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Survival of Myogenic Cells in Freely Grafted Rat Rectus Femoris and Extensor Digitorum Longus Muscles

GREGG D. PHILLIPS, DANYUN LU, VICTOR I. MITASHOV, AND BRUCE M. CARLSON
Department of Anatomy and Cell Biology (G.D.P., D.L., V.I.M., B.M.C.) and Department of Biological Sciences (B.M.C.), University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT **The objective of this study was to determine how long myogenic cells can survive in the central ischemic zone of early free muscle grafts in the rat. The study was conducted on free grafts of a large (rectus femoris) and a small (extensor digitorum longus) muscle. At times ranging from zero hr to five days post-grafting, the central zones were isolated, minced, and implanted under the back skin of mice. After five days the minces were removed and examined histologically for the presence of rat myotubes, which should form only in minces that contain viable myogenic cells. The results show that myogenic cells survive two to four hr in the ischemic centers of the large rectus femoris grafts; after longer post-grafting intervals, rat myotubes did not arise in central zone minces. In grafts of small muscles, myotubes consistently appeared in central zone minces. Since the formerly ischemic central areas of rectus muscle grafts are ultimately replaced by regenerating muscle fibers, we conclude that these regenerating muscle fibers are derived from precursor cells located outside of the ischemic zone.**

INTRODUCTION

A prominent feature of early free muscle grafts is a large central zone of ischemic necrosis, in which obvious degeneration of muscle fibers is a prominent reaction (Carlson et al., 1979; Studitsky, 1977). Except for a thin peripheral rim of muscle fibers which survive the grafting procedure intact (apparently aided by the diffusion of oxygen and nutrients), the muscle fibers develop signs of severe ischemia within hours after grafting. They remain in that state until days, or even weeks, later when they are removed by the action of macrophages associated with the blood vessels that grow into the interior of the graft. Concomitant with the removal of the degenerated muscle is the appearance of activated myogenic cells beneath the persisting basal laminae of the original muscle fibers.

Free grafting is most successful with small muscles. Rarely does a functional graft result if the original muscle weighs over 3 gm (Lavine and Cochran, 1976; Watson and Muir, 1976). In smaller grafts (<500 mg), the ischemic muscle fibers of the central zone are replaced by regenerated muscle fibers; but in grafts of some larger muscles, e.g., the palmaris longus of the monkey (Markley et al., 1978), the original muscle fibers of the central zone are replaced by a core of dense fibrous connective tissue.

For a better understanding of the cellular dynamics of free muscle grafts, it is important to know the fate of the muscle cells that are located in the zone of ischemic necrosis of early grafts. The principal question is whether or not myogenic cells associated with the original muscle fibers in the central zone of a graft are capable of surviving the initial ischemic insult and can then become activated upon revascularization of this region. To investigate this question, we have employed an in vivo double grafting technique. A standard free graft of a rat muscle was first performed. Then at various early intervals after grafting, the central ischemic zone of the original graft was removed, minced, and placed beneath the skin of a host. The presence of myotubes within the mince was the endpoint indicating that viable myogenic cells were present in the region in the original muscle graft from which the mince was taken.

MATERIALS AND METHODS

Free Autografts

Two groups of free muscle autografts were performed. In the first series, extensor digitorum longus (EDL) muscles were grafted in 43 male, 300–350 gm, Sprague-Dawley rats. The other autograft group consisted of 39 adult male rats (150–370 gm) of the F-455 inbred (94–96 generations) strain maintained at the University of Michigan. In these animals, both rectus femoris muscles were orthotopically autografted.

All rats were anesthetized with ether for the grafting operations. The muscles were completely removed from their beds and immediately replaced in their normal orientation. Both proximal and distal ends of the muscle were sutured to the corresponding tendon stumps. Nothing was done to reconnect the severed blood vessels and nerves. Postoperatively, the rats were provided with tetracycline water and rat chow ad libitum.

Rat-to-Mouse Grafts

This part of the experiment involved the use of 127 adult male (25–44 gm) athymic nude mice (*Ho*, Taconic Farms, Germantown, N.Y.), or immunocompetent mice of the C57 and 129 SvSl strains, obtained from colonies

Address reprint requests to Bruce M. Carlson, University of Michigan, Department of Anatomy and Cell Biology, Ann Arbor, Michigan 48109.

The current address for Danyun Lu is National Research Institute for Sports Science, 11 Tiyuguan Road, Beijing, China.

The current address for Victor I. Mitashov is Institute of Developmental Biology, Academy of Sciences of the U.S.S.R., Ulitsa Vavilova, D. 26, 117334 Moscow, USSR.

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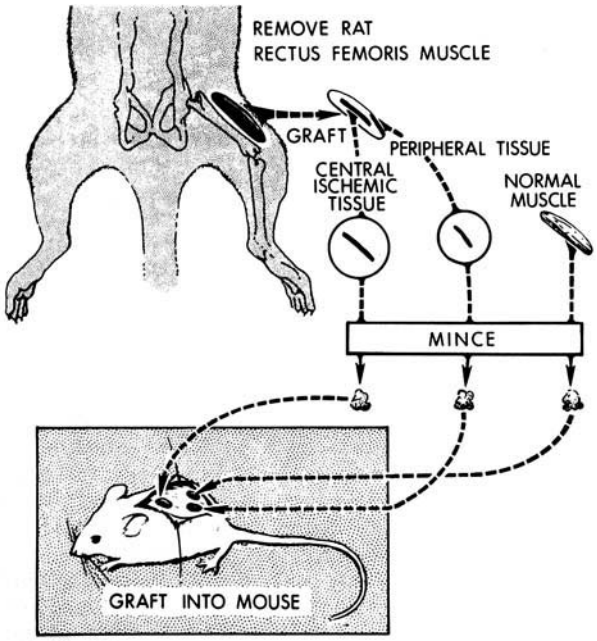

Fig. 1. Rat-to-mouse grafting procedure. Central ischemic and peripheral tissues from a rectus femoris autograft, along with an equivalent amount of non-grafted (normal) muscle, were minced and implanted separately beneath the back skin of a mouse host.

at the University of Michigan. The next stage in the experimental design involved the removal of pieces of test muscle from the rats, mincing them and implanting the minces in separate areas beneath the back skin of host mice (Fig. 1). Free autografts in the rats were removed at 0, 1, 2, 3, 4, 6, 12, or 16 hrs or 1, 2, or 5 days after transplantation. After removal from the rat, the grafted muscles were split longitudinally and the central zones removed, taking special care to avoid contamination from the peripheral region. The pieces of central zone were then minced into 1-mm^3 fragments with iridectomy scissors and implanted beneath the back skin of the host mice. Similarly, muscle from the very periphery of the grafts and pieces of normal ungrafted muscle from the same rat were minced and grafted in different locations beneath the back skin of the same host mouse. Rat-to-mouse grafts were used because, with the ease in distinguishing between rat and mouse myotubes, it was possible to eliminate host contamination of the grafts.

The minces were left in place for four or five days before sacrifice of the mouse host. Before they were removed from the mouse, the minces were photographed. They were then fixed in Bouin's solution, and serial 7-μm paraffin sections were stained with Ehrlich's hematoxylin and eosin for histological examination.

Serial sections of the minces were examined for the presence or absence of rat myotubes, which are readily

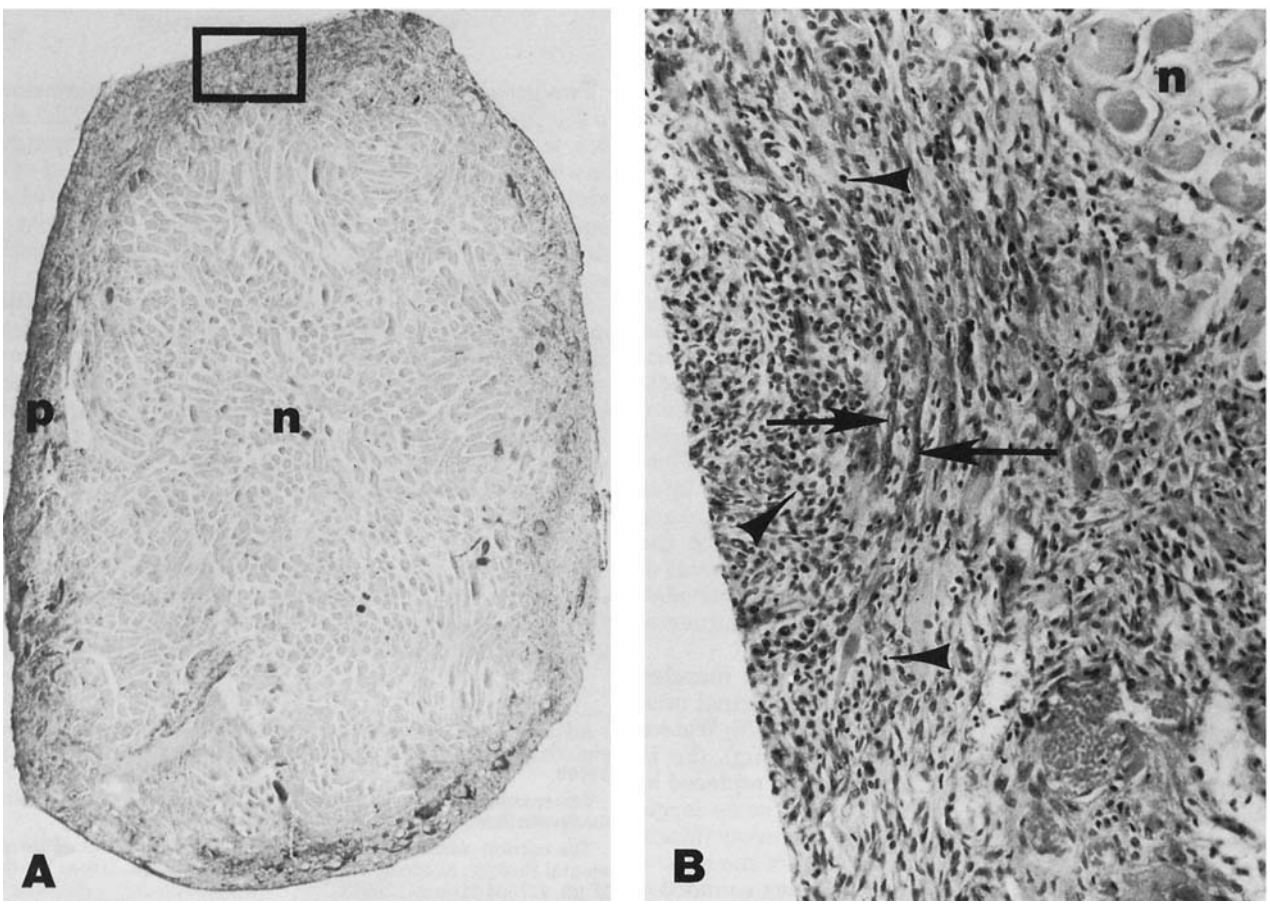

Fig. 2. Five-day rat-to-mouse mince of non-grafted extensor digitorum longus muscle. **a.** Low-magnification photograph of the entire mince showing peripheral basophilia (p) and necrotic muscle fibers (n). H&E, ×28. **b.** High-magnification photograph of the boxed region in **a**, illustrating myotubes (arrows), necrotic muscle (n), and diffuse lymphocytes (arrowheads). H&E, ×197.

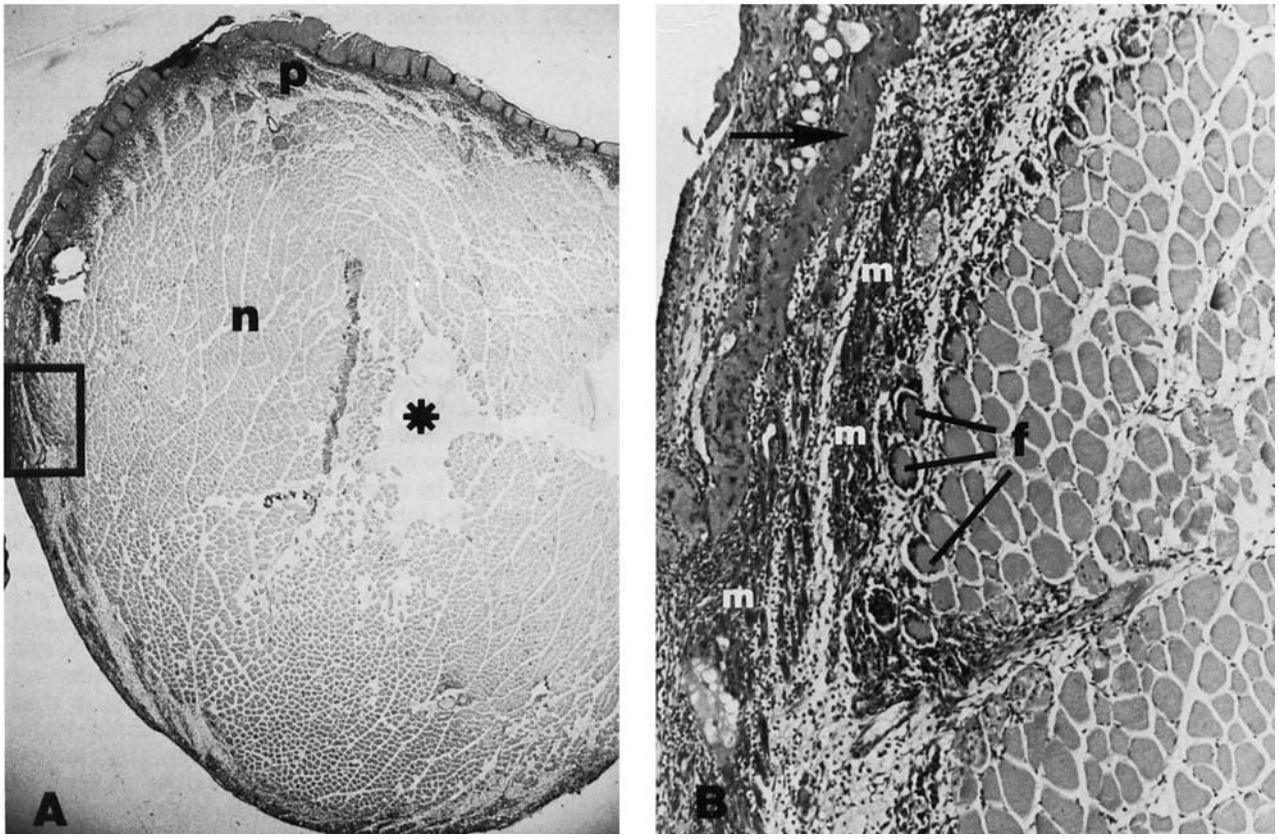

Fig. 3. Five-day rectus femoris autograft displaying zonation characteristic of regenerating muscle. **a.** Low-magnification photograph showing a peripheral basophilic zone (p), a central zone of necrotic muscle (n), and the region from which central ischemic tissue minces were removed (asterisk). H&E, × 13. **b.** High-magnification photograph of the boxed region in **a**, showing tendon (arrow), cell-mediated muscle fragmentation (f), and myotubes (m). H&E, ×77.

distinguishable from mouse myotubes. Mouse myotubes are smaller and have darker staining nuclei which are more round in outline than are those of the rat. The minces of normal muscle served as the first level of control. In order for a given experiment to be valid, it was necessary to demonstrate that myotubes could form from the minces of normal rat muscle, which should contain viable precursor cells for regenerating muscle. A second level of control was served by the minces taken from the peripheral regions of the rat grafts. In most if not all cases, these minces should have also been able to produce rat myotubes. Minces of the central zones of the rat muscle grafts were the test tissue. The presence of rat myotubes in central zone minces would demonstrate that myogenic cells in that part of the rat muscle graft had survived the period of ischemia and were capable of regenerating new muscle fibers.

The central zone minces from the muscle grafts were categorized by the approximate number of myotubes counted in the serial sections of each mince. Minces with 0–12 myotubes were considered to contain few or no surviving myogenic cells; minces with 13–100 myotubes were graded as intermediate; and central zone minces with over 100 myotubes were considered to contain control levels of surviving myogenic cells.

RESULTS

Background Experimentation

Experiments involving the implantation of minced normal rat muscle into mice showed that regeneration was abundant in all cases. For the purposes of this experiment, the use of nude mice as hosts seemed to confer no particular advantage over the use of immunologically competent hosts. Therefore, the use of nude mice was abandoned. As in the case of rats (Carlson, 1970), immunological incompatibility did not seem to interfere with the aspects of early regeneration studied here. Typical examples of rat-to-mouse minces are illustrated in Figures 2 and 6. At five days, the latest that rat minces were kept in the mouse hosts, only small accumulations of lymphocytes were beginning to appear in isolated regions of the minces.

Minces Taken From Rat EDL Grafts

The time course of development of rat EDL muscle grafts has been described many times (Carlson and Gutmann, 1975; Carlson et al., 1979) and will not be repeated in detail here. During the first 48 hr after grafting, from about 95 to 98% of the muscle fibers in the graft fall into a state of ischemic necrosis. The remainder survive as a thin peripheral rim. Typically the

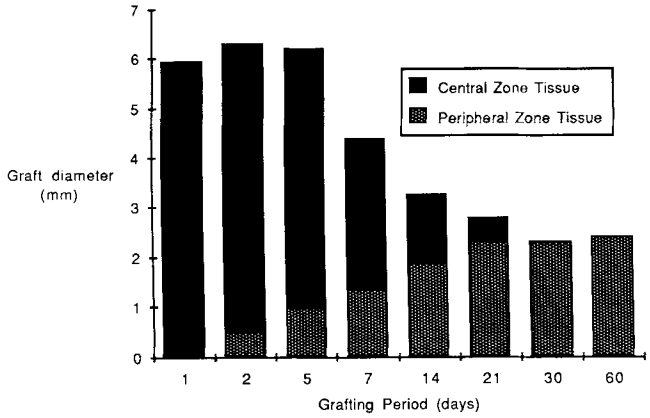

Fig. 4. Graph showing the changing proportions of central vs. peripheral zone tissues in rat rectus femoris muscle autografts.

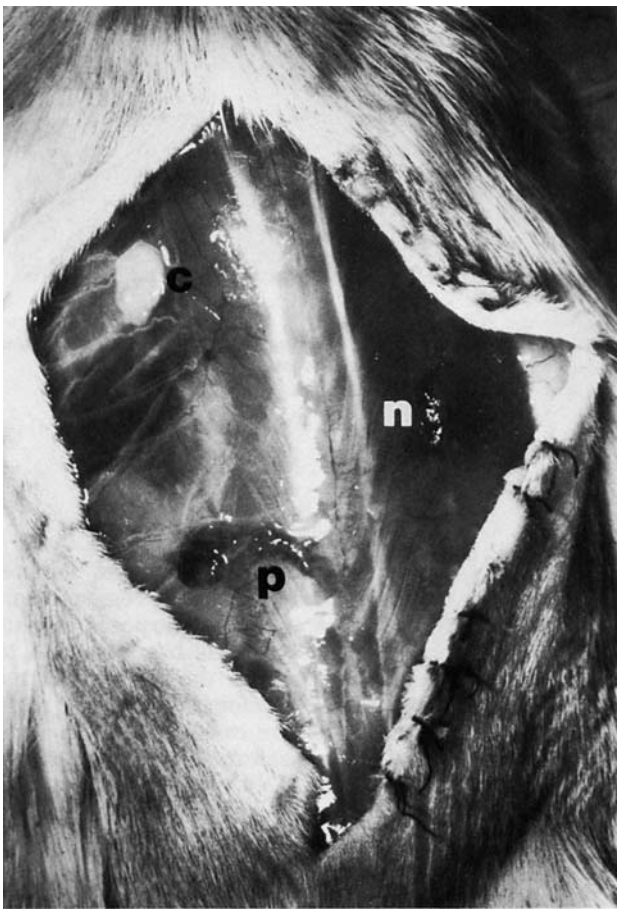

Fig. 5. Gross photograph of rat-to-mouse tissue minces 5 days postgrafting. The mince of non-grafted muscle (n) is hypervascularized and surrounded by fresh hemorrhage. The mince of central ischemic tissue (c) is very pale, and the mince from the peripheral tissue (p) lies between the two extremes. H&E.

rim of surviving muscle fibers is from 2 to 5 muscle fibers thick; but in larger grafts it is not uncommon for up to 50% of the circumference of a cross-section of the graft to contain no intact, surviving muscle fibers. At 48 hr, new blood vessels are just beginning to appear in patches on the periphery of the grafts, and no regenerating myotubes are yet seen.

TABLE 1. Regeneration from central zones of rat EDL grafts

Age of graft	(Total no. of grafts)	Number of central zones exhibiting myotubes: 0–12 myotubes	13–100 myotubes	Over 100 myotubes
Control	(10)	1 (10%)	0	9 (90%)
6 hr	(10)	1 (10%)	2 (20%)	7 (70%)
12 hr	(7)	0	6 (85.7%)	1 (14.3%)
24 hr	(7)	1 (14.3%)	1 (14.3%)	5 (71.4%)
48 hr	(9)	0	1 (11.1%)	8 (88.9%)

Examination of 33 central zone minces from 6- to 48-hr EDL grafts revealed the presence of myotubes in all cases. The relative numbers are given in Table 1. These data, which will be treated in greater detail in the Discussion, could be interpreted to indicate: 1) that myogenic cells survive throughout EDL grafts during the early post-transplantation period; or 2) that there may be a small central core of muscle fibers where viable myogenic cells do not persist after grafting, but contamination by more peripheral areas containing viable muscle precursors obscures its existence. With the relatively small size of the EDL muscle (about 100 mg in these experiments), it was not possible to discriminate between these two options with our in vivo experimental model. We decided, therefore, to use as the original free graft a muscle large enough so that central zone tissue could be removed with no danger of possible contamination by peripheral tissue containing surviving myogenic cells or newly formed regenerating myoblasts. For this purpose, the freely grafted rectus femoris muscle of the rat was chosen.

Rectus Femoris Autografts

Rectus femoris grafts of less than one day have begun to enter into a state of ischemic necrosis, characterized by a pronounced reduction in the number of detectable myonuclei throughout much of the graft. The 24-hr grafts were not at all revascularized; and few macrophages were seen, even in the periphery of the graft. Within 3 to 4 days after transplantation, early revascularization had occurred in parts of the grafts; and the zones of degenerative and regenerative activity began to become evident.

Five days after grafting, the rectus femoris muscles exhibited the zones described earlier (Fig. 3). The peripheral zone was occupied primarily by a broad connective tissue extension of the tendon and sinusoid-like blood vessels, and it contained very few surviving muscle fibers. The transitional or regenerating zone contained macrophages, degenerating muscle fibers, and abundant myotubes, but this zone represented only a small percentage of the total graft. The central ischemic zone took up the majority of the graft and was filled with necrotic muscle. The myofibers had a glassy appearance, and viable myonuclei seemed to be absent. The relative size of the central zones at this time assured us that isolation of the central zone could be accomplished accurately with minimal cellular contamination from the other two zones.

On cross-sections of 31 rectus femoris autografts taken from one to 60 days postoperatively, the mean diameter (mean of greatest and least diameter) and proportion

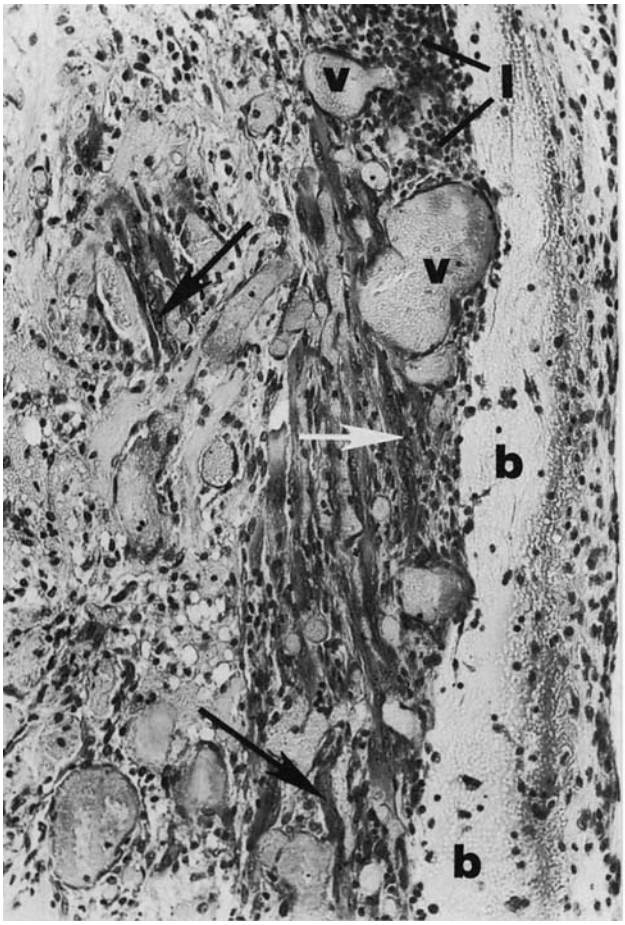

Fig. 6. Five-day rat-to-mouse mince of non-grafted muscle showing moderate lymphocytic infiltration (l), extravasated blood (b), and the association between dilated blood vessels (v) and myotubes (arrows). H&E, × 178.

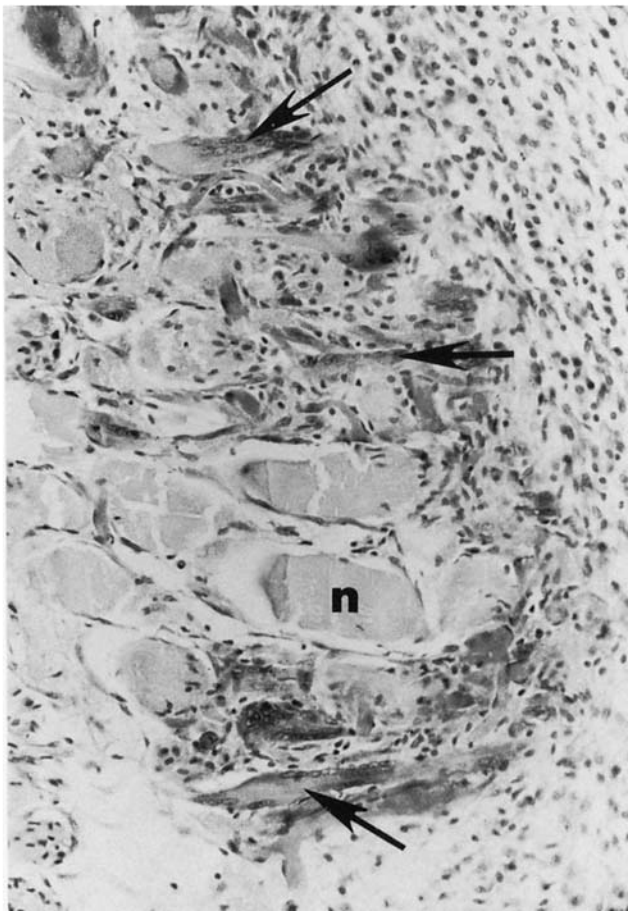

Fig. 7. Five-day rat-to-mouse mince of central ischemic tissue from a 2-hr rectus femoris autograft, showing numerous myotubes (arrows) and necrotic muscle fibers (n). H&E, ×186.

of the diameter occupied by central and peripheral zone tissues were calculated. The increasing proportion of peripheral vs. central zone tissue is illustrated in Figure 4.

Minces Taken From Rat Rectus Femoris Grafts

All rectus femoris minces were examined 5 days after implantation into host mice. Grossly, each of the three types (from central zone, peripheral zone, and normal control muscle) displayed a characteristic appearance (Fig. 5), although there was a definite overlap of these characteristics. Minces from normal ungrafted muscle were relatively well integrated into a homogeneous-appearing mass, with no evidence of the original muscle fragments obvious upon gross examination. Well over 50% of the normal minces were highly vascularized, with dilated and tortuous vessels leading to them from the surrounding mouse tissues. Many of these minces were also characterized by frank hemorrhage, sometimes to the extent that the entire mince appeared bright red.

Peripheral zone minces were, on the whole, similar to minces of normal muscle; but hypervascularization and hemorrhage were less common. On the other hand, peripheral minces were less well integrated, in the sense that in some of the minces it was possible to discern original muscle fragments within the mince.

Central zone minces, especially from grafts older than 2 hr, were usually very pale; and typically one could discern the boundaries of the original minced fragments in large areas of the mince. Only rarely were grossly dilated vessels seen passing into central zone minces.

Histological examination demonstrated that the controls, ungrafted and peripheral minces from all grafting periods, exhibited typical muscle regeneration (Fig. 2). Numerous myotubes accompanied by a vast number of macrophages were observed in the periphery of each mince, demonstrating that viable myogenic cells can give rise to regenerating myotubes under these experimental conditions. Because one can readily distinguish mouse from rat myotubes, we concluded that all of the myotubes observed originated from the rat muscle minces.

Although the immunological incompatibility between the implanted minces of rat muscle and the mouse hosts did not seem to influence regeneration of myotubes within the minces, there were, nevertheless, signs of immunological rejection in the minces. As early as 4 days, and more commonly at 5 days, light infiltrates of small lymphocytes could be seen in scattered locations

TABLE 2. Regeneration from central zones of rat rectus femoris grafts

Age of graft	(Total no. of grafts)	Number of central zones exhibiting myotubes		
		0–12 myotubes	13–100 myotubes	Over 100 myotubes
Control	(5)	0	0	5 (100%)
2 hr	(8)	0	0	8 (100%)
3 hr	(7)	5 (71.4%)	2 (28.6%)	0
4 hr	(18)	13 (72.2%)	3 (16.7%)	2 (11.1%)
6 hr	(8)	7 (87.5%)	1 (12.5%)	0
16 hr	(6)	5 (83.3%)	1 (16.7%)	0
24 hr	(6)	6 (100%)	0	0
2 days	(9)	8 (88.9%)	1 (11.1%)	0
5 days	(10)	9 (90.0%)	1 (10%)	0

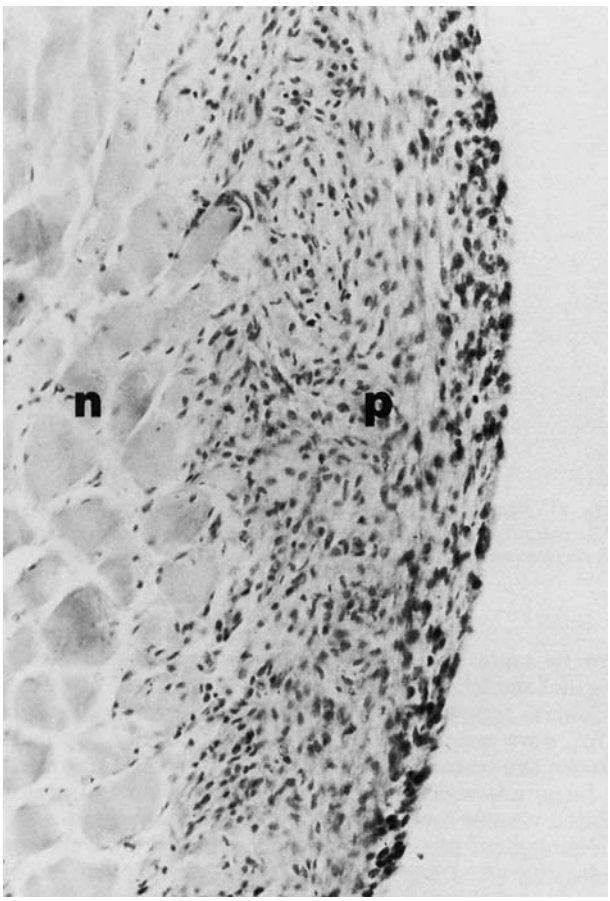

Fig. 8. Five-day rat-to-mouse mince of central ischemic tissue from a 6-hr rectus femoris autograft. Although peripheral basophilia (p) and necrotic muscle (n) are present, there are no myotubes. H&E, ×192.

within some of the minces (Figs. 2, 6). The most prominent reaction was the vascular response, which included vasodilation, engorgement of blood, and localized hemorrhage (Fig. 6). The correlation between areas of dilated vessels and areas of regenerating myotubes was striking. In some minces with a very prominent vascular response, greatly dilated vessels could also be seen in areas where original muscle fibers were still being phagocytized.

The histological findings concerning the central zone minces are summarized in Table 2. Central zone minces from 0- to 2-hr grafts were similar to the controls in that they contained hundreds of myotubes (Fig. 7). Central zone minces from 3- and 4-hr grafts were transitional—some contained no myotubes and others contained abundant myotubes. On the other hand, central zone minces from 6-hr and longer grafts contained few or no myotubes (Fig. 8). The degenerative changes, breakdown of peripherally located necrotic muscle by a large number of macrophages, were similar for all minces regardless of the presence or absence of myotubes.

Blood vessels were found in sections of all central zone minces, but they were not dilated as were the vessels in minces that gave rise to myotubes. In addition, the basophilic periphery of a non-myotube-containing central zone mince was substantially narrower than that seen in normal or peripheral zone minces.

DISCUSSION

The central issue in this study was the ability of potentially myogenic cells to survive in the ischemic center of free muscle grafts. On the basis of morphological studies (Carlson et al., 1979) the following information was at our disposal: 1) the central region of a standard free muscle graft shows the classical signs of severe ischemia or necrosis; 2) within several days or weeks, depending upon the size of the graft, this same region becomes populated with regenerating muscle fibers; and 3) regeneration in a specific area within the central region occurs after blood vessels have grown toward it from the periphery.

With the experimental design used here, the presence of rat myotubes developing within a mince placed beneath the back skin of the host mouse demonstrated the presence of viable myogenic cells in the donor muscle tissue. The absence of muscle fibers regenerating within a similar mince could be interpreted as demonstrating the absence of viable myogenic cells within the mince, although the possibility that the mince contained viable myogenic cells that were unable to develop would have to be considered as well.

The results on the grafted rectus femoris muscle (Table 2) showed that muscle tissue taken from the central region of the graft was able to produce essentially control numbers of regenerating myotubes during the first 2 hr after free grafting. Thereafter, the ability of muscle tissue taken from the same region to produce regenerating muscle fibers declined dramatically to virtually

nothing. This course of events correlates well with previous reports of the progression of muscle damage due to ischemia (Harman, 1947; Harman and Gwinn, 1948; Mäkitie, 1977; Mäkitie and Teräväinen, 1977; Korthals et al., 1985), and it strongly suggests that the muscle fibers in the center of a free rectus femoris graft undergo irreversible ischemic damage, and that this region of the graft consists literally of "dead muscle."

In contrast, one could not come to a similar conclusion from the results of the minces taken from grafts of the EDL muscle (Table 1). There is no question that substantial numbers of myogenic cells were present in minces taken from the central zone of EDL grafts at all time periods sampled. There are two possible explanations for this. One is that because of the relatively small size of the EDL muscle there is enough diffusion of oxygen and nutrients so that even in the center of the graft myogenic cells can survive, although the muscle fibers themselves degenerate. Snow (1977a,b) has shown that satellite cells are better able to withstand ischemia than are myonuclei. The other possibility is that the apparent survival of myogenic cells in the center of EDL grafts is a surgical artifact. The EDL muscle has a small cross-sectional area; and even though removal of the central regions was done with considerable precision, contamination by more peripheral, less ischemic muscle is possible. The reason is that the peripheral zones of EDL grafts are not geometrically regular, and occasionally a tongue of obviously peripheral tissue penetrates relatively deep into the graft. The large cross-sectional area of rectus femoris grafts allows a greater degree of precision in removing purely central zone tissue. On the basis of the experiments performed here, it is not possible to make a definitive interpretation of the results on the EDL grafts.

Nevertheless, it is clear that there is a continuum of reactions that depends upon the size of the muscle. The rectus femoris is a large muscle (about one gm in adult rats), and the evidence points to the death of all muscle fibers and myogenic cells in the central region of free grafts. On the other end of the spectrum, grafts of very small muscles or bundles of muscle fibers do not show evidence of profound ischemia (Faulkner et al., 1983; Weiss and Faulkner, 1983). At some point between these extremes there would be a size of free graft at which irreversible ischemic damage to myogenic cells occurs. It is possible that the EDL muscle is close to this threshold.

Differences in the pattern of revascularization of the minces of different types of muscle must also be taken into account when interpreting the results of the rectus femoris minces that are placed in the mouse. All minces became vascularized, but the degree and appearance of vascularization differed. Minces from the central zone tissue of grafts older than 4 hr were grossly pale, and microscopic examination showed a relatively thick area of basophilia and muscle fiber breakdown around the periphery (Fig. 8). Yet, myotubes were rarely seen in these basophilic areas. At the other extreme, many minces of normal muscle or even peripheral regions of grafts were hypervascularized, with grossly dilated, tortuous vessels and even areas of frank hemorrhage. Microscopic inspection and another set of experiments (Phillips, 1987) demonstrate that all three types of muscle are capable of inducing the ingrowth of blood vessels. However, the presence of myotubes in the peripheral and normal muscle minces may be responsible for the formation of the dilated vessel morphology. Invading endothelial cells may encounter an antigen present on the myotube surface which leads to the formation of a vessel type similar to those in transplanted organs or to damaged vasculature characteristic of graft rejection (Peer, 1959; Bailey, 1970; Guttmann and Lindquist, 1970). Myotubes would present endothelial cells with basement membrane components lacking in central zone minces. According to Madri et al. (1983), endothelial cells in vitro exhibit different behavior dependent on the substratum upon which they are grown. Interstitial collagens promote proliferation with the infrequent formation of tubular structures, but basement membrane components promote tube formation without proliferation of endothelial cells. In the normal and peripheral muscle minces, the basement membranes of the myotubes could promote more rapid blood vessel formation, which in turn would facilitate the rejection of the graft and the observed vascular pathology. Given the proximity of the myotubes to the dilated, tortuous vessels, such a sequence is entirely possible.

The presence of large numbers of regenerating muscle fibers in the formerly ischemic central zones of rectus muscle grafts poses an obvious question. Where do these muscle fibers come from and how do the myogenic cells make their way to the central region of the graft? There is little concrete information that would provide a direct answer, although it has been recognized for a number of years that there is a strong spatiotemporal correlation between the ingrowth of blood vessels into a formerly ischemic area and the presence of concentrations of macrophages and myoblastic cells in that area.

It has been suggested (Bateson et al., 1967; Partridge and Sloper, 1977; Partridge et al., 1978; Sloper et al., 1970; Grounds et al., 1980) that regenerating muscle fibers may arise from myogenic cells that find their way into the systemic circulation. More recent studies (Grounds and Partridge, 1983; McGeachie and Grounds, 1985) have cast doubt on these conclusions; but, as these authors admit, the available evidence does not permit one to make the overall conclusion that no circulating cell is capable of entering a grafted muscle and participating in myogenesis.

Another possibility, which seems more likely in the context of the experiments reported here, is that viable myogenic cells in the periphery of the grafts migrate toward the center as revascularization proceeds. Migration of myogenic cells has been cited by several investigators. Schultz (1978) observed myogenic cells migrating out of the basal lamina into the interstitial space following denervation of rat tibialis anterior and mouse lumbrical muscles. Both Jones (1979) and Lipton and Schultz (1979) reported the migration and fusion of labeled myogenic cells injected into rat and Japanese quail muscles. Bischoff (1979) noticed myogenic cells migrating out of tears in the basal laminae of single muscle fibers in vitro. In a more recent communication, Schultz et al. (1985) described the ability of myogenic cells to migrate parallel to the long axis of a muscle from undamaged areas to injured regions. Although the ability of myogenic cells to migrate has been well documented, it has not been demonstrated that myogenic cells are able to migrate perpendicular to the long axis of a graft

through necrotic muscle to the central ischemic area. Preliminary results from an ongoing study (Phillips, unpublished) suggest that myogenic cells may indeed possess this capability. The present study offers no direct proof of the migration of myogenic cells into the formerly ischemic center of a muscle graft, but the evidence to date suggests that this is the most likely possibility.

The results of grafting central zones of EDL grafts (Table 1) could be interpreted in two major ways. One is that because of possible contamination of the explants by surviving peripheral tissue, the results reflect variations in sampling technique. Both from impressions at the time of surgery and from examination of histological cross-sections of EDL grafts from which the central minces were obtained, this explanation alone seems unlikely to account for the high percentage of central minces that produce large numbers of myotubes. Another explanation involves the relatively large surface area in relation to the center of an EDL graft as opposed to rectus graft. It is possible that better diffusion of oxygen allows cells in the very center of an EDL graft to survive longer (i.e., 6 hr) than they do in a rectus graft. The 12-hr interval may reflect a period where fewer myogenic cells remain viable. The increased percentage of central minces that produced over 100 myotubes after being in grafts in the rats 24 and 48 hr could be a reflection of the early migration of myogenic cells toward the center of the graft, although at 24 hr this would have to occur in advance of vascular ingrowth. Until a direct marker for identifying migrating myogenic cells is obtained, it will not be possible to test this option directly.

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