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Apoptosis in differentiating C2C12 muscle cells selectively targets Bcl-2-deficient myotubes

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Abstract

Muscle cell apoptosis accompanies normal muscle development and regeneration, as well as degenerative diseases and aging. C2C12 murine myoblast cells represent a common model to study muscle differentiation. Though it was already shown that myogenic differentiation of C2C12 cells is accompanied by enhanced apoptosis in a fraction of cells, either the cell population sensitive to apoptosis or regulatory mechanisms for the apoptotic response are unclear so far. In the current study we characterize apoptotic phenotypes of different types of C2C12 cells at all stages of differentiation, and report here that myotubes of differentiated C2C12 cells with low levels of anti-apoptotic Bcl-2 expression are particularly vulnerable to apoptosis even though they are displaying low levels of pro-apoptotic proteins Bax, Bak and Bad. In contrast, reserve cells exhibit higher levels of Bcl-2 and high resistance to apoptosis. The transfection of proliferating myoblasts with Bcl-2 prior to differentiation did not protect against spontaneous apoptosis accompanying differentiation of C2C12 cell but led to Bcl-2 overexpression in myotubes and to significant protection from apoptotic cell loss caused by exposure to hydrogen peroxide. Overall, our data advocate for a Bcl-2-dependent mechanism of apoptosis in differentiated muscle cells. However, downstream processes for spontaneous and hydrogen peroxide induced apoptosis are not completely similar. Apoptosis in differentiating myoblasts and myotubes is regulated not through interaction of Bcl-2 with pro-apoptotic Bcl-2 family proteins such as Bax, Bak, and Bad.

Keywords

Apoptosis; Bcl-2; differentiation of C2C12 cells; myotube

INTRODUCTION

Muscle cell apoptosis accompanies normal muscle development as well as degenerative diseases and biological aging [1-5]. While the role of apoptosis in muscle degradation is not fully understood, it is known that stress conditions, such as oxidative stress [6], denervation [7], mitochondrial dysfunction [8, 9], and accumulation of advanced glycation endproducts [10, 11] enhance muscle cell apoptosis, which may contribute to skeletal muscle atrophy and sarcopenia. Skeletal muscle is very adaptive to stress due to a remarkable regenerative capability attributed to the growth and differentiation of satellite cells. Myogenic differentiation is a complex multi-step process controlled by myogenic transcription factors and also accompanied by the activation of the caspase family of proteases, which play a

central role in the execution of apoptosis [12]. As a result of caspase activation during myogenic differentiation, a fraction of cells undergoes apoptosis rather than forms myotubes or reserve cells; in cell culture, such cells detach from the plates and display an apoptotic phenotype [13, 14].

The C2C12 cell line derived from murine skeletal muscle cells represents a common model to study muscle regeneration and differentiation induced by partial serum withdrawal. It was demonstrated that apoptosis is a distinctive part of the differentiation; in particular, caspase-3, caspase-9 and endoplasmic reticulum (ER) stress-specific caspase-12 are required for activation of differentiation and subsequent cell fusion to form myotubes [13, 15, 16]. Some fractions of C2C12 cells may undergo apoptosis, or terminal differentiation to form myotubes, or reversibly exit the cell cycle to enter a quiescent state, thus forming reserve cells, which show many characteristics of muscle satellite cells. Given that muscle tissue represents a heterogeneous system, a question arises which types of cells are subject to apoptotic elimination in muscle tissue and which mechanisms are involved in the differential sensitivity of cells to apoptosis.

A number of studies demonstrate the potential importance of Bcl-2 family proteins in muscle apoptosis. For instance, muscle-specific overexpression of Bcl-2 in mouse models of laminin α2-deficient dystrophy [4] and oculopharyngeal muscular dystrophy ameliorates disease [17], but not in a dystrophin-deficient model. Exercise training prevents the apoptosis of skeletal muscle, possibly through an increase of the Bcl-2/Bax ratio [18]. Biological aging increases the predisposition of skeletal muscle-derived satellite cells to apoptosis that correlates with a lower amount of Bcl-2 in old relative to young adult animals [19]. An age-associated increase of Bax and decrease of Bcl-2 levels was suggested as a putative mechanism of apoptosis in aging tissue [7, 20]. However, most of these studies were designed to determine the effect of Bcl-2 in animal models of muscle dystrophies and did not target the types of muscle cells and the underlying mechanisms of apoptosis.

The anti-apoptotic protein Bcl-2 can bind the pro-apoptotic protein Bax, inhibiting its homodimerization, which otherwise would lead to the induction of cytochrome c release from mitochondria, followed by caspase activation [21, 22]. Bcl-2 can also regulate intracellular Ca^{2+} levels, where Ca^{2+} released from the ER and taken up by the mitochondria can activate Ca^{2+}-dependent caspases to execute apoptosis [23-27]. In fact, perturbed Ca^{2+} homoeostasis has been recognized as one factor responsible for muscle degeneration [28, 29]. SERCA2B represents a “housekeeping” isoform of the Ca^{2+} pump, which is responsible for Ca^{2+} uptake into the ER in various types of cells and, thus, implicated in the regulation of multiple Ca^{2+}-dependent processes such as protein folding, ATP-production in the mitochondria, and apoptosis [30, 31]. Unfortunately, data on the levels of SERCA2B in muscle tissue or differentiating C2C12 cells are not available. This is especially important in view of a potential role of Bcl-2 with regard to Ca^{2+} homeostasis. For example, Bcl-2 overexpression prevented Ca^{2+} overload and inhibited apoptosis in dystrophic myotubes [32], and in vitro studies have shown that various SERCA isoforms are inactivated upon interaction with Bcl-2 [34, 35], which can be inhibited by HSP70 [36].

Here, we provide a comprehensive analysis of the expression profiles of Bcl-2 family proteins for different types of cells trough different stages of myogenesis in the C2C12 cell model, and determine the sensitivity of these cells to apoptosis. Currently, there are no available data on Bcl-2 family protein expression specifically in myotubes and reserve cells, and on the role of Bcl-2 family proteins in the apoptosis of differentiating C2C12 cells. The only studies related to this matter demonstrated differential immunostaining of pro-apoptotic members of the Bcl-2 protein family (i.e., Bad, Bak and Bax) in mononuclear and multinuclear cells in the C2C12 model and in mouse primary myogenic cell culture, where

Bcl-2 expression is limited to mononuclear, myogenin-negative cells and Bad, Bak, Bax and Bcl-x are expressed in all myogenic cells [37, 38].

We analyzed the differential expression of proteins that can interact with Bcl-2 to control apoptosis through different mechanisms in different muscle cell populations. We focused on the expression levels of the proapoptotic proteins Bax, Bak and Bad and also of SERCA2B and HSP70. We will show that reserve cells express higher levels of Bcl-2 and Hsp70 compared to myotubes, that correlate with their survival during the exposure to pro-apoptotic stimuli, while myotubes, characterized by low levels of Bcl-2 and HSP70, exhibit enhanced sensitivity to apoptosis. The increased apoptotic response of myotubes did not correlate with the levels of the pro-apoptotic proteins Bax, Bak and Bad. Treatment of differentiated C2C12 cells with hydrogen peroxide, a common pro-apoptotic agent, resulted in the preferential loss of myotubes with low levels of Bcl-2, while myotubes with elevated Bcl-2 levels were less sensitive to apoptosis. The overexpression of Bcl-2 in myoblasts prior to differentiation, that resulted in an increase of Bcl-2 expression levels in myotubes, partially protected myotubes from apoptosis. Our results suggest a role of Bcl-2 and potentially HSP70 in the regulation of apoptotic responses in differentiated C2C12 cells and lower importance of the pro-apoptotic proteins Bax, Bak and Bad.

MATERIALS AND METHODS

Cell Culture and incubations

C2C12 mouse skeletal muscle cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). C2C12 myoblasts cells were cultured in growing medium (GM) (DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen)) at 37°C and 5% CO_2 in a humidified chamber. Myogenic differentiation was induced on confluent cultured cells by changing the growth medium to differentiation medium (DM), supplemented with 2% horse serum instead of FBS. DM was replaced every 2 days; the aspirated medium was collected and the detached cells were recovered by centrifugation at 3000 × g for 5 min as described elsewhere [15, 16]. Differentiated C2C12 cells were treated with H_2O_2 (Sigma), or thapsigargin (TG) (Sigma) diluted in DMEM. In these experiments, cells were additionally rinsed with DMEM to remove any detached cells before the addition of either H_2O_2- or TG-containing media. For controls, cells were incubated in the same volume of DMEM without additives. At certain time points the incubation media were recovered from the dish and the detached cells collected by centrifugation as described above. The adherent cells were rinsed with PBS and harvested in a small volume of PBS.

For WB analysis, the adherent and the detached cells were washed twice with PBS and solubilized in a lysis buffer (20 mm Tris, pH 8, 150 mm NaCl, 1% Triton X-100, 10 mm orthovanadate, 10 mm EDTA, 2 mm phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1× protease inhibitor mixture) and then briefly sonicated and incubated at 4 °C for 30 min. The protease inhibitor mixture (Complete, Mini) was from Roche Applied Science (Indianapolis, IN). Afterwards, the cell lysates were centrifuged at 12,000 × g for 10 min at 4°C to remove cellular debris. Protein concentration of each freshly prepared cell lysate was determined with Coomassie Blue Plus protein reagent (Pierce, Rockford, IL, USA).

Isolation of Reserve Cells and Myotubes

After incubation in the differentiation medium, C2C12 cultures were separated into reserve cells and myotubes by a differential trypsinization procedure [39]. Briefly, the myotubes were specifically detached by mild trypsinization (incubation for 3 min in PBS containing

0.025% (w/v) trypsin and 0.1 mM EDTA followed by the addition of excess GM), whereas the reserve cells remained adherent to the dish. These cells were then detached by incubation with higher trypsin concentrations (0.25% (w/v) trypsin and 1 mM EDTA) and reseeded onto a dish in GM. After 30 min of incubation, the reserve cells attached to the culture dish were harvested by scraping off into a small volume of PBS.

Cell imaging

Morphological changes and survival of cells were monitored under an inverted phase contrast microscope (Olympus America Inc., Melville, NY) equipped with a digital camera to obtain photomicrographs, and captured at ×20 initial magnification. For analysis of morphological changes, cell images at different days of differentiation were processed using the ImageJ program located in public domain of the National Institute of Health. To demonstrate a shape of small myoblasts in GM, the images were zoomed in, while to show the formation of long myotubes in DM, the images were zoomed out.

SDS-PAGE and Western Blot analyses

After treatment with 4× SDS loading buffer, proteins extracted from cells were separated on precast Bio-Rad 4-20% gradient gels using tris-glycine running and sample buffers (Invitrogen, Carlsbad, CA). The gels were then electro-blotted onto a PVDF membrane (Millipore, Billerica, MA) prior to Western blot (WB) analysis. The spots were visualized with the ECL or ECL-Plus detection kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's procedure. Rabbit anti-HSP70 polyclonal antibody (SPA-812) was from Stressgen Biotechnologies (Ann Arbor, MI). Mouse monoclonal anti-Bcl-2 (sc-7382), anti-Bax (sc-7480), anti-myogenin (sc-12732), anti-β-actin (sc-81178) and rabbit polyclonal anti-Bak (sc7873), anti-Bad (sc-942), anti-MyoD (sc-304x) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-SERCA2 (MAS-913) and mouse monoclonal anti-SERCA1 (MA3-912) antibodies were from Stressgen Biotechnologies (Ann Arbor, MI). Rabbit polyclonal anti-SERCA2B antibody was generously provided by Drs. J. Enouf and R. Bobe (Inserm, Hopital Lariboisiere, Paris, France) [40]. Polyclonal anti-caveolin 1 (610059) and mouse monoclonal anti-caveolin 3 (610421) were from BD Biosciences (San Jose, CA). Rabbit polyclonal anti-caspase 12 (2202) and mouse monoclonal anti-caspase 9 (9504) were from Cell signaling Technology Inc. (Danvers, MA). Secondary horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies were from Sigma (St. Louis, MO), and HRP-conjugated anti-mouse antibodies were from Pierce Biotechnology (Rockford, IL). The monoclonal anti-Pax7 antibody was obtained from the Developmental Studies Hybridoma Bank, developed by Dr. David R. Sell under the auspices of the National Institute of Child Health & Human development (NICHD) and maintained by The University of Iowa, Department of Biology (Iowa City, IA).

Mass spectrometry analysis

Aliquots of cell lysate containing 100 μg protein, reductively alkylated with 2 mM DTT and 5 mM iodoacetic acid, were precipitated by ethanol, reconstituted in 50 mM ammonium bicarbonate, pH 7.8, and digested overnight with trypsin (sequencing grade from Promega, Madison,WI) at a ratio to protein of 1:20. The resulting peptide samples were introduced into an LTQ-FT hybrid linear quadrupole ion-trap FT-ICR (Fourier-transform ion cyclotron resonance) mass spectrometer (Thermo Finnigan, West Palm Beach, FL) via *capillary* LC using a nanoAcquity UPLC (Waters Corp., Milford, MA). Peptides were separated on a reverse-phase C18 column (Acclaim PepMap300, 300 Å, 5 μm, 15 cm ×300μm I.D.,Thermo, West Palm Beach, FL). A linear gradient was developed from 1 to 40% B in 100 minutes, ramped to 95% B in 8 minutes and held at 95%B for 10 minutes at a flow rate

of 10 μL/min with solvents A (99.9% H_2O, 0.1% formic acid) and B (99.9% acetonitrile, 0.1% formic acid). The nanoAquity UPLC Console (Waters Corp., version 1.3) was used to execute the injections and gradients. The ESI source was operated with a spray voltage of 2.8 kV, a tube lens offset of 160 V and a capillary temperature of 200°C. All other source parameters were optimized for maximum sensitivity of the YGGFL peptide MH^+ ion at m/z 556.27. The instrument was calibrated using an automatic routine based on a standard calibration solution containing caffeine, the peptide MRFA and Ultramark 1621 (Sigma).

A data-dependent acquisition method for the mass spectrometer (configured version LTQ-FT 2.2) was set up using the Xcalibur software (ThermoElectron Corp., San Jose, CA, version 2.0). Full MS survey scans were acquired at a resolution of 50,000 with an Automatic Gain Control (AGC) target of 5×10^5. The five most abundant ions were fragmented in the linear ion trap by collision-induced dissociation with AGC target of 2×10^3 or maximum ion time of 300 ms. The ion selection threshold was 500 counts. The LTQ-FT scan sequence was adapted from a published procedure [41].

For protein identification, MS/MS spectra were analyzed using Mascot (Matrix Science, London, UK; version 2.3) and Sequest (Proteome Discoverer, Thermo Fisher Scientific, San Jose, CA, version 1.3) search engines. The programs were set up to search the Uniprot-sprot and IPI (mouse) databases assuming the digestion enzyme trypsin. Mass tolerances for precursor and fragment ions were 20 ppm and 0.20 amu respectively, and carboxymethylation of cysteine residues was considered as a fixed modification. The Sequest and Mascot results then were imported into a Scaffold program (Proteome Software; version 3.4) for analyzing with the X!Tandem search algorithm (the GPM, thegpm.org; version 2010.12.01.1) and statistical validation of peptide and protein identities. Peptide and protein identifications were accepted if they could be established at greater than 95% probability. Relative quantification of the proteins was achieved using the spectrum counting method [42, 43] and the MS/MS total ion current (TIC) values using the Scaffold reports.

RESULTS

Myogenic differentiation of C2C12 cells

Six days after the onset of C2C12 myoblast differentiation they undergo cell fusion and form multinuclear myotubes (Fig. 1a). This morphological change is accompanied by a gradual increase in expression levels (detected by WB) of muscle-specific proteins such as myogenin, a transcription factor of late stage myogenesis, and SERCA1, the fast-twitch muscle-specific isoform, which can serve as a protein marker of mature myotube formation (Fig.1b). Another muscle-specific protein isoform, caveolin-3 (Cav3), is expressed only during late stage of differentiation, while the ubiquitous caveolin-1 (Cav1) isoform was detected already in myoblasts (i.e., at day 0) with a gradual increase in expression levels during differentiation (Fig.1b).

Next we used WB analysis to study protein expression profiles separately in myotubes and reserve cell subpopulations, fractionated based on their differential sensitivity to trypsin [39, 44]. As expected, myogenin, SERCA1 and Cav-3 were detected in the fraction of terminally differentiated myotubes, in contrast to original myoblasts. Importantly, in the reserve cell fraction of differentiated C2C12 cells none of these proteins were observed, suggesting a high purity of the fractionated cell populations (Fig. 1c). Cav1 expression levels were higher in reserve cells than in myoblasts or myotubes, in good agreement with previously published data on the expression of Cav1 in reserve cells [45].

Expression of apoptosis regulator proteins during myogenic differentiation of C2C12 myoblast cells

With regard to a potential regulatory effect on apoptosis accompanying C2C12 cell differentiation, we analyzed the expression levels of the anti-apoptotic protein Bcl-2, Hsp70, and of the pro-apoptotic proteins of the Bcl-2 family, Bax, Bak and Bad. We did not detect significant changes in the expression levels for these proteins during C2C12 cell differentiation (Fig. 2a); similar results were previously reported [38]. Note that an apparent increase in the Bcl-2 levels for cells incubated in DM (i.e., starting from day 2) relative to myoblasts (incubated in DMEM) is due to non-specific recognition of immunoglobulins, present in the horse serum supplement of DM, by the secondary antibodies used for WB detection (data for respective controls are not shown).

Differences became apparent when these proteins were monitored individually for myotubes and reserve cells (Fig. 2b). The two important protein markers to control the purity of the reserve cell fractions, Pax7 and MyoD (a marker of activated myoblasts), were also monitored. Reserve cells express both Pax7 and MyoD, in contrast to isolated myotubes where neither Pax7 nor MyoD were detected (Fig. 2b). Importantly, Bcl-2 was expressed predominantly in the reserve cell fractions (Fig. 2b), where Bcl-2 levels were significantly higher than in non-differentiated myoblasts. The dramatic difference in Bcl-2 levels between myotubes and reserve cells (Fig. 2c), given that Bcl-2 is co-expressed with Pax7 and Cav1 (Fig. 1c), makes Bcl-2 a useful marker for reserve cells in differentiating C2C12 cells. WB analysis of HSP70 showed a moderate increase of HSP70 expression levels in reserve cells and a significantly lower expression in myotubes. The pro-apoptotic proteins Bax, Bak, and Bad did not exhibit a pronounced difference in the expression levels between myotubes and reserve cells, with a slight prevalence in reserve cells versus myotubes. Our data for Bcl-2 expression in reserve cells and myoblast cells, as well as the expression pattern of pro-apoptotic proteins in these cell types are in a good agreement with previous immunostaining studies [37, 38]. Thus, the most significant difference between the differentiated C2C12 cell fractions is a specifically lower expression of HSP70 and Bcl-2 in myotubes relative to reserve cells.

Expression of SERCA2A and SERCA2B during myogenic differentiation of C2C12 myoblast cells

We monitored SERCA2 expression levels during C2C12 cell differentiation by WB analysis with the help of commercially available anti-SERCA2 antibodies. These antibodies recognize two isoforms, SERCA2B, a “house-keeping” isoform expressed ubiquitously, and SERCA2A, the isoform specific for cardiac and slow-twitch muscle. Both isoforms have identical sequences except for the C-terminal part (Fig. 3a), where SERCA2B contains a 50-amino acid stretch, GKECVQPATKSSCSLSACTDGISWPFVLLIMPLVVWVYSTDTNFSDMFWS, and SERCA2A, instead, a short sequence, AILE [46]. The SERCA2 isoform expressed before differentiation onset (day 0) is most likely SERCA2B (see also below). During differentiation of C2C12 cells, a significant increase in SERCA2 levels is observed (Fig.3b), increasing 2-fold by day 6 incubation in DM (Fig.3c). To reveal which isoform of SERCA2 is upregulated during differentiation, we used anti-SERCA2 antibodies recognizing both isoforms, and a custom-made antibody raised against the C-terminal peptide of SERCA2B (underlined in Fig. 3a), generously provided to us by Drs. J. Enouf and R. Bobe (Inserm, Hospital Lariboisiere, Paris, France). WB analysis with a non-isoform-specific antibody against SERCA2 revealed a significant difference in the expression levels of SERCA2 between myotubes and reserve cells: while the terminally differentiated myotubes expressed very high levels of SERCA2, the reserve cells showed little differences compared to original myoblast cells (Fig.3d). In contrast, the anti-SERCA2B antibody did not reveal significant

changes in the levels of SERCA2B between myoblasts, myotubes and reserve cells at different stages of differentiation (Fig. 3d). Therefore, the data obtained with the non-specific SERCA2 antibodies can be attributed solely to the increase in expression of the SERCA2A isoform in myotubes during differentiation.

Additional evidence for the high expression level of the SERCA2A isoform in myotubes was obtained through the use of proteomic analysis based on the mass spectrometry identification of isoform-specific peptides from the C-terminal sequences of SERCA2A and SERCA2B. The SERCA2A-specific C-terminal tryptic peptide (R)NYLEQPAILE was identified (Fig. 3e), suggesting that the SERCA2A isoform is expressed in C2C12 myotubes, in agreement with the data of transcriptomic analysis performed in developing muscle tissue and C2C12 cells [47, 48]. However, no tryptic peptides from the C-terminal region of SERCA2B were observed in any sample (including original myoblasts) suggesting a low abundance of this isoform relative to SERCA2A in myotubes.

Role of Bcl-2 and HSP70 in apoptosis accompanying myogenic differentiation of C2C12 myoblast cells

To further determine the role of Bcl-2 family proteins and HSP70 in myogenic differentiation and apoptosis of differentiating C2C12 cells we characterized their expression levels specifically in cells undergoing apoptosis, i.e. detached (floating) cells, versus live adherent cells. The detached cells forming during C2C12 cell differentiation represent a small fraction compared to cells adherent to the dish. The relative quantification of apoptotic cells on day 2, based on the amount of protein, show the development of apoptosis during differentiation (Fig. 4a). For comparison, we collected the adherent cells from the dishes at different days of differentiation. Fig. 4a shows that at the time of myotube formation (day 6), the amount of detached cells is similar to that at the early stage of differentiation (day 2), so the formation of mature myotubes does not prevent cells from detachment. A slight loss of adherent cells from the dish was also observed at day 6 (Fig.4a). To characterize the apoptotic process, we analyzed the activities of caspase 9 and caspase 12 during differentiation in both detached and adherent cells (Fig. 4b). The level of active caspase 9 (lower band on WB) was significantly higher in detached floating cells than in the adherent cells, in agreement with previously published results [15]. The highest levels of active caspase 12 (lower band on WB), an ER stress-specific caspase, in the attached cells were observed on day 2 of differentiation (Fig. 4b), while the highest protein levels of caspase 12 in the detached cells were observed at late stage of differentiation (day 6). Since the detached cells cannot be physically separated into myotubes and reserve cells, unlike the attached cells, we monitored the expression levels of myotube markers, such as SERCA1 and Cav-3, in the detached cells. Fig. 4b demonstrates that the myotube marker SERCA1 displayed higher levels in detached cells compared to the adherent cells, reflecting a predominant sensitivity of myotubes to apoptosis accompanying myogenic differentiation of C2C12 cells. The time-course of SERCA2 expression in both adherent and apoptotic detached cells was similar to that of SERCA1, corresponding to the accumulation of predominantly the SERC2A isoform in differentiated myotubes (see above). Of note, we detected intense higher molecular weight bands of SERCA1 and SERCA2 (data not shown), indicative of aggregation in the floating cells, which can be rationalized by the high sensitivity of SERCA Cys residues to oxidation [49]. It is unclear why the levels of Cav-3 were similar in detached and adherent cells. Considering that adherent cells represent a mixture of myotubes and reserve cells, we suggest that apoptotic myotubes detached from the dish may have lower levels of Cav-3 compared to myotubes adherent to the dish. Such feature could reveal a yet uncharacterized role of Cav-3 for the adhesion of myotubes to the dish during differentiation.

WB analysis of Bcl-2 and HSP70 shows diminished levels of these proteins in the apoptotic detached cells as compared to the adherent cells for all times after the onset of differentiation (Fig. 4c). Again, the detached apoptotic cells display the features of myotubes (compare with Fig. 2b), corroborated by the lack of both a reserve cell marker, Pax7, and a marker of activated myoblast, MyoD, in the detached cells over the entire differentiation period (Fig. 4c). Based on these results we conclude that the cells specifically sensitive to apoptosis during differentiation are neither myoblasts nor reserve cells; rather, they represent mature myotubes or myotubes at the early stages of transformation, characterized by low levels of Bcl-2 and HSP70 expression.

The assessment of pro-apoptotic proteins Bax, Bak, and Bad in the detached cells at the late stage of differentiation (day 6) reveals no increase in the protein expression levels; however, an additional lower molecular weight band appears, which is not present in the adherent cells and may represent a nonphosphorylated form of these proteins (more distinctive for Bax) (Fig. 4d). The main band (likely the phosphorylated form of Bax) is slightly larger in the adherent cells relative to the detached cells. The levels of Bak and Bad expression in the adherent cells were much higher than in the floating cells following the elevated levels of these proteins in the reserve cells (Fig. 4b), a subpopulation that is more resistant to apoptosis and adheres to the dish. Thus, these results suggest no significant contribution of pro-apoptotic Bax, Bad and Bak proteins to the sensitivity of C2C12 cells to apoptosis during differentiation.

An important question is whether our observations apply to apoptosis in general, or whether they are specific for apoptosis accompanying C2C12 cell differentiation. Therefore, for comparison, we investigated the sensitivity of differentiated C2C12 cells to H_2O_2, i.e. oxidative stress-induced apoptosis. These results are described in the following.

Apoptotic sensitivity of myotubes and reserve cell subpopulation of differentiated C2C12 cells to H_2O_2

Recent work demonstrated that the incubation of differentiated C2C12 cells with as high as 4 mM H_2O_2 for 24 h led to apoptosis, although no data on which specific cells undergo apoptosis have been provided [20]. Therefore, we explored the differential sensitivity of myotubes and reserve cells to hydrogen peroxide-induced apoptosis. The microscopic images in Fig. 5 show the H_2O_2 concentration-dependent loss of myotubes through detachment upon the treatment of differentiated C2C12 cells with increasing concentrations of H_2O_2. We note that cells detach after 4 h incubation with as low as 0.5 mM H_2O_2 and at 4 mM H_2O_2 virtually no myotubes remain attached. For the maximal concentration of H_2O_2 tested this effect is observed even for lower incubation times (Fig.5f).

WB analysis of the cells remaining on the dishes after incubation with 4 mM of H_2O_2 (Fig. 6a) discloses a marked time-dependent decrease in the levels of myotube markers, SERCA1 and Cav3. In contrast, Bcl-2 levels slightly increase, suggesting an increase of the fraction of reserve cells in the adherent cells (Fig.6a). Therefore, H_2O_2 produces a selective loss of myotubes in a time- and concentration-dependent manner. An increase in the incubation time allows to detect the pro-apoptotic effects of H_2O_2 on myotubes at physiologically more relevant H_2O_2 concentrations such as 200 μM based on the levels of SERCA1, Cav1 and Bcl-2 (Fig.6b); in the same time, the amounts of reserve cells do not significantly change or even increase slightly. The latter effect is due to significantly higher fractions of the reserve cells in the total mass of differentiated C2C12 cells. This can be easily demonstrated by a comparison of SERCA1 and Bcl-2 levels in both adherent and detached cells after H_2O_2 treatment (Fig. 6c).

Proteomic analysis of apoptotic cells: different stimuli result in different myotube-specific protein profiles

In addition to WB detection of selected cell-specific protein markers, we performed a WB analysis of the proteins of the detached cells obtained upon treatment for 4 h with 4 mM H_2O_2. To validate our approach, the detached cells were also produced by the incubation with 1 μM TG, an ER stress and apoptosis inducer, and by mild trypsinization to obtain a pure fraction of myotubes. The myotube content in these samples was monitored through the analysis of the cell-specific protein markers as shown in Fig.7a. The levels of Cav3, a marker of mature myotubes, were similar for apoptotic cells after H_2O_2 treatment and myotubes isolated by mild trypsinization. In contrast, apoptotic cells obtained by TG treatment showed lower levels of Cav3 and higher levels of Bcl-2. Thus, the detached cells obtained by TG-induced apoptosis demonstrated less similarity to the control myotubes, and represent a mixture of myotubes and reserve cells.

These findings were further confirmed by proteomic analysis and relative quantification of muscle-specific proteins by TIC (identified by specific MS/MS analysis) during shotgun LTQ-FT-MS/MS analysis of respective protein digests [42, 43] as described under “Experimental Procedures”. We quantified the TIC values of peptides representing SERCA1, SERCA2, myosin light chain, myosin 3 and troponin T (skeletal muscle fiber-specific protein isoforms) as well as β-tubulin and myosin-9, the non-specific isoforms (Fig. 7B). Generally the levels of muscle-specific proteins (SERCA1, SERCA2, myosin light chain, myosin 3 and troponin T) are similar for H_2O_2-induced apoptotic cells and control myotubes, in contrast to TG–induced apoptotic cells. The latter show different, significantly less myotube-specific protein profiles. These data validate the applicability of the proteomic approach for the identification of myotubes as a selective target for H_2O_2-induced apoptotis in differentiated C2C12 cells.

Protection of myotubes by Bcl-2 against H_2O_2-induced apoptosis

Although the expression level of Bcl-2 in reserve cells is much higher than in myotubes, Bcl-2 is also expressed in myotubes at low levels. To study the impact of these low expression levels of Bcl-2 on the sensitivity of myotubes to H_2O_2-induced apoptosis we analyzed the adherent myotube cells surviving H_2O_2 treatment (Fig.8a). We separated the subpopulations of respective myotubes and reserve cells by mild trypsinization as described above under control of cell-specific protein markers. Fig. 8b shows that the expression levels of Bcl-2 in the population of myotubes resistant to H_2O_2-induced apoptosis are much higher than those in control myotubes, based on representative average Bcl-2 levels for the entire myotube population. The densitometry quantification of the Bcl-2 protein levels for H_2O_2-resistant and average myotubes is presented in Fig.8c. H_2O_2-resistant myotubes also exhibit lower levels of SERCA1 and Cav3 expression, suggesting that they may represent a subpopulation of less mature myotubes, i.e. myotubes at less advanced stage of differentiation.

To test whether higher levels of Bcl-2 expression in myotubes increase their tolerance to H_2O_2-induced apoptosis, we analyzed the effect of Bcl-2 overexpression. Proliferating C2C12 cells (myoblasts) were transfected with a Bcl-2 expression vector and differentiation was initiated 24 h after transfection. First we looked at the effect of Bcl-2 on apoptosis during differentiation. The WB analysis of selected proteins in apoptotic (detached) cells collected at different days of differentiation is shown in Fig. 9a. Bcl-2 overexpression resulted in high levels of the protein in the detached cells collected at days 2 and 4 after the onset of differentiation, when the highest rate of apoptotic death is observed (compare to Fig. 3A), suggesting that Bcl-2 overexpression does not protect these cells from apoptosis accompanying C2C12 cell differentiation. Higher levels of Bcl-2 present in the detached

cells at late stage of differentiation (day 6) also failed to protect these mature myotubes from spontaneous apoptosis (Fig.9a). Importantly, analysis of Bcl-2 expression in myotubes and reserve cells (Fig.9b) confirmed that the myotubes rather than the reserve cells contained significantly elevated levels of Bcl-2 after overexpression. Of note, overexpression of Bcl-2 also resulted in increased levels of pro-apoptotic Bax (both phosporylated and no-phosphorylated forms) in the myotubes, without significant changes in the levels of other pro-apoptotic proteins, Bak and Bad (data not presented).

We further analyzed the effect of Bcl-2 overexpression on the survival of myotubes after H_2O_2-induced apoptosis. In serum-free media without added H_2O_2, lower levels of Cav3 indicate a reduced number of attached myotubes, i.e., an increased apoptotic death due to serum withdrawal in Bcl-2 overexpressing cells (Fig. 9c, lanes 1 and 2). However, sham transfected cells displayed significantly less tolerance to H_2O_2-induced apoptosis based on the Cav3 levels in both the attached and the detached cells (compare lanes 3 and 4, 5 and 6, respectively, in Fig. 9c). WB densitometry quantitation data are presented in Fig. 9D showing a ca. 40% protection of myotubes overexpressing Bcl-2 against H_2O_2-induced apoptosis. Bcl-2 protection occurred despite the higher levels of activaved caspase 9 detected in detached cells (Fig. 9c) potentially due to higher levels of Bax (Fig. 9b) in the Bcl-2 overexpressing myotubes. Taken together, our data reveal a protective effect of Bcl-2 against H_2O_2-induced apoptosis in myotubes but not against apoptosis accompanying myocyte differentiation.

DISCUSSION

The differentiation of skeletal muscle satellite cells represents a complex multi-step process and apoptosis is one of the routes accompanying differentiation [3, 13, 50, 51]. In the current work, we studied the expression profiles of Bcl-2 and pro-apoptotic Bax, Bak and Bad and also of SERCA2B and HSP70, proteins that can interact with Bcl-2 and control apoptosis in different steps of differentiation through diverse mechanism. We performed analysis of protein expression profiles separately for reserve cells, myoblast and myotubes at different stages of C2C12 cell differentiation and correlated them with apoptotic responses of these cell populations. The formation of two distinct cell populations at the late stages of differentiation (day 6) with different morphology, sensitivity to trypsin, and protein markers of reserve cells (Pax7 and Cav1) and myotubes (myogenin, SERCA1 and Cav3) makes their separate characterization a feasible task. WB analysis allows for detection of very low expression levels of Bcl-2 in myotubes, in contrast to the high expression levels of Bcl-2 in the reserve cells (Fig. 2b), that is in good agreement with previous immunostaining studies [37, 38]. In addition, reserve cells express higher levels of Hsp70 and proapoptotic proteins Bax and Bak compared to myotubes (Fig. 2b). We also show that Bcl-2 can be barely detected in myoblasts. Such feature of the reserve cells turns Bcl-2, together with Pax7 and Cav1, into a very useful marker of reserve cell subpopulations during muscle cell differentiation. The activation of reserve cells may induce them to re-enter the cell cycle for transformation to proliferating myoblasts with low Bcl-2 expression, which is a necessary step prior to the formation of myotubes (to replace damaged myotubes) with lowest levels of Bcl-2, and generate a new reserve cell population with high Bcl-2 expression. Since the contribution of different cell fractions may vary over time under different conditions, the knowledge of the expression profile of Bcl-2 in different cells through myogenic differentiation can be helpful for analysis of the effect on myogenic differentiation, and could help to avoid data misinterpretation.

Our data, in accord with earlier publications [12-15], demonstrate spontaneous apoptosis of activated myoblasts at the early stage of differentiation, accompanied by the detachment of apoptotic cells from the culture dish. In contrast, based on the expression of myotube

markers, SERCA1 and Cav3, and on Bcl-2 for reserve cells, we now show that at a late stage of C2C12 cell differentiation, myotubes are specifically sensitive to spontaneous apoptosis leading to the detachment and loss of myotubes (Fig.4). We show higher expression levels of Bcl-2 in reserve cells then in myoblasts (Fig. 2b), whereas myoblast cells display higher expression levels of pro-apoptotic proteins, Bax, Bak and Bad, and therefore even higher ratios of pro-apoptotic to anti-apoptotic proteins compared to reserve cells. This is consistent with earlier published data on the high sensitivity of myoblasts to apoptotic signals [8], and likely represents a common mechanism of apoptotic regulation based on the interaction between pro- and anti-apoptotic Bcl-2 family members [22].

An important question is what could be a reason for the down-regulation of Bcl-2 when activated reserve cells transform to myoblasts? In our study, the expression of MyoD, a transcription factor implicated in the early stage of myogenic differentiation and necessary for the progression of quiescent muscle satellite cells in the cell cycle [50, 52, 53], was detected primarily in C2C12 myoblasts; reserve cells showed significantly lower expression levels, and myotubes contained only trace amounts of MyoD (Fig.2b). The levels of MyoD inversely correlate with the levels of Bcl-2 in myoblast and reserve cells (Fig.2b), and the higher sensitivity of myoblasts to apoptosis upon activation of differentiation shown by us and other studies [54]. It was suggested before that MyoD, in addition to the regulation of differentiation [55, 56], may control apoptosis, and a pro-apoptotic role of MyoD was credited to Pax3 and the miRNA-1 and miRNA-206-mediated down-regulation of Bcl-2 and Bcl-xL [54,57]. Though, further studies on the regulation of Bcl-2 levels specifically in different subpopulations of differentiating C2C12 cells through MyoD and other potential mechanisms will be needed.

However, the underlying mechanism of apoptotic sensitivity of myotubes at a late stage of differentiation (day 6) is apparently dissimilar to mechanisms of apoptosis at early stage of differentiation discussed above. In apoptotic floating myotubes we failed to detect increased levels of Bak and Bad, although Bax exhibited an additional low molecular band in the detached cells, probably corresponding to a non-phospohorylated form (Fig.4d). We also detected a similar de-phosphorylated form of Cav1 in the detached cells (data not shown), which can potentially be rationalized by a general decrease of kinase relative to phosphatase activity in the apoptotic cells. Thus, our results suggest that apoptotic detachment of myotubes during differentiation is not likely regulated by pro-apoptotic proteins of the Bcl-2 family alone. In addition to Bcl-2, the resistance of reserve cells to apoptosis accompanying muscle cell differentiation may also be credited to the higher levels of HSP70 in the adherent cells (Fig.4c).

Recently, it was reported that Bcl-2 and HSP70 may interact with the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2B) in C2C12 myoblasts [36]; specifically in vitro experiments demonstrated a strong effect of Bcl-2 on SERCA activity [34, 35], suggesting that both Ca^{2+} homeostasis, and, therefore, apoptosis in muscle cells might be affected by such interactions. A key observation of the present study is the difference in the expression levels of Bcl-2 and Hsp70 between differentiated C2C12 reserve cells and myotubes while the expression of SERCA2B remained at comparable levels (Fig.3c). It is possible that the low expression levels of Bcl-2 and HSP70 in myotubes result in higher SERCA2B activity and higher ER Ca^{2+} levels available for the execution of apoptosis. Unfortunately, we were unable to measure the specific activity of SERCA2B in myotubes because of the high expression levels of other SERCA isoforms involved in the muscle contraction and relaxation cycle, SERCA1 and SERCA2A. It is tempting to hypothesize that Bcl-2 and HSP70 may work as putative modulators of SERCA2B and Ca^{2+}-regulated apoptosis in myotubes depicted in the scheme in Fig.10. Besides, other potential mechanisms, which could control Ca^{2+} homeostasis and Ca^{2+}-dependent apoptosis, such as changes in the

expression levels of inositol triphosphate receptor (IP3R), previously revealed in model systems and in dystrophic myotubes [26, 32], and the interaction of Bcl-2 with the IP3R [58] have also to be also considered.

Spontaneous apoptosis of mature (differentiated) C2C12 induced by hydrogen peroxide cells was chosen as a comparative model to study apoptotic responses in different cell types. Hydrogen peroxide-induced apoptosis of differentiated C2C12 cells has been described [20]; however, in previous studies no analysis of isolated populations of differentiated cells (reserve cells and myotubes), as well as of adherent and non-adherent cells have been performed. Here, by monitoring the myotube markers SERCA1 and Cav3 and a marker of the reserve cells, Bcl-2, for both adherent and detached cells, we found that H_2O_2-induced apoptotic detachment involves primarily mature myotubes (Figs. 5 and 6). Proteomic analysis of apoptotic (detached) cells after exposure to hydrogen peroxide confirmed the similarity of muscle-specific protein expression profiles of these cells to control myotubes, fractionated by mild trypsinization. In contrast, the apoptotic cells produced by treatment with TG, a specific SERCA inhibitor, represent a mixture of myotubes and reserve cells. The overexpression of Bcl-2 in myoblasts prior to differentiation resulted in higher levels of Bcl-2 expression in the myotubes whereas those in the reserve cells were not significantly altered (Fig. 9b). Importantly, the higher levels of Bcl-2 in these myotubes correlated with an elevated resistance to H_2O_2-induced apoptosis (Fig. 9c and 9d). In contrast, Bcl-2 overexpression did not prevent spontaneous apoptosis of myoblasts at early stages of differentiation (Fig. 9a). These data suggest different control mechanisms for early stages of differentiation and hydrogen peroxide-induced apoptosis. However, one cannot exclude that Bcl-2 overexpression may result in some as yet unidentified changes in cell adhesion and/or myotube formation, suggesting an additional role of Bcl-2 in differentiation besides protection against apoptosis.

Taken together, our results demonstrate that apoptotic responses in differentiated C2C12 cells are likely regulated through expression of Bcl-2 so that the sensitivity of myotubes and the resistance of reserve cells to apoptosis result from low and high Bcl-2 levels, respectively. The downstream mechanism(s) for terminally differentiated cells may involve pro-apoptotic Bcl-2 family members, such as Bax, Bak, and Bad, which may contribute to spontaneous apoptosis during muscle cell differentiation; in addition, proteins implicated in regulation of Ca^{2+}-mediated apoptosis, such as SERCA2b and HSP70, may be involved. Further studies are necessary to prove the physiological relevance of under normal and pathological conditions.

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LIST OF ABBREVIATIONS

DM	differentiation medium
ER	endoplasmic reticulum
GM	growing medium
TG	thapsigargin
TIC	total ion current

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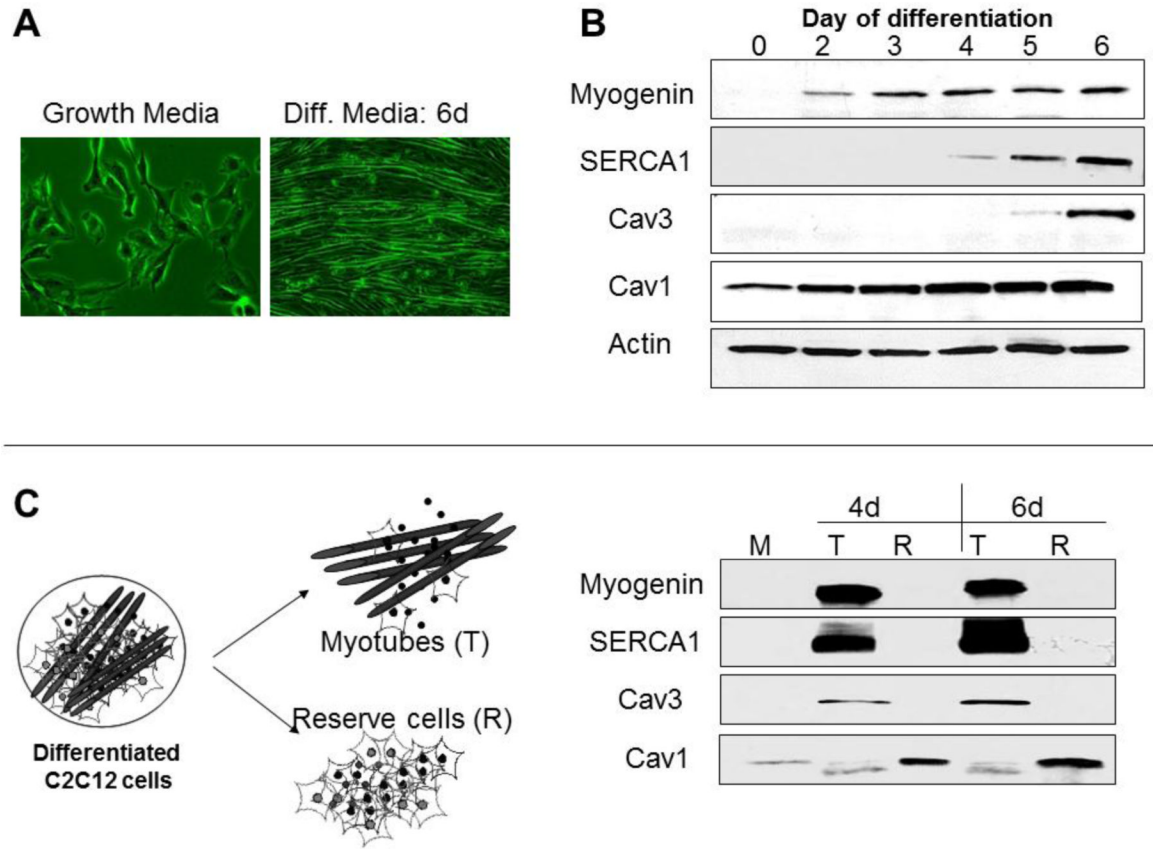

Fig.1. Differentiation of C2C12 myoblasts and myotube formation

(a) – Phase contrast light microscopy of proliferating myoblasts and myotubes formed on day 6 of differentiation. (b) - WB analysis of specific protein marker expression during C2C12 cell differentiation assessed with antibodies against myogenin, SERCA1, Cav3, and Cav1. Actin expression levels are shown as controls for protein load. (c) – Myotubes and reserve cells were separated at days 4 (4d) and 6 (6d) of differentiation, as shown in the schematic representation and described under “Experimental Procedures”, and analyzed by WB with antibodies specific for myogenin, SERCA1, Cav3, and Cav1. The abbreviations used for the cell types are M - proliferating myoblasts; T – myotubes; R - reserve cells.

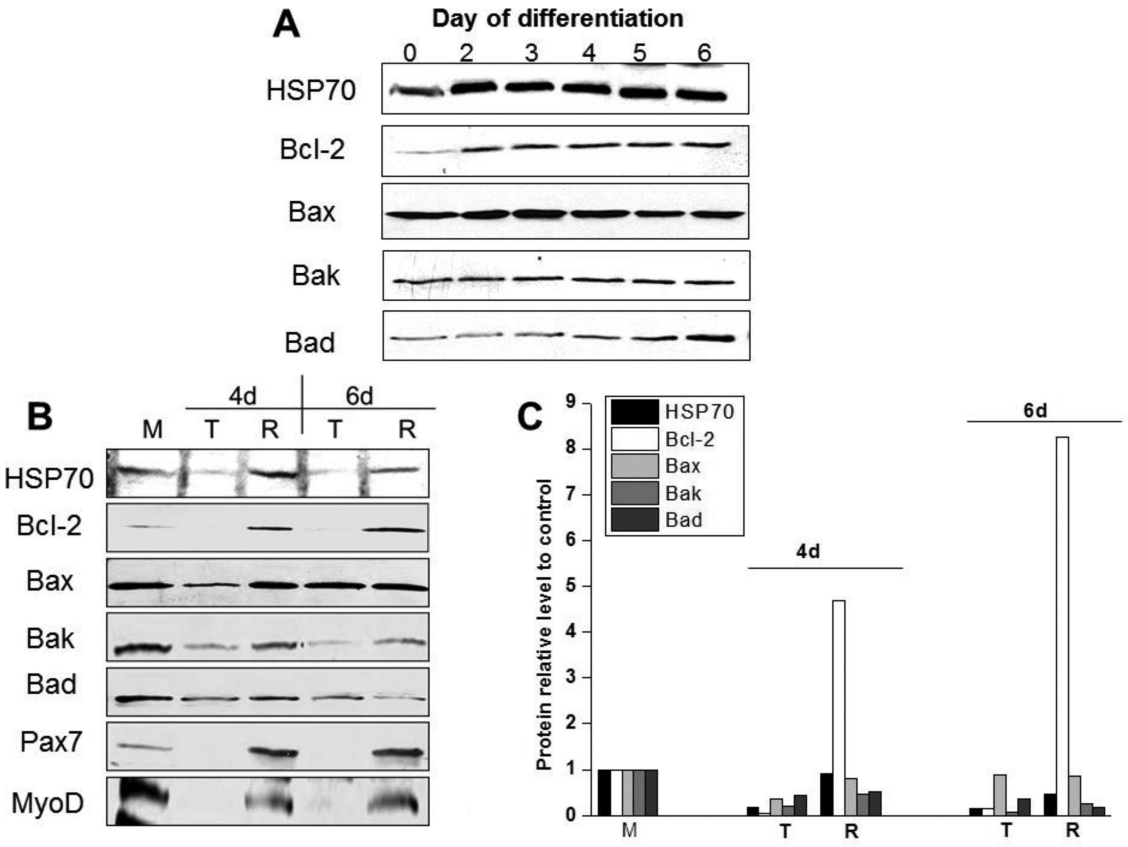

Fig.2. Expression profiles of Hsp70 and Bcl-2 family proteins during myogenic differentiation of C2C12 cells

(a) - Proliferating myoblasts (day 0) and differentiating C2C12 cells were harvested on days 2 - 6 after the onset of differentiation and analyzed by SDS-PAGE and WB analysis with antibodies against Hsp70, Bcl-2, Bax, Bak, and Bad. (b) – Analysis of these proteins separately in myoblasts (M), myotubes (T) and reserve cells (R) along with cell type-specific protein markers Pax7 and MyoD at days 4 (4d) and day 6 (6d) of differentiation. (c) Densitometry analysis of data from (b).

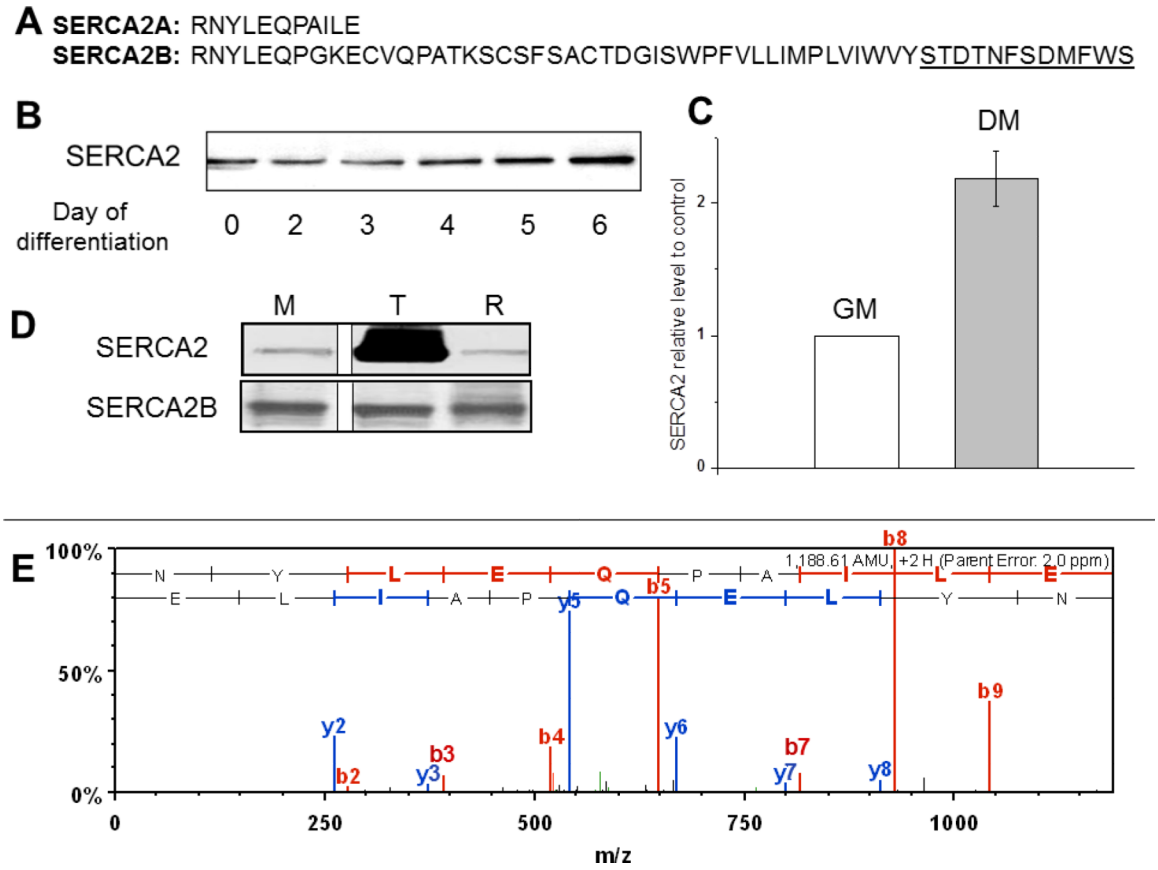

Fig.3. Expression of SERCA2A and SERCA2B isoforms in differentiating C2C12 cells

(a) – Sequence specificity of SERCA2A and SERCA2B in the C-terminal part. (b) – WB analysis of protein expression during C2C12 cell differentiation assessed with antibodies against SERCA2. (c) - Densitometry analysis of WB data of SERCA2 level in GM and in DM after 6 days of differentiation from three independent experiments.(d) – Analysis of SERCA2 isoforms separately in myoblasts (M), myotubes (T) and reserve cells (R): myotubes and reserve cells were separated as described under “Experimental Procedures” at day 6 of differentiation and analyzed by WB with antibodies specific for SERCA2 and SERCA2B (polyclonal custom antibody raised against the C-terminal peptide underlined in panel A). (e) - Capillary LC-LTQ-FT-MS/MS spectrum of the C-terminal tryptic peptide of SERCA2A, identified in the tryptic digests of C2C12 cell lysates.

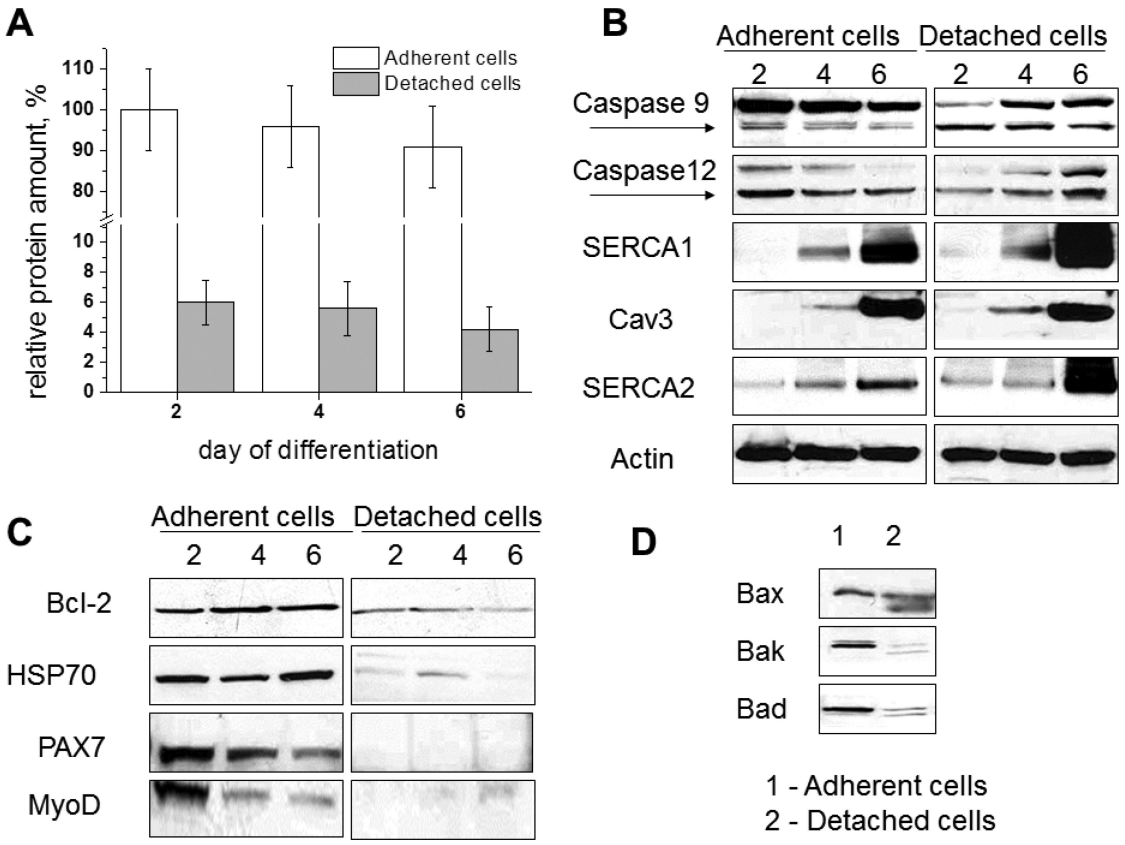

Fig.4. Characterization of apoptotic proteins in the adherent and detached cells during C2C12 differentiation

(a) – Total protein content in harvested cells normalized to the value for adherent cells harvested on day 2. (b) – WB analysis of adherent and detached cells with anti-caspase 9, anti-caspase 12, anti-SERCA1, anti-cav3, and anti-SERCA2 antibodies. Actin expression levels in cells are presented for control of protein load. (c) - WB analysis of adherent and detached cells with anti-Bcl-2, anti-Hsp70, anti-Pax7 and anti-MyoD antibodies. (d) - WB analysis of adherent (lane 1) and detached (lane 2) cells on day 6 of differentiation with anti-Bax, anti-Bak and anti-Bad antibodies.

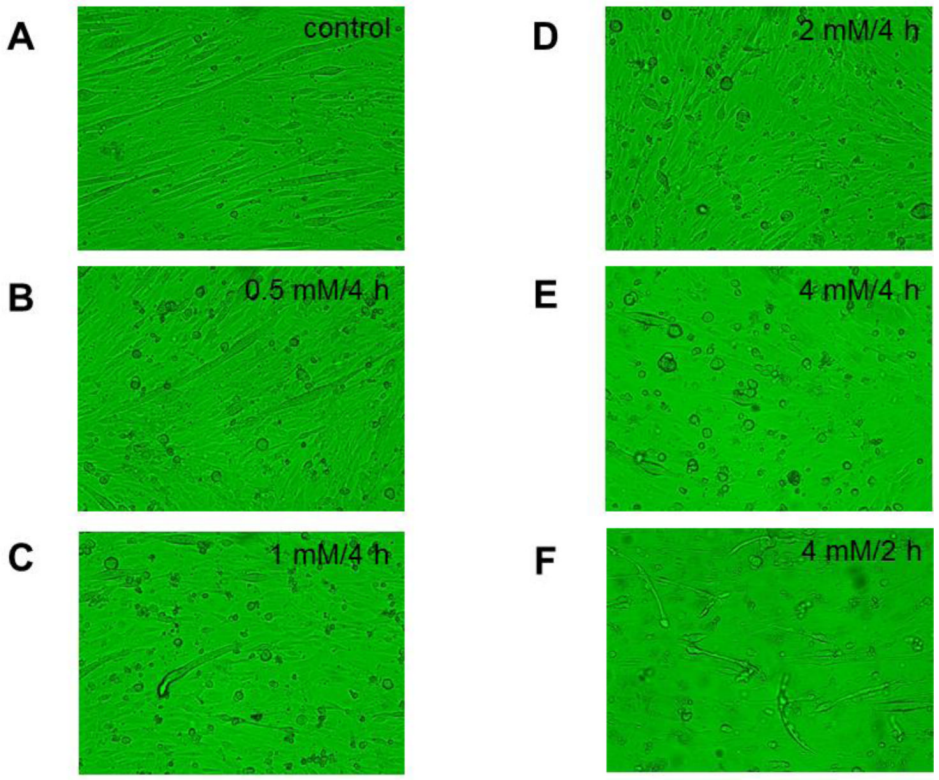

Fig.5. Morphology of differentiated C2C12 cells after H_2O_2 treatment

Phase contrast light microscopy of differentiated C2C12 cells prior to (a) and after exposure to 0.5 (b), 1 (c), 2 (d), and 4 mM H_2O_2 (e) for 4 h or 2 h (f) as described under "Experimental Procedures".

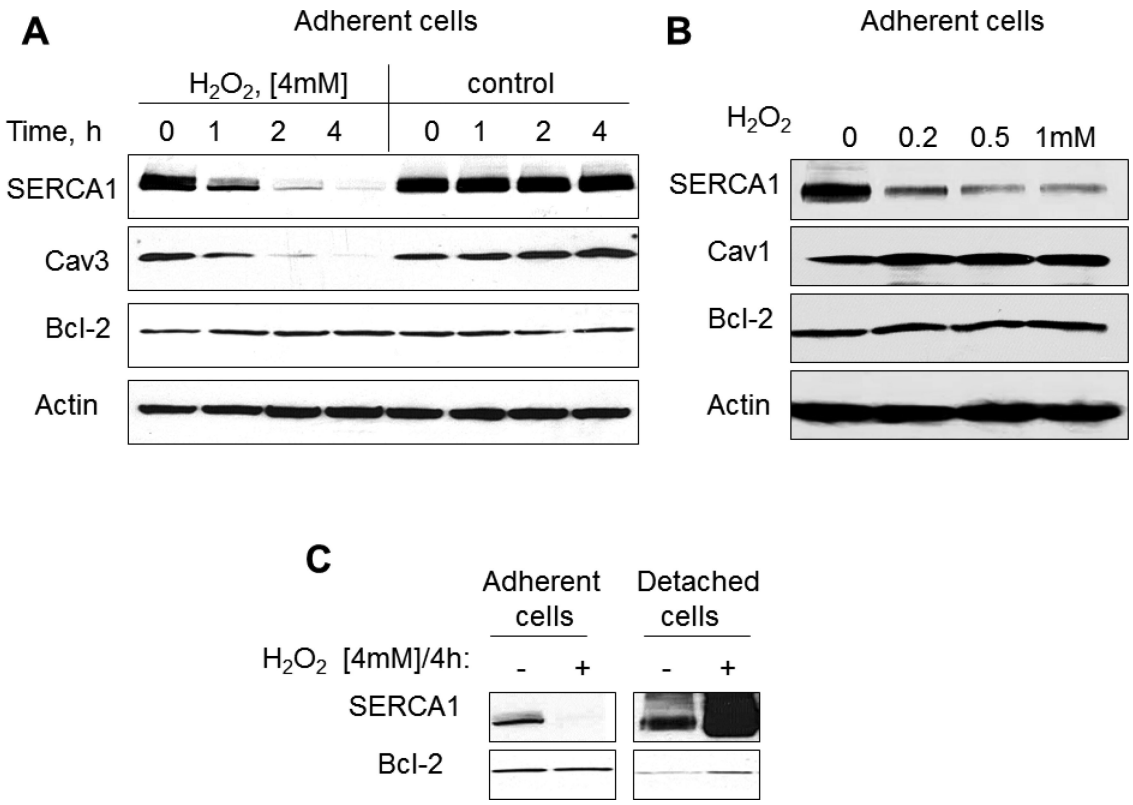

Fig.6. WB analysis of protein markers in differentiated C2C12 cells adherent and detached from the plate after exposure to hydrogen peroxide

Differentiated C2C12 cells were incubated without (control) or with 4 mM H_2O_2 for different times as indicated (a) or incubated overnight with smaller concentrations of H_2O_2 (b), and adherent cells were analyzed by WB with anti-SERCA1, anti-Cav3 and anti-Bcl-2 antibodies. Actin expression levels are presented for control of protein load. (c) Differentiated C2C12 cells were incubated in the absence or in the presence of 4 mM H_2O_2 for 4 h, and expression levels of SERCA1 and Bcl-2 were analyzed by WB separately for adherent and detached cells.

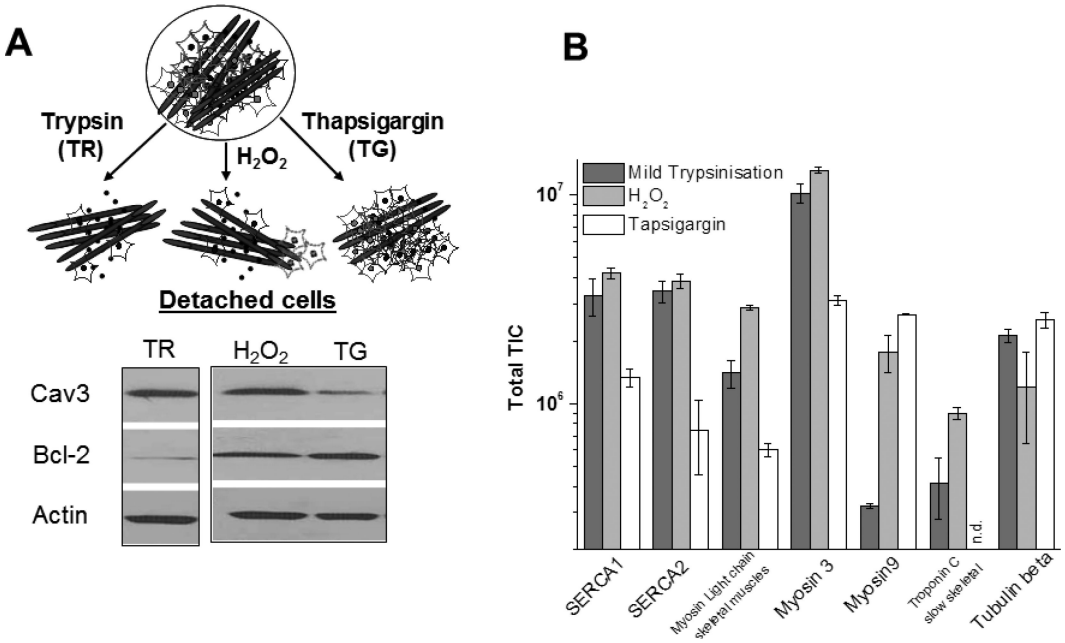

Fig.7. WB and MS analyses of selected proteins in detached cells collected after mild trypsinization to obtain myotubes (T) or incubation of differentiated C2C12 cells with either 4 mM H_2O_2 or 1 μM thapsigargin (TG) for 4 h

(a) – Detached cells obtained after different treatment were harvested as depicted in the inserted scheme and analyzed by WB for Cav3, Bcl-2, and actin. (b) – Differentiated C2C12 cells treated as above were lysed, digested with trypsin, and submitted to a "shotgun" capLC-LTQ-FT-MS/MS analysis coupled to a protein database search, followed by a quantification of peptide TIC values for selected proteins as described under "Experimental Procedures". Protein IDs in the IPI (mouse) database: SERCA1, 00311654; SERCA2, 00468900; Myosin light chain, skeletal muscle, 00224549; Myosin 3, 00380895; Myosin 9, 00123181; Troponin c, slow-twitch skeletal muscle, 00113712; Tubulin beta, 00117352. (Note: error bars on logarithmic scale).

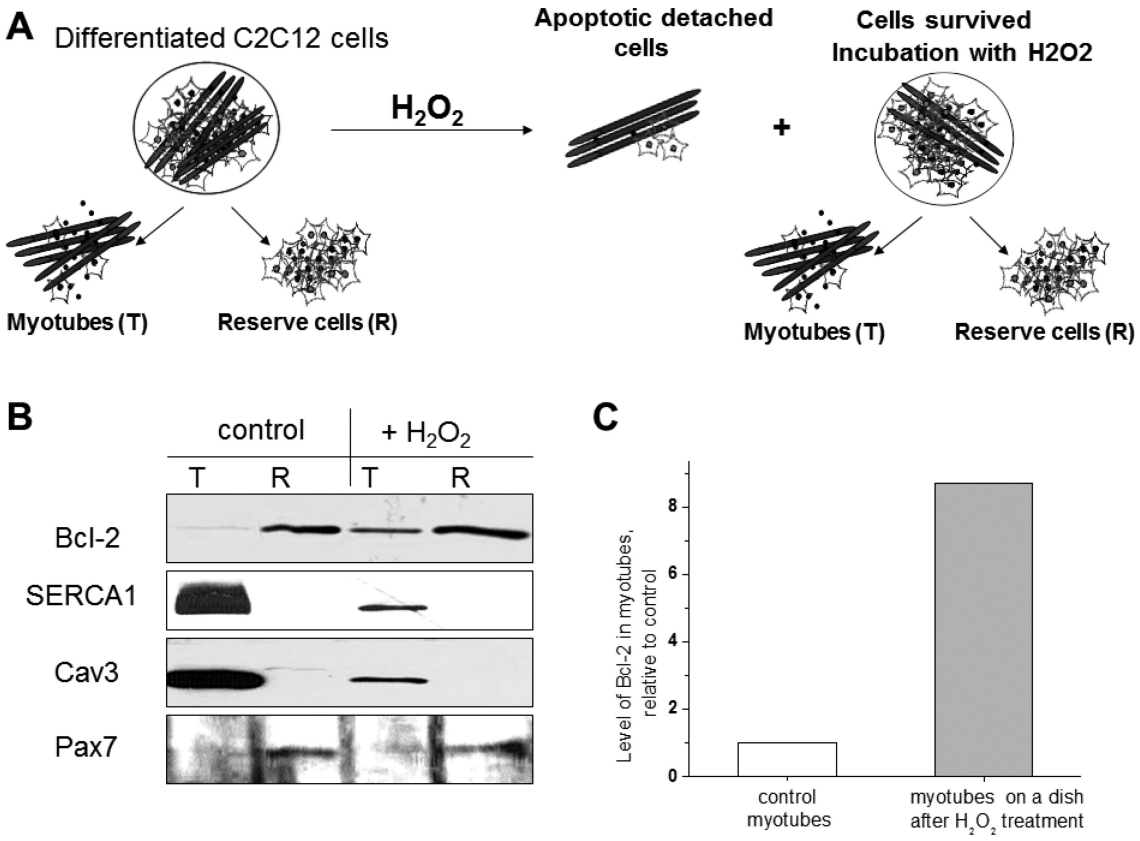

Fig.8. Bcl-2 protects myotubes from H_2O_2-induced apoptosis

Differentiated C2C12 cells were incubated without (control) or in the presence of 4 mM of H_2O_2 for 2 h as shown in the schematic representation, and myotubes and reserve cells were separated by mild trypsinization as described under “Experimental Procedure”. (a) – Scheme of experiment. (b) – Expression profiles of Bcl-2, SERCA1, Cav3, and Pax7 in myotubes and reserve cells analyzed by WB after incubation without (control) or with 4 mM H_2O_2 for 2 h. (c) - Bcl-2 levels in non-treated myotubes (control) and myotubes remaining attached to the dish after incubation with 4 mM H_2O_2 for 2 h, collected by mild trypsinization, and analyzed by a densitometry analysis of the respective blots presented in panel B.

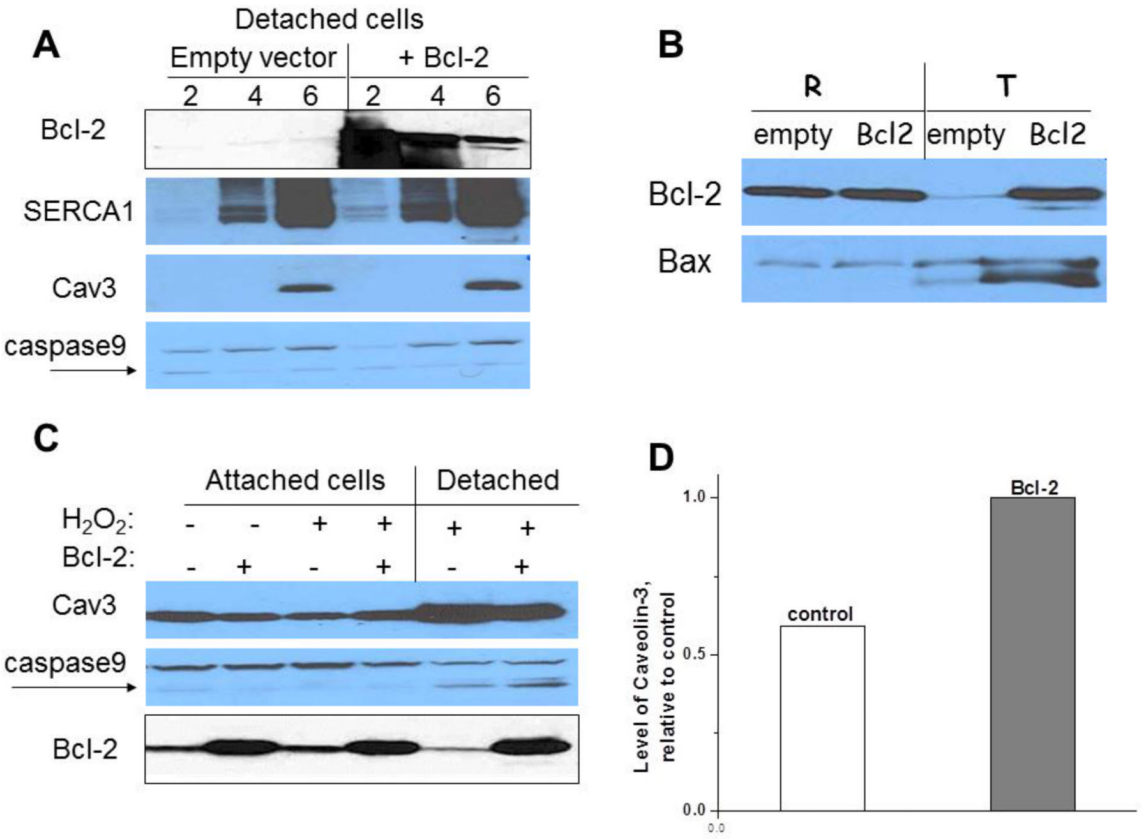

Fig.9. Effect of overexpression of Bcl-2 on myotube apoptosis

Proliferating C2C12 myoblasts were transfected with either empty vector or Bcl-2 expression vector for 24 h before the onset of differentiation. (a) - Cells detached from the dishes at days 2, 4, and 6 after the initiation of differentiation were harvested and analyzed by WB for Bcl-2, SERCA1, Cav3 and caspase 9. (b) - Myotubes and reserve cells remaining attached to the dishes at day 6 after initiation of differentiation were separated via differential trypsinization as described under “Experimental Procedures”, and analyzed by WB with anti-Bcl-2 and anti-Bax antibodies. (c) – Control and Bcl-2 transfected C2C12 cells at day 6 after initiation of differentiation were incubated without or with 4 mM H_2O_2 for 2 hours, followed by WB analysis for Cav3, Bcl-2, and caspase 9 of both adherent and detached cells. (d) - Densitometry quantification of Cav-3 on the blots (marker of myotubes) shown in panel C, normalized to the level of Cav-3 in Bcl-2 transfected cells, remaining adherent to the dish after exposure to 4 mM H_2O_2 for 2 hours.

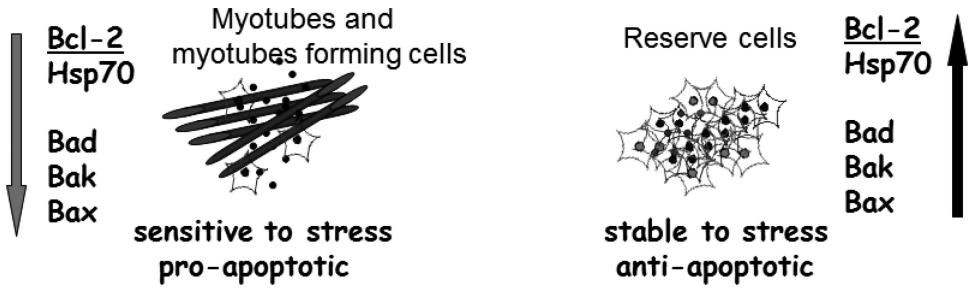

Fig.10.
The interplay between Bcl-2 family proteins and HSP70 to control apoptotic responses of myotubes and reserve cells.