A model of myogenesis in vivo, derived from detailed autoradiographic studies of regenerating skeletal muscle, challenges the concept of quantal mitosis

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Summary. We have recently shown that myogenesis following severe injury is prolonged compared with minor injury (McGeachie and Grounds 1987). In this previous autoradiographic study 44 mice were injected with tritiated thymidine at various times after muscle injury (0 to 120 h), and samples were taken 9 d after injury to determine the percentage of labelled myotube nuclei. In the present study the same experimental data are analysed in detail to reveal how many times labelled muscle precursors divided before fusing to form myotubes.

Additional mice were prepared and samples removed 1 h after injection of tritiated thymidine to determine the maximum grain counts of premitotic nuclei. When a labelled premitotic nucleus divides, each of the two daughter nuclei will contain half of the original label. The grain counts of nuclei resulting from sequential divisions of a maximally labelled premitotic nucleus, forms the basis for our detailed analysis which can reveal how many times a muscle precursor has divided after labelling.

Nine days after injury the autoradiographic grain counts of labelled myotube nuclei were analysed in detail. The results describe an in vivo model of myogenesis which we use to evaluate quantitatively observations derived from tissue culture studies. The analysis shows that, at the onset of myogenesis in regenerating muscle (30 h after injury), muscle precursors divide only twice before fusing to form myotubes. This observation challenges the concept of quantal mitosis as defined by the tissue culture studies of Quinn et al. (1984, 1985).

Key words: Skeletal muscle – Myogenesis – Muscle precursors – Quantal mitosis – Regeneration – Autoradiography – Mouse

Muscle nuclei lying within the cytoplasm of multinucleated skeletal muscle cells are post-mitotic. The formation of new skeletal muscle (during embryogenesis or following muscle injury) results from proliferation of mononuclear muscle precursors which fuse together into young muscle cells (myotubes). In regenerating adult muscle many (possibly all) muscle precursors are derived from satellite cells which lie between the basal lamina and sarcolemma of muscle fibres (Mauro 1961).

The initiation of muscle precursor replication and the duration of proliferation following injury of adult skeletal muscle has been studied autoradiographically using tritiated thymidine (³H-TdR) in mice (McGeachie and Grounds 1987). In the present paper these same autoradiographic data are analysed with respect to the number of times muscle precursors divide before fusing to form myotubes. The rationale for this analysis is outlined in detail in the Results section. The analysis presents the opportunity to evaluate, in an in vivo situation, models of myogenesis which have been derived from tissue culture studies.

Myogenesis has been examined extensively in tissue culture using differentiating embryonic muscle. From such studies of embryonic chick muscle Bischoff and Hölterer (1969) developed the concept of two discrete populations of muscle precursors, separated by a crucial cell cycle (quantal mitosis) which transforms replicating muscle precursors (potential myoblasts) into post-mitotic terminally differentiated cells (myoblasts) ready for fusion. This lineage or quantal mitosis model of myogenesis has been further defined by Quinn et al. (1984, 1985) who propose that, once a stem cell becomes a committed cell, it undergoes a specific number of symmetrical and obligate cell divisions to produce terminally differentiated myoblasts and that, in embryonic chick muscle, stem cells must divide at least four times before forming myoblasts.

In contrast to the quantal mitosis model of myogenesis, it has been proposed that there is a single heterogeneous population of presumptive myoblasts and that there is no quantal cell cycle. Cells will continue to divide until they fuse. Because (in tissue culture) subsequent generations of replicating cells spend progressively longer and longer in the G₁ phase of the cell cycle, and because more cells are generated with time, their chance of fusing together increases (Buckley and Konigsberg 1977; Konigsberg et al. 1978). These two conflicting hypotheses are further discussed by Hölter et al. 1975; Linkhart et al. 1980; and Lee et al. 1984. The average cell cycle time of proliferating muscle precursors of rats and mice has been calculated as 18–21 h from in vivo studies (Zhinkin and Andreeva 1963).
which is similar to 22 h (Yao and Essien 1975), but higher than 12.5 h (Linkhart et al. 1980) derived from in vitro studies; for chick muscle estimates of cell cycle times are 10–17 h (Marchok and Herrmann 1967) and 9.5 to 12 h (Bischoff 1970; Barberie 1971; Zalin 1979) for in vivo and in vitro studies respectively. It appears that the cell cycle time is highly variable, largely due to the G1 phase becoming longer (G1 range 2–15 h) with subsequent cycles of daughter cells (Marchok and Herrmann 1967; Buckley and Konigsberg 1977; Konigsberg et al. 1981; Lee et al. 1984).

The analysis of our autoradiographic data from regenerating skeletal muscle is used to test predictions of the model of myogenesis as outlined by Quinn et al. (1984, 1985).

Materials and methods

Forty-six BALB/c mice (20–25 g) received a slight cut injury across the middle of the tibialis anterior (TA) muscle of the right leg, and an extensive crush injury to the TA of the left leg (described in detail in McCrachie and Grounds 1987).

To label replicating myogenic cells, 3H–Tdr (specific activity 5 Ci/mmol) was injected intraperitoneally at the dosage of 1 μCi/g body weight (25 μCi/mouse), at various times ranging from 0 to 120 h after injury. At different times after 3H–Tdr injection the mice were anaesthetised with ether and killed by cervical dislocation. Samples of cut and crush injured muscle were removed and fixed in 10% formaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight. Samples of intestine were also taken to check the uptake of 3H–Tdr and to determine the autoradiographic exposure time. All tissues were post-fixed in 1% OsO4 for 2 h, washed in phosphate buffer and block stained in 1% p-phenyle nediamine to obviate the need for staining autoradiographs after processing (Dilley and Mccrachie 1983). Tissues were infiltrated and embedded in Araldite. Sections 1 μm thick were cut, placed on glass slides and coated with AR10 (Kodak) autoradiographic stripping film. They were exposed in light-tight boxes at −20°C for 7 weeks, developed in D19 (Kodak), fixed in acid hardener fixer, washed and dried. Sections were viewed and analysed under an oil immersion lens (x100). The analysis involved counting the number of grains per nucleus and the number of labelled myotube nuclei, in the regenerated cut or crush lesions. In some samples where different areas of the lesion had diverse labelling, areas with the highest numbers of labelled cells were used in the analysis; this was done for consistency.

Maximum grain counts of premitotic nuclei

Two mice were injected with a single dose (25 μCi) of 3H–Tdr, one at 72 h and the other at 96 h after injury, and killed one hour after injection (73 h and 97 h after injury respectively). Tissues from muscle lesions were taken for autoradiography to determine the maximal level of 3H–Tdr uptake in labelled premitotic cells. All cells labelled in this lesions had to be premitotic because the one hour that elapsed between injection and sampling would have been insufficient for cells to pass through the post-DNA synthesis (G2) phase and enter mitosis (M phase). Theoretically, these labelled nuclei should have had twice the maximum labelling of nuclei which had time to complete cell division, and did not divide again (such nuclei incorporated into myotubes are examined in the following experiment).

Labelling of myotube nuclei after cut and crush injury

In this major experimental group 44 mice were injected with two doses of 3H–Tdr, one hour apart, at times ranging from 30 to 120 h after injury (Table 1). Mice were killed 9d after injury and muscle lesion samples taken for autoradiography to determine labelling of nuclei within myotubes.

Results

Maximum grain counts of premitotic nuclei

In mice injected 72 or 96 h after injury and killed 1 h later (73 or 97 h) about 500 nuclei were analysed for each lesion and cells were divided into 3 groups for analysis: "cuffing" cells, perivascular and endothelial, and other unidentified cells. "Cuffing" cells were those encircling degenerating muscle fibres: they were probably activated satellite cells. No differences in nuclear grain counts were found between these 3 groups. About 10% of nuclei, of all cell types, were labelled in crush lesions. The numbers of cells labelled in cut lesions were less than in crush lesions, since fewer cells were affected by the cut injury. In addition, maximum grain counts per nucleus were lower in cut than in crush lesions; this was particularly marked in samples at 97 h. The numbers of labelled nuclei, the mean grain counts, and distribution of grain counts in the 73 and 97 h samples are shown in Fig. 1. As an indication of the maximum labelling levels, the 3 highest grain counts in the premitotic nuclei of the 73 h samples were 52, 62, 76 for the cut, and 72, 80, 86 for the crush lesion.

At 97 h the grain counts of premitotic nuclei in both lesions were similar to the 73 h pattern (Fig. 1). The three highest grain counts seen in the cut lesion were 55, 63 and 66. In the central area of the crush lesion maximum grain counts were 79, 89, 91 and at the edge of the lesion slightly higher values of 94, 95, and 101 were counted. The mean values and distribution of grain counts in premitotic nuclei are shown in Fig. 1. (The labelling patterns that would result if such labelled precursors all divided once, twice or three times, are illustrated in Fig. 2.)

Labelling of myotube nuclei after cut and crush injury

Since the highest labelling of premitotic nuclei was around 70 to 101 grains/nucleus, it was anticipated that maximal labelling of post-mitotic myotube nuclei, which had undergone only a single division, would be in the order of 35–50 grains/nucleus. Precisely such grain counts were found in myotube nuclei of mice injected at 72 h, and sampled 9d after injury. In two separate areas of crush-injured muscle from one such mouse, the 3 highest grain counts of myotube nuclei were 33, 34, 43 and 34, 41, 53. Similar values of 35, 39, 44 and 34, 37, 38 were found in crush lesions of two other mice.

In mice labelled at 72 h and sampled 9d after injury, the maximal grain count in a myotube nucleus was 53 grains; this exceptional nucleus must have been the daughter of a precursor which had been labelled with 106 grains prior to cell division. (The highest labelling actually observed in a premitotic nucleus was 101 grains.) If such a
Fig. 1. Distribution of grain counts in premitotic nuclei. Mice were injected with $^{3}H-Tdr$ at 72 or 96 h after injury, and samples of cut and crush lesions taken 1 h later. About 500 nuclei were observed for each lesion. * Indicates the number of labelled nuclei analysed

labelled precursor had divided not once, but twice, the highest grain count of the resultant myoblasts (and subsequent myotube nuclei) would be half of 53 grains i.e. 27 grains. Therefore, all nuclei labelled with more than 27 grains (28+) must have been the result of a single precursor division. Many other myotube nuclei resulting from a single precursor division will have lower grain counts (as is evident from the distribution of label in premitotic nuclei in Fig. 1), but cannot be distinguished from nuclei which have divided several times. Only myotube nuclei with 28+ grains are assured to result from a single division of a labelled precursor; this identifiable group represents only a small sample of a much larger population of nuclei.

When a precursor labelled maximally with 106 grains divides repeatedly, the label is subsequently diluted to 53, 27, 13, 7, 3 and 1 grains, assuming that the $^{3}H-Tdr$ is evenly distributed in the daughter cells. Using the same reasoning as outlined above, nuclei with more than 13 grains (14+) cannot have divided more than 2 times; it should be noted that this group will also include many nuclei which have divided only once. Similarly, nuclei labelled with more than 7 grains (8+) cannot have divided more than 3 times, but will include nuclei which have divided once or twice. This argument forms the basis for the analysis of data in Table 1 (also shown in Fig. 3).

Cells undergoing a maximum of 4 divisions should, strictly, have more than 3 grains; however, for convenience in the analysis this group also includes nuclei with 3 grains. It should be emphasised that numbers of nuclei which have divided only once are an underestimate of the actual number, and this is clearly illustrated in Fig. 2. In contrast, the numbers of nuclei which appear to have divided 4 times also include nuclei which have divided fewer times, and are therefore an over-estimate.

Direct labelling of muscle precursor cells resulting in labelled myotube nuclei 8d after injury was first apparent in mice injected 30 h after injury (Table 1). The data in Table 1 clearly show that from 30 to 48 h after injury nuclei had divided 2 or more times after labelling. After 48 h a greater proportion of nuclei in the regenerates divided only twice, and an occasional nucleus only once: after 72 h, nuclei resulting from single division of a labelled precursor (28+ grains) were conspicuous.

It is clear that labelled precursors which divide once only will produce half as many labelled myotube nuclei as precursors which divide twice, and so on. An estimate of the minimum number of precursors labelled in a tissue sample can be obtained by dividing the "numbers of labelled myotube nuclei" in the regenerates by the "maximum number of divisions after labelling", e.g. such calculations show that 7 precursors were initially labelled in cut lesion samples at 30 and 90 h, but multiple divisions of precursors at the earlier time resulted in 25% of myotube nuclei being labelled, compared with 16% in the lesion labelled at the later time. In mice injected more than 60 h after injury few (4 or less) precursors were labelled in cut lesion samples of most mice (in 3 instances this was higher) indicating that the peak of myogenesis was over by 60 h.

In crush lesions the numbers of labelled myotube nuclei were slightly different and more protracted compared with cut lesions (Table 1). The change of grain counts in crush lesions was similar to that in cut lesions, and there was no delay in the appearance of more heavily labelled myotube nuclei, i.e., the estimated proportion of nuclei which had divided only twice after labelling increased after 48 h, as for cut lesions. Nuclei resulting from the single division of a labelled muscle precursor were more prevalent in crush than in cut lesions; higher proportions of such nuclei were present at 72 h and remained at 108 h. Estimates of numbers of precursors originally labelled in lesions indicate that more precursors were labelled in crush than in cut lesions; furthermore, it appears that similar numbers of precursors were replicating at 48 and 108 h after injury.

Discussion

Maximum labelling of regenerated myotube nuclei should be seen when the total dose of $^{3}H-Tdr$ is available to myoblast precursors throughout the DNA synthesis phase of the cell cycle (S phase), if these labelled nuclei (having divided) do not go through a subsequent cycle of cell division before becoming incorporated into myotubes. Injected $^{3}H-Tdr$ is available to replicating cells for about 30 min after administration. To increase the labelling of DNA during S phase, two doses of $^{3}H-Tdr$ were therefore given one hour apart. The S phase of different cell cycles is of variable length (Cleaver 1967) and has been reported to be 6–7 h for developing rat muscle (Zhinkin and Andreeva 1963) and 7.5 h for mouse muscle cells in vitro (Yao and Esslen 1975; Linkhart et al. 1980). Replicating cells which enter S phase after $^{3}H-Tdr$ is first available, and also those which leave S phase before the second injection of $^{3}H-Tdr$, may not incorporate the maximal amount of $^{3}H-Tdr$ into DNA. Furthermore, incorporation of $^{3}H-Tdr$ into DNA occurs at different rates during various stages of the S phase (Cleaver 1967), so a population of potential myoblasts fully
Table 1. Autoradiographic grain counts in regenerated myoblast nuclei 9d after cut and crush injury. Mice were injected with 2 x (25 μCi) doses of $^3$H - Tdr (one hour apart) at 30 to 120 h after injury. (Where <5% of myoblast nuclei were labelled the data are shown in parenthesis)

<table>
<thead>
<tr>
<th>Maximum number of divisions after labelling</th>
<th>Cut lesion</th>
<th>Crush lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>grains/nucleus</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>3-7</td>
<td>8-13</td>
<td>14-27</td>
</tr>
<tr>
<td>Time after injury of $^3$H - Tdr injection (h)</td>
<td>Myoblast nuclei labelled (%)</td>
<td>Myoblast nuclei labelled (%)</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>52</td>
<td>44 (100)</td>
<td>50</td>
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<tr>
<td>48</td>
<td>75</td>
<td>69</td>
</tr>
<tr>
<td>42</td>
<td>87 (100)</td>
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<td>56</td>
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<td>100</td>
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<td>120</td>
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exposed to both doses of $^3$H - Tdr throughout S phase, will not exhibit equal labelling. Moreover, it is unlikely that replicating muscle precursor cells are completely synchronised in their cell cycle. As a result of the above factors it was expected that a range of grain counts would be found in potential myoblasts labelled in their final cell division. This is clearly shown in our results where a wide range of grain counts was found in premitotic nuclei (Fig. 1).

These data include many cell types in addition to potential myoblasts, and reflect a large asynchronous population of replicating cells. Because of the spectrum of nuclear labelling it is necessary to use the maximum grain counts of premitotic nuclei as the basis for analysing the number of times that a labelled muscle precursor nucleus divides before fusion.

The maximum labelling observed in a premitotic nucleus was 101 grains: these mice received only a single dose (25 μCi) of $^3$H - Tdr. In the subsequent experiment where two doses of $^3$H - Tdr were given one hour apart (a total dose of 50 μCi per mouse), it was anticipated that dividing nuclei might have attained slightly higher labelling levels, which would result in post-mitotic myoblast nuclei possibly containing more than 50 grains/nucleus (half the premitotic level). However, the very small increase in labelling intensity was not significant. In an associated paper (Grounds and McGeachie 1987) we have shown that a two-fold increase in labelling intensity of premitotic nuclei results from a 7-fold increase in $^3$H - Tdr dosage (from 25 μCi to 170 μCi). A value of 53 grains was taken as a conservative maximum for postmitotic labelled myoblast nuclei and formed the basis for our analysis of grain counts of nuclei 9d after injury. It was found that labelling of myoblast nuclei rarely exceeded 50 grains/nucleus; of the 130 myoblast nuclei labelled with 28+ grains, only 8 had more than 50 grains (all in crush lesions) and only 5 of these exceeded 53 grains (values 55, 55, 56, 59, 66).

Myogenesis. It is generally considered that the muscle precursors (satellite cells) in mature skeletal muscle are similar to embryonic muscle stem cells (Campion 1984). When the
Fig. 2. Estimated patterns of grain counts when all labelled precursors undergo 3, 2 or 1 divisions. The patterns are derived from grain counts of premiotic nuclei at 97 h after injury (Fig. 1). There are striking similarities between the pattern resulting from 2 divisions of labelled precursors, and that following labelling at 30 h after cut injury (Fig. 3); and also between the pattern when precursors divide only once and labelling at the end of myogenesis (90 h) in the cut lesion. (These similarities also apply to crush lesions labelled at 30 and 108 h).

The model of Quinn et al. (1984, 1985) is applied to our experimental situation (where $\text{H}^\text{1}$-Tdr was used to label replicating muscle precursors after injury to mature skeletal muscle), it predicts that labelling of muscle precursors in early, compared with late, myogenesis should produce a transition in labelling of myotube nuclei from pattern A to C as illustrated in Fig. 4. For comparison, our experimental data are illustrated in Fig. 3. However, the data in Fig. 3 must also be evaluated against the range of grain counts that will occur in nuclei after $\text{H}^\text{1}$-Tdr injection. A wide range of grain counts was demonstrated in premiotic nuclei after a single injection of $\text{H}^\text{1}$-Tdr. If these premiotic nuclei had subsequently divided only once, the grain counts would be correspondingly halved. The expected patterns of such labelled nuclei after a single, and then a second division, were calculated for cut and crush lesions (from grain counts of premiotic nuclei at 97 h), and the patterns are shown in Fig. 2.

In regenerating muscle, replicating myoblasts were first labelled 30 h after injury; labelling was not seen when $\text{H}^\text{1}$-Tdr was injected 4 h earlier at 26 h, or at times preceding this. Since the minimal cell cycle time of muscle precursors has been reported as 9.5 h in vitro (Bischoff 1970) it seems that division of muscle precursors could not have occurred before 30 h. The data presented in Fig. 3 clearly show that some potential myoblasts (labelled at 30 h) had divided only twice before fusing to form myotubes. It may be that most muscle precursors (labelled in their first cycle of cell division at 30 h), had only divided twice, since comparison of these patterns with the estimated grain count distribution from precursors all undergoing two divisions (Fig. 2) shows marked similarities. It seems likely that some muscle precursors in these lesions had divided 3 or 4 times after labelling, but the analysis cannot distinguish these. In some instances (at 30, 36, 42 and 48 h) it appears that muscle precursors divided 3 rather than 2 times from the onset of myogenesis.

The striking conclusion from our results, that replicating muscle precursors in vivo need divide only twice before forming myotubes, is in direct conflict with the proposal (from tissue culture studies of embryonic chick muscle) that myogenic stem cells must undergo at least 4 obligate divisions before quantal mitosis (terminal differentiation), and fusion into myotubes (Quinn et al. 1984, 1985). The concept of quantal mitosis embodies the idea of a cell lineage, where progressive cell cycles make available regions of the genome that were not available for transcription in the mother cell (Holtzer et al. 1975). This model may function only in muscle growing in tissue culture. Alternatively, it may apply to developing embryonic, but not to adult muscle in vivo; unfortunately this is difficult to test. We consider that our results seriously challenge the concept of quantal mitosis.
(as described by Quinn et al. 1984; 1985) occurring during myogenesis in vivo.

The only way in which our data can fit the model of Quinn et al. (1984, 1985), is to propose that stem cells are not the main source of muscle precursors in mature skeletal muscle. Instead committed cells trapped near the end of the cell lineage must give rise to muscle precursors. To account for our results, none of these committed cells can be trapped in the G1 phase prior to quantal mitosis, although many would have to be in the postmitotic cell cycle. This explanation is compatible with the suggestion that satellite cells may represent a separate cell along the myogenic lineage (Cossu et al., 1983; 1985). Another possibility might be that the number of symmetrical divisions between committed and quantal mitosis of muscle precursors, is less in mice than in chickens (Quinn et al. 1984; 1985). We are currently testing this in vivo and it could also be tested in vitro.

Our results appear to be compatible with the idea that cycling muscle precursors can fuse with another precursor while in G1 phase, or alternatively, re-enter the S phase of the cell cycle (Konigsberg et al. 1978). Conditions in regenerating muscle in vivo are very different to embryonic muscle growing in tissue culture, and are probably more conducive to myoblast fusion. However, our data indicate that in regenerating muscle, precursors divide at least twice before these options are available to them.

The time at which precursors which have divided only once first appeared, was when 3H-TdR was injected 48 h after injury in the cut lesion (and 60 h in the crush lesion) (Table 1, Fig. 3). These precursors had presumably completed the first cell cycle and passed through G2 phase ready to re-enter S phase, in the interval between 30 to 48 h. This indicates that the time elapsed from the first S phase to the next is in the order of 18 h. This estimate of cell cycle length is compatible with observations from in vivo studies that the cell cycle of developing rat muscle is 18-20 h (Zhinkin and Andreeva 1963), and in developing chick muscle ranges from 12-24 h (Marchok and Herrmann 1967).

In regenerating muscle the minimal time for a precursor to replicate twice and be ready in G1 phase to fuse and form myotubes would be less than 66 h. This is based on a calculation of 30 h (1st replication) + 36 h (2×18 h cell cycles), although the 2nd cell cycle time may be shorter than 18 h. The estimated time of myotube formation around 66 h fits observations in the literature that myotubes appear abruptly from 2.5 to 3 days after injury (see Introduction).

Further examination of the patterns of grain counts in Fig. 3 supports the trend predicted from the model of myogenesis as illustrated in Fig. 4. Comparison of grain count patterns in crush lesions labelled at 30, 60 and 90 h after injury shows the predicted transition to a pattern where precursors have undergone fewer cell divisions. However, the transition is neither as marked, nor as consistent in the cut lesions. There is remarkable similarity between the estimated patterns for a single division of all labelled precursors (Fig. 2), and the results of cut and crush lesions labelled near the end of myogenesis (at 90 and 108 h respectively).

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