Muscle Growth Factors, Ubiquitin and Apoptosis in Dystrophic Muscle: Apoptosis Declines with Age in the mdx Mouse

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Abstract

The pathology of Duchenne Muscular Dystrophy (DMD) is characterised by unstable muscle fibres and by increased cell turnover which is due to the absence of functional dystrophin protein. Previously we have used skeletal muscle, primary muscle stem cell cultures (Smith & Schofield, 1994 (a)) and clonal skeletal muscle stem cell lines of the mouse DMD model (mdx) and its congenic (C57Bl) control (Smith et al, paper submitted) to demonstrate that programmed cell death (PCD) and apoptotic morphology is increased in dystrophic (mdx) muscle and in cultured muscle cells (Smith, 1993; Smith et al, 1994 (b); Smith et al, 1995). Here we report that the level of programmed cell death observed in the skeletal muscles of young mdx mice declines significantly during the first year of life but remain substantially greater than levels of PCD observed in age matched control mouse muscles up to 18 months after birth. We found no differences in PCD levels of age matched male compared to female mdx mice. We also found that the degradation pathway protein ubiquitin was elevated in pyknotic dystrophic skeletal muscle stem cells as well as in some differentiating (fused) and dividing (mitotic) skeletal muscle stem cells suggesting a common cell cycle-linked pathway for these events in muscle stem cells.

Key words: ubiquitin, mdx, dystrophin, growth factor, apoptosis, PCD, fibrosis, skeletal muscle.

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Duchenne Muscular Dystrophy [34] is a debilitating heritable disease of skeletal muscle caused by absence of functional dystrophin protein [28]. The role of dystrophin in the physiology of muscle cells remains unclear although its location near the sarcolemma and formation of a transmembrane complex with the dystrophin associated glycoproteins (DAGs) [16] suggests a signalling function. Recently we have shown [38, 40, 41] that programmed cell death (PCD) and apoptosis are elevated in the skeletal muscles of dystrophic (mdx) mice when compared to congenic control animals.

Cell death may either be a gene dependent (programmed cell death, apoptosis) or gene independent (necrosis) process. These two forms of death are morphologically and biochemically distinct. Characteristics of necrosis are cell swelling, formation of large vacuoles and loss of membrane integrity [30] and are usually a result of cytotoxic insult. Programmed cell death on the other hand is a gene directed phenomenon operating under physiological conditions. Apoptosis is defined by a series of distinctive morphological changes which include nuclear condensation and fragmentation; degradation of DNA into oligonucleosomal fragments by means of an endogenous nuclease activity [2] and usually a dependence on protein synthesis [46]. The process of apoptosis occurs rapidly. Apoptotic cells, even in large numbers, do not induce an inflammatory response but they are targets of immediate phagocytosis either by neighbouring viable cells or by macrophages already present in the tissue [46]. Therefore detection of even a small number of apoptotic bodies generally indicates massive cell death [12]. There are several reports suggesting that withdrawal of cytokines stimulates programmed cell death (PCD) in a variety of cell systems [17, 45, 31] and this has led to the finding that individual growth factors can act to prevent PCD and promote survival in many different systems including; kidney [12] skeletal muscle [38, 41] and several cell lines [25, 23]. Ubiquitin has been implicated in the later stages of apoptosis and probably plays a specific role in protein degradation during PCD which may be of particular importance in the rapid pyknosis and death of cytoplasm rich cells such as differentiated skeletal muscle [36, 37].

Reports of apoptosis in muscle were until recently very few although programmed cell death plays an important role in the remodelling of embryonic insect muscle [37]. It has recently emerged however that PCD may have an important role in the pathology of two important skeletal muscle diseases, namely, acute infantile spinal muscular atrophy [19] and the far more common Duchenne muscular dystrophy (DMD). Several lines of evidence have shown that apoptosis can be detected in the skeletal muscles of the mdx mouse [40, 43]. Despite extensive ultrastructural studies of both human and mouse dystrophic muscle however there had been no previous reports of apoptosis in these muscles. Although Coulton et al [13] reported extensive occurrence of nuclei containing single-strand DNA breaks, which with hindsight are consistent with apoptosis [21], in the muscles of mdx mice. It is now clear that apoptosis is also found in normal skeletal muscle but that it is considerably elevated in dystrophic muscle [41].

There is almost nothing known about the process of programmed cell death in either dystrophic or normal skeletal muscle. Over the last 2-3 years however there has been considerable focus on the genes involved in PCD in other systems, in particular in the immune system and in cancer biology [47, 3] as well as in developmental models such as C. Elegans [27], Drosophila [24] and mouse [12]. Some work has also been done on the role of growth factors in controlling PCD [25, 23, 41]. Several key genes have been identified to play a role in PCD in different systems and these probably represent 4 or 5 separate pathways by which cell death can be initiated or modified [42, 9]. The genes which are known to be important in one or more systems are P53 [11], BCL-2 and Bax [35]; Rb-1 [26] and Fas Ligand [10] and one or more of these is likely to be found to be of importance in skeletal muscle.

Materials and Methods

Mice

C57Bl Sc10 and C57Bl (*mdx*) mouse stocks were maintained in house. The C57Bl (*mdx*) mice were generated from a nucleus of animals generously provided by the AFRC unit (Roslin, Edinburgh)

Estimation of cell death in vivo

Animals for use in the study were heavily anaesthetised with Avertin (0.4 ml/10g body weight of freshly prepared 0.6% solution 2,2,2-tribromoethanol in 2-methyl-2-butanol). Following a cardiac injection of heparin (0.1ml, vetdrug) animals were exsanguinated by cardiac perfusion with a vascular saline rinse (8g/l NaCl, .25g/l KCl, 0.05g/l CaCl2, 0.5g/l NaHC03, 0.05M NaHPO4: pH 7.4) and then fixed by perfusion with 4% paraformaldehyde (in phosphate buffered saline). Tibialis anterior muscles were dissected out and postfixed overnight in 4% paraformaldehyde followed by 12 hours in 30% sucrose and cryopreservation in OCT compound (Miles). Frozen muscle sections (7µm) were cut, post fixed in 70% ethanol and incubated at room temperature with propidium iodide and RNAse for 30 minutes as described by Smith *et al* [41]. Slides were washed in PBS and observed and counted under fluorescence illumination at x40 magnification. At least 10 non-adjacent sections were counted from each TA. Results were subject to statistical analysis by ANOVA (analysis of variants).

Cell culture

Dystrophic (*mdx*) and normal (C57Bl/10) skeletal myoblasts were derived from skeletal muscle explants and passaged as previously described [39] Normal (C57Bl/10) and *mdx* derived explants were established in parallel and passaged primary cultures used in the experiments described were all at passage 3. We have previously established that such cultures contain 99-100% pure myogenic stem cells as determined by antigenicity, morphology and ability to fuse in culture (described in detail in Smith & Schofield, [39]). Passaged primary myoblast cultures were routinely cultured in a 50% mix of calcium depleted Dulbecco's minimal essential medium and Ham's F12 medium (DMEM/F12) supplemented with glutamine and 20% fetal calf serum (FCS) as described previously [39]. For immunostaining myoblasts were plated onto glass chamberslides (Labtek).

Immunostaining

Myoblasts cultured on glass chamber slides were washed in Dulbecco's phosphate buffered saline (PBS) and fixed in formal saline (3.7% formaldehyde in PBS) for 45 minutes. Cells were permeabilised at room temperature in PHEM buffer [29] for 2 minutes and then incubated for 1 hour at room temperature with an anti-ubiquitin primary antibody (1/100 dilution, Sigma). Following three PBS washes (15 minutes each) slides were incubated with second antibody (Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit, 1/150 dilution, Sigma) for 1 hour at room temperature. After final washes in PBS slides were incubated in 4, 6-Diamidino-2-phenylindole (DAPI) (1 mg/ml) to counterstain nuclei.

Results

Apoptosis is elevated equally in both male and female mdx skeletal muscles.

We compared the levels of pyknosis in the skeletal muscles of the lower limb of 14, 2 week old male and female *mdx* mice and 12 age matched congenic (C57Bl) controls. As expected we observed an approximately 5 fold increase in percent pyknosis in the muscles of the *mdx* animals compared to normals (Figure 1), which was significant at 95% confidence levels. However we observed no difference between the sexes in either normal or dystrophic animals (Figure 1) suggesting that imprinting effects and sex hormone levels are not important in the control of apoptosis in these skeletal muscles. Statistical analysis of this data by ANOVA confirmed that there was no significant variation between pyknosis levels found in male and female muscles.

Apoptosis in mdx skeletal muscles declines with age

Pyknosis was assayed in propidium iodide stained skeletal muscle sections from dystrophic (*mdx*) mouse skeletal muscle at 2 weeks after birth (n=14), 5 weeks after birth (n=7), 12 months after birth (n=4) and 18 months after birth (n=3) and from normal congenic (C57Bl) control mouse muscle at 2 weeks (n=12), 5 weeks (n=3) and 12 months (n=3). Pyknosis was significantly greater by ANOVA (at 95% confidence levels) in *mdx* muscles than in C57Bl muscles at all ages examined (Figure 2). A significant fall in apoptosis as measured by a decrease in percent pyknosis was observed in the first year of life in the skeletal muscles of the dystrophic mice. This was significant by ANOVA analysis at the 95% level ($p = 0.05$). The detectable percentage of pyknotic cells in the normal muscles remained constant during this time period at approximately 1.5-2% suggesting that the decline was related to the pathology of the dystrophic muscle rather than to its maturation.

Ubiquitin protein is upregulated in pyknotic nuclei

Ubiquitin protein was detected by immunofluorescence staining (Figure 3) of primary dystrophic muscle stem cell cultures. Low level cytoplasmic staining was observed in the interphase cell population, but levels increased where chromatin condensation was observed. This was most striking where cells were apoptosing and pyknotic (Figure 3a, c) but was also observed where cells were in the metaphase stage of cell division (Figure 3b, d) and in some polynucleate, differentiated muscle fibers. In the latter case pyknotic nuclei were often observed suggesting that the fiber itself may be undergoing apoptosis.

Discussion

The skeletal muscles of Duchenne muscular dystrophy (DMD) patients and those of the analogous mutation in the dystrophic (*mdx*) mouse [6] appear to undergo an initial phase of upregulated skeletal muscle fiber turnover which is manifested by cycles of proliferation and cell death. This causes a progressive degeneration and regeneration of skeletal muscle fibres [22] and is followed by a progressive crippling fibrosis in both DMD [34] and, to a lesser extent, *mdx* [7] skeletal muscles. It is this end point of the pathology of DMD which contributes most to the severe disability and early death suffered by DMD patients. The cause of this fibrosis is unknown but may involve the inappropriate expression of muscle growth factors.

In this report it has been demonstrated that apoptosis is consistently elevated in the skeletal muscles of *mdx* mice during the course of its lifetime. However the greatest discrepancy between normal and dystrophic pyknosis levels occurs at the time (2-5 weeks) just prior to the most overt histopathology observed in *mdx* skeletal muscles. This suggests that apoptosis and PCD contribute to that pathology and confirms an earlier report [43] that apoptosis precedes necrosis in these muscles. Consistent with the subsequent recovery of *mdx* skeletal muscle pathology, the levels of pyknosis can be observed to decline with age, but remain consistently elevated above levels observed in normal muscle even in old age (18 months). It has been previously reported that excerised normal and *mdx* skeletal muscle displays increased levels of ubiquitin which can be associated with apoptotic muscle cells and fibers detected by TUNEL labelling [36], here I also show that ubiquitin protein is elevated in pyknotic skeletal muscle cells, but also, to a lesser extent, is associated with other chromatin condensation events during cell division and differentiation suggesting that this protein may play a role in muscle stem cell control. Considerable evidence suggests that this crucial process is regulated by specific peptide growth factors, and that this control may be peturbed in dystrophic muscles.

The growth and terminal differentiation of myoblasts and other committed stem cells has been shown to be under the positive and negative control of peptide growth factors such as the IGFs and the FGFs which upregulate and down regulate, respectively the myogenic regulatory transcription factors MyoD and myogenin [5, 8, 18, 44] which initiate the terminal differentiation pathway of skeletal muscle. Recently we showed [41] that the developmentally expressed, peptide growth factor (IGF-II), which is thought to play a role in mammalian myogenesis, reduces PCD in

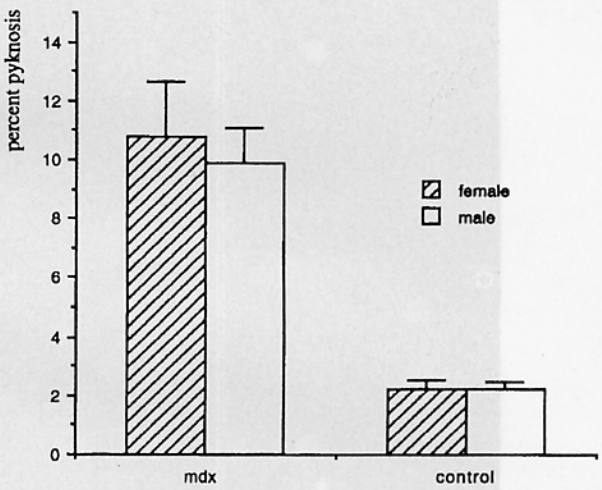

Figure 1: Percent pyknosis in 2 week old, male and female, normal and mdx skeletal muscles stained with propidium iodide after RNase treatment. Demonstrating elevated cell death in dystrophic muscle compared to normal and no difference between male and female. Data was obtained by counting 40 randomly selected separate fields of a minimum of 10 non-adjacent sections per animal. Graph shows the mean and standard deviation of data obtained from 14 mdx and 12 normal (C57Bl) animals.

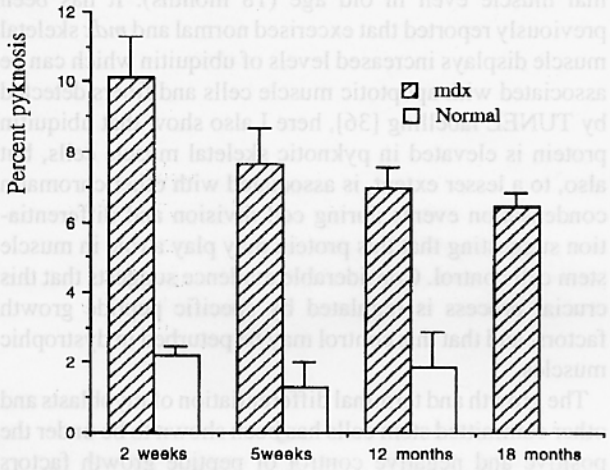

Figure 2: Percent pyknosis normal and mdx skeletal muscles stained with propidium iodide after RNase treatment as above. Demonstrating that elevated cell death in dystrophic muscle compared to normal is consistent throughout life but is greatest in the first few weeks of life after which a decline in cell death levels is observed. Data was obtained by counting 40 randomly selected separate fields of a minimum of 10 non-adjacent sections per animal. Graph shows the mean and standard deviation of data obtained from 28 mdx and 18 normal (C57Bl) animals.

mammalian skeletal muscle myoblasts both in vivo and in vitro. Several other growth factors thought to be important in myogenesis did not have this effect suggesting that IGF-II has a specific role as a survival factor in both normal and dystrophic skeletal muscle.

In the *mdx* mouse several studies [1, 14, 15, 39] have demonstrated an increase in the expression and synthesis of members of the FGF family of peptides in dystrophic skeletal muscle which suggests that one consequence of the loss of muscle dystrophin protein may be inappropriate growth factor action. Both FGFs and TGFbs (which also have been reported to act on muscle, [33]) have been

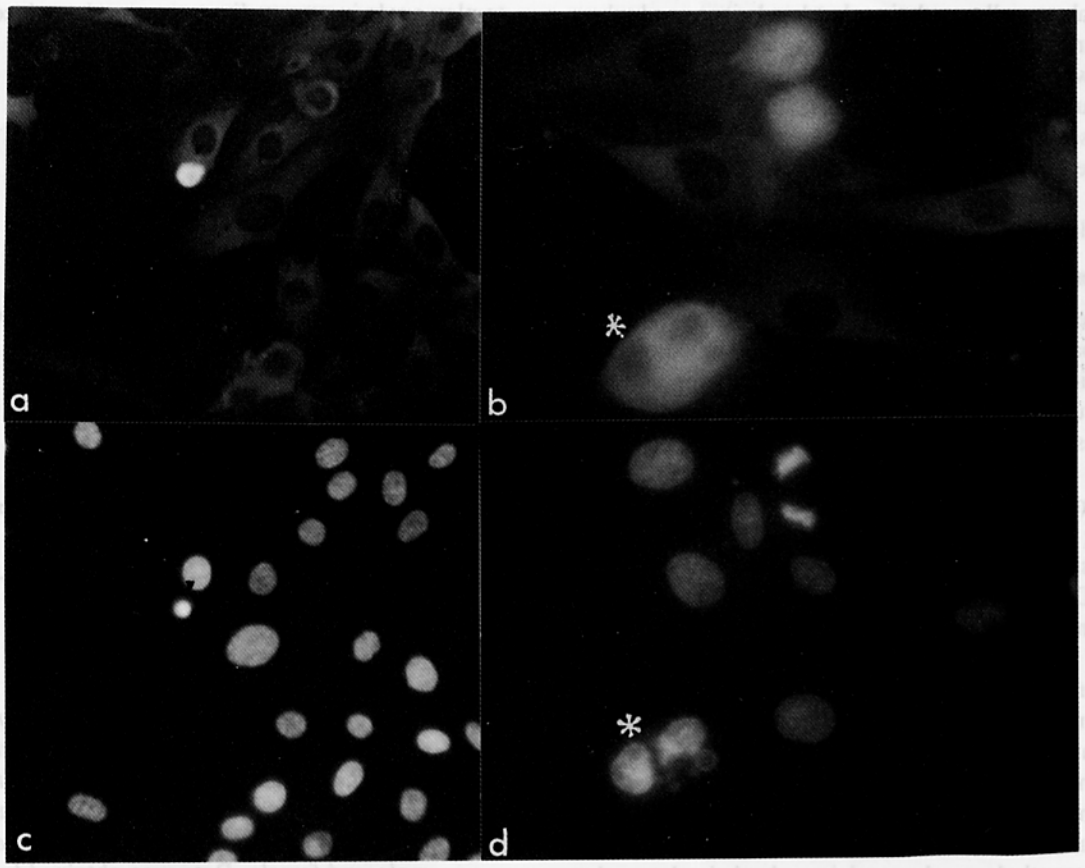

Figure 3: Immunostaining of passaged primary skeletal muscle stem cells demonstrating upregulation of cytoplasmic ubiquitin in (a), (b) pyknotic (arrow) and (c), (d) dividing and fused cells (star). (a) and (c) ubiquitin staining visualised with FITC-conjugated second antibody. (b) and (d) DAPI counterstain showing same fields as (a) and (c) respectively.

previously shown to be involved in fibrosis in other systems [32, 4], particularly in wound healing.

Thus work is currently underway to attempt to distinguish the complex and interconnected pathways by which skeletal muscle growth factors regulate the decision process which determines whether a muscle cell differentiates, dies or divides which ultimately determines the balance between regeneration and degeneration so clearly perturbed in dystrophic muscles.

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