The role of p53 in vivo during skeletal muscle post-natal development and regeneration: studies in p53 knockout mice

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ABSTRACT The tumour suppressor gene p53 is recognised as a central regulator of the cell cycle and apoptosis. Post-natally, p53 mutations are associated with many cancers and mice lacking p53 are prone to spontaneous tumour formation. The present study examines skeletal muscle formation in post-natal mice lacking p53 using two different models of skeletal muscle regeneration. The level of endogenous myogenic cell proliferation in mature skeletal muscle was examined and the time course of muscle regeneration after whole muscle transplantation or crush injury were compared in p53 (-/-) and control C57Bl/6J adult mice, using desmin and proliferating cell nuclear antigen (PCNA) immunohistochemistry and histological analysis. The pattern of inflammation, myoblast proliferation and myotube formation in regenerating p53 (-/-) skeletal muscles appears normal and similar to those in control C57Bl/6J muscle. These data indicate that p53 is not required for the regulation of myoblast proliferation, differentiation and myotube formation in vivo during myogenesis of adult skeletal muscle.

KEY WORDS: p53, knockout mouse, skeletal muscle, regeneration, whole muscle graft, fusion, proliferation

Introduction

Skeletal muscle has an exceptional ability to regenerate after tissue breakdown following mechanical or chemical injury and pathological insult. The regeneration of damaged skeletal muscle follows a series of clearly defined events beginning with myofibre necrosis (Grounds, 1991). This results in inflammatory cells such as polymorphonuclear leucocytes and macrophages being attracted to the site of damage where they phagocytose myofibre debris and also produce many factors that stimulate myogenesis. In response to muscle damage, quiescent muscle precursor cells on the surface of myofibres (called satellite cells) are activated and these are widely referred to as myoblasts. The myoblasts proliferate, exit the cell cycle, differentiate, and fuse together to form multinucleated myotubes; these then fuse with the ‘sealed’ ends of the damaged myofibres to repair the myofibres (Roberston et al., 1993). Re-innervation completes the process of new muscle formation.

The differentiation of myoblasts is characterised by permanent withdrawal from the cell cycle, activation of muscle specific gene expression, and fusion into multinucleated myotubes. The activation, proliferation, differentiation and fusion of myoblasts to form myotubes, in the absence of p53, has been studied in vitro where myotube formation is severely impaired (Porrello et al., 2000). The tumour suppressor gene p53 is a transcription factor commonly considered to be the “gatekeeper for cell replication”, being important for maintaining the quiescent state of cells (Levine et al., 1991).

The development of a viable p53 knockout (-/-) mouse demonstrates that the p53 molecule is not critical during development. The p53 (-/-) mouse is however susceptible to spontaneous tumour formation after birth (Donehower et al., 1992) indicating a basic requirement for the p53 gene post-natally, possibly with particular relevance to preventing accumulation of mutations within the genome (Levine, 1997). In addition, hepatocytes from adult livers of p53(-/-) mice divide at over 2.5 times the rate of normal mice and show altered morphology in vivo (Dumble et al., 2001) supporting the proposal that p53 may play an important role post-natally in some cell types.

This study examines the role of p53 in skeletal muscle of adult mice. It specifically tests the hypothesis that the absence of p53 will result in extended myoblast proliferation, delayed differentiation and subsequently delayed myotube formation, during skeletal muscle regeneration in p53(-/-), compared to wild type control mice.

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Results

Uninjured Post-Natal Skeletal Muscle and Liver

Cell Proliferation. Cell proliferation was examined by PCNA immunohistochemistry in uninjured adult skeletal muscle of p53(-/-) and control mice aged 2, 3, 6, 16 and 32 weeks of age (Fig. 1). The numbers of PCNA positive nuclei per unit area in control and p53(-/-) skeletal muscle are summarised in Fig. 1A. Although PCNA positive cells were also present in areas of connective tissue and blood vessels, these were not included in the quantitative analysis; only nuclei lying within myofibres or intimately associated with the surface of myofibres were counted (Fig. 1B). At 2 weeks of age significant cell proliferation was present in p53(-/-) and control skeletal muscles, but by 3 weeks cell proliferation was considerably reduced in muscles of both strains. In control skeletal muscle this low (background) level of cell proliferation was maintained out to 32 weeks of age. In contrast, in the oldest p53(-/-) skeletal muscle examined (at 32 weeks) a significant increase in cell proliferation was noted (Fig. 1A). We were unable to obtain p53(-/-) skeletal muscle from older mice due to the increasing tumour load and poor health of the animals after this age.

In contrast to skeletal muscle, many proliferating cells were present in the liver of the p53(-/-) mice at all ages, and this was most pronounced in peri-portal regions (Fig. 1C). This pattern of cell proliferation was never observed in livers from control mice. There was marked heterogeneity in the liver histopathology between mice and this did not seem to be age related. However, the pathology was consistent throughout each liver. A detailed analysis of cell replication in the livers of p53(-/-) mice was not done, but these observations accord closely with those of Dumble et al. (2001). The number of PCNA positive cells observed in skeletal muscle was not correlated with the severity of the liver histopathology in individual p53(-/-) mice.

Studies of Regenerating Skeletal Muscle

Whole Muscle Autografts. The morphological changes occurring during regeneration in whole skeletal muscle autografts are extensively described elsewhere (Roberts et al., 1997; White et al., 2000). The main features of this process are the presence of two distinct zones within a transverse section of the EDL graft: (i) the central necrotic zone and; (ii) the peripheral regenerative zone.

Fig. 1. Cell proliferation in post-natal tissues. PCNA immunohistochemistry was used to evaluate cell replication in post-natal TA muscle and liver of p53(-/-) and control C57Bl/6J mice aged 2 to 32 weeks of age. (A) Quantification of cell proliferation in uninjured TA muscles. The number of PCNA positive nuclei was counted in 10 randomly selected non-contiguous fields of view (at 200x magnification) and the mean numbers of positive nuclei are expressed per mm². (B) In longitudinal sections of p53(-/-) muscle, PCNA positive nuclei are associated with myofibres (arrows) and blood vessels (arrowheads). (C) The livers from p53(-/-) mice show many PCNA positive cells around the peri-portal regions. The severity of the liver pathology in p53(-/-) mice ranged from mild, to moderate (shown here), to severe. Scale bar, 50 µm.

Fig. 2. Desmin immunostaining of regenerating whole muscle autografts. Desmin immunohistochemistry on transverse sections of p53(-/-) (A,C) and control C57Bl10/6J (B,D) whole muscle autografts. At day 3, myotubes (arrows) are scarce and small in both p53(-/-) (A) and C57Bl/6J (B) autografts. At day 7, many desmin positive myotubes (arrows) are present and similar numbers of myotubes are seen in both p53(-/-) (C) and control (D) autografts. In all grafts some surviving myofibres (*) which have not undergone necrosis are present near the interface of the graft and the underlying TA muscle. At day 10, myotube formation is essentially complete with myotubes covering the entire area of the p53(-/-) (E) and control (F) grafts. Scale bar, 50 µm.
and the data are summarised in Fig. 3. At all times there was no
counted in a single transverse section of each graft at days 3, 5 and
control C57Bl/6J mice (Fig. 2E,F). The numbers of myotubes were
complete and myotubes filled the entire graft in both p53(-/-) and
both strains (Fig. 2C,D). At day 10, regeneration was essentially
the regenerative zone was approaching the centre of the grafts in
C57Bl/6J mice. At day 7, myotube formation had progressed and
regenerating zone at the periphery of grafts in both p53(-/-) and
control C57Bl/6J autografts at 3, 5 and 7 days after transplantation. The total
numbers of myotubes were counted in a single transverse section of each
graft. Each vertical column represents a single graft. There was no significant
difference in myotube formation between the two strains at any time.

(-inflammatory cells, myoblasts and myotubes), which starts at the
very edge of the graft and progressively moves inwards to replace
the necrotic tissue.

At 3 days after surgery, very few myotubes were present in any
of the grafts examined and there was no significant difference in the
number of myotubes in p53(-/-) compared to C57BL/6J grafts. In three of the four p53(-/-) grafts at 3 days, no myotubes were
apparent, and in the remaining graft only a single myotube was
observed (Fig. 2A). In C57BL/6J mice at day 3, a single graft had
no myotubes and the remaining three had isolated myotubes (Fig.
2B). At day 5, desmin immunostaining was pronounced in myo-
blasts and in “cuffing cells” (activated satellite cells) lining the
contour of the persisting basement membrane of necrotic myofibres.
Many plump desmin positive myotubes were present in the regener-
ating zone at the periphery of grafts in both p53(-/-) and control
C57Bl/6J mice. At day 7, myotube formation had progressed and
the regenerative zone was approaching the centre of the grafts in
both strains (Fig. 2C,D). At day 10, regeneration was essentially
complete and myotubes filled the entire graft in both p53(-/-) and
control C57Bl/6J mice (Fig. 2E,F). The numbers of myotubes were
 counted in a single transverse section of each graft at days 3, 5 and
7 and the data are summarised in Fig. 3. At all times there was no
significant difference in the extent of myotube formation between
p53(-/-) and control autografts. The numbers of myotubes in day 10
and 14 grafts was not quantified, as myotube formation was
essentially complete with myotubes covering the entire area of the
grafts at these times.

Crush Injured Muscles. The regenerative process is more difficult to
quantify after severe crush injury but the pattern of regeneration in
p53(-/-) skeletal muscle after crush injury was indistinguishable
from control C57Bl/6J muscles in the few samples studied. In both
strains, equivalent myotube formation was seen at day 7 and 14
(Fig. 4).

Discussion

Cell Replication in Post-Natal p53 (-/-) Skeletal Muscle is
Normal

Using PCNA immunohistochemistry, no difference in the extent
of cell replication in post-natal skeletal muscle was observed
between p53(-/-) and control mice up to the age of 32 weeks. In
contrast, in the livers of p53(-/-) mice many proliferating blast-like
cells were present; these were not seen in control livers. The
numbers of proliferating cells in p53(-/-) livers did not seem to be
age related and the severity of the pathology was variable. Even in
younger mice that exhibited a severe liver phenotype, cell replica-
lation in the skeletal muscle was the same as control muscle. The
striking difference in pathology between various tissues in post-
natal p53(-/-) mice is the subject of a separate investigation.

The significant increase in cell proliferation in skeletal muscle of
32 week old p53(-/-) mice suggests that even skeletal muscle may
be susceptible to pathology over time. We were unable to analyse
muscle from older mice as most died beyond 32 weeks of age.
While the change in old skeletal muscle might reflect an age-
related instability of the myofibres lacking p53, it might well be an
indirect effect resulting from the declining health of the animals,
due to significant tumour formation by this time.

Regeneration is Unaffected in p53 (-/-) Skeletal Muscle

Skeletal muscle regeneration was examined in p53(-/-) mice to
test the hypothesis that sustained myoblast proliferation and/or
delayed differentiation would result in delayed myotube formation.
Two models of skeletal muscle regeneration were used. In whole
autografts of EDL muscles, the onset and extent of myotube forma-
tion, examined between 3 to 14 days after transplantation, was the same in p53(-/
/-) and control C57Bl/6J grafts at all
times. Significant numbers of
myotubes were first observed at day
5. This accords with the onset of
myotube formation seen in other
strains of mice such as BALB/c and
SJL/J (Roberts et al., 1997; Smythe
et al., 2001; White et al., 2000). The
pattern of cellular events during re-
generation was indistinguishable
between p53(-/-) and control muscle
autografts. Similarly, after crush in-
jury where there was severe disrup-
tion to muscle architecture, myotube

Fig. 3. Quantitation of myotube formation in whole muscle autografts.
Numbers of desmin positive myotubes in transverse sections of p53(-/-) and
C57Bl/6J autografts at 3, 5 and 7 days after transplantation. The total
numbers of myotubes were counted in a single transverse section of each
graft. Each vertical column represents a single graft. There was no significant
difference in myotube formation between the two strains at any time.

Fig. 4. Myotube formation after crush injury in p53 (-/-) and control skeletal muscle. Desmin
immunohistochemistry on muscles from p53(-/-) (A) and control C57Bl/6J (B) at 14 days after crush injury.
Large myotubes (arrows) with striations are apparent in injured muscle from both strains. Scale bar, 50 µm.
formation was essentially the same at 7 days in p53(-/-) and control mice and was complete at 14 days in both strains. There are some fundamental differences between the dynamics of skeletal muscle regeneration in these two models of injury. Regeneration occurs in whole muscles grafted within the persisting architecture of the basement membrane, but the grafted muscle is removed from both vascular and nervous supply. This is in stark contrast to crush injury where there is significant disruption to the muscle structure, but the blood and nervous supply remains relatively intact (Mitchell et al., 1993). While regeneration can occur in the absence of a basement membrane (Caldwell et al., 1990), the presence of this extracellular matrix scaffold facilitates regeneration. However, the pattern of myotube formation was not affected in either the whole muscle graft or crush injury models in the absence of p53.

The two events of cell proliferation and differentiation are mutually exclusive since myoblasts exit the cell cycle prior to differentiation and subsequent myotube formation. We have previously shown using the whole muscle graft model, that in MyoD(-/-) muscle where myoblast proliferation is sustained for 2-3 days (compared with controls) this results in a 2-3 day delay in myotube formation (White et al., 2000). In regenerating whole muscle grafts of desmin(-/-) mice myotube formation was delayed by 1 day (Smythe et al., 2001). However, no such delay (or enhancement) in myotube formation was seen in regenerating p53(-/-) muscles. These observations provide strong evidence that there is no defect in the regulation in vivo of myoblast proliferation and fusion in the absence of p53.

The behaviour of myogenic cells during myogenesis of developing skeletal muscle appears to be essentially the same as in regenerating adult muscle. The most striking difference between myogenesis during embryogenesis and in post-natal muscle is the presence of inflammatory cells during regeneration (Grounds, 1991). The apparently normal development of skeletal muscle in p53(-/-) mice (Donehower et al., 1992) and the ‘normal’ regeneration shown here in post-natal p53(-/-) muscle indicates that p53 is either not a significant regulator of muscle cells during development or regeneration, or that some other factor is compensating for the absence of p53 in these null mice. Two members of the p53 family, p63 and p73, show weak but transcriptionally active heterotypic interactions (Davison et al., 1999; Di Como et al., 1999). The p63 and p73 share homology with p53 within the activation, DNA binding and oligomerisation domains. Furthermore, when over-expressed, p63 and p73 are able to transactivate some p53 target genes (Kaelin, 1999; Oren, 1997). Mice lacking p63 or p73 have more marked developmental defects than those seen in p53(-/-) mice (Mills et al., 1999; Yang et al., 2000). However, these papers do not describe embryonic skeletal muscle development in p63 and p73 null mice. Thus it is not unreasonable to predict that in vivo, p63 or p73 may compensate for the lack of p53. While in vitro evidence suggests that p63 and p73 cannot successfully substitute for the role of p53 in muscle differentiation (Porrello et al., 2000), this does not exclude the possibility of such compensatory mechanisms in the more complex in vivo situation.

### A Possible Role for p53 in the Molecular Regulation of Muscle Differentiation

Cell cycle withdrawal is associated with increased expression of the cdk inhibitor p21 (a p53 inducible gene) which in turn inhibits the phosphorylation of retinoblastoma protein (pRb) and further promotes cell cycle withdrawal. In cultures of primary skeletal muscle and myogenic cells lines it has been shown that pRb co-operates with the skeletal muscle specific transcription factor MyoD to promote the expression of late markers of differentiation (Chen et al., 1996; Gu et al., 1993; Novitch et al., 1996) through activation of MEF2 (Novitch et al., 1999). Active pRb is required for proper functional activity of MyoD during skeletal muscle differentiation (Gu et al., 1993; Novitch et al., 1996; Novitch et al., 1999). Within the promoter region of pRb there are p53 binding sites (Osifchin et al., 1994) and p53 binds the Rb promoter in skeletal muscle cells (Porrello et al., 2000), indicating that such transcriptional control mechanisms may play a role during cessation of replication and the onset of differentiation in myoblasts. Expression of p53 increases during differentiation and fusion of skeletal muscle cells in vitro (Haley u., 1995; Soddu et al., 1996; Tamir and Bengal, 1998), and p53 is thought to co-operate with MyoD in the transcriptional induction of muscle creatine kinase (MCK), a gene that is induced during muscle differentiation. While these tissue culture studies using both primary p53(-/-) skeletal muscle cells and dominant negative p53 myogenic cells lines strongly support a role for p53 in myogenesis this is clearly not manifested in the in vivo environment.

In stark contrast to the excellent myotube formation in vivo observed in regenerating adult p53(-/-) muscle in the present study, myotube formation in vitro is impaired in myogenic C2C12 cells expressing a dominant negative p53 protein and in primary myoblasts derived from p53(-/-) muscle. After growth factor withdrawal in vitro, these myoblasts exit the cell cycle but fail to effectively form myotubes (Porrello et al., 2000). These p53 negative myoblasts do not up-regulate pRb and have a reduced MyoD activity.

In the above in vitro studies differentiation was induced by growth factor withdrawal. Differentiation of skeletal muscle cells can alternatively result from increasing cell-cell contact which occurs as cultures reach confluence (Martelli et al., 1994). It has been proposed that induction of cell cycle (cdk) inhibitors, either by cell-cell contact (involving p27) or growth factor withdrawal (involving p21), maintains the activity of MyoD in the differentiated myotube (Martelli et al., 1994; Porrello et al., 2000). Interestingly, Porrello et al., (2000) report as ‘unpublished observations’ that confluent cultures of both primary p53(-/-) myoblasts and dominant negative p53 C2C12’s up-regulate Rb in a p53 independent manner. There is intimate cell-cell contact in vivo in developing and regenerating p53(-/-) muscle where effective myotube formation is observed. Therefore, Rb may be upregulated independent of p53 in vivo and the critical role of cell contact may possibly account for the differences between in vivo and in vitro results.

This study emphasises the importance of following up initial in vitro observations with carefully considered in vivo studies to test overall biological significance. A striking difference between the
responses of liver and skeletal muscle to the absence of p53 is evident in vivo. The complex regulation of biological processes in the in vivo environment, including the intimate contact between many cell types and inherent flexibility and redundancy of many signalling networks, can quite frequently compensate for the absence of a single factor considered in isolation. For these reasons, the present study clearly demonstrates no essential role for p53 during both development and regeneration of skeletal muscle in vivo.

Materials and Methods

Animals

p53(-/-) and C57Bl/6J mice were obtained from specific pathogen free colonies held at the Animal Resources Centre, Murdoch, Western Australia. These colonies were established from mice generously supplied by Prof. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA, USA). Female p53 (+/+) mice were crossed with male p53(-/-) mice to generate p53(-/-) and p53(+/-) offspring. These mice are difficult to breed and thus the supply of experimental mice was restricted and only limited numbers of p53(-/-) mice were available for the present study. The p53 gene status was assessed individually using PCR (Jacks et al., 1994). Experiments were conducted in strict accordance with guidelines of the Animal Ethics Committee of The University of Western Australia and the National Health and Medical Research Council, Australia. All mice were housed in individual cages under a 12-hour day/night cycle and allowed access to food and water ad libitum.

Surgical Procedures

Autografts of whole muscles were made in both legs of 15 p53(-/-) and 10 C57Bl/6J mice. The transplantation procedure for whole muscle grafts has been described in detail previously (Roberts et al., 1997; White et al., 2000). In brief, mice were anaesthetised with a gaseous mixture of halothane, N2O and O2, and the extensor digitorum longus (EDL) muscles were removed from both hind legs of each mouse and were relocated over the tibialis anterior (TA) muscles of the same leg. Each EDL autograft was sutured proximally to the distal tendon of the quadriceps femoris muscle and distally to the distal tendon of the TA and the skin closed with 6.0 braided silk sutures. Grafts were left for 2 to 14 days before sample collection and tissue processing. Regeneration was also studied in two p53(-/-) and two C57Bl/6J muscle after crush injury, as described previously (Mitchell et al., 1992).

Tissue Collection and Processing

All mice were killed by cervical dislocation. For studies on regenerating muscles, samples were taken at 3, 5, 7, 10 and 14 days after grafting and at 7 and 14 days after crush injury (Table 1). For grafted muscles, both the EDL graft with the underlying (undamaged) TA was dissected and for crush injuries the TA was taken. In addition, for p53(-/-) and control mice at various ages (2, 6, 8, 16 and 32 weeks) the TA and liver were removed: at least two samples were taken at each time. In addition for p53(-/-) and control mice after crush injury, two C57Bl/6J muscle after crush injury, as described previously (Roberts et al., 1992).

Immunohistochemistry

Antibodies. The primary antibodies used were a polyclonal rabbit anti-desmin (DAKO Corporation, Carpinteria, CA, USA), and rabbit polyclonal anti-PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The biotinylated secondary antibody used was a donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and the biotin conjugate was detected with horseradish peroxidase conjugated avidin D (Vector Laboratories, Burlingame, CA, USA).

Immunohistochemical Staining. The cytoskeletal protein desmin is an excellent marker for identifying activated muscle precursor cells and early myotubes in vivo (Lawson-Smith and McGeachie, 1998). Desmin is an intermediate filament that is rapidly up-regulated in activated myoblasts and in mature striated muscle desmin is associated with the Z disks (Smythe et al., 2001). Desmin was used to identify myoblasts and myotubes within the muscle grafts. Desmin immunohistochemistry in paraffin sections required high temperature antigen retrieval in citrate buffer pH 6.0 (Lawson-Smith and McGeachie, 1998) and the procedure was the same as described by White et al., 2000. Replicating cells in grafts were stained in paraffin sections using PCNA immunohistochemistry as for desmin staining except that antigen retrieval was not required for PCNA detection (White et al., 2000).

Analysis of Tissues

Histological analysis and counts of desmin and PCNA positive cells were performed using Image Pro Plus 4.0 (Media Cybernetics, Silver Spring, ML, USA). The pattern of regeneration in transverse sections of whole muscle grafts is uniform throughout the length of the graft (White et al., 2000), thus all analyses were based on a single representative transverse sections from the mid region of each graft. For desmin stained sections the number of positive myotubes within an entire transverse section were counted and tagged to avoid double counting. The number of myotubes in each graft was corrected for the area of regenerating tissue. The level of significance between p53(-/-) and control grafts at each time was tested with a Student’s t-test. A result was deemed to be significantly different if P<0.05.

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References


