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Caspase-3-Mediated Cyclic Stretch-Induced Myoblast Apoptosis Via a Fas/FasL-Independent Signaling Pathway During Myogenesis

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ABSTRACT

Skeletal muscle cells are exposed to mechanical stretch during embryogenesis. Increased stretch may contribute to cell death, and the molecular regulation by stretch remains incompletely understood. The aim of this study was to investigate the effects of cyclic stretch on cell death and apoptosis in myoblast using a Flexercell Strain Unit. Apoptosis was studied by annexin V binding and PI staining, DNA size analysis, electron microphotograph, and caspase assays. Fas/FasL expression was determined by Western blot. When myoblasts were cultured on a flexible membrane and subjected to cyclic strain stress, apoptosis was observed in a time-dependent manner. We also determined that stretch induced cleavage of caspase-3 and increased caspase-3 activity. Caspase-3 inhibition reduced stretch-induced apoptosis. Protein levels of Fas and FasL remained unchanged. Our findings implicated that stretch-induced cell death is an apoptotic event, and that the activation of caspase cascades is required in stretch-induced cell apoptosis. Furthermore, we had provided evidence that caspase-3 mediated cyclic stretch-induced myoblast apoptosis. Mechanical forces induced activation of caspase-3 via signaling pathways independent of Fas/FasL system. J. Cell. Biochem. 107: 834–844, 2009.

KEY WORDS: CYCLIC STRETCH; CELL DEATH; APOPTOSIS; CASPASE-3; MYOBLAST

Skeletal muscle formation or myogenesis is a complex and highly regulated process that involves the determination of multipotential mesodermal cells to give rise to myoblasts, proliferating myoblasts withdraw permanently from the cell cycle, express muscle-specific genes, and fuse into multinucleated myotubes [Perry and Rudnick, 2000]. Previous studies established experimental conditions for in vitro fusion of myoblast cell lines by lowering the concentration of mitogen [Nadal-Ginard, 1978]. These myogenic cell lines (e.g., mouse C2C12 and rat L6) can be induced to differentiate by withdrawal of mitogens, such as serum [Wu et al., 2000]. Myogenesis is required for growth, maintenance, and repair of injured muscle fibers [Parker et al., 2003].

Some earlier studies [Wedhas et al., 2005] demonstrated that embryonic muscle precursor cells undergo temporally regulated disintegration during myogenesis, a process later referred to as programmed cell death or apoptosis [Glucksmann, 1951]. Subsequent in vitro studies further revealed that a large fraction of myoblasts undergo apoptosis during the differentiation of myoblasts, whereas the terminally differentiated myotubes are relatively resistant to apoptosis [Walsh, 1997]. It was recently shown that the activity of caspase-3, a key apoptotic serine protease, plays an important role during myogenic differentiation. Genetic deletion of caspase-3 in mice and in vitro treatment of myogenic cells with caspase-3 inhibitor suppressed myoblast fusion and myotube

Jiaqiang Liu, Jing Liu, and Jing Mao contributed equally to this work.

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formation [Kuida et al., 1996; Fernando et al., 2002]. Although myoblast apoptosis was reported in the literature more than 50 years ago [Nakanishi et al., 2007], the cause of apoptosis and the mechanism that initiates caspase activity during differentiation were largely unknown.

In recent years, accumulating evidence suggests a significant role of physical forces in the development and maintenance of skeletal muscle and in the onset and perpetuation of several myofiber diseases [Proske and Morgan, 2001; Rando, 2001]. Mechanical stress is recognized as an important extracellular stimulus that promotes cellular growth and survival, influences metabolic processes (including gene expression), and governs tissue architecture in various cell types [Ruoslahti, 1997; Lew et al., 1999; Gillespie and Walker, 2001]. Although these studies have established the significant role of mechanical force in activation of quiescent satellite cells, initiation of cell proliferation, differentiation, and survival [Sastry et al., 1996, 1999], skeletal muscle remodeling requires coordinate regulation of cell proliferation and cell death or apoptosis during embryonic and neonatal muscle development. However, the cause of apoptosis and the mechanism of initiation of caspase activation during stretch-induced differentiation of myoblast remain largely unknown.

We have been focusing on identifying the trigger of the role of apoptosis and caspase activation during stretch-induced differentiation of myoblast. Using an in vitro system to subject myoblast to cyclic mechanical stretch, we tested (a) whether mechanical stretch caused an increase in the number of cell death, (b) which stretch-induced form of cell death, apoptosis, or necrosis, was dominant? (c) whether caspase-3 mediated cyclic stretch induced myoblast apoptosis, and (d) whether stretch caused activation of Fas/FasL system that have been implicated in the regulation of myoblast cell death and apoptosis.

METHODS

CELL CULTURE

L6 rat skeletal muscle myoblasts (American Type Culture Collection, CRL-1458) were cultured in Eagle's minimum essential medium (MEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma) (both from Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and nonessential amino acids in a 5% CO_2 atmosphere at 37°C.

APPLICATION OF CYCLIC STRETCH

The cells were stretched as previously described [Kook et al., 2008], with a modification according to our previous study [Yuan et al., 2006]. Briefly, L6 rat skeletal muscle myoblasts were seeded in GM at 3×10^5 cells/well on Bioflex Collagen Type I-coated 6-well plates (Flexcell International Corporation, NC) and grown to 70% confluence (2–3 days). When the cells had reached 80% confluence, the medium was switched to DM. The cells were then exposed to cyclic stretch involving 1 s of 20% stretch alternating with 1 s of relaxation using a computer-controlled vacuum stretch apparatus (FX-4000T Tension Plus System; FlexCell International Corporation). Cells were harvested after different durations of cyclic exposure. Control cells also were plated on elastomer plates to avoid variations based on attachment stratum.

ANALYSIS OF CELL DEATH

Lactate dehydrogenase (LDH) activity was measured using a cytotoxicity detection kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. From each well, 100 μl of medium was removed and the remaining cells were lysed by adding 100 μl of 1% Triton X-100 solution. The samples were incubated in the dark for 30 min with buffer containing NAD+, lactate, and tetrazolium. LDH converts lactate to pyruvate, generating NADH. The NADH then reduces tetrazolium (yellow) to formazan (red), which was detected by fluorescence (490 nm) using a BIO-Tek spectrofluorometer plate reader with KC4 analysis software. LDH release was expressed as a percentage of the LDH in the medium relative to the total LDH lysate.

ANALYSIS OF APOPTOSIS

Annexin V binding and propidium iodide staining. Annexin V labeling and FACS analysis were performed as described previously [Mayr et al., 2000]. In brief, the cells were harvested, stained with FITC-conjugated annexin V and propidium iodide (PI) using the Apoptosis Detection Kit (R&D Systems, Minneapolis, MN) after continuously stretched for 6, 12, 24 h, and analyzed by flow cytometry (Epics XL; Beckman-Coulter, Kreefeld, Germany). Each data point represents $n = 4$ cell isolations with triplicate measurements.

STAINING OF NUCLEAR DNA WITH HOECHST 33342

The procedure was performed as described by Cattaruzza et al. [2000], with a modification according to Niesler et al. [2006]. L6 rat skeletal muscle myoblasts, grown on Bioflex membranes were incubated with fixation buffer (5% formaldehyde in 145 mM NaCl, 10 mM HEPES × KOH, pH 7.5) for 20 min at room temperature. After this period, myoblasts were stained with Hoechst 33342, a fluorescent nuclear binding dye, which allows clear distinction between apoptotic and normal cells on the basis of nuclear morphology (chromatin condensation and fragmentation). Hoechst 33342 [prepared in phosphate-buffered saline (PBS)] was added to the culture medium to a final concentration of 50 μg/ml and the cells were incubated for a second period of 20 min. Thereafter, the buffer was discarded, membranes were cut out with a scalpel, and mounted upside down on a microscope slide with 10 μl of mounting buffer (50% glycerol in Hank's balanced salt solution). Cells were evaluated by fluorescence microscopy according to the following grading system: normal nuclei (blue chromatin with organized structure) and apoptotic nuclei [bright fluorescent chromatin which is highly condensed or fragmented; Niesler et al. 2006]. Six randomly selected fields per coverslip were captured using a fluorescence microscope (Nikon ECLIPSE E400) and digital camera (Nikon DXM1200). The number of apoptotic nuclei, as well as the total number of nuclei in each field of view, was determined using Simple PCI version 4.0 (Compix, Inc., Imaging Systems, Sewickley, PA). The scoring was performed blind. The apoptotic index (AI; percentage of apoptotic nuclei per field) was calculated as the number of apoptotic nuclei divided by the total nuclei counted in

one field multiplied by 100. The mean ± SD AI was calculated from the 18 fields (3 samples; 6 fields per sample) using unpaired Student's *t*-test.

ULTRASTRUCTURAL ANALYSIS

To characterize further the observed cellular alterations as apoptotic versus necrotic, stretch-exposed and control cells were studied by electron microscopy. After 24 h of exposure to cyclic stretch, the cells were fixed with 1.25% glutaraldehyde in 0.15 M sodium cacodylate buffer, post-fixed with 1% osmium tetroxide, and dehydrated through graded ethanols. After removal of chambers and gasket, slides were covered with Epox 812 resin and placed over a resin-filled slide-duplicating mold in a 60°C oven to polymerize overnight. Ultrathin sections were cut from the resulting cell monolayer, stained with uranyl acetate/lead citrate, and viewed using a JEM-2000EX TEM transmission electron microscope at a voltage of 80 kV.

WESTERN BLOT ANALYSIS OF CASPASE-3 CLEAVAGE

After cyclic stretch, myoblast cell cultures were washed twice with cold PBS (4°C, pH 7.4). Then myoblast cell cultures were harvested in ice and lysed in 1% nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 12 μg/ml phenylmethylsulfonyl fluoride (PMSF), 30 μl/ml aprotinin, and 1 mM leupeptin dissolved in PBS. The suspension was incubated in ice for 20 min with vortexing every 5 min. Cellular debris was pelleted by centrifugation for 30 min at 13,000 rpm (Heraeus centrifuge) at 4°C, supernatants were collected, and protein concentration was measured.

To determine whether the observed apoptosis was caspase mediated, cleavage of procaspase-3 was assayed by Western blot analysis of cell lysates. Protein lysates (40 μg/lane) were size fractionated by NU–PAGE Bis–Tris (4–12%) gel electrophoresis (Novex, San Diego, CA) and transferred to polyvinylidene difluoride membranes. Western blots were hybridized consecutively with polyclonal antibodies against the 19-kDa cleaved caspase-3 (Cell Signaling Technology, Beverly, MA) and against the 32-kDa procaspase-3 (H-277). Secondary antibodies were conjugated with horseradish peroxidase, and blots were developed with an enhanced chemiluminescence (ECL) detection assay (Amersham Pharmacia Biotech, Piscataway, NJ). Band intensity was expressed as the integrated optical density (IOD) of relevant bands normalized to the IOD of actin bands. Each experiment was performed in triplicate.

ANALYSIS OF CASPASE-3 ACTIVITY

Colorimetric assays (Biosource International, Camarillo, CA) were used to determine caspase-3 activity in stretched and nonstretched cells. Briefly, 2×10^6 cells were incubated with 100 μl of lysis buffer for 10 min at 4°C. Cell lysates were centrifuged at 10,000 g for 1 min, and the supernatants were collected. The supernatant was incubated with 2× reaction buffer (containing 10 mM DTT) and 5 μl of caspase-3 substrate (DEVD-pNA) for 2 h at 37°C. Colorimetric reaction was measured at 405 nm. Each experiment was performed in triplicate.

WESTERN BLOT ANALYSIS OF APOPTOSIS-RELATED FAS/FASL EXPRESSION

The effects of cyclic stretch on Fas/FasL protein levels were evaluated by Western blot analysis of cell lysates, as described in detail elsewhere [Sandri et al., 2001] using polyclonal rabbit anti-Fas and monoclonal anti-FasL antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After fractionation by NU–PAGE Bis–Tris (4–12%) gel electrophoresis, protein lysates (40 μg/lane) were transferred onto a nitrocellulose membrane. Bands were identified by ECL detection, and their intensities were quantified densitometrically. Band intensity was expressed as the IOD of relevant bands normalized to the IOD of actin bands. Each experiment was performed in triplicate.

RESULTS

ASSESSMENT OF CELL DEATH BY LDH CYTOTOXICITY ASSAY

To investigate the effects of cyclic stretch on cell death in differentiating L6 myotubes, LDH cytotoxicity assay was performed to determine cell death after continuously stretched for 6, 12, and 24 h (Fig. 1). Control cells (0% elongation) were cultured on similar plates and kept in the same incubator without mechanical strain. This assay does not distinguish between the mode of cell death, that is, apoptosis or necrosis. As shown in Figure 1, LDH activity in supernatants of unstretched cells remained relatively stable for the first 12 h of culture. Subsequently, LDH release increased slowly at 24 h exposure than 6–12 h exposure in control unstretched cells, but there was no significant difference between them. It was concomitant with, and likely secondary to, visible cellular overgrowth for 24 h ($P > 0.05$).

From as early as 6 h of exposure, the LDH release of stretched cells was significantly higher than that of control cells ($P < 0.05$). After continuously stretched for 24 h, the LDH activity was 50% higher in stretched groups than in unstretched controls ($P < 0.01$) (Fig. 1).

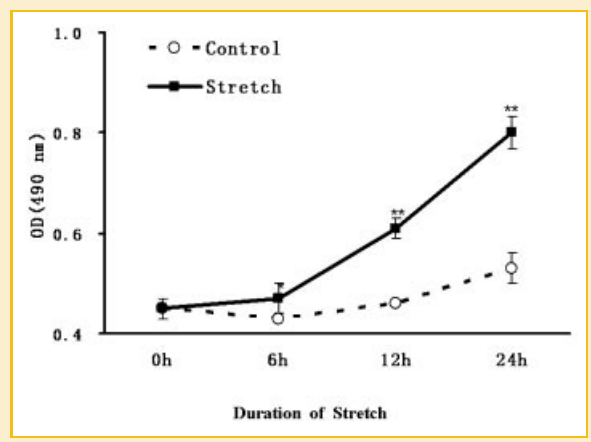

Fig. 1. Lactic acid dehydrogenase (LDH) cytotoxicity assay. Cell death was tested by LDH assay in myoblast cell cultures exposed to control or stretched conditions for the indicated time periods. Results represent means ± SE. $^{*}P < 0.05$ versus corresponding unstretched control; $^{**}P < 0.01$ versus corresponding unstretched control. OD, optical density.

These data suggested cyclic stretch could stimulate cell death during myotube formation.

CYCLIC STRAIN STRETCH-INDUCED SKELETAL MUSCLE CELLS APOPTOSIS

Apoptosis is an active process of cell suicide that is essential for successful organogenesis during development and normal physiological homeostasis throughout adulthood. Previous studies indicate that apoptosis is often associated with cell differentiation [Nakanishi et al., 2007], and myoblast apoptosis is an example of developmental apoptosis in which a subpopulation of myoblasts undergoes apoptosis around the time that myoblast fusion occurs as differentiation begins.

Differentiation of cultured L6 myoblast can be induced by transfer from GM to DM, the former containing 20% fetal bovine serum and the latter 2% horse serum. To explore the possibility that mechanical stress directly stimulates skeletal muscle cells apoptosis, myoblasts were cultivated on a flexible membrane under differentiation conditions, then subjected to cyclic stretch. Concomitantly, a subpopulation of myoblasts undergoes apoptosis. Apoptotic cells were identified by double labeling with annexin V and PI. PI labels all dead cells, including necrosis and final stages of apoptosis, whereas cells entering apoptosis are only stained by annexin V.

A representative plot of a flow cytometry analysis of stretched cells for annexin V binding and PI staining was shown in Figure 2. Cells negative for PI and annexin V were regarded as viable cells (Fig. 2, lower left quadrant). PI-negative cells, which bind annexin V, were defined as cells early in the apoptotic process (Fig. 2, lower right quadrant). PI-positive cells with annexin V binding represent late apoptotic/necrotic cells (Fig. 2, upper right quadrant). PI-positive cells without annexin V binding (Fig. 2, left upper quadrant) were labeled as necrotic cells.

The plots of Figure 3A demonstrate the changes in the percentage of viable (annexin V-negative and PI-negative) cells over the experimental time in response to cyclic stretch. The percentage of viable cells after continuously stretched for 6 h was not significantly different from unstretched controls. However, a significant decrease in the percentage of viable cells occurs after continuously stretched for 12 and 24 h ($62 \pm 6\%$ vs. $81 \pm 4\%$ at 12 h; $56 \pm 7\%$ vs. $83 \pm 3\%$ at 24 h). This decrease may be explained by increase in early apoptotic (Fig. 3B) and late apoptotic/necrotic cells (Fig. 3C), as well as a result of subsequent an early increase in necrotic cells (Fig. 3D). A significant increase in early apoptotic cells in comparison with unstretched controls (Fig. 3B) was found after continuously stretched for 6 h ($15 \pm 1.5\%$ vs. $11 \pm 1.2\%$ at 24 h), and it reached the maximum at 24 h. ($27 \pm 2.0\%$ vs. $12 \pm 1.4\%$ at 24 h). Cyclic stretch resulted in an early increase of necrotic cells at 12 h and a second peak at 24 h ($14.7 \pm 1.3\%$ vs. $5.4 \pm 0.7\%$ at 12 h and $117.6 \pm 1.1\%$ vs. $5.1 \pm 0.4\%$ at 24 h) (Fig. 3D). In contrast, the number of cells in early apoptosis increased continuously starting at 6 h. Cells in late apoptosis/necrosis increased at 12 h and rose to a maximum at 24 h.

These findings indicate that mechanical stretch, which had been shown to be a well-described proliferative stimulus during myogenesis [Iwanuma et al., 2008], can induce apoptotic changes in myoblast. Although stretch-induced apoptosis in differentiating L6 cells continued to be observed after continuously stretched for 24 h in DM, apoptosis in differentiating muscle tissues of embryonic mice is a transient event, observed for only a day or so after the onset of myotube formation at embryonic day 13.5 [Ontell and Kozeka, 1984].

To confirm further that stretch-induced myoblast undergo apoptotic cell death, chromatin condensation and nuclear morphology were evaluated by staining with the fluorescent DNA binding dye, H 33342. As shown in Figure 4, exposure to cyclic stretch indeed resulted in an increase in their apoptotic indices by 12% (12 h) and 26% (24 h), respectively, as determined by Hoechst 33342 staining of apoptotic nuclei.

By electron microscopy, the nuclei of nonstretched cells at 24 h showed small chromatin aggregates evenly dispersed over the nucleus (Fig. 5A). The cytoplasm showed variably sized electron-dense organelles, consistent with surfactant-containing lamellar bodies. Cells exposed to cyclic stretch for 24 h showed highly characteristic condensation of dense chromatin in one or two central or paracentral nuclear foci (Fig. 5B).

CASPASE-3 IS CLEAVED IN STRETCH-INDUCED APOPTOSIS IN MYOBLAST

Among many caspases, the downstream effector, caspase-3, has been shown to play a pivotal role in the terminal, execution phase of apoptosis induced by a variety of stimuli. To ascertain the involvement of the caspase pathway in the observed stretch-induced apoptotic cell death, we studied the processing of caspase-3, by Western blot analysis of whole cell lysates using antibodies specific for either procaspase or the caspase-3 split products. As seen in Figure 6A, cyclic stretch treatment induced cleavage of procaspase-3 in a time-dependent manner. Levels of 19-kDa caspase-3 cleavage products were negligible in control cells at all time points but increased significantly in stretched cells from 6 h, concomitant with decreasing levels of the 32-kDa procaspase-3.

The effect of cyclic stretch on the proteolytic activity of caspase-3 (key executioner caspase) was studied by colorimetric assays using specific caspase substrates. Figure 6B summarizes caspase-3 activities measured in cell lysates of L6 cells after continuously stretched for 6, 12, and 24 h. Cyclic stretch induced a significant two- to three fold increase of caspase-3 proteolytic activity at 12 h which was sustained at 24 h (Fig. 6B).

CASPASE-3 INHIBITION ELIMINATES STRETCH-INDUCED CELL DEATH AND APOPTOSIS IN MYOBLAST

To examine whether caspase-3 activation is involved in stretch-induced apoptotic cell death, the extent of stretch-induced cell death in skeletal muscle cells mediated by caspase-3 was examined by addition of 100 μM of the general caspase inhibitor zVAD to cells 1 h before stretch. After continuously stretched for 24 h, caspase-3 activity was eliminated in the cells pretreated with zVAD (Fig. 7C), with decreases in cell death (Fig. 7A) and reductions in apoptosis (Fig. 7B) to unstretched controls, confirming that stretch-induced apoptotic cell death is caspase-cascade dependent.

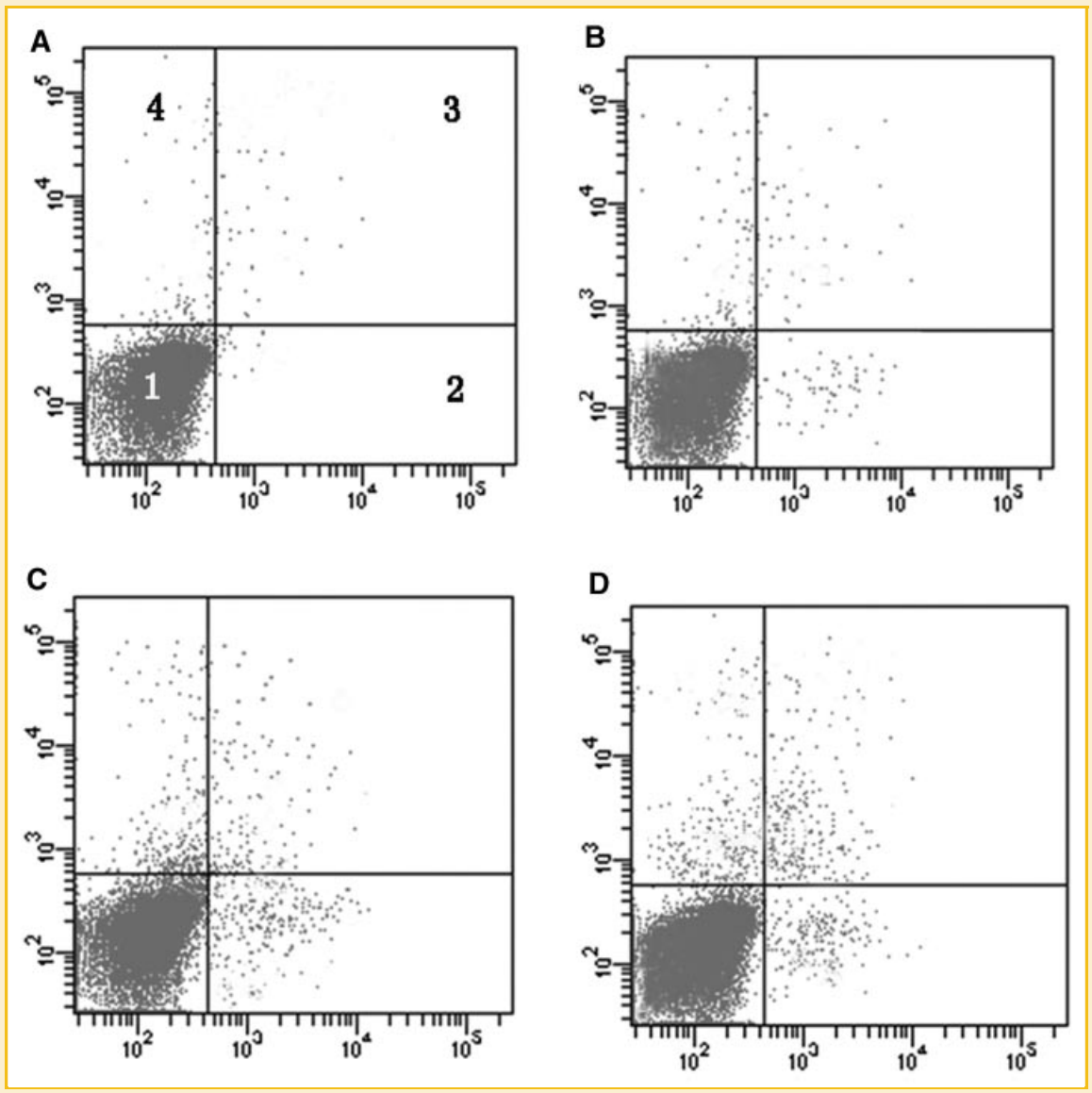

Fig. 2. Determination of apoptosis and necrosis in stretched cells. Cells were stretched, harvested after continuously stretched for 6, 12, and 24 h, stained with annexin V and PI, and analyzed by flow cytometry. The figure shows a representative set of plots of flow cytometry analysis of the following groups: (A) static control, (B) 6 h, (C) 12 h, and (D) 24 h. Intensity of PI staining (*y*-axis) is plotted versus FITC intensity (*x*-axis). In all four plots, viable cells are seen in the left lower quadrant (1: annexin V-negative/PI-negative); early apoptotic cells in the right lower quadrant (2: annexin V-positive/PI-negative); late apoptotic/necrotic cells in the right upper quadrant (3: annexin V-positive/PI-positive); and necrotic cells in the left upper quadrant (4: annexin V-negative/PI-positive).

CASPASE-3 ACTIVATION IS INDEPENDENT OF FAS/FASL EXPRESSION

To assess if activation of the Fas/Fas ligand (FasL) system was involved in activation of caspase-3 in stretch-mediated myoblast apoptosis. During myogenesis, we studied the protein expression of Fas-related apoptotic signaling molecules. Western blot analysis of Fas/FasL expression in myoblast lysates was shown in Figure 8A after continuously stretched at 20% elongation for 6, 12, and 24 h. Densitometry analysis was shown in Figure 8B. Protein levels of Fas and FasL remained unchanged. These data suggested that Fas/FasL is not involved in stretch-induced caspase-3 activation in stretch-mediated myoblast apoptosis.

DISCUSSION

Apoptosis is a cellular suicidal program by which damaged or no longer needed cells are individually eliminated to maintain healthy homeostasis in multicellular organisms [Shiokawa et al., 2002]. Thus, apoptosis during muscle development is considered to be essential for normal skeletal muscle development by eliminating cells with defects or undergoing damage during differentiation. Mechanical stimuli is implicated as a major influence on a number of fundamental cellular processes, including differentiation, growth, and apoptosis [Huang and Ingber, 2000; Farge, 2003]. The activation of apoptosis by mechanical forces has been shown in several cell types [Cigola et al., 1997; Cattaruzza et al., 2000]. But to our

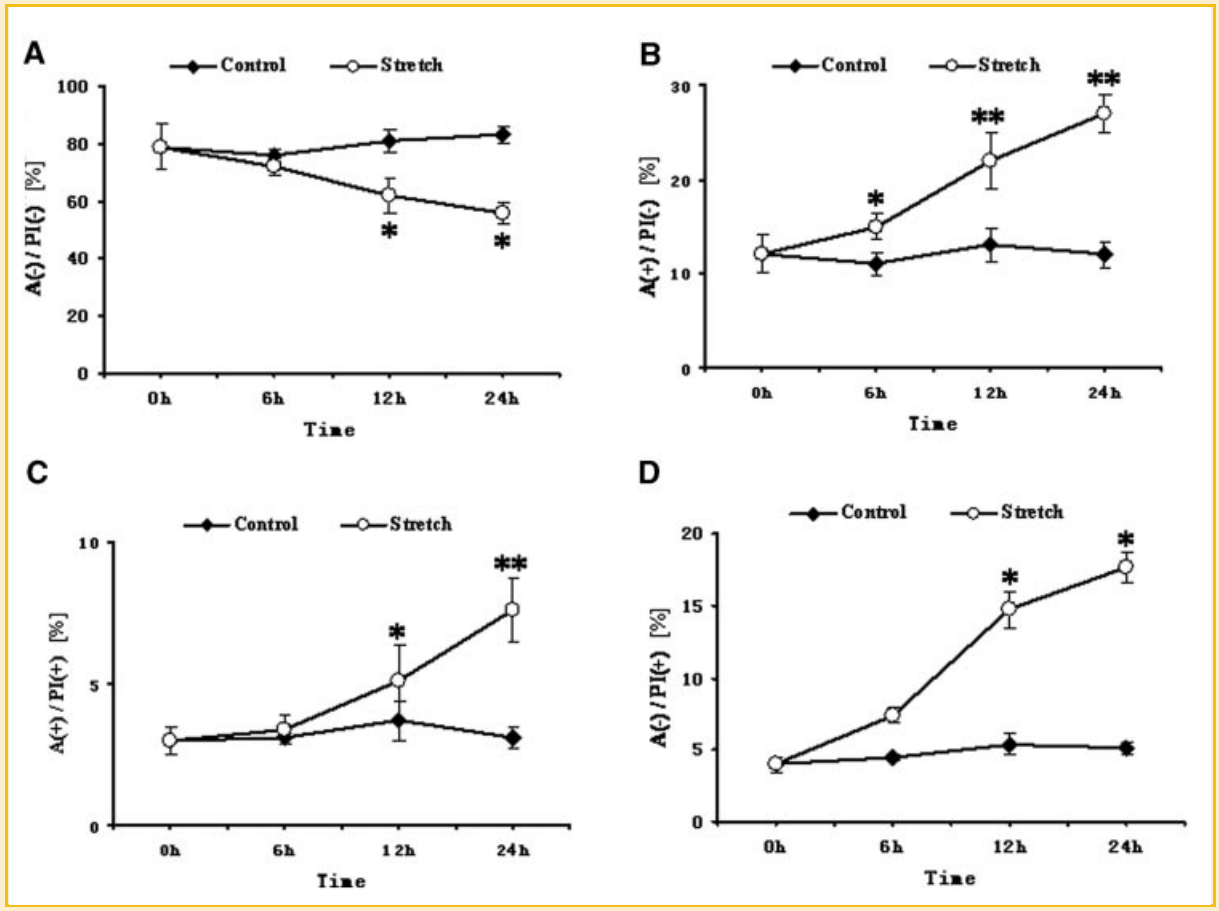

Fig. 3. Time-dependent apoptosis of myoblast stimulated by cyclic stretch. Myoblast were treated with 20% elongation for 0, 6, 12, and 24 h, harvested, and stained with annexin V-FITC and PI 16 h after the onset of mechanical stress. Flow cytometry data of cells subjected to cyclic stretching and cells from static cultures was shown. A: The percentage of viable cells; (B) the early apoptotic cells; (C) late apoptotic/necrotic cells; and (D) necrotic cells. The results represent the mean ± SD of three experiments. $^{*}P < 0.05$ versus control.

knowledge, this is the first report to show an time-dependent apoptosis shift in skeletal muscle cells in response to mechanical stretch during myogenesis. In this study, we attempt to investigate the possible effect of cyclic stretch on LDH release, apoptotic death, and necrotic death of the myoblast during myogenesis. The L6 cells were chosen to exclude the interaction of other cell types over paracrine mechanisms with myoblasts. The stimulus for the cell death and apoptosis should be only mechanical stimuli.

In this study, we hypothesized that mechanical forces are important in inducing cell apoptosis during myogenesis. To test this hypothesis, our first approach was to assess the effect of stretch on myoblast cell death. We found that the stretching pattern chosen (20% elongation in membrane surface area) led to an increase in LDH release starting at 12 h. This stretching pattern also resulted in a significant increase in the percentage of apoptotic and necrotic cells. The percentage of viable cells consequently fell with time in an almost linear fashion (Fig. 3A). It indicated that there were two mechanisms of cellular damage recognizable from the time course of LDH release: the appearance of apoptosis or necrosis. Apoptosis increased early at 6 h (Fig. 3B), with no increase in the number of necrotic cells (Fig. 3D). However, at 12 and 24 h the number of necrotic cells increased with the apoptosis increased greatly (Fig. 3B,D). Both the necrosis and apoptosis peaks at 24 h. A possible interpretation that would fit our data was maybe the apoptosis led to necrosis, which in turn increased the percentage of dead cells (cells in late apoptosis or necrosis) with further time.

To determine the contribution of apoptosis to cell death during myotube differentiation, apoptosis was distinguished by annexin V binding and PI staining, Hoechst staining, electron micrograph, and caspase-3 activation. Flow cytometry analysis for annexin V binding and PI staining was frequently used to quantify viable, early apoptotic, late apoptotic/necrotic, and necrotic cells. To strengthen our flow cytometry data, we chose three additional methods with predominance for early apoptosis and late apoptosis/necrosis, respectively. Caspase-3 activity, which detects early stages of apoptosis [Vermes et al., 2000], was chosen because this enzyme has been shown to play a pivotal role in the execution phase of apoptosis induced by a variety of stimuli. We do recognize cyclic stretch treatment-induced cleavage of procaspase-3 in a time-dependent manner. Also, we found that the

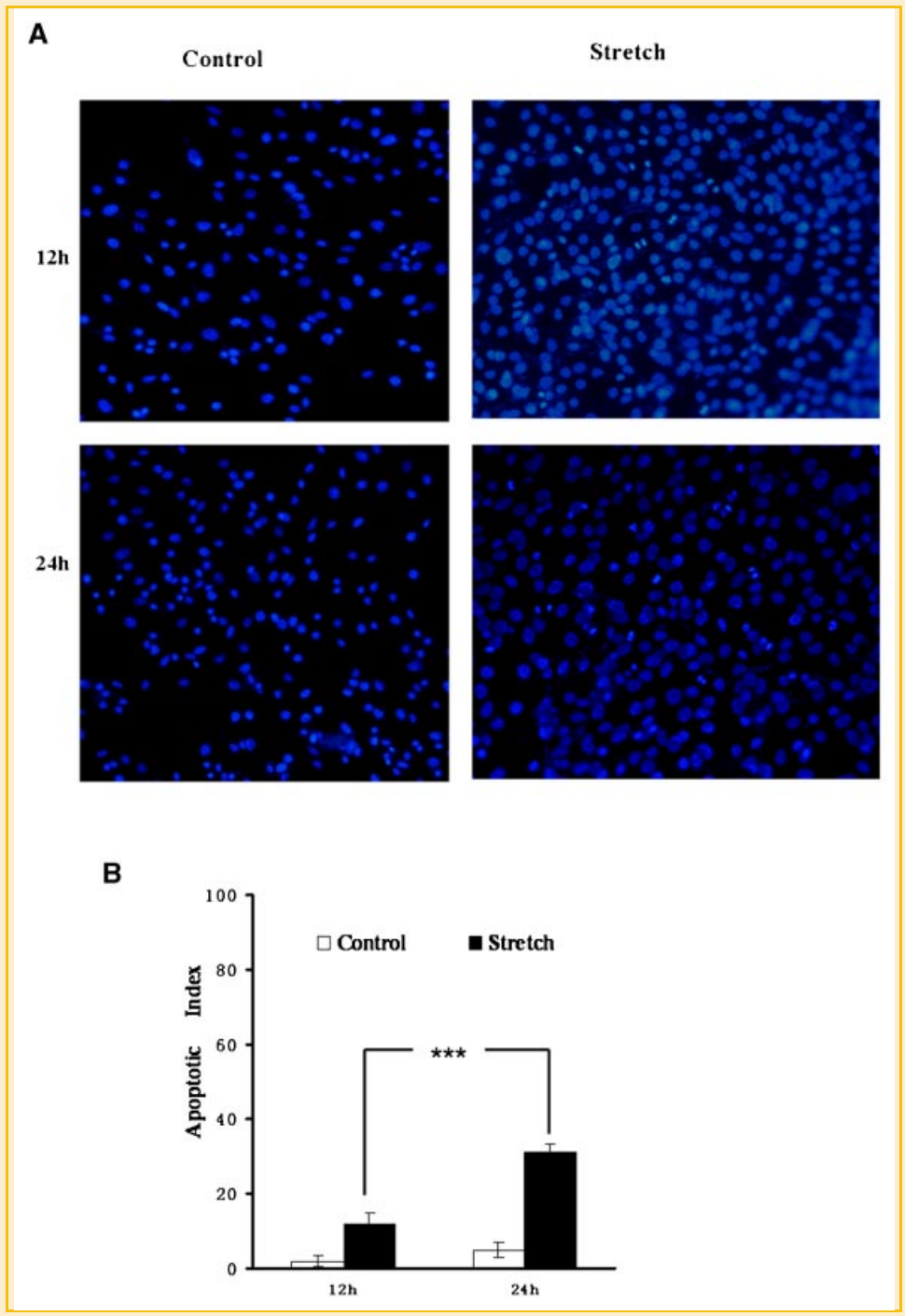

Fig. 4. Chromatin condensation in myoblast exposed to cyclic strain. Cells were incubated with the test compounds at the indicated concentrations and stretched for 12 and 24 h at 0.5 Hz and 20% elongation. Hoechst staining was used to identify both normal and apoptotic nuclei after cyclic stretch. The top panel shows the different images taken from a representative experiment; the bar chart below summarizes the data from six independent experiments with different batches of myoblast. The apoptotic index (AI) was calculated as the percentage of apoptotic nuclei per total nuclei number per field. The mean ± SD of percentage AI was calculated using unpaired Student's *t*-test from six randomly selected fields per treatment. The experiment was repeated three times. $^{***}P < 0.0001$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

expression of cleavage caspase-3 parallels the information from the annexin V-positive/PI-positive cell fraction detected by flow cytometry analysis. Our findings are in agreement with those reported by Morrow et al. (2005) who demonstrated that cyclic strain decreases proliferation and increases SMCs apoptosis. But it was in contrast with those findings from Estrada et al. [2006], who concluded that mechanical stretch maybe an anti-apoptotic stimulus in urinary bladder SMC.

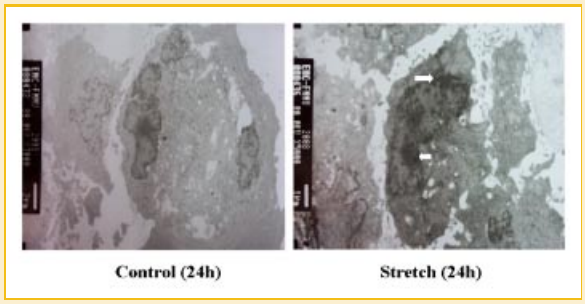

Fig. 5. Electron microscopic findings. A: Myoblasts grown for 24 h under nonstretched conditions showing small chromatin aggregates, evenly dispersed over the nucleus. Cytoplasmic organelles are consistent with surfactant-containing lamellar bodies. B: Myoblasts after continuously exposed to cyclic stretch for 24 h showing fragmentation and condensation of chromatin characteristic of apoptosis (arrow). Bar = 1 μm.

The initiation and execution phases of apoptosis involved activation of a family of cytoplasmic aspartate-specific cysteine proteases known as caspases [Green, 1998]. Caspases cause cell death by degrading critical structural elements and activating proteolytic enzymes. Caspase-3 is seen as one of the key executioners of apoptosis, being responsible for the cleavage of crucial substrates in the final degradation phase. Activation of caspase-3 requires proteolytic cleavage of its inactive zymogen into active p17 and p12 subunits. To confirm our flow cytometry findings, we used Western blot detection of the active (cleaved) caspase-3 isoform. Mechanical stretch did not affect caspase-3 activation before 6 h. Cyclic stretch consistently increased activated caspase-3 accumulation after continuously stretched for 6 h. Next, the activity of caspase-3 was evaluated. Cyclic stretch induced a significant two- to threefold increase of caspase-3 proteolytic activity at 12 h which was sustained at 24 h. To examine whether caspase-3 activation was involved in inducing programmed cell

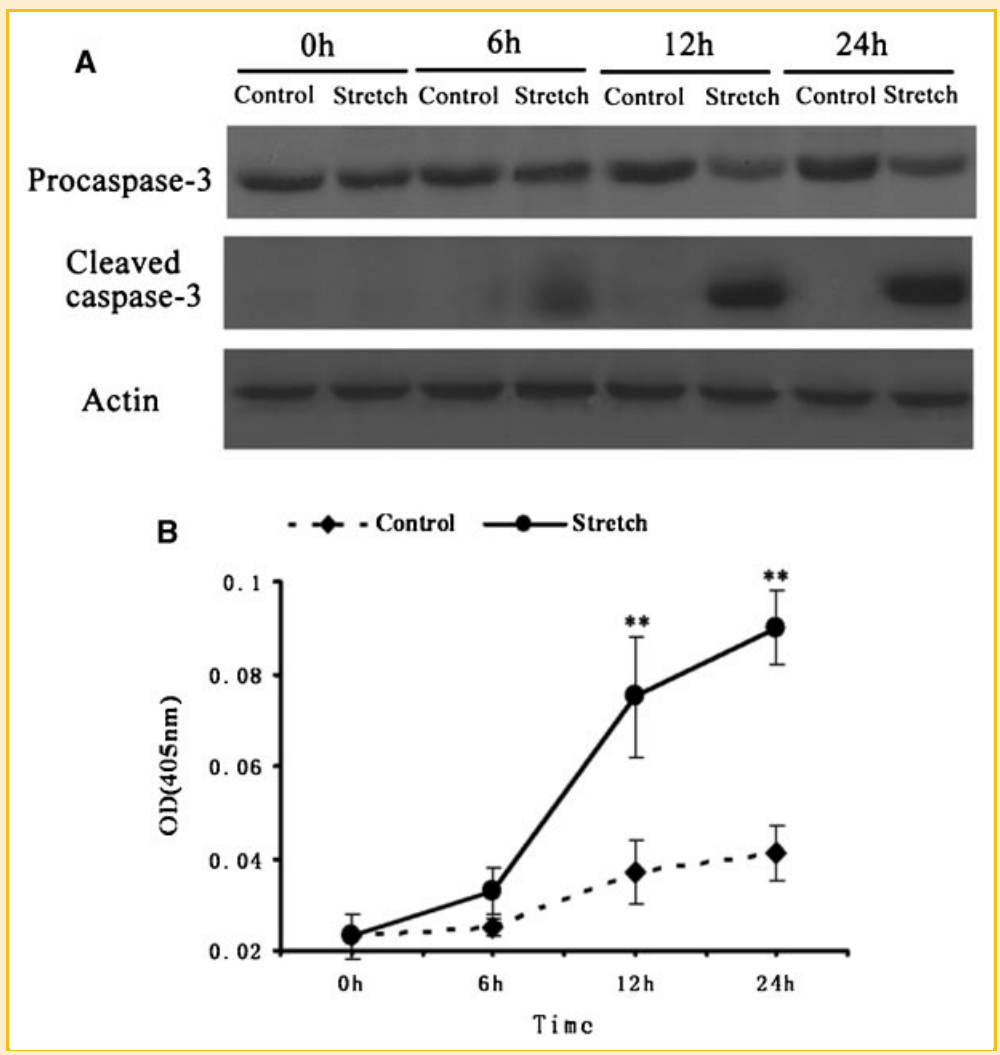

Fig. 6. Analysis of caspase-3 function and processing. A: Western blot analysis of caspase-3 cleavage. Exposure to cyclic stretch correlated with the appearance of the 19-kDa subunit of caspase-3, concomitant with decreasing levels of 32-kDa procaspase-3. B: Analysis of proteolytic activity of caspase-3 in cell lysates of myoblast exposed to cyclic stretch and compared with controls grown in ambient conditions. Values represent mean ± SD of at least three experiments. $^{**}P < 0.001$ versus corresponding unstretched controls.

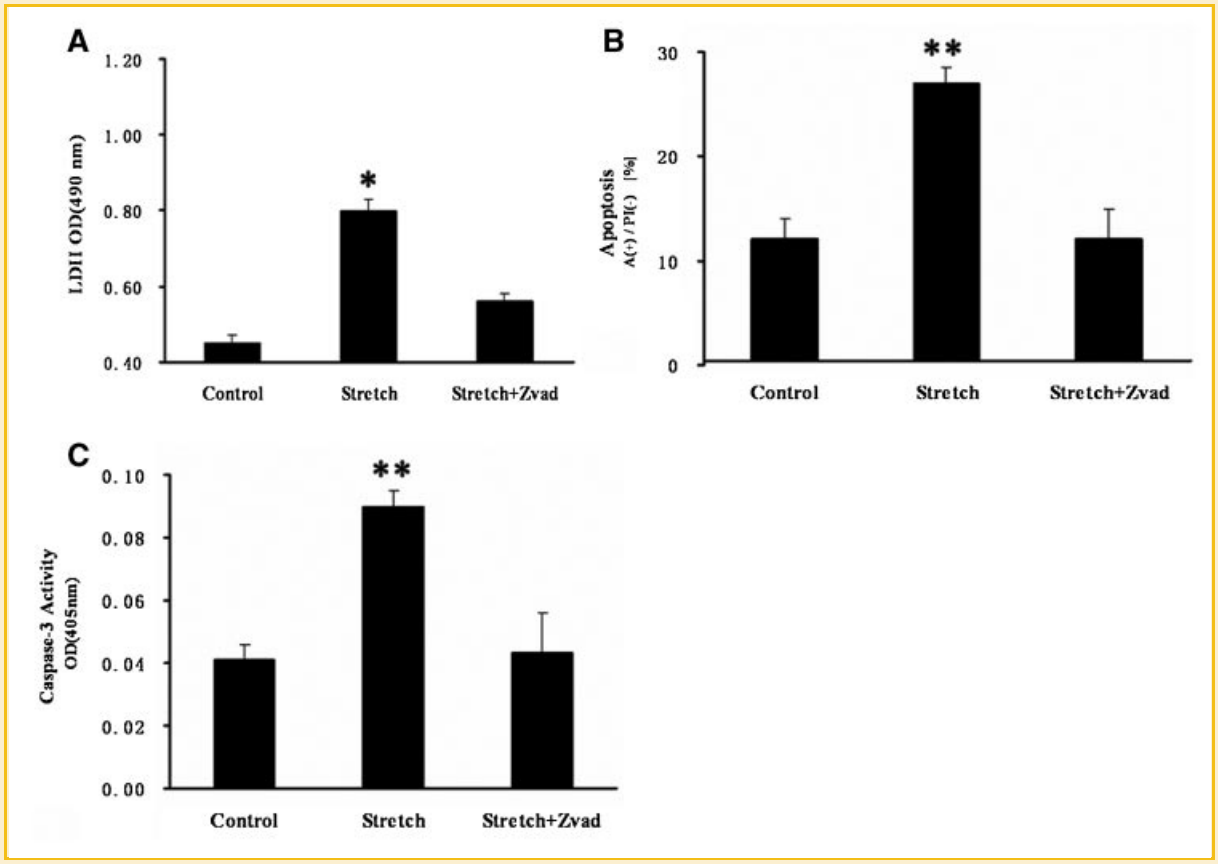

Fig. 7. Cyclic stretch causes caspase-dependent apoptosis in skeletal muscle cells. Skeletal muscle cells were stretched at 20% elongation continuously for 24 h. In some cases, 100 μM zVAD peptide was added 1 h before stretch. A: Assessment of cell death by LDH cytotoxicity assay. B: Percent apoptosis was determined by stained with annexin V-FITC and PI. C: Relative fluorescence for caspase-3 activity using the caspase-3 substrate DEVD-AFC. Graphs (A) and (B) are single representative experiments with three replicate samples, and error bars represent SD. Data in (C) are averages of three duplicate samples from one experiment. $^{*}P < 0.05$, $^{**}P < 0.005$ versus unstretched control.

death, myoblasts were pretreated with the general caspase inhibitor zVAD and exposed to cyclic stretch in a similar manner. Our results indicated that stretch induced procaspase-3 activation. Caspase-3 inhibition reduced stretch-induced apoptosis, which supported the conclusion that stretch-induced cell death was an apoptotic event and that the activation of caspase cascades was required in stretch-induced cell apoptosis.

Previous studies [Nagata and Golstein, 1995] had demonstrated that the Fas/FasL death signaling system is an important developmental regulator of apoptosis. Stimulation of Fas by its natural ligand FasL or Fas-activating antibody results in its trimerization and the recruitment of two key signaling proteins: the adapter protein Fas-associated death domain (FADD, also called MORT-1) and the initiator cysteine protease caspase-8, which form the death-inducing signaling complex. Subsequent activation of the effector caspases through mitochondria-dependent or mitochondria-independent pathways results in activation of caspase-3, the key effector caspase. In the present study, to evaluate the potential involvement of Fas-mediated signaling in the stretch-induced activation of caspase-3 during myogenesis, we studied the protein expression of Fas-related apoptotic signaling molecules after stretch. There was no significant difference in Fas and FasL expression between unstretched and stretched samples. These data suggest that mechanical forces induced activation of caspase-3 in stretch-induced myoblast apoptosis via signaling pathways independent of Fas/FasL, which should be further investigated in the future. Our findings provide a crucial insight into the controversial role of Fas-mediated signaling in apoptosis during myogenesis.

Previous studies indicated that cell cultures contained at least two types of living cells during myogenesis: stress-resistant cells that formed large myofibers and stress-sensitive cells that survived standard differentiation conditions but were eliminated by apoptosis after pre-treatment [Nakanishi et al., 2007]. On the basis of the present study, we propose that appropriate cyclic stretch-induced myoblast apoptosis controls the quality of differentiating myoblasts, so that only apoptosis-resistant cells go through terminal differentiation to form muscle tissue; apoptosis was the predominant mode of cell death in response to mechanical stimuli. Caspase-3 mediated cyclic stretch-induced myoblast apoptosis. Mechanical forces induced activation of caspase-3 via signaling pathways independent of Fas/FasL system, which should be further investigated in the future. Taken together, these results support that the positive effects of cyclic stretch include elimination of vulnerable cells, thereby excluding these cells from myotube formation and augmenting resistance to apoptosis. Quality control

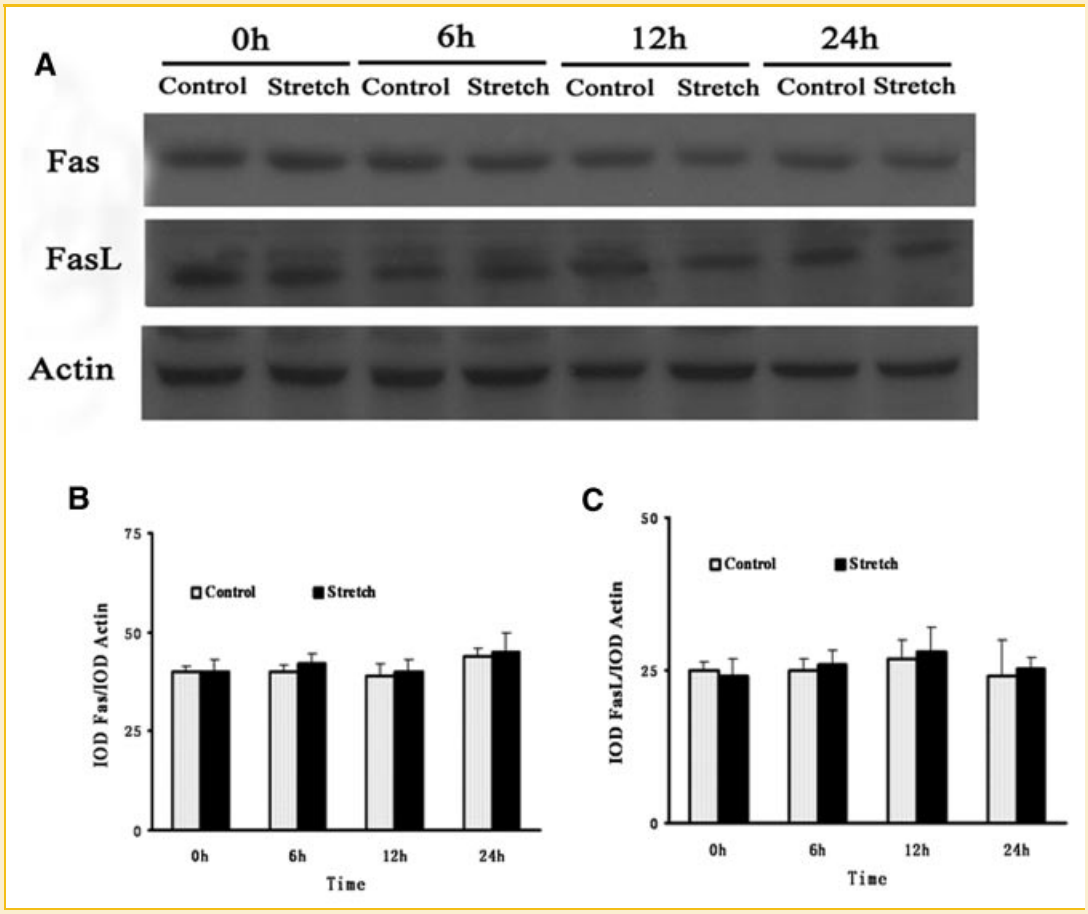

Fig. 8. Cyclic stretch did not alter the expression of Fas/FasL proteins in skeletal muscle cells. Skeletal muscle cells were stretched at 20% elongation continuously for 6, 12, and 24 h. Total cell protein was collected and analyzed by Western blotting. Fas/FasL and actin were detected by specific monoclonal antibodies against Fas, FasL, and actin. All Fas and FasL protein densitometric values were normalized to actin protein. A: Representative Western blot. B: Densitometric values. Values are means ± SD from four different experiments. Student's paired *t*-test was applied.

by apoptosis during stretch-induced myoblast differentiation would be a novel role for apoptosis in the sense that apoptosis controls the quality rather than the number of cells.

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