The timing between skeletal muscle myoblast replication and fusion into myotubes, and the stability of regenerated dystrophic myofibres: an autoradiographic study in mdx mice

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ABSTRACT

In mdx mice, a model for Duchenne muscular dystrophy, the timing between the replication of myoblasts and their incorporation into myotubes was determined autoradiographically. Thirty-eight mdx mice aged 23 d were injected with tritiated thymidine to label myoblasts replicating early in the dystrophic process. At intervals from 8 h to 30 d after injection the tibialis anterior muscles were removed, processed for autoradiography and analysed for labelled central myonuclei (derived from the progeny of myoblasts which had been labelled at 23 d). At 8 h after injection there were no labelled central myonuclei, showing that the labelled myoblasts had not fused within this time. At 1 d, 2% of central myonuclei were labelled, at 2 d, up to 32% were labelled, at 3 d ~ 60% were labelled, and at 4 d the labelling peaked at 74%. In the 27 mice sampled from 5–30 d after injection, the levels of central myonuclear labelling varied enormously: from 1–63%. However, there was a consistent decrease in the numbers of labelled central myonuclei with time. This may have been due to dilution of the relative numbers of labelled myonuclei due to other, nonlabelled, myoblasts replicating after the availability of tritiated thymidine, and fusing. It was also possible that labelled myofibres underwent subsequent necrosis and were eliminated from the muscle. The proposal that a regenerated myofibre can undergo a subsequent cycle of necrosis and regeneration was supported by evidence of some necrotic myofibres with labelled and unlabelled central nuclei. These results have implications for understanding the cellular biology and pathology of dystrophic muscle, particularly in relation to myoblast transfer therapy as a potential treatment of Duchenne muscular dystrophy.

Key words: Skeletal muscle; muscular dystrophy; myogenesis; muscle necrosis.

INTRODUCTION

Skeletal muscle regenerates by the proliferation of mononuclear myogenic precursor cells (myoblasts) that ultimately fuse and become incorporated into multinucleated myotubes, which later mature into myofibres. This process occurs during the embryonic histogenesis of muscle and in postnatal muscle regenerating in response to injury, or in myopathies such as Duchenne muscular dystrophy (DMD).

One of the most thoroughly investigated experimental animal models for muscular dystrophy is the mdx mouse, which is a genetic and biochemical homologue for DMD as both lack the subsarcolemmal protein, dystrophin. The lack of dystrophin results in cycles of skeletal muscle necrosis and regeneration. In mdx mice, this dystrophic process starts around 19–21 d after birth and is very active over the next 5 wk, and continues at a lower rate throughout the life of the mouse (Coulton et al. 1988a, b; Partridge et al. 1988; Beilharz et al. 1992; McGeachie et al. 1993). The pathology of the dystrophy varies markedly between mice and humans, with mice having an apparently sustained capacity for regeneration, whereas the muscles of DMD in young male humans are replaced by fat and connective tissue. The reasons for this marked species difference have not yet been elucidated (Grounds & Yablonka-Reuveni, 1993). However, more recently, closer examination of mdx mice, particularly older animals, is now revealing changes more reminiscent of the human myopathy (Partridge, 1997). Knowledge of the
cellular events involved in muscle regeneration has become increasingly important for understanding the biology of muscular dystrophy, particularly in relation to the advent of myoblast transfer therapy as a potential treatment for DMD (reviewed by Morgan, 1994; and commentaries by Grounds, 1996; Partridge et al. 1997). This therapy attempts to introduce normal (dystrophin-positive) myoblasts into dystrophic muscles so that they can fuse with host myofibres to replace the missing gene product, dystrophin. Clearly, the proliferation and fusion of myoblasts is of central importance for this therapy.

The present study was designed to investigate aspects of the timing of the fusion process and to determine the following in mdx muscles: (1) the minimum time that elapses between the replication of myoblasts and the fusion of their progeny into myotubes; and (2) the long-term pattern of myonuclear labelling after the pulse labelling of a cohort of replicating myoblasts, with the objective of trying to determine whether regenerated labelled dystrophic myofibres were ‘protected’ from subsequent necrosis, as was proposed by Karpati et al. (1988). To our knowledge, this proposal has not been tested directly.

Replicating myoblasts in muscles of mdx mice were labelled with tritiated thymidine (\(^{3}H\)-TdR) at 23 d of age, shortly after the onset of active muscular dystrophy (Beilharz et al. 1992; McGeachie et al. 1993), and sampled at various times (up to 30 d) after \(^{3}H\)-TdR injection. Autoradiographic analysis of tissue sections quantitated the proportions of labelled central nuclei within myotubes and myofibres. These labelled central myonuclei were the progeny of the labelled myoblasts which had fused into myotubes.

Pioneering autoradiographic studies by Moss & Leblond (1970, 1971) showed that, in growing muscle, satellite cells are the primary source of myoblastic cells. As the resultant myotubes matured, they retained their nuclei in the centre of the fibre for many months after being formed (McGeachie et al. 1993). These nuclei will be referred to as ‘central myonuclei’. The presence of central nuclei in muscle fibres is clear evidence that they have undergone a cycle of regeneration. In addition, the term ‘myoblast’ will be used to describe all mononucleated myogenic cells, before fusion into myotubes.

MATERIALS AND METHODS

Animals and labelling procedures

A total of 38 mdx mice, from 4 litters, were obtained from the Australian Neuromuscular Research Institute in Perth, Western Australia. Because of the need for so many mice and the limited availability of mdx mice, the litters were collected over a period of several months. All mice were aged 23 d at the start of the experiment: this is the time when muscle necrosis and regeneration are established (McGeachie et al. 1993). On d 23, each mouse received 2 intraperitoneal injections, 4 h apart, of 1 \(\mu\)Ci/gram body weight \(^{3}H\)-TdR (Amersham International; specific activity 5 Ci/mmole). This regime was designed to label the populations of myoblasts which were replicating within the hour following each injection.

The DNA synthesis (S) phase is considered to be about 6–8 h long. Thus the progeny of these labelled myoblasts could be traced with time as they became incorporated into myotubes and more mature myofibres where the labelled central nuclei were detected autoradiographically (Figs 1, 2). At 8 h to 30 d after the first \(^{3}H\)-TdR injection, each mouse was killed with halothane anaesthesia followed by cervical dislocation. The tibialis anterior (TA) muscles were removed from both legs of each mouse, together with samples of small intestine (to check the autoradiographic exposure and labelled cell distribution with time after injection). All tissues were fixed by immersion in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight followed by postfixation in 1% OsO\(_{4}\) in phosphate buffer for 1 h. Tissues were washed in 70% ethanol and block stained in 1% paraphenylenediamine in 70% ethanol for 1 h (Dilley & McGeachie, 1983). Tissues were embedded in a 1:1 Araldite/Epon mixture and 1 \(\mu\)m transverse sections cut for autoradiography. Sections on glass slides were coated with Kodak NTB-2 dipping emulsion, exposed for 2 wk, developed in Kodak D19, fixed in acid-hardener fixer, washed and dried.

Analysis

All analyses of autoradiographs were performed ‘blindly’, the microscopist (JMcG) did not know from which animal (litter or time after injection of \(^{3}H\)-TdR) the muscle samples were taken. Transverse sections were viewed with a light microscope, using a \(\times 100\) oil immersion objective. For both TA muscles of each of the 38 mdx mice, at least 500 central myonuclei were examined for the presence of autoradiographic grains. The minimum number of grains considered to represent labelling was 2 because this is consistent with the standard protocols in our laboratory where the background in these preparations is very low. The data from both legs were pooled for individual mice.
From these data the percentages of labelled central myonuclei were calculated and used to measure the distribution of labelling with time after injection of $^3$H-TdR. The highest labelling levels (grains/central myonucleus) were also recorded for the 3 most heavily labelled such nuclei for each mouse.

RESULTS

All the 76 (38 × 2) muscles showed clear evidence of dystrophy, with randomly distributed patches of muscle necrosis and regeneration, but there was a very considerable degree of variability in the extent of dystrophy both within and between individual mdx mice. The proportions of myofibre profiles with central nuclei in these dystrophic muscles increased with time, although these data were not collected for this present study. This was consistent with the appearance of other mdx muscles that have previously been analysed in detail in this laboratory (Grounds & McGeachie, 1989; McGeachie et al. 1993).

Examples of autoradiographically labelled central myonuclei are shown in Figure 2a. The labelling data are given in the Table and displayed graphically in Figures 3 and 4. In the earliest muscle sampled, at 8 h after injection of $^3$H-TdR, there was no labelling of central myonuclei, showing that the replicating myoblasts had not had sufficient time to pass from S phase of the cell cycle and through the G2 and M phases, to become fused into myotubes (see Fig. 1). However, by
Fig. 2. Examples of autoradiographic labelling in muscle nuclei. (A) Two examples of labelling in both central and peripheral (PMN) muscle nuclei in mdx muscle. Unlabelled nuclei are also shown. (B) A large necrotic-labelled myofibre, focused on the labelled nuclear areas on the left and focused on the tissue on the right. The irregular blotch in the midleft of the central area in both prints is an artefact in the preparation. These necrotic-labelled myofibres were extremely difficult to photograph because of the apparently rapid breakdown of the cells and the dispersion of the label. The autoradiographic background is very low in these preparations and normally grains were very rarely seen over the sarcoplasm.

24 h, 2% of central myonuclei were labelled in 1 of the 3 mice sampled at this time, whilst the other 2 had no such labelling. This shows that some myoblasts had completed the cell cycle and had become incorporated into myotubes within 24 h. By 2 d, both mice had labelled central myonuclei, and at 3 and 4 d the level in 1 mouse reached as high as 74% (Table, Fig. 3). From 5–30 d the levels of central myonuclear labelling varied enormously, from 1–63% (Table, Fig. 3).

There was a consistent trend towards a general decrease in the percentages of labelled central myonuclei over time (Fig. 3). This decrease was noted from about 2 wk for litters C and D. It occurred earlier with litter A. Litters A and B had overall much lower labelling levels compared with C and D.

During the analysis it was noticed that a few myofibres with labelled central nuclei were undergoing necrosis (Fig. 2b). They were not seen in muscles
Fig. 3. Data for the percentages of labelled central myonuclei in the mdx mice with time after injection of $^3$H-TdR at 23 d of age. The data for each of the 4 litters used in this study are presented separately (see Table).

Fig. 4. Data for the relative (%) highest 3 grain counts over labelled central myonuclei for each of the litters used in this study. The highest grain counts for each litter are shown on the 100% line and these levels were used to compare the other grain counts for each mouse in each litter.

sampled within 13 d of labelling, i.e., they were evident from 10 d after the peak level of incorporation of labelled myoblasts into myotubes. The necrotic fibres with labelled myonuclei were too sparse to count in any meaningful way, but this suggested that some of the myotubes formed around 27 d of age in the mdx mice did not survive, but instead underwent subsequent necrosis from about 2 wk after being formed. Furthermore, a subsequent analysis of the presence of necrotic fibres with unlabelled central nuclei revealed that there were a few such cells in muscles from 16–30 d after injection of tritiated thymidine. As for
Table. Autoradiographic labelling levels of central myonuclei of mdx mice at different times after the injection of $^{3}\text{H-TdR}$, at 23 d of age

<table>
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<th>mdx mouse number</th>
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* Labelled central myonuclei are expressed as percentages of all centrally situated (nonperipheral) myonuclei, both labelled and unlabelled.

labelled central nuclei in necrotic fibres, there were too few to make any firm conclusions or to conduct any meaningful analysis.

Grain counts in labelled myotube nuclei

These were undertaken as part of the routine analytical protocol in our laboratory because the counts often reveal insights into the distribution of labelling levels with time after the injection of $^{3}\text{H-TdR}$, and made possible interpretations of cell generations (Grounds & McGeachie, 1987, 1989). Before reporting the data on grain counts in the present mdx experiment it is pertinent to describe the salient features of the labelling in myoblasts with time after injection of $^{3}\text{H-TdR}$, because the interpretation is rather complicated.

When $^{3}\text{H-TdR}$ is injected in vivo, it is available to all cells synthesising DNA in preparation for mitosis, for a period of about 1 h. Cells (in this case myoblasts) in the most active phase of DNA synthesis will incorporate maximal amounts of $^{3}\text{H-TdR}$, compared with myoblasts which are just commencing, or just completing DNA synthesis. Thus some myoblasts will incorporate less label than those which are synthesising DNA for the whole period of its availability (of about 1 h). Correspondingly, when all of these cells have passed through mitosis, there will be a wide distribution of labelling levels (grain counts) in the autoradiographs, the highest levels being in those cells which were in maximal DNA synthesis during the full availability of label. Another factor which will further dilute the eventual labelling in myonuclei is the number of mitotic divisions of the labelled myoblasts which occur in vivo prior to fusion.

Based on the above information, the grain counts in the present experiment were recorded. The only meaningful data were the maximal levels of labelling because, as explained above, there was a very wide distribution of labelling levels. All labelled myonuclei received their label during the 1 h period following each injection of $^{3}\text{H-TdR}$ (at 23 d of age) and these cells either fused into myotubes shortly after the mitotic division following labelling, or they may have undergone subsequent divisions (with halving of the label with each cell division) before fusing. Once incorporated into myotubes the labelled myonuclei are fixed in the myofibre for the life of that cell.

If the maximal grain counts of myonuclei had been very high during the first few days (after labelling), but decreased with time, then it could be concluded that these labelled myonuclei were relatively unstable over time. On the other hand, if the maximal grain counts of myonuclei were relatively similar throughout the 30 d time course of the experiment, then the labelled myoblasts which had fused shortly after dividing once had a similar life-span as other labelled myoblasts. This was the basis of the interpretation of the grain count data.

In each mdx mouse the 3 highest labelling levels (grains per central myonucleus) were recorded, and the highest individual level was determined; this latter maximum labelling level was determined as a 100% reference point, and was used to compare the labelling levels for each animal within each litter. It was necessary to analyse the 4 different litters separately because they were collected over many months (because of the shortage of mdx mice at the time) and thus processed in different batches. The relative levels of labelling of the 3 heaviest grain counts for each animal were calculated as percentages of the maximal level (100%) for that litter.

The results are shown in Figure 4. Whilst litter A showed a consistent decrease over time, this was not clearcut for the other litters. The peak level of labelling intensity (highest grain counts) varied between litters: A, 2 d; B, 15 d; C, 4 d; D, 26 d. Moreover, the relative levels of labelling in the 4 litters were generally high throughout the time period of the study, indicating
that many heavily labelled myonuclei were formed early in the timecourse of the experiment and survived for the 30 d after injection of \(^{3}H\)-TdR. Whilst the grain count data show that many of the newly-formed myotubes are clearly stable, these data do not exclude the possibility that some labelled myofibres subsequently succumbed to necrosis and, indeed, the data for litter A support this notion (see the Discussion below).

**DISCUSSION**

**Timing of myoblast fusion into myotubes**

The primary objective of this study was to determine the minimum and peak times between the replication of a cohort of myoblasts, labelled as a result of a pulse of \(^{3}H\)-TdR, and their incorporation into myotubes.

The data from this relatively simple experiment show that fusion of myoblasts into myotubes does not occur at 8 h after the labelling of replicating myoblasts. By 24 h, there is only minimal incorporation of myoblasts into myotubes, and it is not until 48 h that any substantial incorporation takes place. Since the onset of the dystrophic process of muscle fibre necrosis and subsequent regeneration in mdx muscles is known to be around 19 d after birth (McGeachie et al. 1993), some of the replicating myoblasts at 23 d will already have had the opportunity to divide one or more times before being labelled by the \(^{3}H\)-TdR. In contrast to similar autoradiographic studies after experimental muscle injury, where there is a clear ‘time-0’ for the activation of myoblasts (McGeachie & Grounds 1987, 1990, 1995; Grounds & McGeachie, 1987, 1989, 1992), this study was not designed to measure the onset of myogenic cell replication, but rather to label a mixed population of recently activated myoblasts in order to ascertain the minimum time it takes for them to fuse into myotubes after DNA synthesis. It is now recognised that myoblasts in regenerating mouse or chicken muscle in vivo can fuse after a single mitotic division (Grounds & McGeachie 1987, 1989); or it may be that they can even fuse without first replicating, although this cannot be assessed by autoradiographic studies. They thus react opportunistically to the environment, fusing when conditions are appropriate. There is no reason to think that this is not also the case in the regenerative phase of muscular dystrophy.

By 3 and 4 d after injection of label there were substantial numbers of labelled myotube nuclei (up to 74% in one case), indicating that most labelled myoblasts had fused within this time. It is well established that in adult mouse muscle regenerating after experimental injury, myoblast replication and fusion are essentially completed by 7 d (Grounds & McGeachie, 1987, 1989; McGeachie & Grounds, 1987, 1990, 1995). Such is also the case for mdx muscle regenerating after a crush injury (Grounds & McGeachie, 1992). In the present study, the data for peak incorporation of myoblasts into myotubes indicate that if donor myoblasts were introduced into a regenerating system, as occurs in myoblast transfer therapy, then there would be quite a narrow window of time in which the introduced donor myoblasts would have the opportunity to fuse with host myoblasts, or with newly formed myotubes, or with the host myofibres.

**Are regenerated dystrophic myofibres protected from subsequent necrosis?**

This was the second and much more problematic objective of the present study. One of the major difficulties in studying mdx mice is the enormous variability within and between animals. For example, the necrotic and regenerative foci vary between muscles, even within the same animal. Such was the case in the very extensive study we reported previously (McGeachie et al. 1993). Moreover, in the present study, the levels of labelling in the 4 different litters were markedly different. Because of limitations in the numbers of animals in any given litter, as well as the limited numbers of litters available, it was necessary to collect and process samples from the 4 litters over many months. Nevertheless, there were foci of necrosis and regeneration to varying degrees in all 76 tibialis anterior muscles sampled from the 38 mice in the study.

If regenerated dystrophic myotubes are indeed protected from subsequent necrosis (Karpati et al. 1988) then the labelled muscle nuclei would retain high maximal counts of \(^{3}H\)-TdR with time after incorporation into myotubes, that is, these labelled nuclei would survive in the myotubes and in the myofibres and would not be lost by subsequent necrosis. Thus it would be predicted that the maximal grain counts in labelled central myonuclei at later stages of the experiment (up to 30 d after injection of \(^{3}H\)-TdR) would remain as high as those in the period of maximal fusion, at 3–4 d.

The maximal grain count data for 3 of the litters show that the labelled central myonuclei did in fact retain reasonably high levels of label up to 30 d after injection, although this was quite variable (Fig. 4), these data therefore generally support the ‘survival’
hypothesis. While many of the newly formed myotubes are stable, these data do not exclude the possibility that some of the regenerated myofibres subsequently underwent necrosis and, indeed, the data for litter A support such a proposal. Furthermore, that the latter occurs is demonstrated by the appearance of labelled nuclei in necrotic myofibres. A formal possibility is that the labelled myonuclei had moved within the dystrophic myofibre away from the original site of fusion with the resealed end of the damaged myofibre, into a part of the myofibre that was ‘naive’ and had not yet succumbed to a cycle of focal necrosis and regeneration. We do not know of any in vivo data that support such movement of myonuclei within mature myofibres. It is of interest to note that in mature normal (Robertson et al. 1993) and dystrophic (Morgan et al. 1990) muscle regenerating in vivo, there is good evidence that the newly formed myotubes (e.g. containing labelled myonuclei) do not fuse with the resealed/damaged myofibres until about a week after injury: this implies that at least 7 d should elapse before any potential movement of labelled myonuclei within a myofibre (i.e. away from the site of fusion) could even be considered to occur.

Analysis of the relative proportions of labelled central myonuclei show a clear decrease over time and, at first glance, these data also support the idea that some labelled myofibres are lost over time, presumably due to subsequent necrosis. However, there is an alternative explanation which involves dilution of the overall numbers of labelled central myonuclei. The scenario is complicated by the fact that in this experiment, only a small cohort of myogenic cells was pulse labelled (at 23 d) and their progeny followed subsequently. Since progressive cycles of necrosis and regeneration continue to occur in the dystrophic muscles, many other myogenic cells which were proliferating at times before and after the availability of label, would complete the myogenic cycle and fuse into myotubes, thus adding to the population of unlabelled myonuclei. The relative levels of labelled myonuclei with time will always be diminished and complicated by this factor.

Autoradiographic studies in experimental models of muscle necrosis and regeneration show that the necrotic process and phagocytosis of cellular debris occurs within 2 d after damage, myoblasts replicate between 1 and 7 d and myotubes are first formed by 3–4 d after injury (Grounds & McGeeachie, 1987, 1989, 1992; McGeeachie & Grounds, 1987, 1990, 1995). These data from experimental injuries to muscle are similar to those in the present mdx study, where endogenous necrosis and regeneration occur in the dystrophic muscle. Therefore, it would be predicted that in the present experiment, if the necrosis and regeneration occurred in a cyclical fashion (within a single myofibre), a second wave of central myonuclei (unlabelled in this case) could be evident from about 10 d after the injection of $^3$H-TdR, that is, there would be a predictable drop in labelled myonuclei some 10 d after injection of label. Such a trend was apparent for each litter of mice examined (see Fig. 3), and it is therefore concluded that some myotubes in mdx muscle (at least in the early stages of the disease) survive for about a week or so after being formed, before undergoing a subsequent cycle of necrosis.

In conclusion, while many regenerated myofibres are stable within the period of time studied (4 wk after the onset of the dystrophic process), the appearance of labelled myonuclei in necrotic myofibres is compelling evidence that the regenerated dystrophic myofibres are not necessarily protected from subsequent cycles of necrosis and regeneration.

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