THE ONSET OF MYOGENESIS IN DENERVATED MOUSE SKELETAL MUSCLE REGENERATING AFTER INJURY

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Abstract—Denervation of skeletal muscle stimulates increased turnover of muscle nuclei and connective tissue cells. The present investigation tests whether denervation “primes” myogenic precursor cells, so that the onset of DNA synthesis in muscle precursors after traumatic injury occurs earlier than in innervated muscle. The left legs of 29 male BALBc mice were denervated, and 1 week later small incisions were made in the tibialis anterior muscles of both legs (denervated and innervated). At specific times after injury each mouse was injected once with tritiated thymidine to label replicating muscle precursors. Muscle lesions were sampled 10 days after injury (when all precursors had fused to form myotubes) and prepared for autoradiography. The presence of labelled myotube nuclei showed that muscle precursors had been synthesizing DNA at the time when [\textsuperscript{3}H]thymidine had been injected.

Our data suggest that very few precursors were proliferating in denervated muscle within 30 h after injury, and the onset of myogenesis at 30 h was essentially the same in denervated and innervated muscle. The retrospective analysis indicates that there were also similar proportions of muscle precursors proliferating at different times after injury, in regenerating lesions of both denervated and innervated muscle. Thus, denervation does not stimulate an earlier regenerative response in injured skeletal muscle.

When skeletal muscle is denervated fibre atrophy is conspicuous. In addition, there are ultrastructural changes\textsuperscript{14,15} and biochemical changes in muscle nuclei with respect to histone composition\textsuperscript{16} and phosphorylation of nuclear proteins.\textsuperscript{17} Although the nuclei of denervated muscle are affected, it is not known whether they become “primed”, as was proposed by Studitsky et al.,\textsuperscript{18} who considered that muscle regeneration was improved in grafts of denervated muscle due to increased muscle “plasticity”. A number of other studies have shown that following injury or mining of denervated muscle, the early myoblastic stages of the regenerative response were accelerated (reviewed by Carlson).\textsuperscript{19} Denervation does appear to increase the early regenerative potential of small free whole muscle grafts,\textsuperscript{4} although more recent studies indicate that pre-denervation does not contribute to the long term success of small muscle transplants.\textsuperscript{6,19}

In the present paper we test the possibility that denervation “primed” (or activates) the nuclei of muscle precursors in mature skeletal muscle. It is considered that these muscle precursors are the satellite cells which lie between the sarcolemma and basal lamina of muscle fibres,\textsuperscript{2} and there is ample evidence that a substantial increase in numbers of satellite cells occurs in denervated skeletal muscle.\textsuperscript{13,12,24,27} Moreover, this increase is associated with increased proliferation and turnover of muscle and connective tissue nuclei.\textsuperscript{16-19,21,22}

We compare the onset of DNA synthesis in muscle precursors in denervated and innervated muscle of adult BALBc mice regenerating after a simple cut injury, using an autoradiographic model of myogenesis \textit{in vitro}. Previous studies with this model show that the onset of DNA synthesis in muscle precursors of innervated BALBc mouse muscle is at 30 h after injury.\textsuperscript{28} If denervation does “prime” satellite cells, we hypothesize that muscle precursors would be activated earlier than 30 h in regenerating lesions of denervated muscle.

EXPERIMENTAL PROCEDURES

The right legs of 29 inbred male BALBc mice (from the Animal Resources Centre, Murdoch University, Western Australia) aged 6–8 weeks were denervated by removing 5 mm of sciatic nerve high in the thigh. Seven days after denervation the tibialis anterior muscles of the right (denervated) and left (control innervated) legs were injured by a delicate cut inflicted with a razor (described in detail by McGeachie and Grounds\textsuperscript{29}).

To label replicating myogenic cells, tritiated thymidine (specific activity 5 Ci/mmol; Amersham, England) was injected intraperitoneally at a dosage of 1 \textmu g/b weight at times ranging from 12 to 72 h after injury (Table 1). Each mouse received only one injection of [\textsuperscript{3}H]thymidine. At 10 days after injury mice were anesthetized with ether and killed by cervical dislocation. The rationale for this experimental regime was based on the fact that when [\textsuperscript{3}H]thymidine is injected, it is available to replicating cells for about 30 min.\textsuperscript{3} [\textsuperscript{3}H]Thymidine, which is not incorporated into DNA during this time, is metabolized and excreted. Cells synthesizing DNA in preparation for mitosis at the time of [\textsuperscript{3}H]thymidine injection, incorporate it into their nuclei and pass the label on to subsequent daughter cells: this label becomes progressively diluted with each cell division. Muscle precursor cells which incorporate [\textsuperscript{3}H]thymidine carry the label through myoblast and myotube nuclei. Therefore, the presence of labelled myotube nuclei in regenerating muscle 10 days after injury indicates that muscle precursor cells had been synthesizing DNA at the time of [\textsuperscript{3}H]thymidine injection. Thus, a retrospective analysis of the initiation and duration of muscle precursor replication can be achieved.

All samples were fixed in 10% formaldehyde, post-fixed in 1% osmium tetroxide, block-stained with p-phenylene-diamine, embedded in Araldite and 1 \mu m sections processed for
Table 1. Proportions of myotube nuclei labelled, and distribution of grain counts, in muscle samples removed from denervated and innervated anterior muscle 10 days after cut injury

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<td>72</td>
<td>14 90 25 — (19)</td>
<td>35 32 44 — (6) 36 42 33 15 10 8</td>
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<td>31</td>
<td>57 28 12 3 — (40)</td>
<td>42 34 21 3 29 31 32 34 3</td>
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Fig. 1. Muscle regeneration 10 days after a minor cut injury to (A) denervated and (B) innervated (control) muscle. Note the atrophy of intact denervated muscle fibres adjacent to the lesion in (A), and the prevalence of myotubes in both lesions.

DISCUSSION

In lesions of both denervated and innervated muscle, the onset of myogenesis at 30 h, and the low proportion of myotube nuclei labelled (10 days after injury) when \([\text{H}]\)thymidine had been injected up to 42 h after injury, is similar to previous experiments using BALBc mice. Autoradiographic studies of regenerating innervated muscle of adult chickens also indicate that few muscle precursors are replicating before 30 h after injury. However, in innervated muscles of adult male and female Swiss mice the onset of myogenesis is at 24, rather than 30 h after injury. When EDL muscles in BALBc mice are autotransplanted the initiation of myogenesis occurs somewhat later, at 48 h, due to diffusion barriers between the host and transplanted muscle, and the lack of revascularization of the transplant. In the present experiment, in samples injected up to 72 h after injury there was no difference between the proportions of labelled myotube nuclei in regenerated lesions of denervated and innervated muscle. Our data suggest
that the response of satellite cells to injury is similar in denervated and innervated muscle. The results do not support the original hypothesis that denervation "primes" satellite cells and stimulates the early initiation of myogenesis in denervated muscle after injury.

The small proportion (less than 6%) of highly labelled (6–15 grains) myotube nuclei seen in denervated muscle which had been injected with [3H]thymidine before the onset of myogenesis (30 h), can be accounted for by the labelling of a small number of satellite cells activated by denervation, and their subsequent incorporation into myotubes. The proliferation of satellite cells in response to denervation is well documented.16–19,21,22 Within 2 days after denervation 10% of satellite cells are proliferating and they have been calculated to reach levels of "14–40%" by 3–4 days.21 These derived values appear high, since it has been shown in outbred Swiss mice injected once daily with [3H]thymidine over the first 7 days after denervation, that 11% of muscle nuclei and 41% of connective tissue nuclei become accumulatively labelled.16 In BALB/c mice (from the same inbred colony as used
in the present experiment) injected once daily with [3H]thymidine during the first 7 days after denervation, there is an accumulated labelling level of 3.5% of muscle nuclei. This level of turnover is maintained over the first 4 weeks after denervation. By comparison, innervated control muscles in the same mice have consistent labelling levels of 0.1% of muscle nuclei (accumulatively over 7 days). The calculated daily turnover of muscle nuclei in denervated muscle would be 0.5%, compared with only 0.014% in innervated muscles. Therefore, in the present experiment, a single injection of [3H]thymidine at 7–8 days after denervation, i.e. within 30 h of injury, should label less than 1% of these proliferating satellite cells. It seems likely that these labelled proliferating satellite cells in denervated muscle would be incorporated into myotubes formed after injury, and would result in about 2–4% of myotube nuclei being significantly labelled, if the labelled satellite cells divided only 2–3 times before fusing into myotubes. This number of muscle precursor divisions before fusion is known to occur in BALBc mouse muscle regenerating after injury.

One problem inherent in [3H]thymidine labelling experiments is the variable uptake of [3H]thymidine due to cell cycles not being synchronized, and to differences in DNA synthesis during the S phase of the cell cycle. This means that autoradiographic grain counts in post-mitotic myotube nuclei could be either the result of an initial low uptake of [3H]thymidine at the time of injection, or alternatively be due to progressive dilution of an initially high label with a number of mitotic divisions. The authors are mindful of such problems with these experiments. Nevertheless, the grain counts of post-mitotic myotube nuclei 10 days after injury clearly show the onset of DNA synthesis in muscle precursors, and also give some indication of the relative proportions of precursors proliferating in denervated and innervated muscle at different times after injury.

It appears that about 30 h may represent a minimum time for quiescent muscle precursors of BALBc mice to become activated after injury, and it is not known whether this time interval can be shortened experimentally. Similar autoradiographic studies in chickens and Swiss mice, indicate that a minimum of 24 h must elapse after injury before quiescent muscle precursors start to synthesize DNA. This time interval may be slightly shorter in rats, since it has been shown that satellite cells of growing rats start to synthesize DNA at 20 h after injury, and observations in tissue culture also imply that the onset of DNA synthesis in satellite cells of adult rat muscle is around 20 h (i.e. 2–4 h before mitosis, reported at 24 h).

CONCLUSION

Although denervation of skeletal muscle causes an increase in satellite cells and connective tissue cell
turnover, it does not "prime" the general population of muscle precursors to start synthesizing DNA more rapidly after injury than in innervated muscle. Moreover, denervation does not appear to enhance the process of muscle regeneration compared with innervated muscle, as indicated by the proportions of labelled myotube nuclei, and hence muscle precursors proliferating, at different times after injury.

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REFERENCES


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