A non-apoptotic role for caspase-9 in muscle differentiation

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Summary

Caspases, a family of cysteine proteases most often investigated for their roles in apoptosis, have also been demonstrated to have functions that are vital for the efficient execution of cell differentiation. One such role that has been described is the requirement of caspase-3 for the differentiation of skeletal myoblasts into myotubes but, as yet, the mechanism leading to caspase-3 activation in this case remains elusive. Here, we demonstrate that caspase-9, an initiator caspase in the mitochondrial death pathway, is responsible for the activation of caspase-3 in differentiating C2C12 cells. Reduction of caspase-9 levels, using an shRNA construct, prevented caspase-3 activation and inhibited myoblast fusion. Myosin-heavy-chain expression, which accompanies myoblastic differentiation, was not caspase-dependent. Overexpression of Bcl-xL, a protein that inhibits caspase-9 activation, had the same effect on muscle differentiation as knockdown of caspase-9. These data suggest that the mitochondrial pathway is required for differentiation; however, the release of cytochrome *c* or Smac (Diablo) could not be detected, raising the possibility of a novel mechanism of caspase-9 activation during muscle differentiation.

Key words: Apoptosis, Caspase-9, Bcl-xL, Muscle, Differentiation, shRNA

Introduction

The differentiation of skeletal muscle is a complex multi-step process involving events that bring about proliferation of myoblast progenitor cells, withdrawal of these cells from the cell cycle, commitment to a myogenic phenotype and subsequent differentiation into the multinucleate syncytia, of which mature muscle cells are comprised (reviewed by Anderson, 1998; Grounds, 1991; Lluis et al., 2006). Although much is known about the transcriptional control of the myogenic process, and the signalling pathways involved (Chun et al., 2000; Perdiguero et al., 2007a; Perdiguero et al., 2007b), there is still much to be understood about how these events are initiated and controlled.

The caspases comprise a family of cysteine proteases and some members of the family are responsible for causing apoptosis. Of these, caspase-3, caspase-6 and caspase-7 are classed as 'effectors', cleaving a wide range of intracellular proteins and being responsible for causing many of the changes typical of apoptosis (Alnemri, 1997; Salvesen, 2002). Effectors are expressed as zymogens and are activated when cleaved by 'initiator' caspases such as caspase-8 and caspase-9. Initiators are also expressed as zymogens but are activated by recruitment into large multi-protein complexes. Different apoptotic signals activate different initiators, but all the initiators activate a common set of effectors. Thus, during apoptosis, different pro-apoptotic signals are integrated and amplified through a proteolytic cascade of initiator and effector caspase activity that kills the cell (Boatright and Salvesen, 2003; Shi, 2004; Slee et al., 1999).

It is therefore remarkable that the apoptotic effector caspase, caspase-3, not only appears to be required for the differentiation of muscle progenitor cells into myofibres but apparently is sufficient for this differentiation (Fernando et al., 2002). It is unknown how caspase-3 is activated during differentiation and therefore we investigated how caspase-3 was activated during this process to test whether a non-apoptotic or an apoptotic pathway is involved. To accomplish this we used the C2C12 mouse myoblast cell line. These mononuclear cells fuse into multinucleate myotubes under low-serum conditions and, hence, are a useful model in which to study the events during muscle differentiation.

We show that caspase-3 activation and myotube formation is inhibited by a reduction of caspase-9 expression or by Bcl-xL overexpression, showing the involvement in myoblastic differentiation of two genes that are normally involved in the mitochondrial apoptotic pathway. These data are important because they show that two genes that usually regulate and/or control commitment to apoptosis can be employed for the very different purpose of myoblastic differentiation.

Results

Suppression of myoblast differentiation using a broad-spectrum caspase inhibitor

Previous research has shown that caspase-3 has a role in skeletal-muscle formation, demonstrating a transient increase in caspase-3-like activity associated with myoblastic differentiation (Fernando et al., 2002). Using the C2C12 mouse myoblast cell line, we sought to confirm these findings by culturing the cells in low-serum-containing media.

When C2C12 cells are induced to differentiate, a proportion of the cells undergo apoptosis rather than becoming myoblasts (Huppertz et al., 2001). To study caspase activity during differentiation it is therefore important to ensure that assays for caspase activation and caspase activity were not influenced by the presence of apoptotic cells. An early event of apoptosis is the

detachment of cells from the substratum and we observed that some cells (<10%) detached from the culture plate during differentiation. The nuclear morphology of these floating cells was apoptotic, as assessed by DAPI staining (Fig. 1A). To minimise the effect of any apoptotic cells on our experiments, we washed our plates extensively to remove non-adherent cells before analysing caspase activity in differentiating cultures. DAPI staining showed the nuclear morphology of the adherent cells (Fig. 1A) and that >99.8% of these cells had normal nuclear morphology.

The activity of caspase-3 in adherent C2C12 cells was assessed next. Induction of differentiation in C2C12 resulted in an increase in caspase-3-like activity (DEVDase activity) in adherent cells over a 5-day differentiation time course (Fig. 1B). The activity was transient, being clearly detectable by 12 hours (data not shown) and achieving maximal activity (~600 AFU/minute/mg) at 48 hours. By comparison, the activity in C2C12 cells that were treated with staurosporine (both adherent and detached cells) was 1100 AFU/minute/mg and approximately 25% of the cells were apoptotic in staurosporine-treated cultures. Activation of caspase-3 involves proteolytic processing of the pro-form to smaller subunits, an event that is detectable by immunoblot (Fig. 1C). Processing of the 32-kD pro-form of caspase-3 was seen to follow a similar profile to the caspase-3-like activity over the time course, with the appearance of the p17 form detectable by 3-6 hours (data not shown) and maximal detection at 48 hours (Fig. 1C). Thus, two independent assays demonstrate that caspase-3 is activated upon induction of myoblast differentiation.

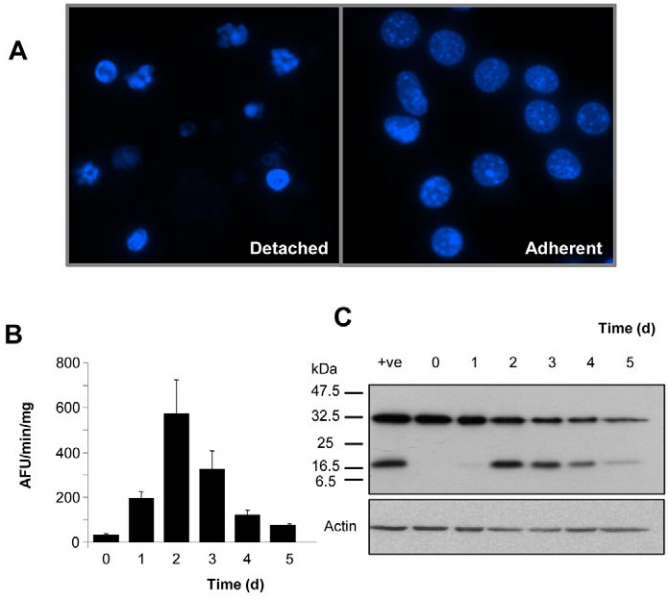

Fig. 1. Non-apoptotic morphology of adherent C2C12 cells and caspase-3 activity in the adherent cells. C2C12 cells were induced to differentiate over a 5-day time course. (A) DAPI-stained nuclear morphology of adherent and detached C2C12 cells after 2 days of differentiation. (B) Caspase-3-like activity was assayed using the fluorogenic substrate DEVD-afc over a 5-day differentiation period. Enzyme activity is expressed as an increase in arbitrary fluorescent units per minute per milligram of protein (AFU/min/mg). The histogram shows the mean ± s.d. of three independent experiments. (C) Immunoblot for caspase-3 processing over a 5-day differentiation period. Staurosporine-treated (1 μM for 4 hours) C2C12 cells (+ve) were used as a positive control for caspase-3 cleavage.

In order to test the functional role of caspase-3 in myoblast differentiation, a broad-spectrum caspase inhibitor, Q-VD-OPH (Caserta et al., 2003) was added to the cells prior to induction of differentiation. Addition of 30 μM Q-VD-OPH significantly inhibited caspase-3-like (DEVDase) activity (Fig. 2A) and caspase-3 processing (data not shown) over the 5-day period in comparison with untreated controls. Two differentiation-related phenotypes were assayed at each time point to assess the effect on differentiation into myotubes: the appearance of myosin heavy chain (MyHC) (Fig. 2B) and the number of cell-fusion events. Cell fusion was severely impaired by the inhibitor (Fig. 2C). Despite a reduction in cell fusion, Q-VD-OPH had a much lesser effect on the elongation of the myoblasts into spindle-shaped cells and the expression of MyHC, changes normally occurring prior to myotube formation (Fig. 2B).

If the caspase inhibitor increased the number of cells in the culture by blocking apoptosis, but these rescued cells were unable to differentiate, the conclusion that Q-VD-OPH inhibited fusion would be incorrect. Therefore, the number of cells per field was determined in both inhibitor-treated and untreated controls, and found to be no different (Fig. 2D). This excludes skewing of the data that could be attributable to cytoprotective effects of the inhibitor.

Thus, caspase-3 is required for efficient cell fusion during myoblast differentiation, findings that are consistent with an earlier report (Fernando et al., 2002). However, it is unknown how caspase-3 is activated in differentiating cells and we therefore set out to test whether apoptotic or non-apoptotic pathways were involved.

Two major caspase-activation pathways that drive apoptosis have been characterized; the death-receptor pathway, in which caspase-8 is the initiator caspase (Peter and Krammer, 2003), and the mitochondrial pathway, in which caspase-9 is the initiator caspase (Riedl and Salvesen, 2007). To test which initiator caspases were involved in myoblastic differentiation, the activation of caspase-1 caspase-2, caspase-8 and caspase-9 were assessed by immunoblot to detect caspase processing. Initiators caspase-1, caspase-2 and caspase-8 showed neither a decrease in the pro-form nor the appearance of a cleavage product over the differentiation time course (data not shown). By contrast, blotting for initiator caspase-9 revealed its processing from the 48-kDa pro-form to the 35- and 37-kDa forms (Fig. 3A). This processing was also accompanied by the appearance of caspase-3 activity, making caspase-9 a strong candidate for the initiator caspase that is activated during myoblast differentiation.

Caspase processing, however, is not a certain indicator of caspase activity so, in order to obtain evidence of caspase-9 activation, we performed affinity labelling using z-VAD–fmk–biotin (Faleiro et al., 1997; Tu et al., 2006). Cells were incubated in growth or differentiation medium containing biotinylated caspase inhibitor for 12 hours, after which cells were lysed and labelled caspases purified using streptavidin beads. Labelled caspases were then detected by immunoblotting. The whole-cell lysates (input samples) showed that 37- and 35-kDa processing products of caspase-9 were detectable. Caspase-9 processing was similar in cells cultured in growth medium or differentiation medium, most probably because, at 12 hours, the differentiation process is just beginning, making detection of increased processing more difficult. However, caspase-9 was only labelled by the inhibitor in cells incubated in differentiation medium, showing that caspase-9 was active only under differentiation conditions (Fig. 3B). Whereas caspase-9 processing resulted in more of the 35-kDa species than the 37-kDa species (Fig. 3A), the 37-kDa form was labelled more than the 35-

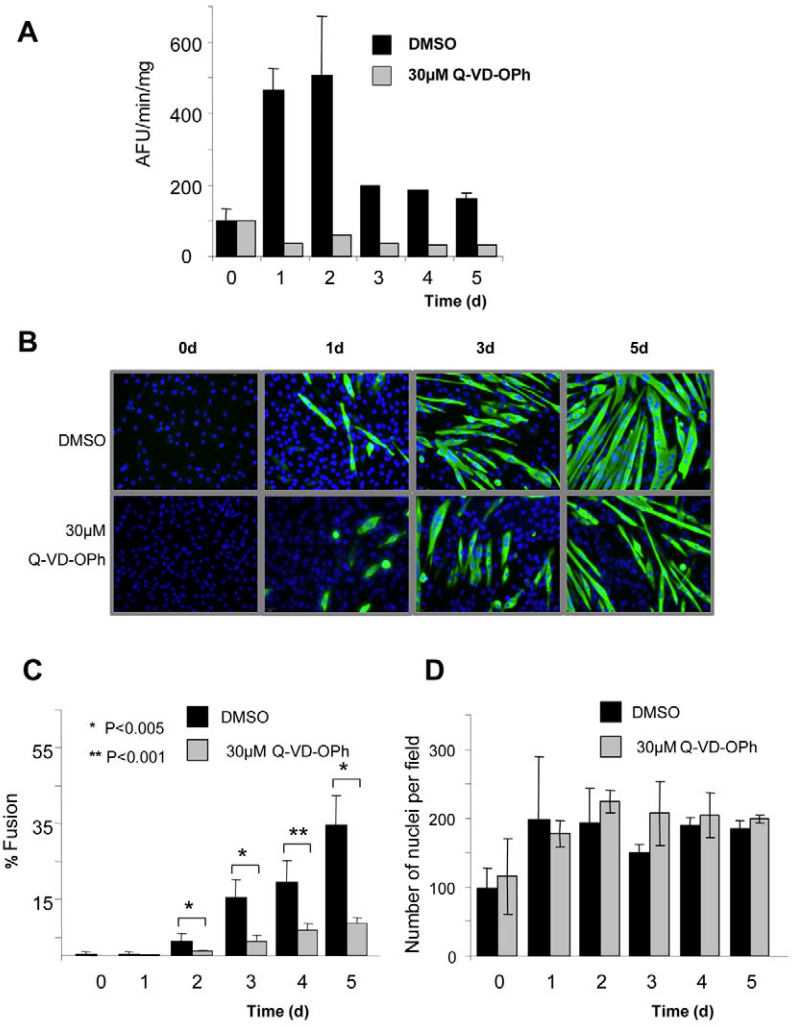

Fig. 2. A caspase inhibitor impairs differentiation. A broad-spectrum pharmacological caspase inhibitor, Q-VD-OPH, was used at 30 μM to inhibit caspase activity in differentiating C2C12 cells. Cells were incubated in differentiation media for the indicated time, and caspase-activity assays were conducted and MyHC expression analysed. The histograms show the mean ± s.d. of three independent experiments. (A) Caspase-3-like activity in differentiating C2C12 cells in the presence (grey) or absence (black) of 30 mM Q-VD-OPH. Enzyme activity is expressed as an increase in arbitrary fluorescent units per minute per milligram of protein (AFU/min/mg). (B) MyHC (green) expression during differentiation with DNA counterstaining with Hoechst 33342 (blue). (C) The degree of muscle-cell differentiation was determined by scoring cell fusion (see Materials and Methods). (D) The number of nuclei per field was determined for each treatment.

kDa form. This is consistent with the report that the 37-kDa form has eightfold higher protease activity than the 35-kDa form (Zou et al., 2003). Caspase-1, caspase-2 and caspase-8 were not labelled (data not shown), making these caspases poor candidates for the role of initiator caspase during myoblast differentiation.

shRNA-mediated knockdown of caspase-9

If caspase-9 is the initiator caspase during myoblast differentiation, then prevention of caspase-9 activation or activity is predicted to have the same effect on differentiation as inhibition of caspase-3 activity. To test the importance of caspase-9 in myoblast differentiation, we reduced caspase-9 levels in C2C12 cells using adenoviral (Ad)-vector-mediated delivery of a small hairpin RNA (shRNA) construct against murine caspase-9 (McDonnell et al., 2003). The construct was delivered at an MOI of 2500 to C2C12 cultures at 24 hours prior to the induction of differentiation using low-serum media. Within 2 days of transduction (day 1 of differentiation), caspase-9 expression was reduced to approximately 50% of normal expression levels (Fig. 4A), with a further reduction in expression over the subsequent 2 days. The knockdown was specific in that no reduction in expression levels of caspase-2, caspase-3 or caspase-8 was detectable (data not shown). Expression and processing of caspases-3 and caspase-9 were also unaffected in control differentiated cells, which were transduced with AdNull vector, containing no transgene, or a nonsense-shRNA Ad vector (Fig. 4A and data not shown) at the same MOI. The delivery of AdNull and nonsense vectors also excluded the possibility that interferon-induced changes altered differentiation patterns.

Delivery of caspase-9 shRNA resulted in a significant reduction of caspase-3-like activity (DEVDase) in the cells (Fig. 4B). Furthermore, caspase-9 and caspase-3 processing was inhibited (Fig. 4C). A profound decrease in myoblast fusion was seen following caspase-9 knockdown (Fig. 4D), whereas cell fusion to form myotubes was unaffected by delivery of an AdNull vector or nonsense-RNA Ad vector (Fig. 4E and data not shown). Thus, caspase-9 is required for caspase-3 activation and efficient cell fusion during myoblast differentiation

Bcl-xL overexpression in C2C12 myoblasts

If caspase-9 is initiating caspase-3 activation, it is possible that the mitochondrial pathway activated during apoptosis is serving a very different role in myoblast differentiation. The mitochondrial pathway is inhibited by anti-apoptotic proteins of the Bcl-2 family (van Delft and Huang, 2006). Therefore, in order to test the role of the mitochondrial pathway in muscle-cell differentiation, an Ad vector was designed to overexpress Bcl-xL, which inhibits the activation of caspase-9 by preventing cytochrome *c* release from mitochondria (Jurgensmeier et al., 1998).

Prior to induction of differentiation, cells were transduced with an adenoviral vector encoding Bcl-xL (AdBcl-Xl; Fig. 5A) at an MOI of 250. Control cells were transduced, at the same MOI, with

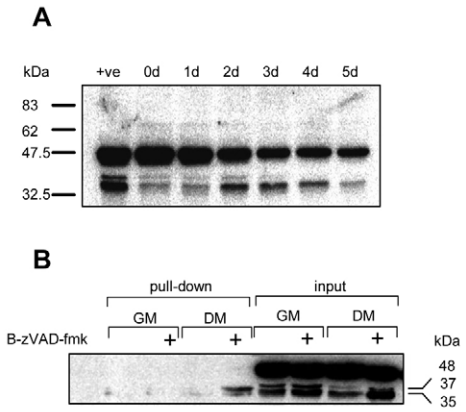

Fig. 3. Caspase-9 is activated during differentiation. (A) Caspase-9 processing was detected by immunoblotting over a 5-day differentiation time course. Staurosporine-treated (1 μM for 4 hours) C2C12 cells (+ve) were used as a positive control for caspase cleavage. (B) Affinity labelling of the 37-kDa band of processed caspsase-9. C2C12 cells were cultured for 12 hours in growth media (GM) or differentiation media (DM) in the presence or absence of 50 μM biotin-VAD-fmk. Labelled caspases were affinity purified and detected by immunoblot.

an AdNull vector, produced by deletion of the *Bcl-xL* gene from the construct. Transduction with Bcl-xL vector resulted in a significant reduction in DEVDase activity, in comparison with AdNull-transduced cells, at 1 day, 2 days and 3 days after induction of muscle differentiation (Fig. 5B). Caspase-3 and caspase-9 processing, which peaked at day 2 in the AdNull-transduced cells, in a manner very similar to non-transduced differentiating myoblasts, was abolished in the cells overexpressing Bcl-xL (Fig. 5C). Bcl-xL expression was shown to attenuate myotube formation and the number of fusion events was significantly reduced in comparison with control cells (Fig. 5D,E). As with the caspase inhibitor, possible skewing of fusion-event results owing to cytoprotective effects of Bcl-xL was ruled out by ensuring that the total nuclei numbers per well were not significantly different between AdBcl-xL-transduced cells and those transduced with AdNull (data not shown).

MyHC expression

Not all changes associated with differentiation were affected by caspase inhibition. MyHC is not expressed in myoblasts but its expression increases as these cells differentiate. At early time points during differentiation, mononucleate MyHC-expressing cells were observed, indicating that MyHC expression was not dependent on cell fusion. At later time points the number of nuclei per MyHC-expressing cell was seen to be increased in a caspase-dependent manner (Fig. 6A) as the myoblasts fused to form myotubes. However, the average number of MyHC-expressing cells was not significantly affected by Q-VD-OPH treatment, Bcl-xL expression or caspase-9 shRNA expression (Fig. 6B), showing that MyHC expression was not caspase dependent. The current data do not reveal whether MyHC expression is upstream of caspase-dependent changes or part of an independent, parallel pathway during differentiation.

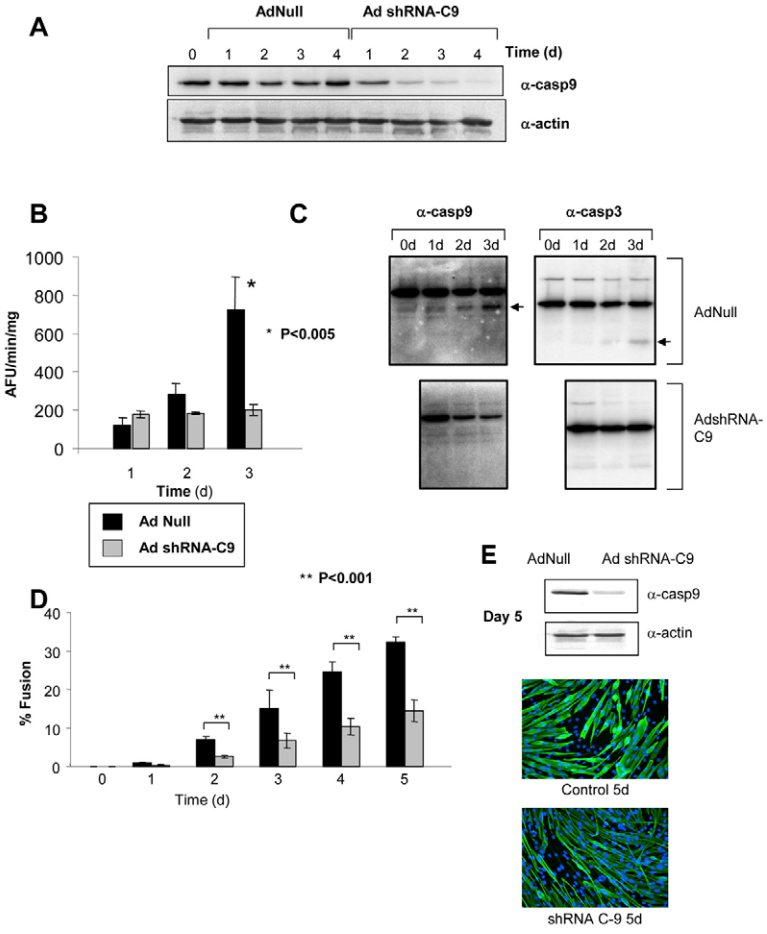

Fig. 4. shRNA against caspase-9 impairs muscle differentiation. C2C12 cells were transduced with either an adenoviral vector encoding shRNA targeted against caspase-9 (AdshRNA-C9) or the same vector without the shRNA (AdNull). Caspase-9 levels were assessed by immunoblot (A) and the effect of shRNA on caspase-3-like activity was assessed by DEVD-afc (B). Enzyme activity is expressed as an increase in arbitrary fluorescent units per minute per milligram of protein (AFU/min/mg). (C) The effect of AdshRNA-C9 knockdown on caspase processing was determined by immunoblot. Arrows indicate the processed forms of caspase-9 and caspase-3. (D) The effect of AdshRNA-C9 knockdown on cell fusion (see Materials and Methods) was assessed. The histograms show the mean ± s.d. of three separate experiments. (E) Top, western blot for caspase-9 in stained samples. Bottom, MyHC (green) staining with DNA (blue) counterstaining (Hoechst 33342).

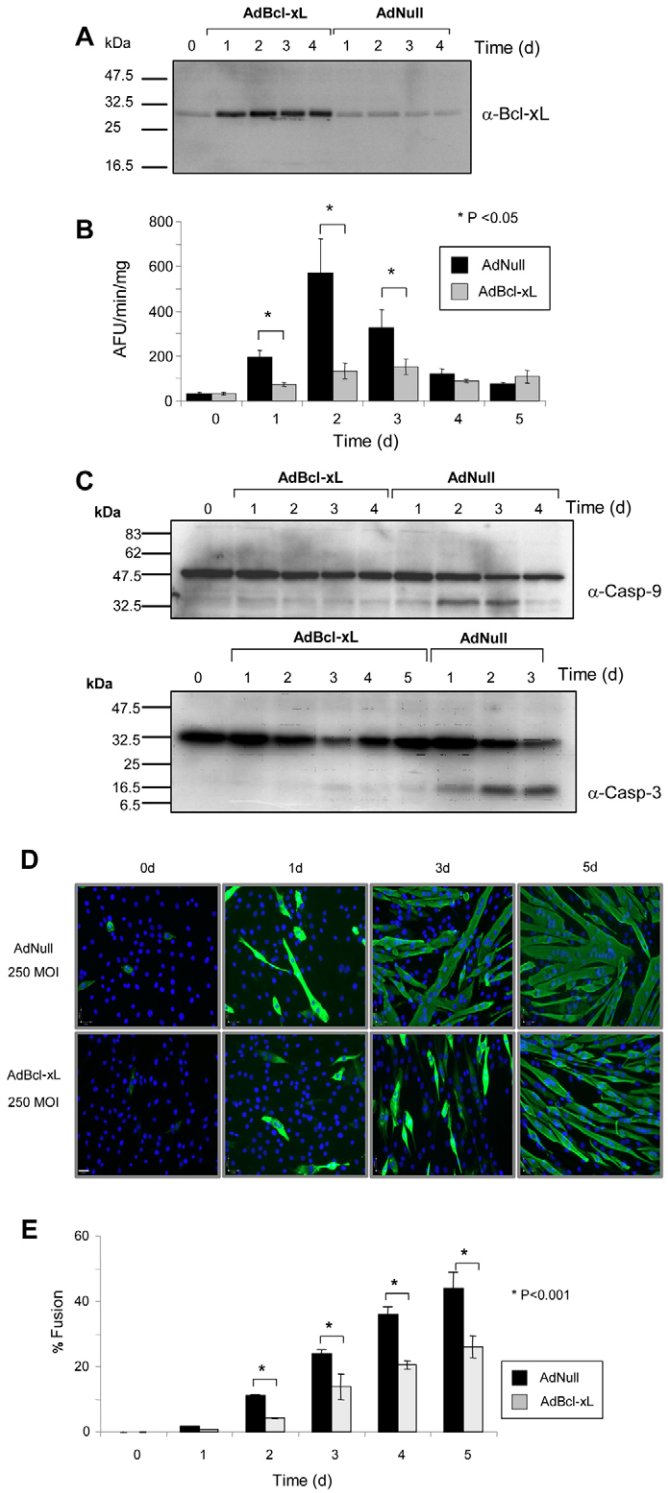

Fig. 5. Bcl-xL overexpression impairs differentiation. C2C12 cells were transduced with either an adenoviral vector encoding the anti-apoptotic protein Bcl-xL (AdBcl-xL) or with the same vector without the transgene (AdNull). Bcl-xL expression was assessed by immunoblot (A) and caspase-3-like activity was assayed using DEVD-afc (B). Enzyme activity is expressed as an increase in arbitrary fluorescent units per minute per milligram of protein (AFU/min/mg). The effect of Bcl-xL expression on caspase processing (C) and on MyHC expression (D) was assessed by immunoblot and immunohistochemistry. MyHC is shown in green whereas DNA counterstaining (Hoechst 33342) is shown in blue. (E) The degree of muscle-cell differentiation was determined as before (see Materials and Methods). The histograms show mean + s.d. of three separate experiments.

Apoptotic mitochondrial events during muscle differentiation

The dependence of differentiation on caspase-9 and the inhibition of differentiation by Bcl-xL suggest that the mitochondrial pathway that is normally associated with apoptosis is being activated in differentiating cells and that this event is required for cell fusion. To test this idea we investigated whether the pro-apoptotic molecules cytochrome *c* and Smac (also known as Diablo) were released from mitochondria in differentiating cells and whether the mitochondrial membranes of these cells depolarized during differentiation.

To assess the release of cytochrome *c* and Smac, cell lysates from differentiating cells were prepared, followed by the preparation of cytoplasmic and mitochondrial fractions. Immunoblotting showed that, whereas an apoptotic stimulus (staurosporine) triggered the release of both these proteins, release of cytochrome *c* and Smac was not detectable in differentiating cells (Fig. 7A).

Activation of the mitochondrial pathway during apoptosis is also associated with mitochondrial-membrane depolarisation (Bossy-Wetzel et al., 1998). To test whether depolarisation occurred during differentiation of C2C12 cells, cells were stained with TMRE and changes in mitochondrial membrane potential assessed by flow cytometry (Fig. 7C). Whereas staurosporine induced an obvious depolarisation of mitochondrial membranes, no depolarisation was seen in differentiating cells, despite the high levels of caspase activity that were associated with differentiation.

Thus, although the effects of Bcl-xL overexpression and caspase-9 shRNA implicate the mitochondrial pathway in differentiation, we found no direct evidence of mitochondrial changes occurring in the differentiating cells.

Discussion

The process of myoblast fusion within the musculature is a complex one and, although much is known about the transcription factors involved, and which signalling pathways are implicated, the process that allows the extensive reassembly of cytoplasmic and membrane components of the cell is poorly understood. Fernando et al. (Fernando et al., 2002) provided evidence that an apoptotic caspase, caspase-3, was required for skeletal myoblast differentiation, and research on the effects of apoptosis repressor with caspase recruitment domain (ARC) on cardiac-muscle fusion has suggested a role for caspase-3 in cardiomyocyte differentiation (Hunter et al., 2007). Although these results clearly implicate the effector caspase in differentiation, neither report identified components of the pathway that are responsible for caspase-3 activation. Here, we set out to resolve whether caspase-3 was activated during differentiation by an apoptotic pathway or a specific differentiation pathway.

Our data show that an initiator caspase, caspase-9, is activated in differentiating cells and is required for caspase-3 activation and cell fusion. Several lines of evidence support the conclusion that caspase-9 is the initiator of caspase activation during myoblast differentiation. First, its processing shows a similar temporal profile to that of caspase-3, suggesting that it might be responsible for activating caspase-3. By contrast, the processing of other known apoptotic initiator caspases, caspase-1, caspase-2 and caspase-8, was not detectable. We have not excluded a role for the other mouse initiator, caspase-12, which might have importance in this process, given the effect of endoplasmic reticulum (ER) stress on myoblast differentiation (Nakanishi et al., 2007). Second, the processing of caspase-9 suggests autocatalytic proteolysis, consistent with caspase-9 being the initiator. Autocatalytic processing of caspase-9 generates p35 and p12 subunits, which together form a tetramer, $(p35/p12)_2$, which activates caspase-3 (Zou et al., 2003). The p37

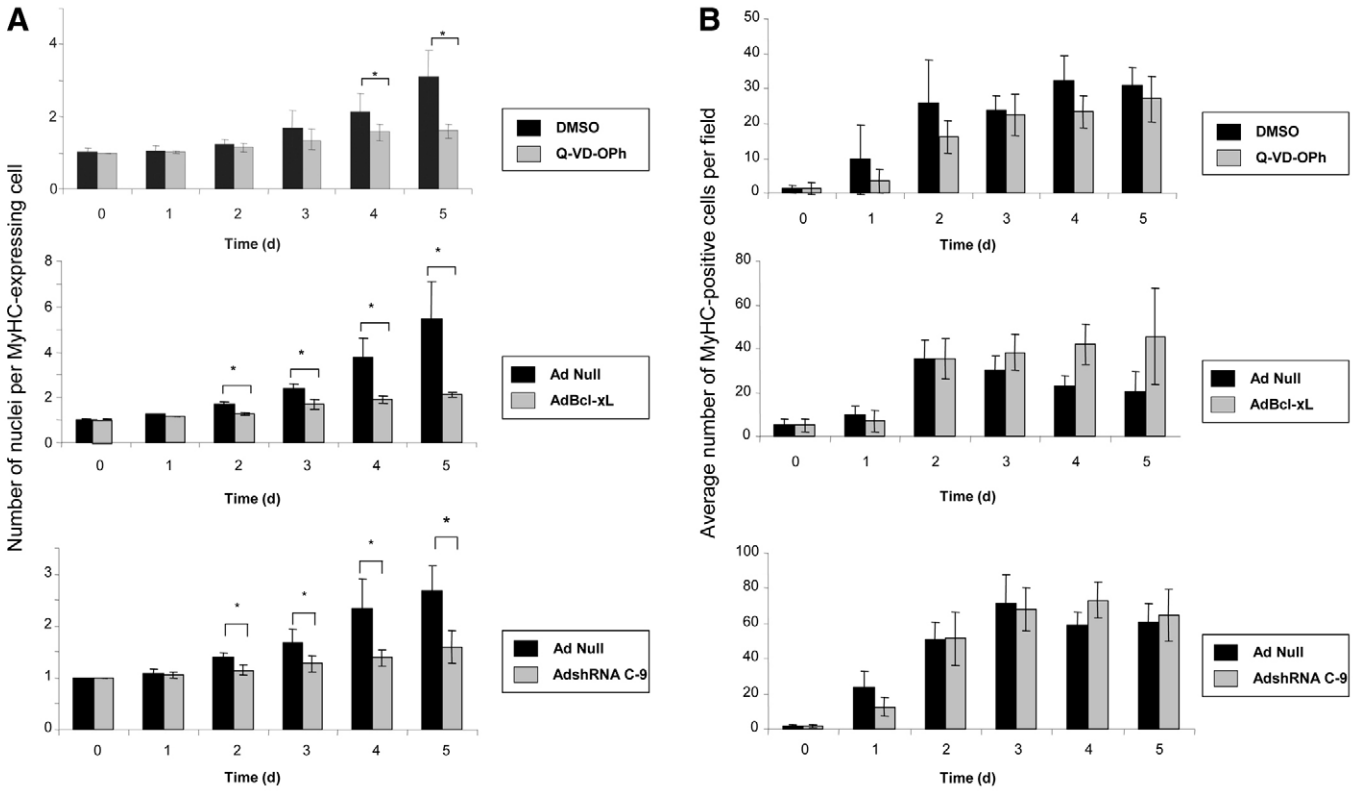

Fig. 6. Number of MyHC-expressing cells per field. C2C12 cells were induced to differentiate after the indicated treatments, then harvested and fixed in graded methanol. Fixed cells were immunostained for MyHC expression. The number of nuclei per MyHC-positive cell (A) and the number of MyHC-positive cells per microscopical field (B) were scored. The histograms show the mean ± s.d. of three independent experiments. **P*=0.005.

subunit and p10 subunits of caspase-9 are generated when caspase-3 cleaves caspase-9 in a positive-feedback loop. During differentiation, caspase-9 was processed to generate the p35 and p37 subunits, with p35 being the major product (the p12 and p10 subunits are not detectable with the antibody used). Finally, reduction of caspase-9 levels with a specific shRNA prevented caspase-3 activation. These findings are consistent with a model in which muscle differentiation requires caspase-9 as the initiator of caspase-3 activity.

Caspase-9 is the initiator of the mitochondrial or intrinsic apoptotic pathway and this pathway is controlled by members of the Bcl-2 family. We found that overexpression of Bcl-xL compromised differentiation in the same way as reduction of caspase-9 protein using shRNA technology. Together, these data suggest that caspase-3 is activated through the mitochondrial apoptotic pathway during muscle differentiation

However, our investigation of apoptotic mitochondrial events in differentiating cells did not reveal detectable cytochrome *c* release, Smac release or mitochondrial depolarisation and these data are inconsistent with activation of the mitochondrial pathway. Thus, it is possible that some other caspase-9-dependent, Bcl-xL-sensitive pathway is involved. Interestingly, primary myoblasts are known to undergo caspase-9-dependent but Apaf1-independent apoptosis (Ho et al., 2004) and, during this apoptosis, caspase-9 is processed to both the p37 and p35 forms (Ho et al., 2007). Although these data raise the possibility of a novel caspase-9 activator in muscle cells, the Apaf1-independent apoptosis requires Smac release from mitochondria (Ho et al., 2007), which is an event that we cannot detect in differentiating cells. Because the mitochondrial pathway is dependent on Apaf1, we attempted to reduce Apaf1 expression by shRNA-based approaches in C2C12 cells. However, we failed to consistently and significantly reduce Apaf1 expression and, at this time, we can neither implicate nor exclude a role for Apaf1 in muscle differentiation.

Studies from caspase-3 and caspase-7 double-knockout mice suggest a different explanation that reconciles a role for caspase-9 and Bcl-xL; no detectable mitochondrial events occurred in these mice. Lakhani et al. (Lakhani et al., 2006) have suggested a positive-feedback mechanism in the mitochondrial pathway; this mechanism involves very low levels of cytochrome *c* release triggering caspase-3 activation, which subsequently induces the large, rapid release of cytochrome *c* that is associated with apoptosis. There is also a potential role for the involvement of BH3-only proteins during muscle differentiation (Shaltouki et al., 2007), because p53 both regulates myotube formation (Porrello et al., 2000) and expression of BH3-only proteins such as Noxa and Puma. This raises the possibility that these ‘killer’ proteins have broader functions than currently suspected.

Although all of the strategies that we employed to manipulate caspase activity and activation had very profound effects on caspase activity, cell fusion was only delayed and not abolished. These data suggest the existence of redundant caspase-dependent and -independent pathways for cell fusion. This implies that more than one pathway is activated in parallel and that either can induce myotube formation. The protease calpain might be part of this caspase-independent pathway because it is known to be important in myotube formation (Balcerzak et al., 1995; Temm-Grove et al., 1999) and shares some substrates with caspases (Vanags et al.,

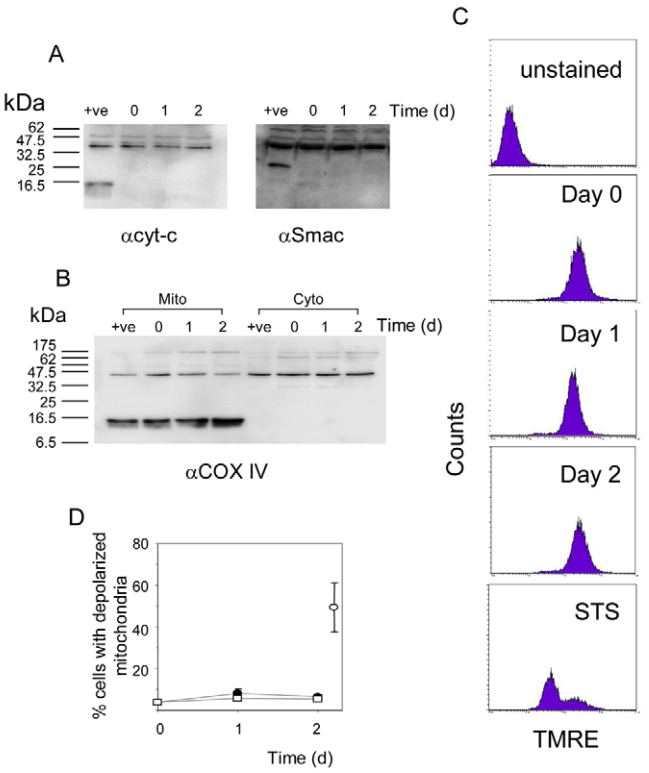

Fig. 7. Mitochondrial changes in apoptotic but not differentiating cells. C2C12 cells were treated with staurosporine (+ve) to induce apoptosis or were induced to differentiate. Differentiating cultures were washed to remove non-adherent cells. Cells were then harvested, lysed and centrifuged to obtain cytosolic and mitochondrial subcellular fractions. (A) The presence of mitochondrial proteins in the cytosolic fractions was then detected by immunoblotting. (B) Mitochondrial contamination of subcellular fractions was assessed by immunoblotting for COX IV. The results shown are typical of three independent experiments. (C,D) Alternatively, cells were harvested and stained with TMRE to detect $\Delta\psi m$ by flow cytometry. The results shown are typical of three independent experiments.

1996). Other markers of cell differentiation, besides cell fusion, such as myogenin expression, were minimally affected by caspase inhibition (data not shown) or, similar to MyHC expression, were not affected at all. Thus, it is clear that caspase activity plays an important but restricted role in the differentiation process.

Our findings have important implications for the fields of cell death, differentiation and muscle disease. Signals leading to caspase-3 activation are usually considered to commit a cell to death. The data shown here confirm that caspase-3 activity can be harnessed for differentiation, and add to our understanding by showing that caspase-3 is activated by a caspase-9-dependent and Bcl-xL-sensitive process. Thus, caspase-3 and caspase-9 are not just agents of cellular disassembly but help decide the fate of differentiating myoblasts. Discovery of which caspase substrates are cleaved in differentiating myoblasts and which are not will be key to understanding how these cells survive the activation of the intrinsic apoptotic pathway and use this pathway to complete myotube formation.

Materials and Methods

Myoblast cell culture and differentiation protocol

C2C12 myoblasts (ECACC) were maintained in culture and induced to undergo differentiation as previously described (Fernando et al., 2002).

Caspase-activity assay

Caspase activity was assayed as previously described (Martin and Fearnhead, 2002). Enzyme activity was expressed as arbitrary fluorescence units per minute per mg of protein (AFU/minute/mg).

Affinity labelling active caspase-9

Cells were treated with 50 μM biotinylated VAD-fmk for 12 hours in either their normal growth medium or differentiation medium. Cell extracts were prepared as previously described (Faleiro et al., 1997). The extracts were subsequently incubated with streptavidin-conjugated sepharose beads for 2 hours at 4°C. The beads were then washed four times with high-salt PBS (400 mM NaCl) before being resuspended in Laemmli loading buffer and heated to 95°C. The proteins were separated by SDS-PAGE and caspase-9 detected by immunoblotting.

Adenoviral vector production and transduction

The AdBcl-xL and AdNull adenoviral vectors were generated, propagated and purified as described before (Fallaux et al., 1996; Ritter et al., 1999). The AdshRNA-C9 and AdshRNA-eGFP vectors were generated using the ViraPower adenovirus expression system (Invitrogen). They encoded shRNA constructs targeted against the caspase-9 and jellyfish enhanced green fluorescent protein (eGFP) genes, respectively, under the control of the human U6 promoter (Behrend et al., 2005; Zwacka et al., 1998). shRNA sense and antisense oligos were synthesized to target a 21-nucleotide sequence within the mouse caspase-9 sequence (5′-AAGCAGGAT-CCAGAGGCTGTT-3′) as described (McDonnell et al., 2003).

For all viral transductions, cells were seeded at 2.5×10^4 cells/cm^2 in 24-well plates and cultured overnight in growth medium before being transduced with the viral vectors for a further overnight incubation. The medium was then changed to differentiation medium.

Immunoblotting

Briefly, the cells were lysed and protein concentrations determined by Bradford assay to ensure equal protein loading in gels. The primary antibodies used were against caspase-3 (Cell Signaling, 1:1000), caspase-9 (Cell Signaling, 1:1000), β-actin (Cell Signaling, 1:1000), COX IV (Cell Signaling, 1:1000), cytochrome *c* (BD Bioscience, 1:1000) and Smac (Adrain et al., 2001). Bound primary antibodies were visualised using chemiluminescence detection of HRP-labelled secondary antibodies (Pierce, 1:5000) and a CCD camera.

Cell fractionation

Cells were collected by centrifugation (350 ***g***, 5 minutes, 4°C) and resuspended in 100 μl of ice-cold lysis buffer (80 mM KCl, 250 mM sucrose and 200 μg/ml digitonin in PBS). The samples were incubated on ice for 5 minutes and then centrifuged at 10,000 ***g*** for 5 minutes at 4°C. The recovered pellet and supernatant were then assayed by immunoblot for the release of mitochondrial-intermembrane-space proteins.

Mitochondrial-membrane depolarisation

To detect changes in mitochondrial-membrane potential ($\Delta\Psi m$), cells were stained with tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes) (Farkas et al., 1989). Briefly, 1×10^6 cells/sample were plated and treated appropriately, after which time samples were collected and washed where appropriate. The samples were then resuspended at 2×10^6 cells/ml in growth medium containing 10 μM TMRE for 30 minutes at 37°C. They were then pelleted, washed once in DMEM, resuspended in DMEM at 1×10^6 cells/ml and analysed by flow cytometry.

Immunocytochemistry

Briefly, cells were washed in PBS, fixed in graded concentrations of methanol and stained as previously described (Megeney et al., 1996). MyHC MF20 mAb (Development Studies Hybridoma Bank, 1:100) was detected with FITC-conjugated anti-mouse IgG Alexa-Fluor-488 (Invitrogen, 1:200) and nuclei were stained with Hoechst 33342 (2 μg/ml).

Quantification of muscle-cell differentiation

The degree of muscle-cell differentiation was determined by a fusion-event scoring system. The percentage number of fusion events was calculated in ten random microscope fields using the following formula:

$$\%\ \text{fusion events} = [(N_M\text{-}M)/N_T]\times100,$$

where N_M=number of nuclei in MyHC-positive cells, M=number of MyHC-positive cells and N_T=total number of nuclei.

Counts were verified by two independent observers.

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