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Initiation and Duration of Myogenic Precursor Cell Replication in Transplants of Intact Skeletal Muscles: An Autoradiographic Study in Mice

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ABSTRACT Skeletal muscle fibers are capable of regeneration following ischemia, traumatic injury, or transplantation. Although the time course of the regenerative process has been studied in detail histologically, little is known about the time of activation of myogenic precursor cells which are primarily responsible for muscle regeneration.

This study was designed to determine the initiation, peak proliferative activity, and cessation times of muscle precursor replication in small skeletal muscle transplants. Forty-eight young male BALB/c mice had the extensor digitorum longus muscles of both hind legs autotransplanted to a different site in the same leg. At various time intervals after transplantation (from 24 hours to 14 days), mice were injected once with a small dose of tritiated thymidine in order to label proliferating myogenic precursor cells.

The transplants were allowed to regenerate for 14 days, before being removed, processed for autoradiography, and analysed by light microscopy. The presence of labelled myotube nuclei in regenerated transplants showed that myogenic precursors had been replicating at the time of tritiated thymidine injection. Myogenic precursor replication was initated late on the second day (42–48 hours) after transplantation, peaked after 6 days, and was complete within 10 days.

That skeletal muscle has the capacity to regenerate following trauma is now widely documented, as are the morphological changes inherent in the regenerative process (Schmalbruch, 1976, 1985; Allbrook, 1981; Carlson and Faulkner, 1983). Regeneration results from the proliferation of mononuclear precursor cells which fuse to form multinucleated young muscle cells called myotubes.

The precursor cell primarily responsible for regeneration is almost certainly the satellite cell of skeletal muscle: these are undifferentiated cells lying between the basal lamina and the plasmalemma of muscle fibers (Mauro, 1961). Although the time course of the regenerative process has been followed (in vivo) by using various histological techniques (Carlson and Gutmann, 1975; Mastaglia et al., 1975; Snow, 1977a,b; Hansen-Smith and Carlson, 1979), little is known of the timing of satellite cell activation following injury.

Recent studies using autoradiographic techniques have enabled the onset of DNA synthesis in muscle precursor cells to be determined after mild (cut) and severe (crush) injuries to skeletal muscles (McGeachie and Grounds, 1987). It was shown in both types of lesions that myogenic cells first started to replicate 30 hours after injury.

In the present study we use the same autoradiographic techniques to investigate myogenesis in transplants of small intact extensor digitorum longus (EDL) muscles in mice. In such transplants the vascular and nervous supply are both completely disrupted, in contrast with cut and crush lesions where the vascular supply remains relatively intact. The initiation, peak activity, and termination times of myogenic precursor cell replication were determined autoradiographically for intact skeletal muscle transplants.

MATERIALS AND METHODS

Animals and Surgical Procedures

The animals used were 48 young (6–8 wk) mature male BALB/c mice (20–25 g). Mice were anesthetized via intraperitoneal injections of pentobarbitone sodium (Nembutal) at a dosage of 40 mg Nembutal/kg of body weight.

The transplantation procedure involved the removal and implantation of EDL muscles from both hind legs of each animal. This was done by locating and severing the distal tendon of the EDL, and with the proximal tendon still intact, the muscle was bluntly dissected from its bed. The proximal tendon then had a loop of 7/0 braided silk suture (Ethicon) tied around it. The tendon was severed and immediately sutured to the distal tendons of the quadriceps femoris muscle of the same leg. The EDL was laid longitudinally over the tibialis an-

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terior (TA) muscle and was extended to its normal (preexcision) length before a loop of 7/0 braided silk suture was tied around the distal tendon of the EDL and anchored to the distal tendon of the TA muscle. A total of 96 autotransplants were inserted.

Autoradiography and Sample Analysis

At different time intervals after EDL transplantation 6–^{3}H–thymidine (^{3}H-TdR; specific activity 5.0 Ci/mmol, Amersham International, U.K.) at a dosage of 1 μCi/g of body weight was injected intraperitoneally into each mouse. The times of injection were 24, 36, 42, 48, 54, 60, 72, 84, 96, 108, 120, 144, 156, 168, 192, 216, 240, 264, 288, 312, and 336 hours. Only one injection of ^{3}H-TdR was given to each individual mouse; ^{3}H-TdR is available for about 1 hour after injection, and it is taken up and incorporated into the nuclei of cells synthesizing DNA during this period.

Fourteen days after transplantation (when muscle precursors have fused to form myotubes), all mice were anesthetized with a lethal dose of pentobarbitone sodium (80 mg/kg) before being perfused with 20 ml of a heparinized saline solution, followed by 20 ml of 10% phosphate-buffered formal saline solution. After perfusion, the transplanted muscle and part of the TA muscle were removed, cut transversely in half, and immersed overnight in fixative at 4°C.

Tissues were postfixed in 1% OsO_4 for 1 hour, washed in 0.1 M phosphate buffer, and block-stained in 1% paraphenylenediamine in 70% ethanol for 1 hour to preclude tissue staining after autoradiography (Dilley and McGeachie, 1983). Tissues were infiltrated and embedded in Araldite before 1-μm whole sections were cut from the central area of each transplant (midway between the proximal and distal tendons), placed on glass slides, and coated with Kodak AR10 autoradiographic stripping film. The slides were placed in light-tight boxes and exposed for 10 weeks at −20°C before being developed in Kodak D19, fixed in acid hardener-fixer, washed, and dried.

Autoradiographs were examined for the presence of labelled myotube nuclei by using a 100× oil-immersion lens. The presence of labelled myotube nuclei in the regenerating EDL transplants 14 days after injury indicates that the nuclei of the myogenic precursor cells were synthesizing DNA at the time of ^{3}H-TdR injection some days previously.

At each of the 21 time intervals studied, a minimum of 800 myotube nuclei in four EDL transplants were blind counted, and the percentages of labelled myotube nuclei were determined. In samples which had been injected at 36, 48, 60 and 72 hours after transplantation at least 1600 myotube nuclei in eight EDL transplants were counted.

RESULTS

Histological Appearance

The transplants showed obvious signs of regeneration 14 days later (Fig. 1). The transplant consisted of small, immature, densely packed myotubes, with characteristic centrally located nuclei and prominently stained sarcoplasm (Fig. 2a). At the periphery of the transplant, small muscle fibers with peripheral nuclei were observed, interspersed between the myotubes. These peripheral muscle fibers were of a smaller diameter than those seen in both the TA and normal EDL muscles.

Large, thin-walled veins and small capillaries could be seen within the transplant. These blood vessels were clear of erythrocytes, indicating that vascular connections had been made with surrounding tissues because the vascular perfusion had cleared the vessels (Fig. 1).

Initiation and Duration of Myogenesis

Autoradiographs of myotube nuclei needed to have at least three photographic grains before they were considered labelled. Examples of labelled myotube nuclei are shown in Figure 2b.

The number (mean percentages) of labelled myotube nuclei in samples injected with ^{3}H-TdR at different times after transplantation is shown graphically in Figure 3. The results show that DNA synthesis in myogenic precursor cells was initiated late on the second day after transplantation (42–48 hours), peaked at about 6 days (144 hours), and was essentially completed by 9–10 days (216–240 hours).

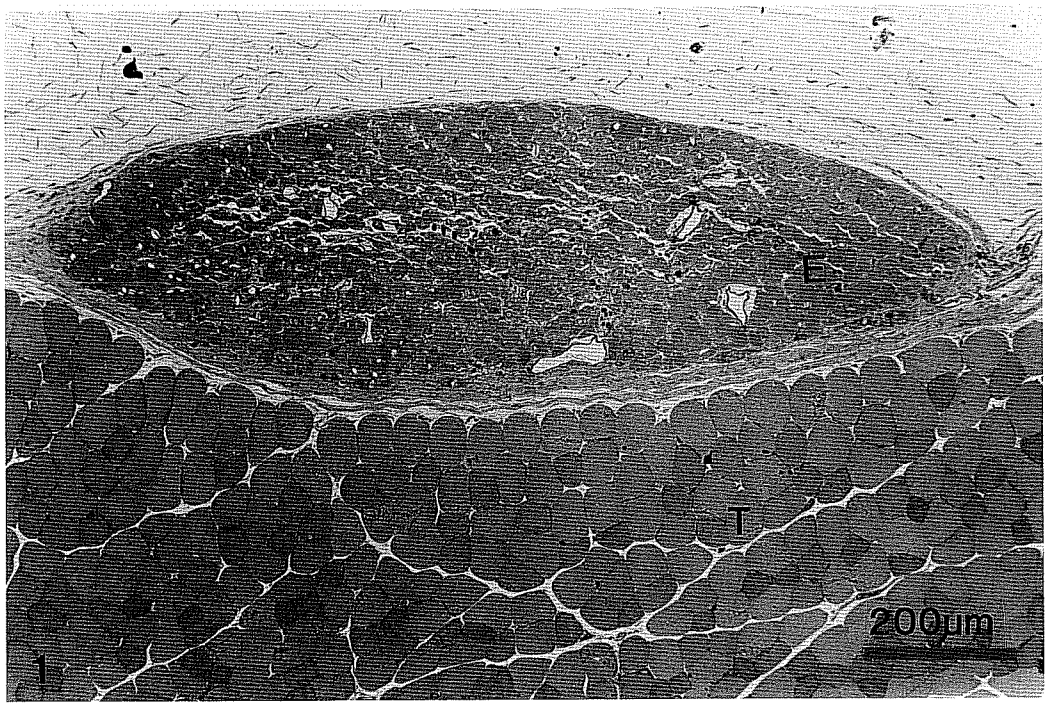
DISCUSSION

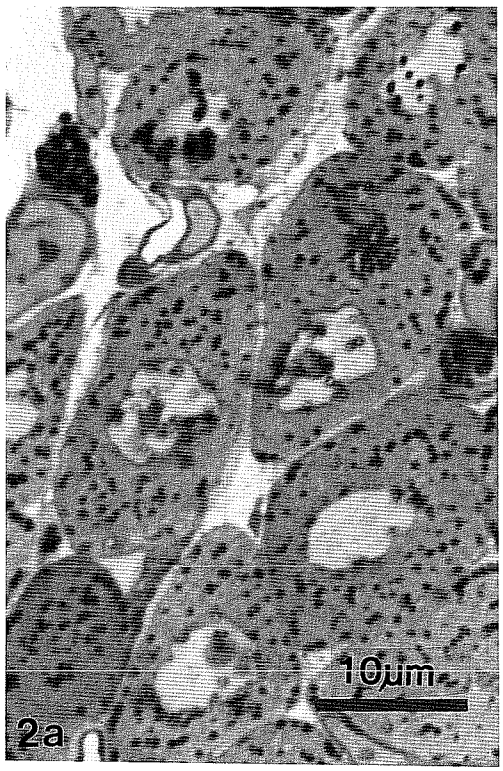
The aim of this study was to determine the initiation, duration, and cessation times of muscle precursor replication in small intact muscle transplants. DNA synthesis in these cells (measured by ^{3}H-TdR uptake) began late on the second day (42–48 hours) after transplantation, peaked at 6 days, and was complete by 9–10 days.

The histological appearance of transplanted EDL muscles (which had been allowed to regenerate for 14 days) agrees with observations made by Carlson and Gutmann (1975), Hansen-Smith and Carlson (1979), and Gulati et al. (1982). Transplants consisted of a peripheral region of myotubes with some large surviving muscle cells, and an inner region containing small, immature myotubes, with characteristic centrally located nuclei (see Figs. 1,2a).

There was no precise information available in the literature on the time course of muscle precursor replication in free muscle transplants. Carlson and Gutmann (1975) studied freely grafted rat soleus and EDL muscles with the light microscope and found myotubes 3–4 days after grafting. In a more detailed EM study of transplanted rat EDL muscles, Hansen-Smith and Carlson (1979) found myoblasts 3 days after transplantation (although criteria used to identify myoblasts were not defined). These reports do not account for the activation of myogenic precursor cells; they only identify times when histological features of myogenic cells could be distinguished. These morphological observations indicate that proliferation of myogenic precursor cells occurs some time prior to 3–4 days (when myotubes were first identified) but give no information about the onset or duration of myogenic cell proliferation.

Our autoradiographic studies show that precursor replication starts between 42 and 48 hours after transplantation and continues until about 10 days. The initiation time in our EDL transplants is 12–18 hours later than in muscle traumatized by cut or crush injuries (McGeachie and Grounds, 1987), where myogenic





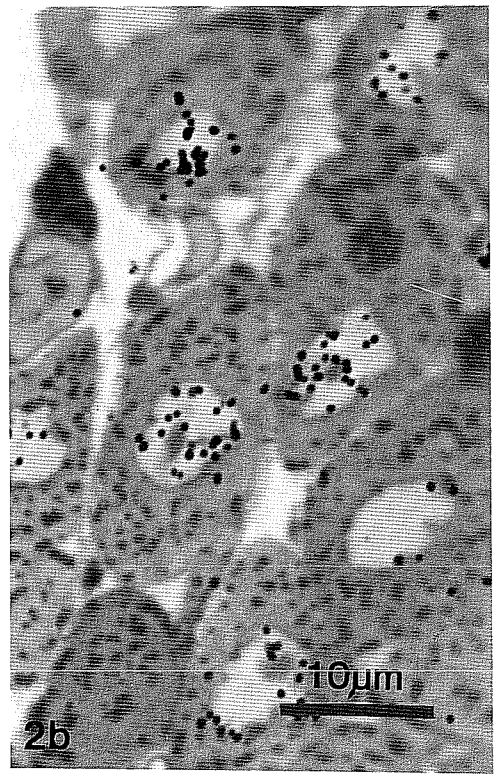


Fig. 1. A low-power photomicrograph of a 1-µm transverse histological section showing a transplanted EDL muscle (E), which has been allowed to regenerate for 14 days after transplantation. Also shown is part of the underlying TA muscle (T).

Fig. 2. High-power photomicrographs (TS) of a transplanted EDL muscle 14 days after transplantation. This animal received a single injection of ^{3}H-TdR 120 hours after transplantation. The tissue in focus (**a**) shows myotubes with characteristic centrally located nuclei. The photomicrograph (**b**), of the same field, shows autoradiographic grains in focus over labelled myotube nuclei, indicating that DNA synthesis was occurring at the time of ^{3}H-TdR injection (120 hours).

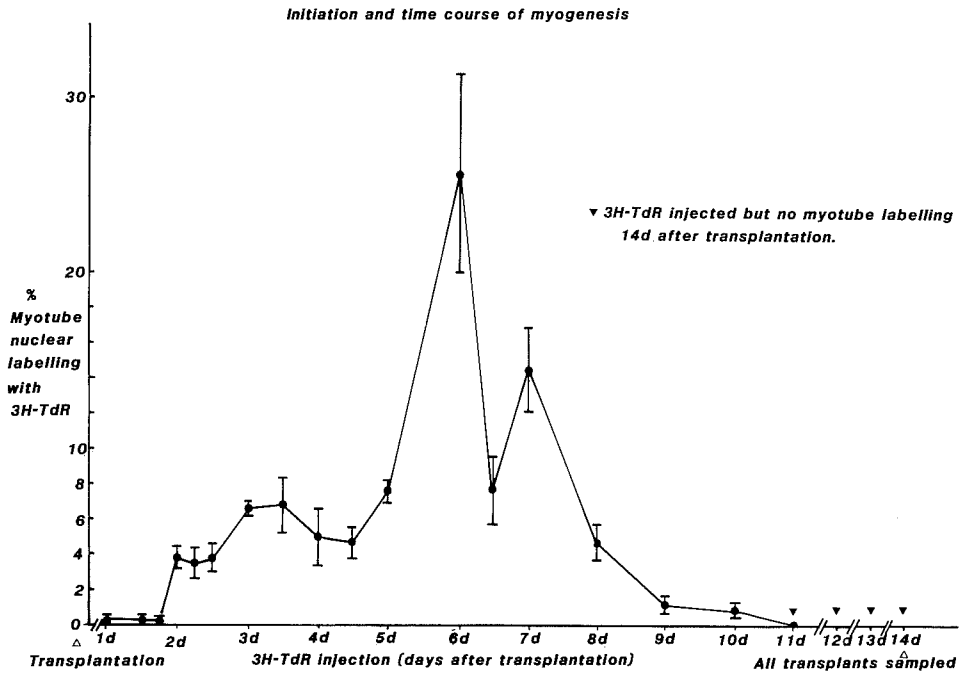


Fig. 3. The initiation, peak, and cessation of myogenesis in transplanted EDL muscles, shown as mean percentages (± SEM) of labelled myotube nuclei with time after ^{3}H-TdR injection. All 96 transplants were analysed for the presence of labelled myotube nuclei 14 days after transplantation.

precursor cell replication started 30 hours after injury. In our transplants peak levels of myogenic precursor ^{3}H-TdR incorporation occurred at 6 days after injury (Fig. 3). By comparison, peak levels in injured muscle were 48 hours (2 days) in cut lesions and 72 hours (3 days) in the crush lesions (McGeachie and Grounds, 1987). Other morphological studies were unable to detect a peak of either the proliferation of myogenic precursor cells or myotube formation in free muscle grafts, although they showed that myotube formation was completed by 7–9 days (Carlson and Gutmann, 1975) and 5–6 days (Hansen-Smith and Carlson, 1979). In the present study, the replication of myogenic precursor cells was essentially completed by 9–10 days after transplantation. This was much later than in cut and crush injuries, where cessation of DNA synthesis occurred at 4 and 5 days, respectively (McGeachie and Grounds, 1987). The reasons why muscle precursor replication stops by 5 days in injured muscle, even when regeneration is incomplete, is curious because the results from whole muscle transplants clearly show that myogenic precursor cells have the capacity to replicate for at least 9 days.

A similar sequence of myogenic events occurs in developing embryonic muscle but over a much longer time span compared with muscle transplants: in embryonic muscle there is continual muscle precursor cell replication and myotube formation. The severely restricted time span of myogenesis in regenerating adult muscle may be attributable to the presence of adjacent mature muscle and the rapid formation of connective tissue barriers surrounding new myotubes. Embryonic muscle is, by contrast, more fluid and plastic, and not under the same physical constraints.

The differences in timing of myogenic precursor cell replication between these transplant studies and others using traumatized muscle are probably accounted for by the more rapid revascularization of cut or crush-injured muscle where the inflammatory response promotes capillary ingrowth to the lesion. Moreover, traumatized muscle is in continuity with surrounding healthy muscle and its vascular supply. By comparison, in EDL transplants no vascular continuity exists and the transplant is separated from surrounding tissues by layers of fascia: consequently revascularization is retarded. There is considerable evidence that a vascular nutrient supply is necessary for myogenic precursor cell activation to commence (Carlson and Faulkner, 1983). This supply is in much closer proximity to regenerating muscle in an injury than in muscle transplants, where all nutrients have to diffuse from surrounding tissues (through fibrous connective tissue) to initiate myogenesis. The revascularization of transplanted muscle may also influence the progress of myogenesis. For example, in interpreting data for the peak levels of precursor replication at 6 days, and cessation at 9–10 days, there are some limitations. The "peak" level at 6 days may, in fact, represent the earliest time at which maximal revascularization of the EDL transplants occurs, and ^{3}H-TdR is available to all potential myogenic cells via the circulation.

When complete revascularization of the transplant occurred, at some time later than 72 hours, increased numbers of myogenic precursor cells were exposed to ^{3}H-TdR and labelling levels increased accordingly. After 6 days the numbers of replicating cells decreased although the vascular supply had been reestablished. The apparent "cessation" of myogenic precursor cell

replication at 9–10 days after transplantation cannot have been due to the fact that myogenic cells labelled at 9–10 days had insufficient time to fuse into myotubes by 14 days (the time of sampling). The cell-cycle time of muscle precursors ranges from 9.5 to 22 hours and has been shown to decrease with progressive cell cycles (Grounds and McGeachie, 1987a). Since myogenic precursor cells have probably divided several times before being labelled at the end of myogenesis, and previous studies have shown that myogenic cells may fuse into myotubes after only two or three cell divisions (Grounds and McGeachie, 1987a), the majority of myogenic cells labelled 10 days after transplantation would have fused to form myotubes, or fused with preexisting myotubes, within 2 days (two to three cell cycles). Due to the restricted availability of ^{3}H-TdR (because of the lack of vascular connections) to myogenic cells replicating in the first few days after transplantation, their ^{3}H-TdR uptake was very low. With subsequent cell divisions this label is diluted beyond the sensitivity of our autoradiographic analysis. For example, a premitotic labelling level of 6 grains would be diluted to 3 in each daughter nucleus after the first division: with a subsequent division of these cells the resultant daughter nuclei would have fewer than 3 grains and therefore not be included in our analysis. This is in accordance with well-accepted convention in autoradiographic analysis. Thus the myotube nuclei resulting from these myogenic precursors would not appear to be labelled 14 days after injury, when transplants were analyzed. Although the observed numbers of replicating precursors are almost certainly lower than the actual numbers, the time of myogenic precursor cell activation is not affected.

Could the initiation and regulation of myogenic precursor cell replication be due to growth factors such as fibroblast growth factor (FGF), the somatomedins, or transforming growth factor beta (TGF beta)? It has been shown that FGF (Gospodarowicz et al., 1986; Strohman and Kardami, 1986; Bischoff, 1986) and the somatomedins (reviewed Florini, 1987) stimulate the proliferation of myogenic cells and that TGF beta inhibits myoblast fusion (Massague et al., 1986) in tissue culture, although there is no direct evidence that they play a similar role in muscle transplants in vivo. We have shown that the onset of myogenic precursor replication occurs prior to revascularization and that ^{3}H-TdR must have diffused across connective tissue barriers for incorporation into myoblasts. It is possible that growth factors could have diffused from the blood in a similar manner. In addition, it is likely that some growth factors were released locally within the transplanted muscle from cells or basement membranes (Folkman et al., 1988). It is not known whether such growth factors were involved in initiating and regulating myogenic cell proliferation in the transplant.

In an associated study (Roberts et al., 1989) the premitotic labelling of myogenic precursor cells was examined in similar transplants during the first 72 hours after insertion; ^{3}H-TdR was injected into mice with transplants, and tissues removed 1 hour later before labelled cells had time to pass through the G2 and M phases of the cell cycle. In the transplants examined at 12 and 24 hours there were no labelled premitotic cells of any type: at 36 hours one labelled endothelial cell was detected, and from 48 to 72 hours labelled presumptive myogenic (satellite) cells were observed closely surrounding necrotic muscle fibers. This evidence confirms the findings from our present study that myogenic precursor cells in muscle transplants start to proliferate at the end of the second day after transplantation.

Does ^{3}H-TdR reutilization contribute to the labelling pool of myogenic precursors in these experiments? When ^{3}H-TdR is injected it is available in the blood for 30–60 minutes (Cleaver, 1967). Cells synthesizing DNA in preparation for mitosis at that time incorporate ^{3}H-TdR and carry it through to their daughter cells and successive generations; ^{3}H-TdR that is not incorporated in the first 60 minutes is metabolized and excreted (Cleaver, 1967). Some rapidly dividing cells, such as in the bone marrow, also have a short life span of 3–4 days, and when they die they release their nuclear components, including ^{3}H-TdR, which becomes available for reutilization (McGeachie and Grounds, 1985, 1987). However, ^{3}H-TdR is only available for reutilization in any significant amounts (detectable by autoradiography) if large or repeated doses have been used. This phenomenon was investigated in detail in a recent study by Grounds and McGeachie (1987b). Therefore, in the present study, where each mouse received a single small dose (1 μCi/g body weight) of ^{3}H-TdR, the amount available for reutilization 3–4 days later (which is trivial compared to the initial dose) would have been too insignificant to affect the autoradiographic results.

In summary, the autoradiographic techniques used in this experiment enable the determination of the initiation and duration of DNA synthesis in myogenic precursor cells to be made much more precisely than is possible by using standard morphological techniques. Evidence is presented to show that myogenic precursor cells in these small transplants are proliferating as early as 42–48 hours after transplantation, at least 24 hours prior to revascularization. These findings may have important implications in the postoperative management of clinical muscle transplantation.

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