Applications of an Autoradiographic Model of Skeletal Muscle Myogenesis In Vivo

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INTRODUCTION

Skeletal muscle cells can regenerate following traumatic injury (1, 6, 7, 10, 11). Some, possibly all, myogenic cells are derived from satellite cells of skeletal muscle which act as precursors for new muscle cell formation (26). Histologically, new muscle cells are not recognisable in a regenerating muscle until three to four days after injury when myoblasts commence fusing into multinucleated myotubes. This fusion event occurs at similar times following different types of injuries (22, 23, 24, 25, 42). However myogenic events prior to myotube formation, from the time of injury until three to four days later are poorly understood. when does DNA synthesis start in the once quiescent myogenic precursor cells (satellite cells)? What is the time course of myogenic precursor cell proliferation after injury?

One of the problems with investigating specific events during early myogenesis is the difficulty in identifying specific cells in the inflammatory tissue following traumatic injury. For example, proliferating mononuclear cells abound in the first few days after injury and it is extremely difficult to distinguish between young fibroblasts and myoblasts, and even bone marrow derived cells such as macrophages. It is not until myoblasts have started to produce muscle specific proteins, usually some days after injury, that they can be identified as being of the myogenic lineage. Even muscle satellite cells can only be identified with suitably orientated electron micrographs.

The specific question we asked is "When do myogenic precursor cells first

start to proliferate after injury?" To investigate this we employed the technique of tritiated thymidine (^{3}H-TdR) injections followed by autoradiography.

As a brief background to this technique it is important to point out that ^{3}H-TdR is incorporated into DNA of replicating cells and that this occurs during the S phase of the cell cycle, some 8-12 hours prior to mitosis (8). Once ^{3}H-TdR is incorporated into a cell it is passed on to the nuclei of daughter cells following mitosis, and through subsequent generations, being diluted with each cell division. Thus it is possible to quantitate the number of cell divisions by measuring the nuclear ^{3}H-TdR.

Furthermore, it is fortunate that when ^{3}H-TdR is injected, it is available to cells synthesizing DNA (for mitosis) for about 30 minutes (8), thus a cell population may be pulse labelled. Cells sampled within one hour after injection will be premitotic because insufficient time has elapsed for them to have passed from the DNA synthesis phase through to the preparation for mitosis.

Applying this technique to regenerating muscle it will be seen that when ^{3}H-TdR is injected into an animal following muscle trauma, and the tissues sampled one hour later, this will identify cells which are replicating at the time of ^{3}H- TdR injection (Fig 1). However, in the early stages of regeneration (first 48 hours) myogenic precursor cells are essentially indistinguishable from other proliferating cells. When myotube formation occurs, at 3-4 days after injury, the cells are post-mitotic and do not incorporate ^{3}H- TdR (45).

We determined the timing of myogenic precursor cell proliferation by injuring leg muscles in mice, injecting ^{3}H- TdR once only into the animal at a specific time post- injury, and leaving muscles to regenerate to the advanced myotube stage at 10 days after injury. If myogenic precursor cells had been synthesizing DNA at the time of injection they would have incorporated ^{3}H-TdR, and this would have been carried through to daughter cells and subsequent generations, until fusion, when myotube nuclei would be labelled. This could be detected by autoradiography (Fig 2). Thus by comparing labelling patterns of myotube nuclei, with the time of injection of ^{3}H-TdR, we could determine the initiation, peak activity and duration of myogenic precursor cell replication in skeletal muscle following different types of injury.

Initially we set out to resolve a dilemma that arose as a result of conflicting evidence for the existence of circulating muscle precursor cells. Bateson, et al (3) injected mice with ^{3}H-TdR *prior* to inflicting a crush injury and subsequently found labelled myotube nuclei in the regenerated muscle. Because the ^{3}H-TdR was not available directly to myogenic precursor cells after injury it was claimed that the labelled cells in regenerated muscle had been derived from the circulation, possibly as a result of bone marrow cells being labelled at the time of injection (prior to injury). Conversely, Bintliff and Walker (4) used a similar experimental design with a small cut lesion (rather than a crush) and did not find labelled myotube nuclei in the regenerate.

We proposed that the difference in these results may have been, in part, accounted for by the different injuries (cut or crush) used by the two groups: myogenesis might have been delayed or prolonged after a severe crush (3) compared with a minor cut lesion (4). Thus after crush injury, precursors might
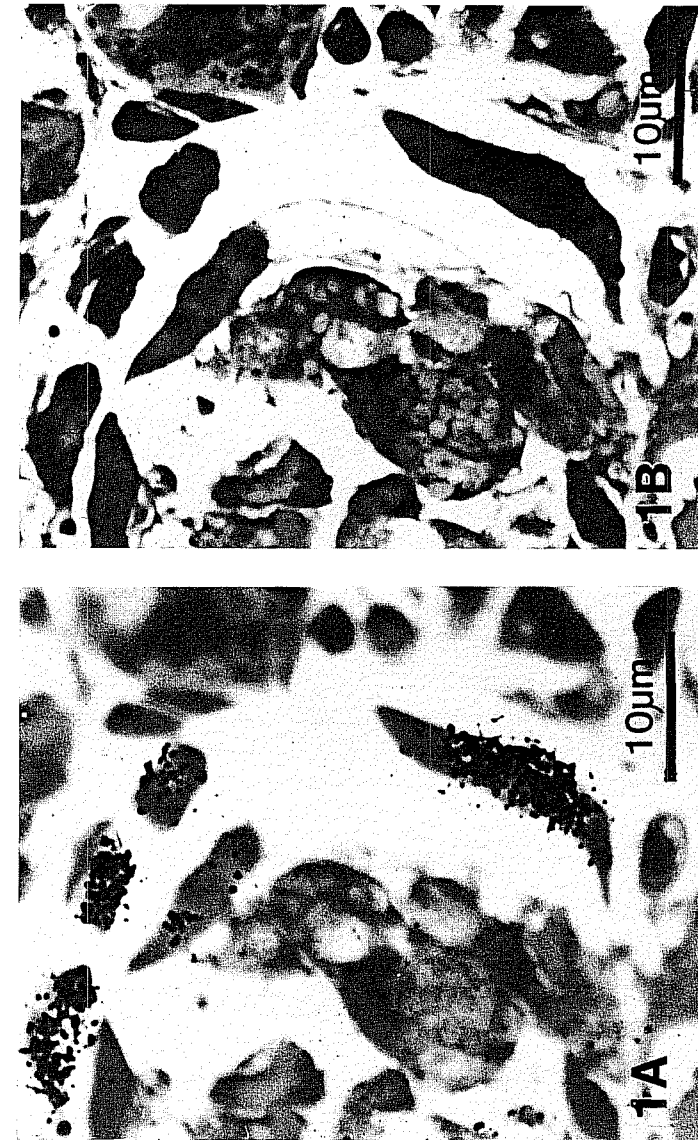

FIG. 1A.   An autoradiograph of labelled and unlabelled cells (possibly muscle precursors or satellite cells) in degenerating muscle fibres 73 hours after a crush injury to mouse skeletal muscle. These labelled nuclei are premitotic because ^{3}H-TdR was injected one hour previously. Such cells will carry the label through to daughter cells and subsequent generations. If they are myogenic-precursors, they will fuse to form myotubes, with labelled myotube nuclei, as shown in Figure 2.

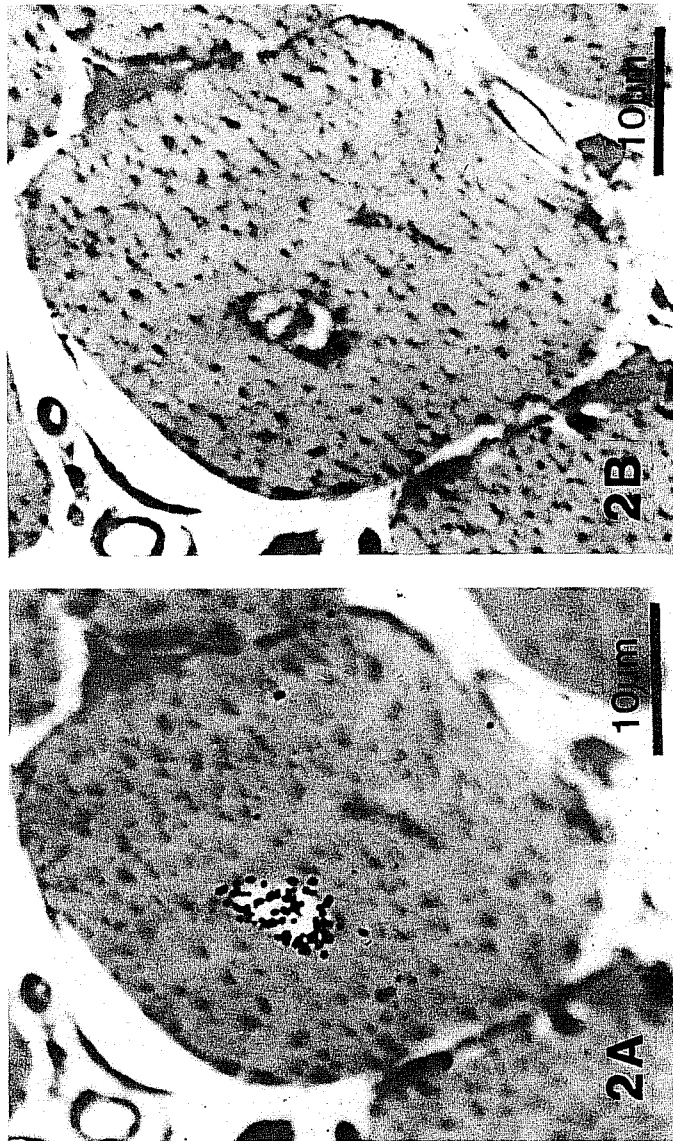FIG. 1B.   The same field as in A but with the tissues in focus.

FIG. 2A. A labelled myotube nucleus in mouse muscle 10 days after a crush injury. ^{3}H-TdR was injected at 48 hours after injury. The label was incorporated into myogenic precursor cells proliferating at this time, and after fusion resulted in labelled myotube nuclei.

FIG. 2B. The same field as in A but with the tissue in focus.

still be proliferating at a time when some of the heavy preinjury dose of ^{3}H-TdR, used by Bateson et al, might be available for reutilisation. Our autoradiographic results (15, 30, 31) show that ^{3}H-TdR reutilisation does account for the heavy labelling reported by Bateson et al (3). The time course of myogenic cell proliferation in skeletal muscle regenerating after different injuries, and details of our autoradiographic model of myogenesis in vivo are described below.

CUT AND CRUSH LESIONS (EXPERIMENT 1)

This is the first experiment in a series of 14. The other 13 experiments (2-14) are described later under "Other Related Experiments".

MATERIALS AND METHODS

Six to eight week old male BALB/c mice (20-25g body weight) were used in all experiments. Forty-four mice were anaesthetised with ether and 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. The skin of the right leg was opened and a small oblique incision made with a razor blade across superficial fibres of the TA. The cut was about 1mm deep and 4mm long; haemorrhage was minimal. A fine suture (10.0 Ethilon) was placed at either end of the cut for future identification of the lesion site. In the left leg, one arm of a small pair of artery forceps was inserted down the side of the tibia beneath the muscle, and the TA crushed 3 times transversely. The crush was about 3mm wide, 4mm long and extended throughout the depth of the muscle. Although the crush was severe, longitudinal continuity of the muscle belly was maintained. Skin wounds overlying the lesion were closed and sutured.

AUTORADIOGRAPHY AND ANALYSIS

To label replicating myogenic cells ^{3}H-TdR (specific activity 5 Ci/mmol) was injected intraperitoneally at the dosage of 1 uCi/g body weight (20-25 uCi/mouse) at various times ranging from 0-120 hours after injury. Each mouse received only one injection. Ten days after injury 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 taken to check the uptake of ^{3}H-TdR and to determine the autoradiographic exposure time. All tissues were postfixed in 1% OsO4 for 2 hours, washed in phosphate buffer and block-stained in 1% p-phenylenediamine to obviate the need for staining autoradiographs after processing (9). 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 with an oil immersion lens (x100). The analysis involved counting the number of myotube nuclei and calculating the percentages which were labelled in the regenerated cut or crush lesions. (An extensive analysis of grain counts in these myotube nuclei is presented in 14).

RESULTS

At 10 days after injury the appearances of the cut and crush lesions in BALB/c mice were quite different.

Macroscopically it was difficult to see the cut, whereas the crush often had calcified zones. Light microscopy revealed a thin border of about 100 myotubes with little intervening connective tissue on the surface of the cut muscle. Crush lesions were large and had a heterogeneous appearance: there were areas showing excellent regeneration with many myotubes, but the central region was usually fibrotic and contained necrotic muscle fibres and calcified areas (32). Extensive fibrosis in crush lesions in muscle was also described by Le Gros Clark in 1946 (24).

0-26 hours

The autoradiographic analysis showed that in the eleven mice injected at 0 to 26 hours there was low labelling (only 3-5 grains/nucleus) in 2-25% of myotube nuclei examined 10 days after injury. Further autoradiographic studies have shown that this low labelling is attributable to reutilisation of ^{3}H-TdR (15).

30 hours later

The labelling in myotube nuclei of cut and crush lesions 10 days after injury is shown in Table 1. Direct labelling of myogenic precursor cells resulting in labelled myotube nuclei 10 days later (with a labelling intensity ranging from 3 to 66 grains per nucleus), was first apparent in mice which had been injected 30 hours after injury (Table 1). There was variation in results from different mice injected at the same time. Initiation of muscle precursor replication was delayed in some mice: there was no significant labelling of lesions in 1 of 4 mice injected at 30 hours, neither in 1 of 4 mice injected at 36 hours.

Muscle precursor proliferation was generally more advanced in the cut compared with the crush lesions (Table 1, Fig 3). In cut lesions there appeared to be two different phases to labelling. In mice injected 30 to 60 hours after injury a mean value of $30 \pm 4\%$ (SEM) of myotube nuclei were labelled at 10 days. After this time similar proportions of labelled myotube nuclei were seen in only 1 of 3 mice injected at 72 hours, and 1 of 2 mice at 78 hours. In the second shorter phase, from 72 to 90 hours, fewer myotube nuclei (mean $13 \pm 3\%$) were labelled.

In crush lesions the labelling pattern was slightly different and more protracted (Table 1, Fig 3).

TABLE 1. Percentages of labelled myotube nuclei in regenerated tibialis anterior muscle after cut and crush injury. Mice were injected with ^{3}H-TdR at 30 to 120 h after injury. Each mouse received only one injection. Muscle samples were removed 10 days after injury and processed for autoradiography.

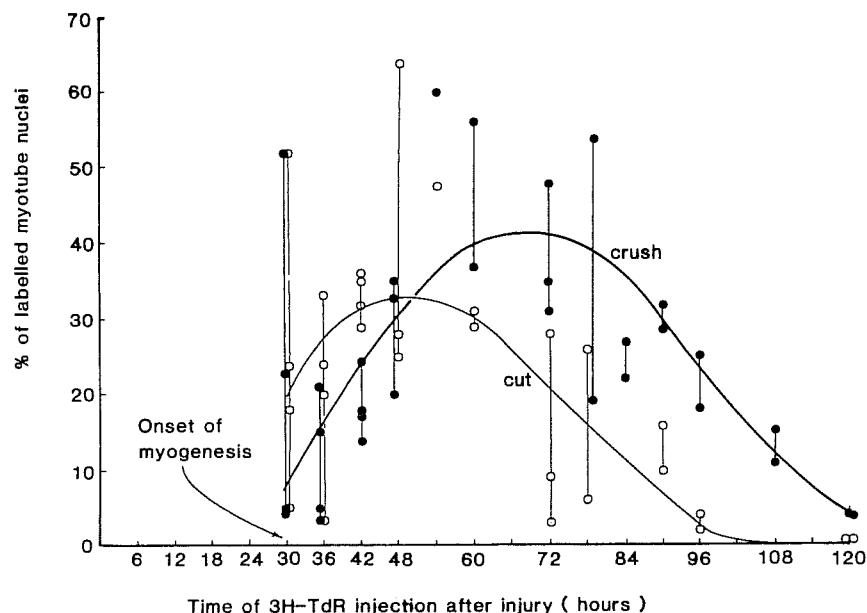| Time after injury of 3H-TdR injection (h)*	% of labelled myotube nuclei	
	Cut	Crush
30	5	5
	52	50
	18	23
	25	5
36	3	3
	24	21
	33	5
	20	15
42	32	17
	36	18
	29	14
	35	22
48	25	35
	28	20
	64	33
56	49	61
60	29	37
	31	56
72	28	48
	9	35
	3	31
78	6	19
	26	54
84	11	27
	11	22
90	10	29
	16	32
96	4	18
	2	25
108	0	11
	0	15
120	1	4

FIG. 3.   The patterns of muscle precursor replication in cut or crushed muscle. All samples were analysed for labelled myotube nuclei at 10 days after injury. 3H-TdR had been injected at the various times after injury shown on the horizontal axis: each mouse received only one injection of ³H-TdR.

OTHER RELATED EXPERIMENTS

Since results of the cut and crush injury experiments were so informative we applied these techniques to other muscle injuries under different conditions to see whether the timing of myogenic cell replication (particularly the initiation of myogenic precursor cell proliferation) differed, or could be modified. These experiments are described briefly below, and summarised in Table 2. (The first experiment of the whole series, 1-14, is the cut and crush work, described above).

2.   Different Strains of Mice

We compared the pattern of myogenic cell proliferation found in BALB/c mice with that of inbred Swiss (SJL/J) mice, in which muscle regeneration had been shown to be more vigorous (13). Twenty three male Swiss mice received crush lesions, and were analysed as above (16).

3.   Male and Female Mice

As an extension to the study of crushed muscle in Swiss mice experiment 2., we compared myogenesis in 15 inbred female Swiss mice (SJL/J) with that in the 23 Swiss males above (16).

4.   F₁ Hybrid (Swiss x BALB/c) Mice

Comparison of the onset of DNA synthesis in myogenic precursors of BALB/c and Swiss mice showed that precursor proliferation was consistently initiated 6 hours earlier in Swiss than in BALB/c mice. The genetic influence on this process was examined by crush injuring 11 male and 10 female F1 hybrid (SJL/J x BALB/c) mice (16). Studies on F2 mice are currently in progress.

5.   Sequential Study

To investigate the precise cellular events responsible for the strikingly different regeneration in BALB/c and Swiss mice, sequential samples were examined between one and ten days after crush injury (35).

6.   Chicken Muscle Myogenesis

Our data from the cut and crush injury experiments in BALB/c mice fortuitously enabled quantitation of the number of myogenic precursor cell divisions that occurred between the onset of myogenesis and fusion of myoblasts into myotubes (14). Our results challenged the concept of the cell lineage model of myogenesis supported by Quinn et al (39, 40, 41). This aspect is dealt with in more detail in the paper by Dr Grounds in this volume. A series of ten chickens were injured with small cut or crush lesions in the pectoralis and the anterior and posterior latissimus dorsi muscles (to compare myogenesis in fast and slow muscles). It was necessary to repeat the experiments in adult chickens in order to employ the same model used by Quinn et al, who did all their experiments on chick muscle in vitro. Details of our experiments are published elsewhere (17).

7.   Transplants of Intact Whole Muscle

Small extensor digitorum longus muscles (EDL) were transplanted onto tibialis anterior muscles in 48 BALB/c male mice and the pattern and timing of myogenic precursor proliferation determined as above (43). In these grafts the neural and vascular supplies are completely disrupted, although the muscle fibres are essentially intact. See also Fig 4.

TABLE 2. Summary of the Range of Experiments Carried out Using the Autoradiographic Model of Myogenesis-in-Vivo

| Experiment	Species/Strain	Age & Sex	References
1 Comparison of a minor (cut) and severe (crush) injury	BALB/c	8 weeks male	McGeachie & Grounds, 1987 Grounds & McGeachie, 1987a
2 Strain comparison crush injury	BALB/c + SJL/J	8 weeks male	Grounds & McGeachie, 1989a
3 Sex comparison crush injury	SJL/J and female	8 weeks male	Grounds & McGeachie, 1989a
4 F₁ hybrids crush injury	F₁(SJL/J x BALB/c)	8 weeks male	Grounds & McGeachie, 1989a
5 Sequential study crush injury	BALB/c + SJL/J	8 weeks male	Mitchell et al, 1989a
6 Model of myogenesis cut and crush injury	Chicken white leghorn	Adult female	Grounds & McGeachie, 1989b
7 Transplants of whole intact EDL muscle	SJL/J	8 weeks male	Roberts et al, 1989a
8 Transplants of minced TA muscles	BALB/c + SJL/J	8 weeks male	Grounds & McGeachie, 1989c
9 Predenervation cut injury	BALB/c	8 weeks male	Grounds & McGeachie, 1989
10 Preinjury crush injury	BALB/c + SJL/J	8 weeks male	Morlet et al, 1989
11 Comparison of old and young mice crush injury	BALB/c	40+4 weeks	Morlet et al, 1989
12 Dexamethasone crush injury	BALB/c	8 weeks male	Mitchell et al, 1989b, c
13 Fibroblast growth factor in crush injury	BALB/c	8 weeks male	Mitchell et al (manuscript in preparation)
14 Titanium metal implants	BALB/c	8 weeks male	McGeachie et al (manuscript in preparation)
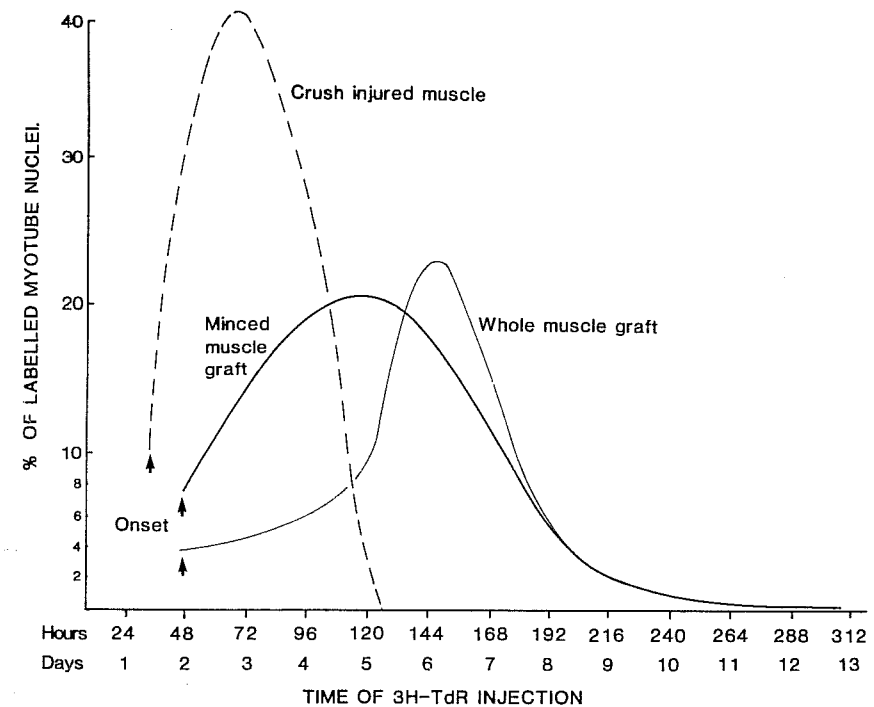
FIG. 4. A comparison of the time of onset, peak and cessation of myogenic precursor cell proliferation in BALB/c muscle regenerating after crush injury, or transplantation of intact EDL muscle, or minced TA muscle.

8. Transplants of Minced Muscle

This model of muscle regeneration has been used extensively for some decades and it was of interest to compare the pattern of precursor proliferation with that seen in transplants of whole intact muscles, and also in injured muscle. In 22 BALBc and 23 Swiss (SJL/J) mice the tibialis anterior muscle was removed, minced into fine fragments and placed into the original site, and myogenesis investigated as above (18). See also Fig 4.

9. Denervated Muscle

There is evidence that pre-denervated muscle has substantially increased numbers of satellite cells which turnover at relatively high levels (27, 28, 29). It has been proposed that denervation "primes" skeletal muscle, and it was considered that (satellite cells) in denervated muscle might commence DNA synthesis more rapidly after injury than in innervated muscle. We tested this hypothesis by predenervating 29 BALB/c male mice one week prior to a cut injury and treating them as for the cut and crush experiment above (33). See also Fig 5.
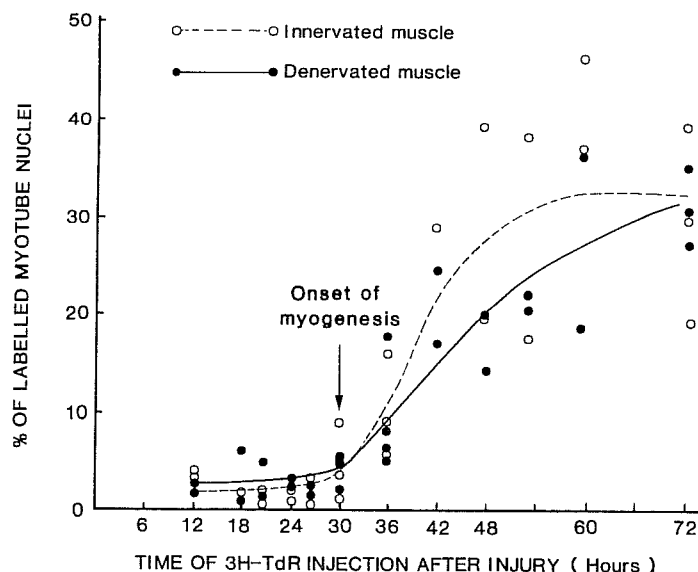
FIG. 5.   The proportions of labelled myotube nuclei in muscle lesions 10 days after a cut injury to denervated and innervated muscle. ³H-TdR was injected at the times shown on the horizontal axis. The onset of myogenesis in both denervated and innervated muscle occurs at 30 hours after injury.

10. Reinjured Muscle

More rapid regeneration has been observed if muscle is regenerating after a recent injury (20). It was therefore proposed that after a previous recent cycle of regeneration, myoblastic cells might be primed to respond more rapidly to a subsequent injury than quiescent muscle precursors responding to a single isolated injury. A rather complicated series of experiments was conducted on 55 mice: 15 reinjured 7 days after an initial crush injury; 15 reinjured 28 days after a similar injury; and 25 had EDL transplants, (as in 7), reinjured at 7 or 28 days. All 55 mice were examined 10 days after the final injury (38).

11. Myogenesis in Old Versus Young Mice

Would the onset of myogenesis be delayed in muscle regenerating in old, compared with young animals? A series of 28, "old" mice, aged 40 weeks, and 37 "young" male BALB/c mice aged four weeks received small crush lesions, and the tissues were treated and analysed as for the cut and crush lesions above. Details of this experiment are in preparation for publication.

12. Dexamethasone

Experiments show that Dexamethasone stimulates the proliferation of myogenic precursors of neonatal muscle and myogenic cell lines grown in tissue culture (19). We investigated whether Dexamethasone similarly stimulated proliferation of myogenic precursors in mature muscle, regenerating in vivo. A series of different doses of Dexamethasone was administered to 46 BALB/c mice at different times after crush injury (34, 36, 37).

13. Fibroblast Growth Factor (FGF)

This growth factor stimulates myogenic and satellite cell proliferation in tissue culture (2, 5, 12). We hypothesised that FGF might enhance myogenic precursor cell replication and perhaps initiate myogenesis at an earlier time in vivo. Forty male BALB/c mice with crush lesions were used and given different doses of FGF subcutaneously over the wound, at different times after injury (34).

14. Metallic Implants of Titanium or Stainless Steel

Titanium metal implants are now used extensively for the anchorage of prosthetic devices into bone. These "oseo-integrated" implants are extremely successful and do not invoke scar tissue formation, as occurs with other metals. We tested the interface between titanium and regenerating muscle, and compared its influence with stainless steel, to determine whether myogenic precursor cell replication would be modified.

DISCUSSION OF RESULTS

Our model for quantitating myogenic precursor cell proliferation in vivo has now been used for 14 different experiments, and the data generated have given us a profound insight into the cell proliferative events that occur in skeletal muscle regenerating after injury. The model is easy to use and the techniques of autoradiography are quite standard, facilitating repeatable experiments. The data provided from our initial studies have resolved the dilemma on published evidence for the existence of a circulating myoblast precursor cell being derived from the bone marrow. With our model, not only have we been able to measure the timing of myogenic cell proliferation (32), but we have also provided a substantial body of data on the numbers of cell divisions that occur during myogenesis (14, 17). Data from our in vivo model seriously challenges the long established cell lineage model of myogenesis, where it has been claimed that myogenic precursor cells must go through a series of obligate mitotic divisions before fusing into a myotube. Evidence for the cell lineage model of myogenesis has been derived entirely from tissue culture studies (39, 40, 41).

ONSET OF DNA SYNTHESIS

One of the most striking results of our series of experiments with this model is the very rigid onset of myogenic precursor cell proliferation in regenerating muscle. Initially we proposed that myogenesis might commence later, or be prolonged in crushed muscle compared with cut muscle, due to the more extensive tissue damage in crushed muscle which would induce a greater inflammatory response. However the initiation of myogenic precursor replication occurs in BALB/c mice consistently at 30 hours after a minor (cut) or severe (crush) injury there being no detectable difference between the two types of injury (Fig 3).

When we had established this initiation time of 30 hours in BALB/c mice it generated other hypotheses, based on sound experimental evidence, to test whether the initiation time of myogenic precursor cell proliferation could be modified. Prior denervation was such a case, where the increased number and turnover of satellite cells (myogenic precursors) might possibly prime the muscle for a more rapid response to injury. The data show that BALB/c mice myogenic precursor replication occurs at 30 hours in both innervated and denervated mice, (Fig 5) and similar numbers of precursors are proliferating at the same times after injury (33).

Reinjury of muscle, which appears to hasten the process of muscle regeneration, did not promote an earlier onset of myogenic precursor cell proliferation. This was tested comprehensively with a complex set of three different experiments, using both crush lesions and regenerated EDL isografts (38).

The onset of proliferation was not affected by either titanium or stainless steel implants. We have attempted to accelerate the onset of DNA synthesis in muscle precursors of mature muscle following injury by administering Dexamethasone and Fibroblast Growth Factor in different doses and combinations, at different times after injury. Dexamethasone did not alter the timing of myogenic cell replication: onset at 30 hours for BALB/c and 24 hours for Swiss mice (34, 36, 37). The experiments with FGF are currently being analysed, but preliminary results indicate that FGF does not affect the initiation time of precursor cell proliferation.

Our results show that genetically determined factors influence the timing of myogenic precursor cell replication. Grounds (1986) reported that regeneration was more successful and extensive in Swiss compared with BALB/c mice and the hypothesis that this may have been related to an earlier onset of cell replication was supported by our experiments (Fig 4) (16). This occurs at 24 hours in Swiss mice (male and female), consistently 6 hours prior to the onset in BALB/c mice.

The data from F1 (SJL/J x BALB/c) mice show that myogenic precursor replication commenced at 30 hours after injury, and indicate that the vigorous regeneration of Swiss mice is not a dominant trait (16). However the earlier initiation of precursor replication (and possibly, shorter cell cycle time) in Swiss mice cannot alone account for the superior muscle regeneration of this strain. Other factors such as widespread, synchronised activation of large areas of precursors also appear to be important.

MUSCLE TRANSPLANTS

In all muscle transplants the onset of myogenesis was delayed, presumably because of the time taken to re- establish vascular connections. However in minced muscle grafts myogenic precursor cell proliferation still commenced at 36 hours in Swiss mice, 12 hours earlier than similar grafts in BALB/c mice, where the onset of DNA synthesis was seen at 48 hours (Fig 4) (18). Considering the severity of the mincing procedure, the availability of ^{3}H-TdR to regenerating muscle by 36 hours after injury is remarkably rapid and efficient.

In transplants of whole intact muscle the EDL is totally devascularised and there are substantial connective tissue barriers (fascial coverings of the grafted EDL and underlying TA muscles) to revascularisation. However, the onset of myogenesis occurred at 48 hours in BALB/c mice as in minced muscle grafts (Fig 4) (43).

This evidence is remarkable in that revascularisation of the EDL isograft in this situation does not occur until 72 hours after transplantation, indicating that diffusion of ^{3}H-TdR occurs in these extreme situations (44). These findings are quite contrary to the established view that no myogenesis occurs until revascularisation has been established (21).

It is interesting that, although myogenic precursor cells of mature BALB/c muscle can initiate DNA synthesis at 30 hours after trauma (as shown from the injury experiments), they do not start to proliferate until 48 hours in transplanted muscles, implying that extrinsic factors are involved in controlling cell proliferation. It is also noteworthy that muscle transplants show that muscle precursors can continue to proliferate for many days, and yet the same cells presumably cease proliferating in the incomplete regeneration seen the central fibrous zone of crush injured BALB/c muscle.

What implications does this information have for the potential therapy and management of severe muscle injuries, muscle transplantation and some myopathies. It is probable that appropriate therapies could be introduced to enhance the cell proliferative and regenerative processes? Our model enables the quantitative assessment of such potential therapies. Such procedures might have particular importance in minimizing the accumulation of fibrous connective tissue in regenerating muscle.

We are investigating ways of modifying the regenerative process by trying to induce myogenesis earlier, and also trying to prolong cell proliferation both directly and by temporarily delaying fusion of precursors into myotubes. In addition we are investigating the genetic influences controlling different aspects of myogenesis so that we can gain greater insights into the poorly understood mechanism of skeletal muscle regeneration. The clinical application of this information has never been more relevant than at the present time, with the exciting new developments in Myoblast Replacement for the therapeutic reconstitution of diseased muscles, particularly in Duchenne muscular dystrophy.

ACKNOWLEDGEMENTS

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DISCUSSION

Professor Peter K Law:
Thank you Associate Professor McGeachie, the paper is now open for discussion.

Dr Donald S Wood:
I would be interested to hear your comments on what actually triggers the myogenesis cycle.

Associate Professor John K McGeachie:
As far as genetic factors are concerned we found a genetic difference between two strains, BALBc and Swiss mice, and it makes a difference of 6 hours in the initiation of myogenesis. As for environmental factors, we have tried a number of experiments but so far we cannot modify the timing of myogenesis. We have used very differing injuries. With Dexamethazone, Chris Mitchell used a whole range of doses at different times after injury and nothing would change the timing of myogenesis. This was based upon some really sound evidence from tissue culture (2). That is the sort of thing we are looking at, at the moment.

Dr Donald S Wood:
Could it be that the genetic program activated in regeneration differs according to the circumstances causing the need for regeneration? For example, can we conclude that just because the defective gene is the same in the mdx mouse and in Duchenne muscular dystrophy that the genes activated during regeneration are the same?

Associate Professor John K McGeachie:
It is very interesting, as I said, that in the mdx mice the regeneration cycle just keeps going on and on, yet in our muscle injury models, also in mice, muscle regeneration stops very abruptly 4 or 5 days after injury.

Professor George Karpati:
Dr A G Engel had shown some years ago (4), that in necrotic muscle fibre segments, the complement cascade becomes active with the ultimate generation of complement 9. There has been a suggestion that it may be the molecular spark that perhaps triggers satellite cell activation during regeneration. The study of regeneration in decomplemented animals could be, therefore, informative. Have you done that?

Dr Miranda D Grounds:
No. I have not.

Associate Professor John K McGeachie:
In our model we can quantitate the very early stages of myogenesis. By the time one can actually see evidence of myogenesis (myotube formation), virtually all myogenic cell replication has ceased. In severe lesions where necrotic fibres persist for more than 6 or 7 days the histological appearance would suggest continued myogenesis, but our autoradiographic studies show that myogenic cell replication ceased at 4-5 days after injury. There is another aspect of this model which I would like to add briefly. Not only have we been able to look at the timing of early myogenic events, but we also quantitated the number of myogenic precursor cell replications that occur before fusion. That has given us a profound insight into the myogenic lineage, and we have been able to challenge the long-established views that have come from in vitro work, on the number of cell divisions that a muscle cell must go through before it fuses into a myotube. Miranda Grounds will talk more about that.

Professor Peter K Law:
I wonder if you can elaborate more on reinjury after transplantation. That seems to be a very interesting topic.

Associate Professor John K McGeachie:
Yes. Thank you Peter. The evidence we used for that was Gulati's (1986) (3) work. He found, in reinjured muscle, that myotubes appeared a day or so earlier than after a single injury. That was the basis of our work: we assumed that myotubes would have formed, and fusion would have occurred at a set time after myoblast proliferation. Therefore working backwards we hypothesised that the earlier myotube formation in reinjured muscle was due to earlier myogenic cell proliferation, because the cells were "primed". The satellite cells had gone through the previous cycle of regeneration and were thus primed for a more rapid response to injury. We tried three different experiments. We used the model of a EDL muscle graft (which is a lovely model of myotubes). We let the muscles regenerate and then injured the myotubes either 7 days or 28 days after transplantation. Myogenic precursor cells began proliferating in BALBc mice at 30 hours, after the reinjury, just as for a single injury. Likewise, we put in single injuries, left them for 7 or 28 days and reinjured them. No matter what we did, at 30 hours myogenic precursor cell replication started. Therefore, our hypothesis was not supported: the earlier appearance of myotubes in reinjured muscle (3) is not due to an earlier onset of myogenesis. It must be due to more rapid myoblast maturation and fusion into myotubes.

Dr Olav M Sola:
The response of muscle to injury or stimulation may often occur about 24 to 36 hours after the event. Loss of a snapping claw of the shrimp (Alpheus) results in onset of transformation of the pincer claw to become a large snapping claw within 24 hours. Denervation of the rat left hemidiaphragm precipitated initial changes at about 30 hours. Marked changes are present in stretched latissimus dorsi muscle pedicles in less than 36 hours.

Professor Lawrence Austin:
This may be quite a common phenomenon. In regeneration of peripheral nerve you

see the same sort of thing. There is a standard pattern of RNA changes in the cell body of the affected neurone which follow a very precise time pattern and changes in axonal transport and so on (1). There is also another very well known variation on this called a prelesion injury, in which you injure once and injure again some time later closer to the neuronal cell body. The first injury always accelerates the rate of change of the various parameters that are measured after the second injury, and it seems to change all of these parameters, so it may be quite a common cellular response.

Associate Professor John K McGeachie:
But our reinjury did not really seem to make any difference you see. This is the interesting thing.

Professor Lawrence Austin:
No but you had a difference of the time period.

Associate Professor John K McGeachie:
Yes.

Professor Peter K Law:
Are there any more questions? Thank you John McGeachie. The next paper is by Dr Miranda Grounds entitled "Factors Controlling Muscle Regeneration In Vivo."

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Factors Controlling Skeletal Muscle Regeneration in Vivo

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The genetic, X-chromosome linked human myopathy Duchenne muscular dystrophy (DMD) appears to be extremely similar to the X-linked muscular dystrophy of mdx mice (31), and the X- linked muscular dystrophy in dogs (12). Skeletal muscles of humans, mice and dogs lack dystrophin (12, 30) and persistently degenerate. In humans and dogs the DMD muscle fails to regenerate successfully and is replaced by fatty and fibrous connective tissue (10). In contrast there appears to be excellent continuous muscle regeneration in mdx mice (1, 9, 13). It is not known whether possible differences in muscle loading or in muscle fibre length related to the animal's size, or in the relative ageing processes in the three species can alone account for the progressive dystrophy in human and dogs, and the effective muscle regeneration in mdx mice. The important question arises as to why is muscle regeneration successful in mdx mice but not in DMD of humans and dogs?

Identification of factors responsible for the striking difference in regenerative capacity of dystrophic muscle in these species, might lead directly to a treatment for the chronic muscle wasting seen in DMD (even without correction of the underlying gene defect). The possible manipulation of conditions controlling muscle regeneration presents an alternative approach to potential treatment of DMD: this is complementary to the theoretically attractive possibility of genetically replacing the defective gene for dystrophin (55).

The processes involved in skeletal muscle regeneration after injury or transplantation are well documented (8): they involve revascularisation, cellular infiltration and phagocytosis of necrotic muscle by macrophages, proliferation of