NCL-MHCf, and NCL-MHCs are available from Novocastra Laboratories, UK. The specificity of these three antibodies previously has been described [11, 12].

Additional monoclonal antibodies, F1.652 and A4.840 (Developmental Hybridoma Bank, University of Iowa), were used to confirm the specificity of the NCL-MHC antibodies. F1.652 is derived from hybridomas prepared from mice immunized with human MHC from human leg muscle at the 15th week of gestation and reacts with embryonic and fetal (neonatal) MHC. A4.840 is derived from mice immunized with human MHC from adult human quadriceps muscle and is specific for the slow MHC. Preparation of the antibodies and demonstration of their specificity have been outlined previously [13, 14]. Sequential investigation of the staining patterns of developing muscles in rats revealed the specificity of NCL-MHCs to be similar to that of A4.840. The specificity of NCL-MHCd was quite similar to that of F1.652, although F1.652 detected a small number of fibers negative for NCL-MHCd. In this manuscript, the fibers positive for NCL-MHCd are referred to express the developmental MHC that means both embryonic and fetal (neonatal) MHCs.

Immunocytochemistry. Following air drying, 10-μm-thick sections were blocked with 2% horse serum in PBS for 20 min and incubated with primary antibodies specific for MHC for 1 h at room temperature. Sections were further incubated with biotinylated horse anti-mouse antibody (rat absorbed, Vector Laboratories, CA) followed by biotin-avidin-peroxidase complex diluted as per Vectastain ABC kit (Vector Laboratories) instructions in 1% horse serum in PBS for 30 min at room temperature. Between each step the sections were washed for 30 min in three changes of PBS. Development was carried out by incubation with 0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB), 0.01% H_2O_2 in PBS for 5 min, and the sections were mounted in Permount (Fisher).

RESULTS

At least four rats with successful operations were analyzed at each time point for each group (nerve-intact EDL graft, aneural EDL graft, nerve-intact soleus graft, and aneural soleus graft). The findings were quite consistent within groups.

General Morphology

Following nerve-intact or aneural grafting, the majority of muscle fibers in both the EDL and the soleus muscles underwent ischemic necrosis followed by muscle fiber degeneration. In both muscles, the presence of persisting, adult fibers that did not degenerate was noted in a 0-to-6-cell-thick rim around the periphery of the muscle.

On Day 7, necrotic fibers which had not as yet been phagocytosized still were observed in the center of the grafts, whereas a number of regenerating fibers were seen just under the most peripheral layers of persisting fibers (Fig. 1). A centripetal gradient of degeneration/

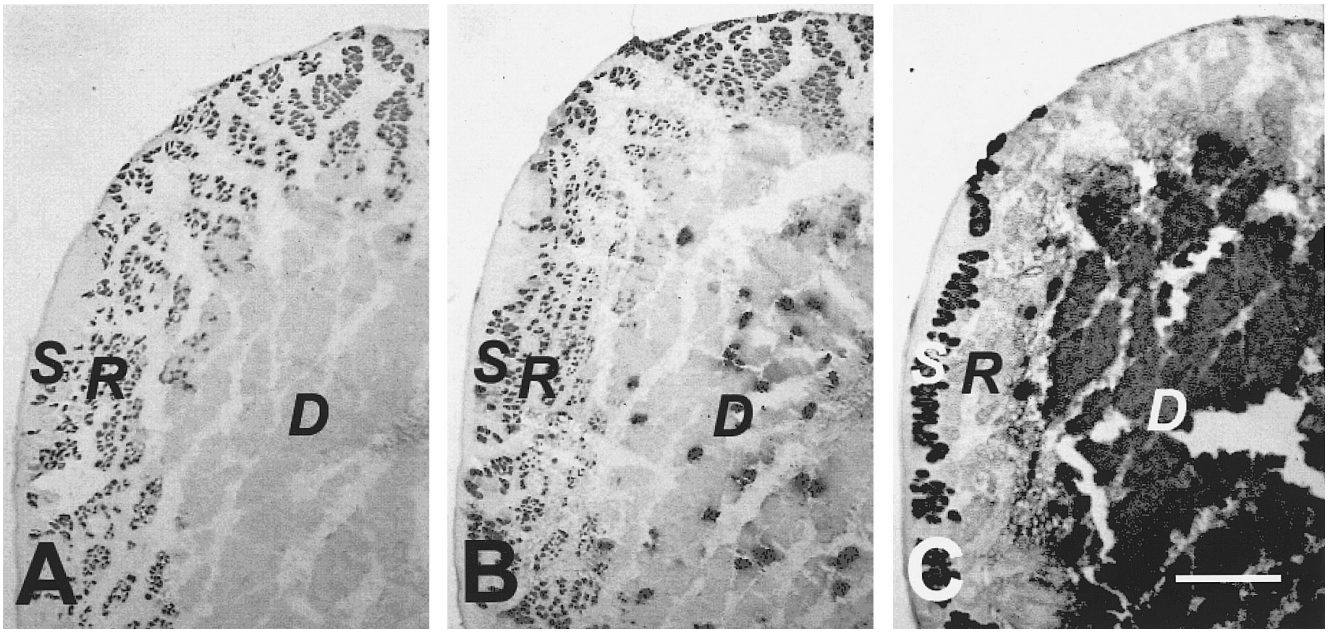

FIG. 1. Adjacent sections of a nerve-intact at Day 7 of the soleus muscle stained with antidevelopmental (A), antifast (B), and antislow (C) myosin heavy chain (MHC) antibodies. A biotinylated secondary antibody and avitin-biotin complex were used to visualize binding of the primary antibodies in this and all subsequent figures. Three centripetal layers are easily distinguishable from each other; "*S*", "*R*", and "*D*" indicate a series of centripetal layers that are the surviving layer (*S*) in the periphery, the regenerating layer (*R*) in the middle, and the degenerating layer (*D*) in the center, respectively. The surviving fibers are normal in size and usually express one of the adult MHCs, slow or fast MHC, without expressing developmental MHC. Most of the regenerating fibers show fast and developmental MHC without expressing slow MHC. Bar = 500 μm.

regeneration from the periphery inward toward the center of the graft was seen and, as a result, all stages of degeneration and subsequent regeneration can be observed in a single section during the second week. By the end of the second week, the regenerating fibers located next to the persisting fibers increased in size and, in nerve-intact grafts, were nearly indistinguishable in size from the persisting fibers (Fig. 2). By Day 14, all necrotic fibers were phagocytosized by infiltrating macrophages, and even the central core was filled with regenerating fibers. In nerve-intact grafts, regenerating fibers gradually enlarged and matured inward from the peripheral region. By Day 28, in nerve-intact grafts, even regenerating fibers in the central core had matured and formed fascicles with as minimal perimisium and endomisium as seen in a normal muscle (Fig. 3). In aneural grafts, on the other hand, the regenerating fibers did not increase in size and remained atrophic even on Day 28 (Fig. 4). Furthermore, persisting fibers in the periphery became atrophic due to persistent denervation.

MHC Expression in Nerve-Intact Grafts of EDL and Soleus

In general, the sequential changes in MHC expression during the degeneration/regeneration processes of soleus nerve-intact and aneural grafts essentially are quite similar to those of EDL nerve-intact and aneural grafts, except for the changes derived from the distinct fiber-type compositions of the two muscles.

On Day 7, regenerating fibers were beginning to form muscle fascicles from the periphery inward toward the intermediate layers (Fig.1). Most regenerating fibers expressed fast MHC as well as developmental MHC. The exceptions were small cells expressing only developmental MHC and suspected to be activated satellite cells. Activated satellite cells were seen around the persisting fibers as well as around regenerating fibers. Some of the relatively mature regenerating fibers, especially those located close to persisting fibers in the peripheral region, demonstrated slow MHC as well as embryonic and fast MHCs. The number of regenerating fibers expressing slow MHC increased with time. These findings suggest that regenerating fibers underwent innervation and subsequent fast to slow fiber-type transformation. In the EDL, a small number of persisting fibers expressed only slow MHC while most expressed only fast MHC. By contrast, in soleus most persisting fibers expressed only slow MHC (Fig. 1), suggesting that they were expressing their "original" MHC without undergoing fiber-type transformation. No persisting fibers were stained for developmental MHC.

At Day 14, regenerating fibers in the peripheral areas increased in size and it was difficult to distinguish them by size from persisting fibers in the periphery, while those two populations can be distinguished from each other by expression of developmental MHC. All fibers other than persisting fibers in the periphery expressed developmental MHC. Some of the regenerating fibers expressed slow MHC, but these fibers always simultane-

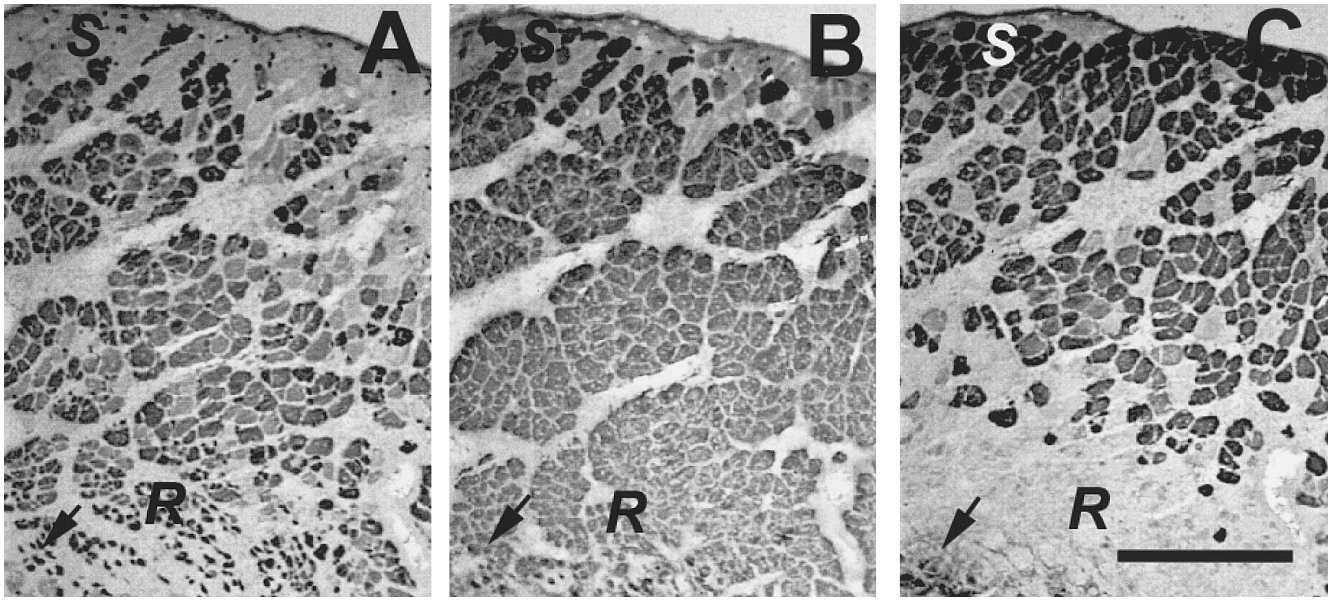

FIG. 2. Adjacent sections of a nerve-intact graft at Day 14 of soleus stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. Developmental-negative fibers in the surviving areas (*S*) are surviving fibers which express fast or slow MHC, or both. The developmental-positive regenerating fibers in the peripheral areas are similar to the surviving fibers in size and can be distinguished from the surviving fibers only by expression of developmental MHC. The regenerating fibers expressing all of the three MHCs (fast, slow, and developmental) are large in size and located only in the periphery. The regenerating fibers (myotubes) which are not matured in size and express fast and developmental MHC without expressing slow MHC are located more centrally (*R*). In the central core (arrows), there are some degenerating fibers around which activated satellite cells are fusing each other to form new myotubes. Bar = 500 μm.

ously expressed fast and developmental MHC. The number of regenerating fibers expressing slow MHC increased with time. Regenerating fibers in the central area, however, did not express slow MHC, suggesting that slow MHC expressed in regenerating fibers was induced by innervation (Fig. 2). In EDL, almost all regener-

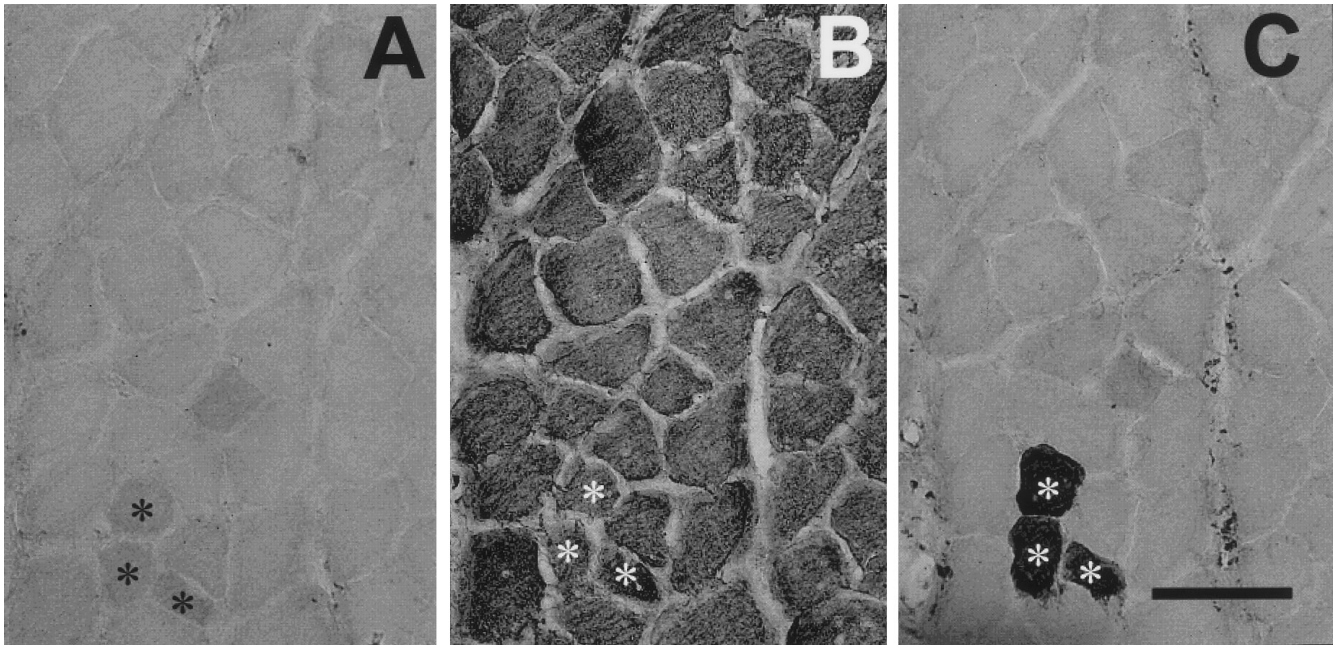

FIG. 3. Adjacent sections of a nerve-intact graft at Day 28 of the extensor digitrum longus muscle (EDL) stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. Most, if not all, of the regenerating fibers become mature and do not express developmental MHC. Almost all regenerated fibers express adult MHC, slow or fast. Asterisks indicate the regenerated fibers which express both slow and fast MHCs. These slow-positive regenerated fibers cannot be found in aneural grafts. Bar = 100 μm.

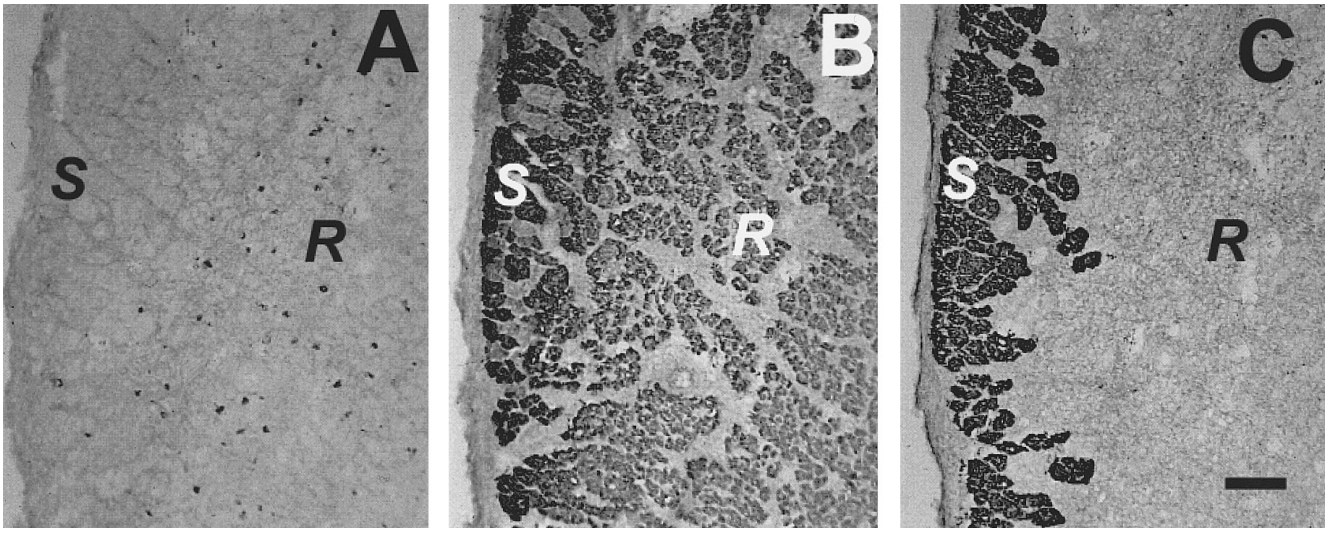

FIG. 4. Adjacent sections of an aneural graft at Day 28 of soleus stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. Only the fibers located in the surviving areas (*S*) can express slow MHC, while no fiber express it in the regenerating areas (*R*). The surviving fibers become atrophic by denervation and the regenerating fibers remained small in size without receiving innervation. Small developmental-positive fibers are occasionally seen in aneural grafts at Day 28, while those are rarely seen in nerve-intact grafts at Day 28. Some of the surviving fibers express only slow MHC, while others are positive for both slow and fast MHC, presumably because of fiber-type transformation induced by denervation. Bar = 100 μm.

ated fibers which already had lost developmental MHC were found to express fast MHC only (Fig. 5). These findings suggest that fibers undergoing fast-to-slow fiber-type transformation tend to mature more slowly with respect to MHC expression than the rest. A number of activated satellite cells were observed all over each section including around the persisting fibers. This suggests that, in addition to the activated satellite cells derived from necrotized fibers, satellite cells of persisting fibers also are activated, possibly mediated by diffusive factors, and thereafter migrate to the regenerating areas and become involved in the regeneration process.

During the second and third week, muscle regeneration progressed into the central core, and peripheral regenerating fibers matured and no longer expressed developmental MHC. Activated satellite cells still were seen around persisting or regenerating fibers. Most regenerating fibers, as well as persisting fibers, expressed slow MHC in soleus. Five MHC expression patterns were observed: (a) fast only, (b) fast and slow, (c) fast, slow, and developmental, (d) fast and developmental, and (e) developmental alone (Fig. 6). It is suggested that, in Fig. 6, the large "a" fiber expressing fast MHC alone is a mature fiber innervated by a fast motoneuron, whereas the large mature "b" fiber showing fast and slow MHCs and the relatively large immature "c" fiber showing all three MHCs are in the process of fiber-type transformation from fast to slow induced by innervation of a slow motoneuron. The small "d" and "e" fibers, respectively, are suspected to be a myotube expressing fast and developmental MHCs and an activated satellite cell expressing only developmental MHC.

As regenerating fibers developed during the third and fourth week, the intensity of staining for developmental MHC became weakened. All fibers became enlarged and mature by Day 28, and indistinguishable from normal muscle fibers (Figs. 3 and 7). None, if any, regenerated fibers expressed developmental MHC. Regenerated fibers expressed either only fast or only slow MHC, except for a small number of fibers expressing both fast and slow MHC (Figs. 3 and 7).

MHC Expression in Aneural Grafts of EDL and Soleus

During the first week, the differences in MHC expression and morphological characteristic between nerve-intact and aneural grafts are difficult to detect. In both EDL and soleus, some regenerating fibers expressed developmental and fast MHCs, but none expressed slow MHC.

At Day 7, all regenerating fibers, other than small cells corresponding to activated satellite cells, stained positive for both developmental and fast MHCs (Figs. 8 and 9). None were positive for slow MHC (Figs. 8 and 9). Activated satellite cells were seen not only around the regenerating fibers but also around the persisting peripheral fibers (Fig. 8) as seen in nerve-intact grafts.

At Day 14, small regenerating fibers did not express slow MHC but always developmental MHC and frequently also fast MHC. Some persisting fibers expressed both slow and fast MHC presumably due to fiber-type transformation induced by denervation.

At Day 21, activated satellite cells were still seen around persisting or regenerating fibers (Fig. 10). Slow MHC was expressed only in the persisting fibers, a few of which also expressed fast MHC (Fig. 10). Most of the regenerating fibers differentiated, lost developmental MHC, and as a result expressed only fast MHC (Fig. 10). The regenerating process proceeded at a slower pace in aneural grafts than in nerve-intact grafts.

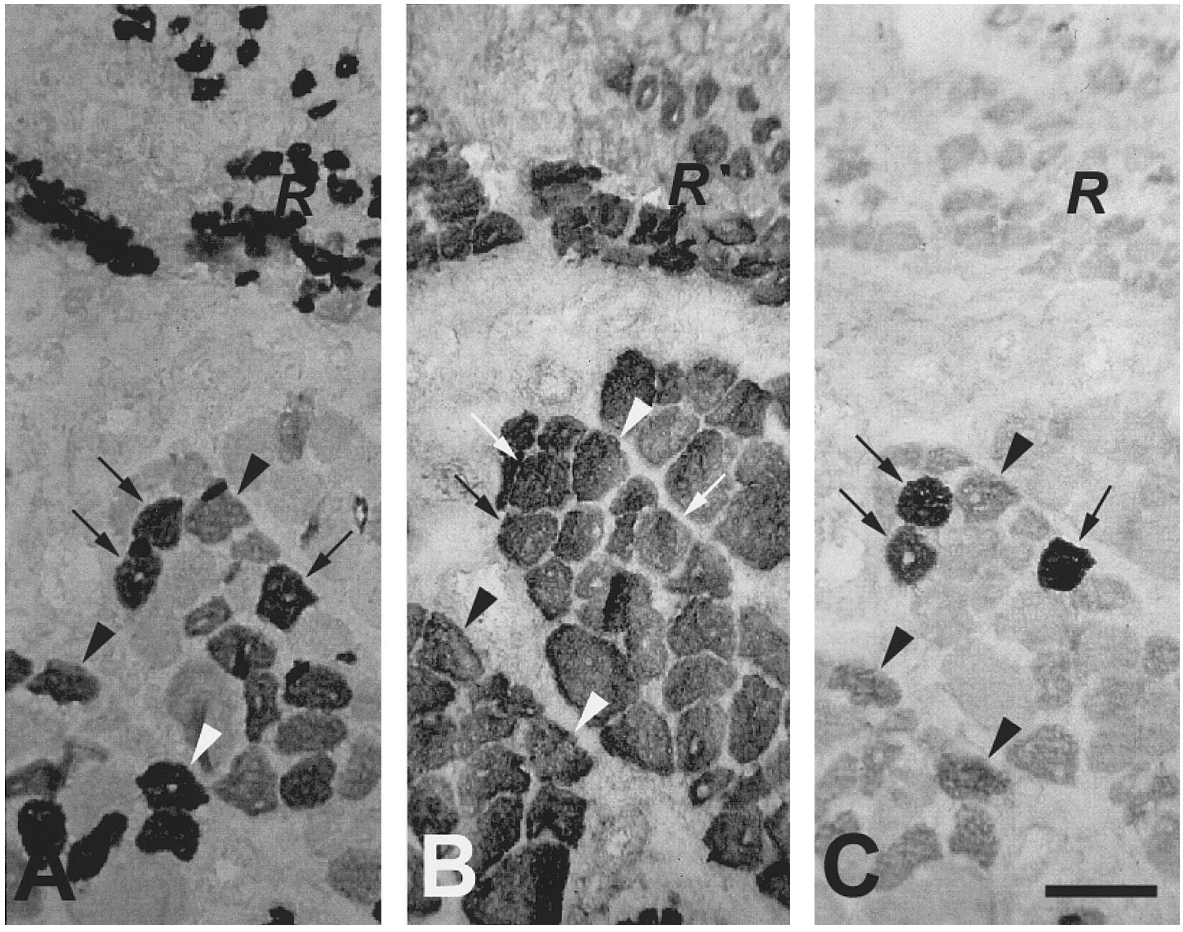

FIG. 5. Adjacent sections of a nerve-intact graft at Day 14 of EDL stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. All of the large regenerated fibers which are packed compactly and form muscle fascicles and most of the small regenerating fibers in the regenerating areas (*R*) express fast MHC. Some of the large regenerated fibers still express developmental MHC, some of which also stain with the antislow antibody lightly (arrowheads) or intensely (arrows). This finding suggests that the fibers undergoing fiber-type transformation probably induced by innervation of slow motoneuron usually lose developmental MHC expression later than those which do not undergo fiber-type transformation because of innervation by fast motoneuron. Bar = 50 μm.

At 4 weeks, although all regenerated fibers were atrophic, most had already lost developmental MHC and expressed only fast MHC, suggesting they already matured in view of MHC expression (Figs. 4 and 11). No regenerated fibers expressed slow MHC in fast EDL and slow soleus. In contrast, some persisting fibers expressed slow MHC, and frequently fast MHC also, probably because they are in the process of slow-to-fast fiber-type transformation induced by denervation (Figs. 4 and 11). Small cells expressing developmental MHC were seen in aneural grafts, but not detected in nerve-intact grafts at day 28 (Figs. 3 and 7).

The transitions of MHC expression patterns in regenerating and persisting fibers as observed in this study are summarized in Table 1, together with those in developing muscle fibers of embryonic and neonatal rats.

DISCUSSION

Muscle Regeneration Models in This Study

Nerve-intact grafts and aneural grafts were used in the present study to experimentally induce muscle degeneration/regeneration and to examine neural influence on MHC expression patterns during regeneration. Although there is a brief period of denervation, the intact endoneurial tubes and short distance required for axonal regeneration in nerve-intact grafts allow for rapid axonal ingrowth and muscle fiber reinnervation [10]. Regenerating fibers in nerve-intact grafts supposedly receive neural input as early as the myotube fusion phase of regeneration, whereas in aneural grafts, even regenerated fibers which have already lost developmental MHC remain denervated. On the other hand, while innervation of at least some persisting fibers may be maintained in nerve-intact grafts, all remain denervated in aneural grafts.

Primary, Secondary, and Adult Myoblasts (Satellite Cells) and Their MHC Expressions

It has been shown that, in view of MHC expression, two kinds of myogenic precursor cells appear sequentially in fetal development, which have been referred to as "embryonic" and "fetal" myoblasts, early and late myoblasts [16], or primary and secondary myoblasts [9]. Each myoblast type appears to be committed to

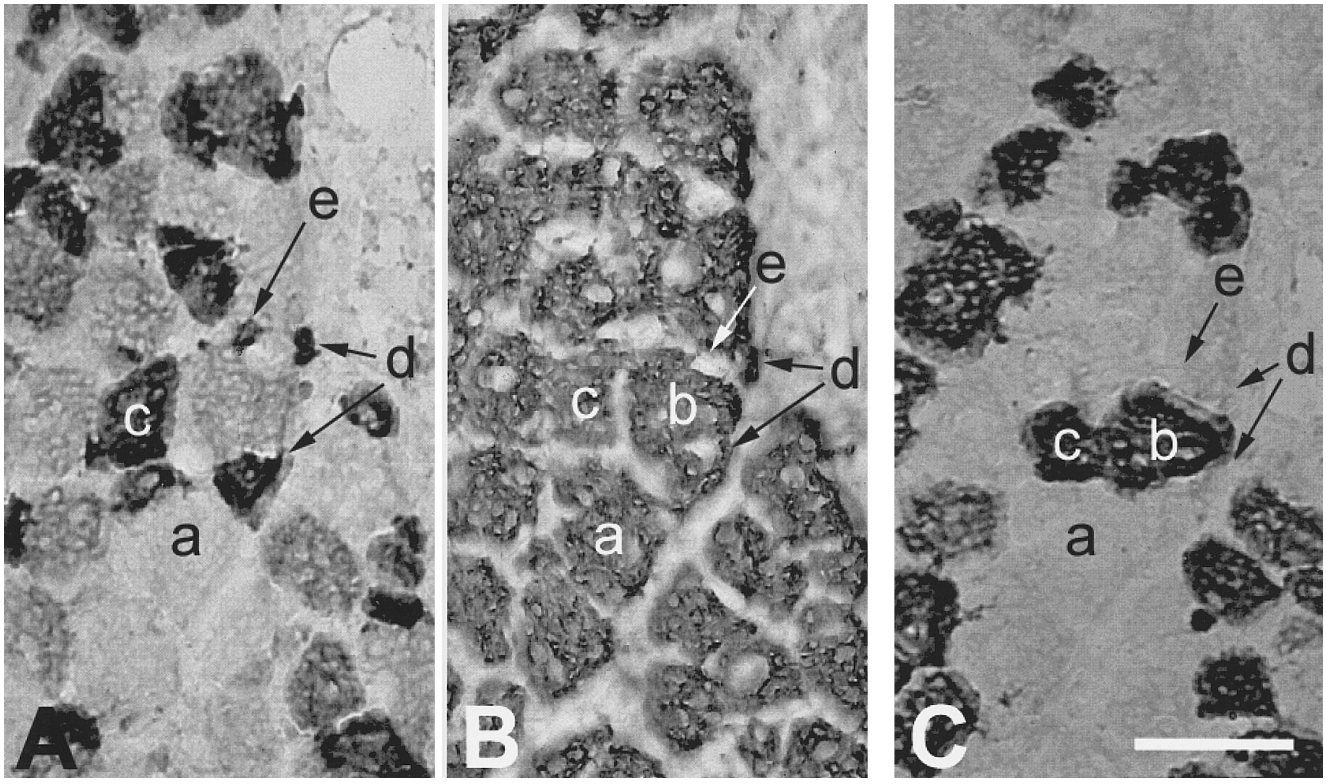

FIG. 6. Adjacent sections of a nerve-intact graft at Day 21 of EDL stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. Five kinds of MHC expression pattern are seen: (a) developmental−, fast+, slow−; (b) developmental−, fast+, slow+; (c) developmental+, fast+, slow+; (d) developmental+, fast+, slow−; and (e) developmental+, fast−, slow−. Bar = 50 μm.

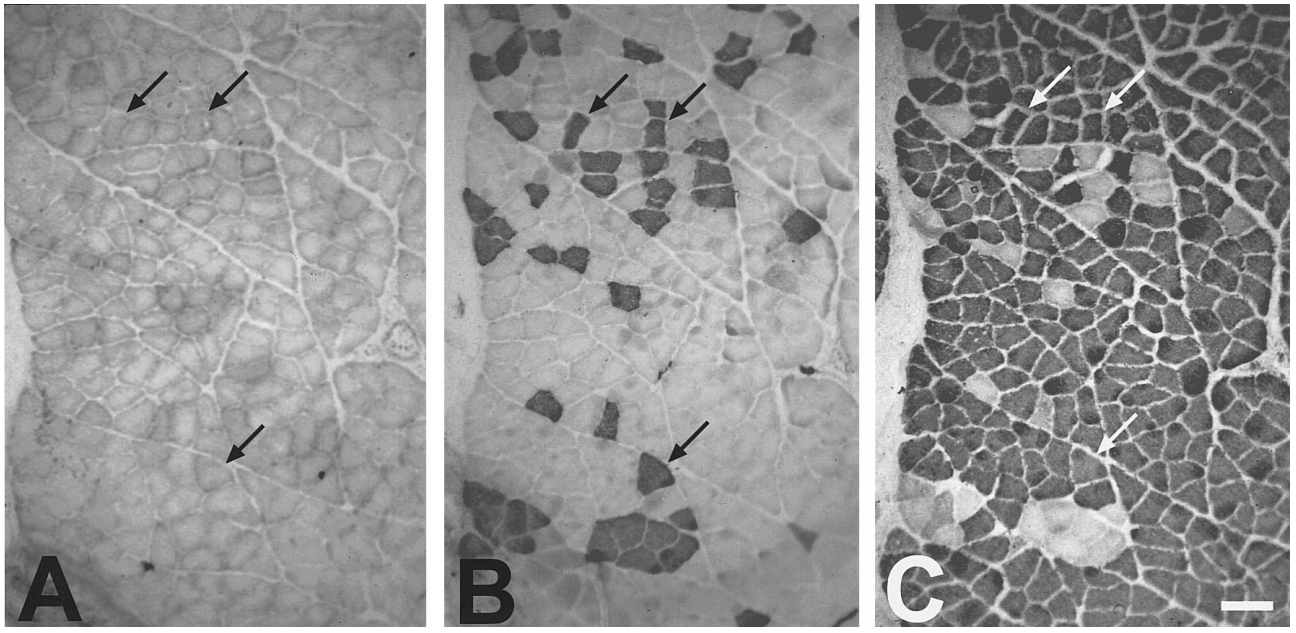

FIG. 7. Adjacent sections of a nerve-intact graft at 4 week of soleus stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. All fibers matured and were distributed in fascicles with minimal perimisium and endomisium just like normal muscles. Most of the fibers express one of the two adult MHCs, slow or fast MHC, without expressing developmental MHC. There are a few fibers expressing both slow and fast MHCs (arrows). Bar = 100 μm.

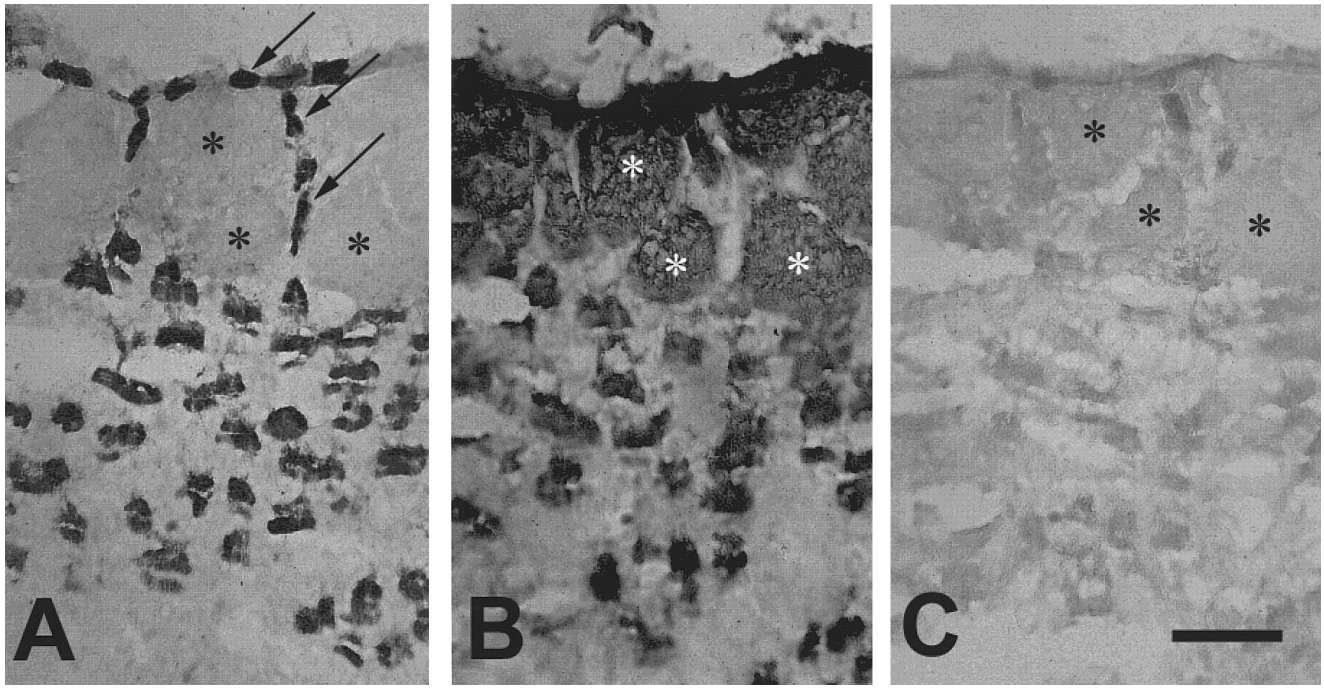

FIG. 8. Adjacent sections of an aneural graft at Day 7 of EDL stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. The surviving fibers (asterisks) express original MHC without expressing developmental MHC, while the regenerating myotubes usually express developmental and fast MHC without expressing slow MHC. The satellite cells of the surviving fibers (arrows) differentiated, migrated to the regenerating areas, and fused with other activated satellite cells derived from the degenerating fibers to form new myotubes. Bar = 50 μm.

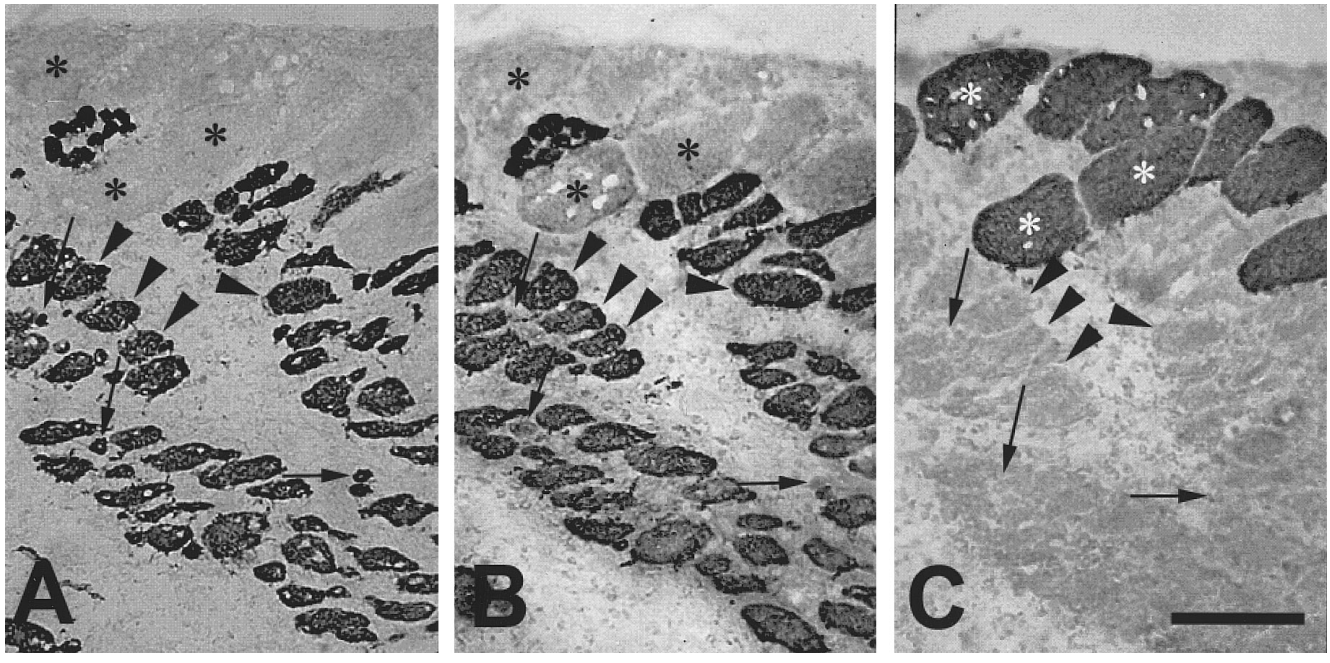

FIG. 9. Adjacent sections of an aneural graft at Day 7 of soleus stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. Large surviving fibers (asterisks) in the periphery do not show developmental MHC, but express original MHC, predominantly slow MHC in soleus. Slow-to-fast fiber-type transformation of the surviving fibers induced by denervation is not yet prominent at 1 week after aneural graft. The regenerating fibers express fast MHC as well as developmental MHC (arrowheads). Small developmental-positive cells (arrows) appear to be activated satellite cells which do not yet show fast MHC. Bar = 100 μm.

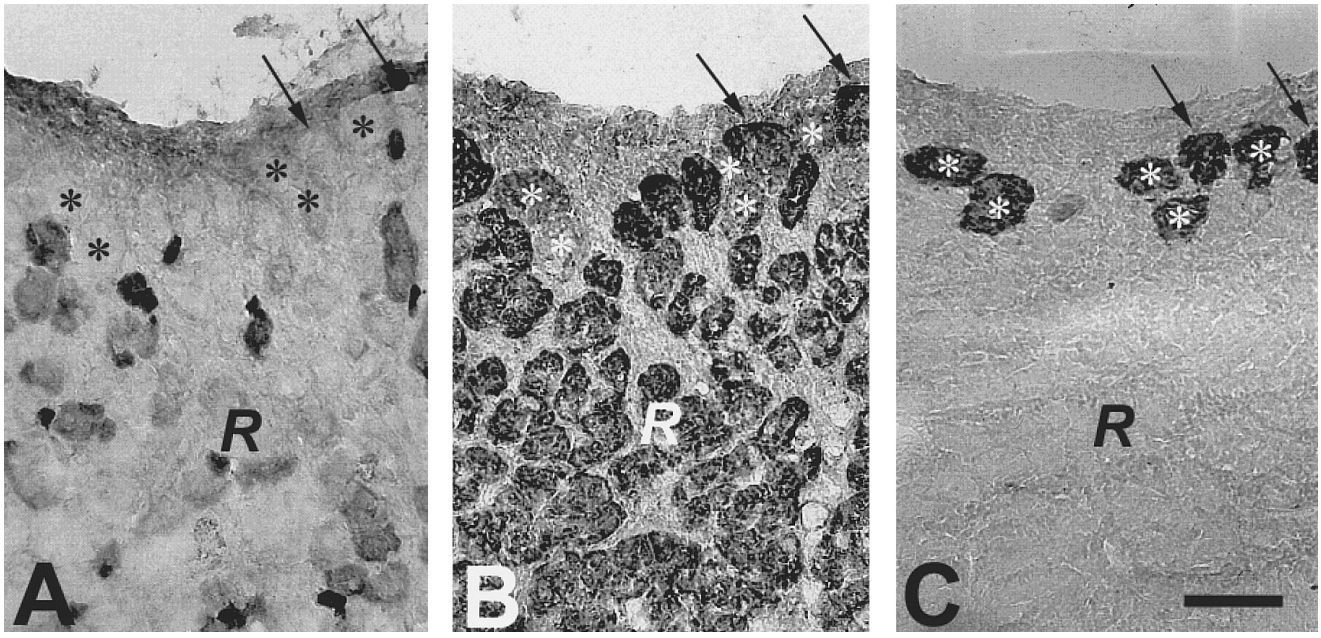

FIG. 10. Adjacent sections of an aneural graft at 3 week of soleus stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. The surviving fibers become atrophic and are round-shape as well as the regenerating fibers most of which became mature in view of MHC expression and already lost developmental MHC. All regenerating fibers express fast MHC, but not slow MHC in aneural grafts (*R*). Only some of the surviving fibers can express slow MHC, some of which express only slow MHC (asterisks) and the others express both slow and fast MHC (arrowheads). Bar = 50 μm.

distinct cell lineages. In rodents, primary myocytes (differentiated myoblasts) and myotubes derived from embryonic (primary) myoblasts express embryonic and slow MHC. By contrast, secondary myocytes and myotubes derived from fetal (secondary) myoblasts express embryonic and fast MHCs. This pattern has been observed both *in vitro* and *in vivo* [16–18]. As a secondary event, some myotubes subsequently undergo transition

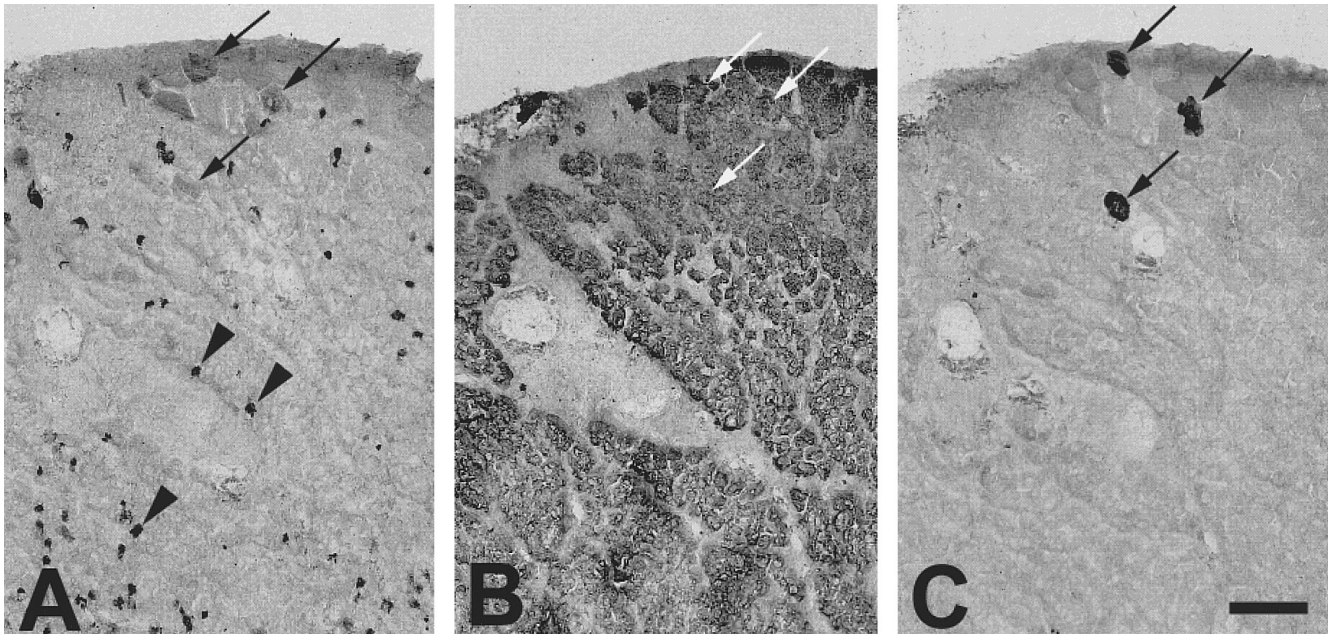

FIG. 11. Adjacent sections of an aneural graft at Day 28 of EDL stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. Almost all fibers including the surviving fibers express fast MHC. Only a few surviving fibers located in the periphery express slow MHC (arrows), and they usually express also fast MHC. Small developmental-positive cells are scattered across the section (arrowheads). Bar = 100 μm.

TABLE 1

Transition of Myosin Heavy Chain Expression in Developing Muscles, Nerve-Intact Grafts, and Aneural Grafts in Rats

Developing fibers	(A) emb → emb · slow (primary myotube) → **slow** emb · slow · fast → emb · fast → **fast**
	(B) emb → emb · fast (secondary myotube) → **fast** emb · fast · slow → emb · slow → **slow**
Regenerating fibers in nerve-intact grafts	emb → emb · fast → **fast** emb · fast · slow → fast · slow → **slow**[a]
Regenerating fibers in aneural grafts	emb → emb · fast → **fast**
Persisting fibers in nerve-intact grafts	fast → **fast** slow → **slow** (slow → slow · fast → fast)[b]
Persisting fibers in aneural grafts	fast → **fast** slow → **slow** slow · fast → **fast**[c]

[a] Some regenerating fibers in nerve-intact grafts undergo transformation from fast to slow induced by innervation by a slow motoneuron.
[b] Few denervated fibers in nerve-intact grafts undergo transformation from slow to fast as seen in aneural grafts.
[c] Some denervated slow fibers undergo fiber type transformation from slow to fast.

of MHC composition in response to environmental factors such as innervation [16]. Secondary myotubes are formed around a primary myotube in a rosette arrangement underneath the basement membrane of the core primary myotube. Prior to the investigation of nerve-intact and aneural grafts, we reconfirmed these sequential changes during fetal development in morphological and MHC expression using the same series of specific anti-MHC antibodies as used in this study (Figs. 12 and 13).

Difference in MHC Expression between in the Presence and in the Absence of Innervation

Myofibrillar protein expression is influenced by environmental factors such as innervation, hormone, growth factors, and muscle mechanical activities [19, 20]. Of these factors, innervation appears to have the greatest influence on myofibrillar protein expression. The results of the present study show a striking difference in MHC expression patterns during experimentally induced regeneration between the presence and the absence of innervation in both fast and slow muscle. Four weeks after grafting, slow fibers are abundant in nerve-intact grafts, in contrast with only the periphery in aneural grafts (Figs. 3, 4, 7, and 11). The induction of slow MHC of regenerating fibers was seen only in the presence of motor innervation, suggesting that neural input, possibly tonic pattern impulse from a slow motoneuron, may be indispensable for regenerated fibers to

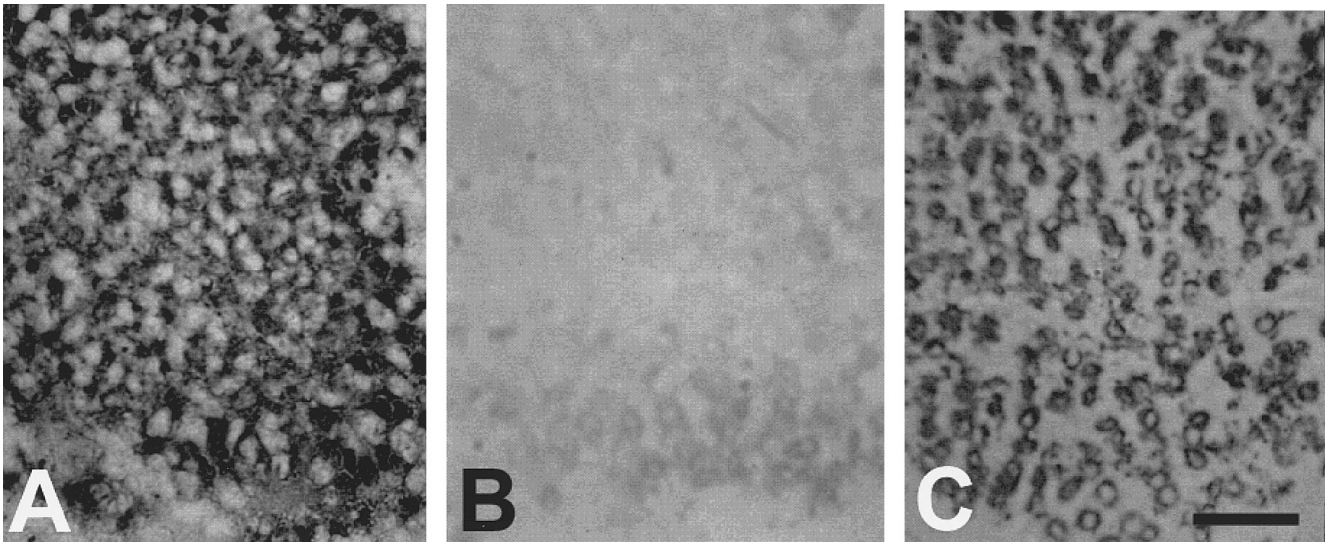

FIG. 12. Adjacent sections of hindlimb muscles of a rat fetus at Gestation Day 15 stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. At Gestation Day 15, all myoblasts (early or primary myoblasts) and myotubes (primary myotubes) express developmental MHC (A), and most of them also express slow MHC (C), while none of them express fast MHC (B). Bar = 50 μm.

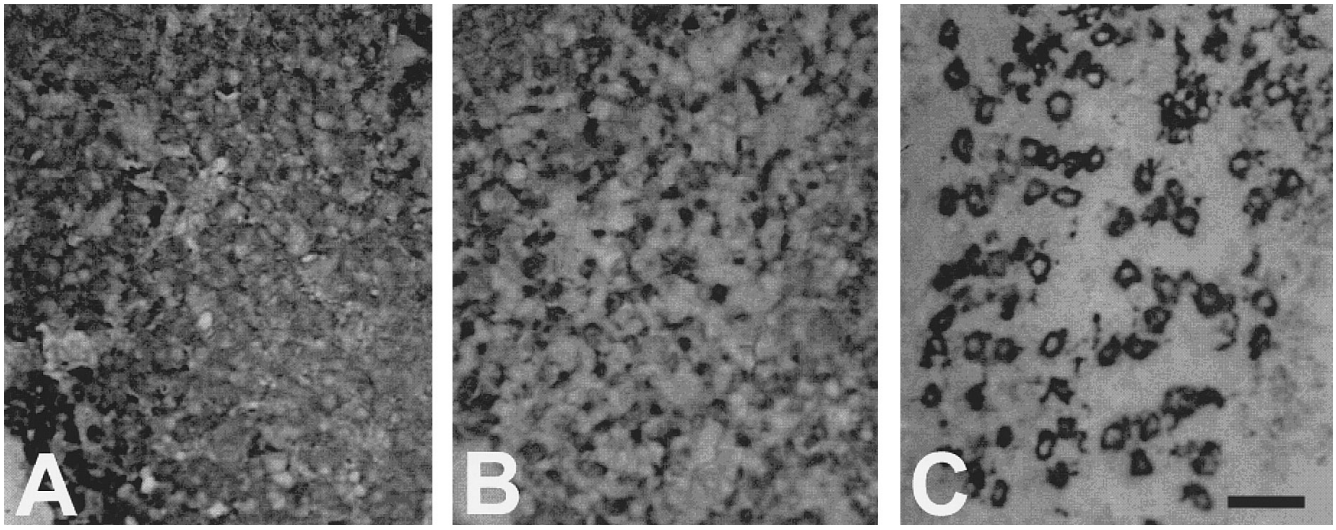

FIG. 13. Adjacent sections of hindlimb muscles of a rat fetus at Gestation Day 19 stained with antidevelopmental (A), antifast (B), and antislow (C) MHC antibodies. At Gestation Day 19, secondary myoblasts and secondary myotubes which express fast MHC as well as developmental MHC but not slow MHC appear around primary myotubes which express developmental and slow MHCs in a rosette arrangement. Bar = 50 μm.

express slow MHC. Satellite cells from slow rat soleus muscle [the present study, 21] and slow chicken anterior latissimus dorsi muscle (ALD) [22] give rise to fast fibers during regeneration in the absence of nerve. While neonatal (fetal) MHC does not disappear from most of noninnervated regenerating fibers in chicken pectoral muscle even at 8 weeks after cold injury [23], in the present study induction of fast MHC and suppression of developmental MHC in regenerating fibers occurred even in the absence of nerve. However, small cells expressing developmental MHC which appear to be activated satellite cells were still seen in regenerating areas of aneural grafts at 4 weeks after surgery.

The transformation of persisting fiber MHC composition, i.e., suppression of slow MHC and induction of fast MHC, was seen in some fibers of aneural grafts. This slow-to-fast transformation generally is observed in a majority of denervated fibers in rodents, suggesting innervation is necessary for these fibers to express slow MHC. Previous studies using newborn rats demonstrated that innervation is not required for the appearance of adult fast MHC [24], and that induction and maintenance of slow myosin are nerve-dependent [25], although the initial fiber phenotype pattern can be established without innervation [26].

Heterogeneity of Satellite Cells (Difference in MHC Expression between Regenerating Fibers in Fast EDL and Those in Slow Soleus)

There have been a number of studies which indicate the possibility of heterogeneity of satellite cells. It has been shown that there is a difference in number and mitotic behavior [27] and acetylcholinesterase regulation [28] of satellite cells between fast and slow skeletal muscles in rodents. It has been shown that jaw and limb satellite cells are committed to distinct cell lineages, each programmed to express a different subset of genes, superfast/slow or fast/slow, respectively [29]. In contrast, Hartley *et al.* [30] found that satellite cells from fast and slow avian muscles are indistinguishable on the basis of their expression of embryonic MHC isoforms. Similarly, there is no evidence of satellite cell diversity among rat soleus (slow), tibialis anterior (fast), and diaphragm (fast and slow) muscle [31].

In the present *in vivo* study, satellite cells with similar developmental origins (such as within limb muscles) showed identical MHC expression in two regenerating muscles with distinct fiber-type compositions. Almost all, if not all, regenerating fibers expressed developmental and fast MHC, but never slow MHC under aneural condition in both fast-twitch EDL and slow-twitch soleus. Under neural condition, on the other hand, although some regenerating fibers expressed slow MHC as well as developmental and fast MHC, all small regenerating fibers which likely were not yet innervated, showed developmental and fast MHC, but not slow MHC. These results suggest that satellite cells in adult fast and slow muscles are intrinsically equivalent, at least in view of MHC expression *in vivo.* The findings of this study conflict somewhat with those of some *in vitro* studies, which suggest that there are two kinds of populations of adult satellite cells which differ in MHC expression when grown in culture [32], and also that some satellite cells express slow MHC under appropriate culture condition [33]. However, phenotypic differences observed *in vitro* can reflect previous interactions with other cell types and environmental factors in culture, and these differences are not necessarily due solely to intrinsic factors [27]. Cell-cell interactions can affect phenotype, and diffus-

ible factors that are secreted by fibroblasts are likely to affect the regulation of satellite cells. It is possible that regenerating fibers in aneural grafts could express slow MHC even *in vivo* under regulation by factors other than innervation, such as diffusible growth factors.

Difference in MHC Expression between Regenerating Fibers and Persisting Fibers

Another striking difference in MHC expression patterns was observed between regenerating fibers and persisting fibers in aneural grafts. The regenerating fibers do not express slow MHC, but some persisting fibers maintain expression of slow MHC. Since a few persisting fibers in EDL aneural grafts and most in soleus aneural grafts still express slow MHC even 4 weeks after denervation, they appear to be an intrinsically different population from regenerating fibers which cannot express slow MHC without innervation. Soleus, in contrast with other muscles, is considered to have a number of fibers derived from primary myoblasts, which can maintain slow MHC even after denervation. Persisting fibers expressing slow MHC 4 weeks after denervation are possibly committed to slow and derived from primary myoblasts, while the other population of persisting fibers may be committed to fast and derived from secondary myoblasts.

Persisting fibers are originally derived from either primary myoblasts or secondary myoblasts, thereafter satellite cells participate in the process of growth of these fibers, while regenerating fibers are derived from only satellite cells. It has been revealed that the progeny of clonally derived satellite cells carrying a reporter gene can fuse with existent fibers of different types without affecting the phenotype of the existing fibers with which they fuse [34]. Regenerating fibers do not express slow MHC without innervation, and their MHC expression pattern is quite similar to that of persisting fibers committed to fast, which are derived from secondary myoblasts and satellite cells.

Secondary myotubes are formed under the basement membrane of the primary myotube, and subsequently secondary myotubes are separated as individual fibers. At the time of this separation, some secondary myoblasts which have not participated in myogenesis possibly remain as satellite cells of primary myotubes, although the time point when satellite cells become a distinct class of myoblasts in rodents has not been clarified. The satellite cells thus are a population distinct from primary myoblasts, but may be intrinsically the same population as secondary myoblasts in view of MHC expression.

ACKNOWLEDGMENTS

The monoclonal antibodies, F1.652 and A4.840, were obtained from the Developmental Studies Hybrydoma bank maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, Maryland, and the Department of Biological Sciences, University of Iowa, Iowa City, Iowa, under Contract N01-HD-6-2915 from the NICHD. This work was supported by Grant NS34380-02 from the National Institutes of Health, Bethesda, Maryland.

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