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Research article

THE AGE-DEPENDENT INDUCTION OF APOPTOSIS-INDUCING FACTOR (AIF) IN THE HUMAN SEMITENDINOSUS SKELETAL MUSCLE

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Abstract: To assess the dependence on age of the expression of apoptosis regulatory proteins in the human semitendinosus muscle, we measured the expression levels of several apoptosis-related genes, including apoptosis-inducing factor (AIF), Bax, Bcl-2, caspase-3 and heat shock protein 70 (HSP70), using RT-PCR, immunohistochemistry and TUNEL assays. We found that the DNA fragmentation was proportional to the age of the tissues sample donors. The expression levels of AIF were significantly elevated (by 10 to 25%) in semitendinosus tissue samples from older individuals, but the Bax, Bcl-2, caspase-3 and HSP 70 levels remained almost constant. This data suggests that the morphological and functional changes observed in aged human semitendinosus muscle correlates with the apoptosis of muscle cells through the induction of AIF.

Key words: Human skeletal muscle, Semitendinosus, Apoptosis, Aging, Apoptosis-inducing factor

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Abbreviations used: AIF – apoptosis inducing factor; Bcl-2 – B-cell leukemia/lymphoma 2; caspase-3 – cysteine-aspartic acid protease-3; HSP70 – heat shock protein 70

INTRODUCTION

The normal aging of human skeletal muscle is accompanied by muscle loss, and apoptosis appears to play an important role in this process. However, the role of skeletal muscle apoptosis as a causative mechanism in the aging of the human semitendinosus muscle has not yet been investigated. Skeletal muscles in the human body increasingly lose mass during the aging process, and several studies have demonstrated the role of apoptosis in this loss of muscle mass and function in the normal aging of skeletal muscle [1-6]. In addition, skeletal muscle aging is associated with an increased liability to injury, inflammation and oxidative stress, all of which trigger the apoptotic process [7-9]. The damaging effects of muscle degradation and function are greater in aged muscles, and recent studies on mammals demonstrated that aged skeletal muscles have increased amounts of apoptotic cells [10, 11].

Apoptosis occurs via specific signaling pathways, eventually leading to DNA fragmentation, nuclear condensation, proteolysis and cell fragmentation [12, 13]. In general, apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway. The intrinsic pathway is triggered by cellular stress, specifically mitochondrial stress caused by factors such as DNA damage and heat shock. The relative ratios of the various bcl-2 proteins can largely determine how much cellular stress is necessary to induce apoptosis. Following its formation, the complex activates caspase-9, an initiator protein. The activated caspase-9 works together with the complex of cytochrome c, ATP and Apaf-1 to form an apoptosome, which activates caspase-3, the effector protein that initiates degradation. The extrinsic pathway is initiated through the stimulation of death receptors, such as the Fas receptors, located on the cell membrane [14-20]. The release of apoptosis-inducing factor (AIF) and endonuclease G from the mitochondria [21-24] can produce DNA fragmentation and apoptosis, following their translocation to the nucleus [1, 25]. This provides an alternative and more direct cell death pathway. In the mitochondrial pathway, p53 can induce apoptosis by regulating the proteins of the Bcl-2 family [26] as a response to cellular stress or DNA damage. AIF is a mitochondrion-derived pro-apoptotic factor that, following its release and translocation to the nucleus, works independently of caspase activity [27]. Its increased levels in aged gastrocnemius muscles suggest that apoptotic potential increases with age [28].

Heat shock proteins (HSPs) play a role as anti-apoptotic proteins by inhibiting apoptosome formation and also by acting as an antagonist of AIF [29]. The other major pathway in apoptosis is a caspase-dependent mechanism, in which the initiator caspases (caspases-8 and -9) activate the effector caspases (caspases-3, -6, and -7), and are responsible for beginning the cascade to cell disassembly [18, 30, 31].

In this study, we chose to examine and compare the level of apoptosis in the muscles of 10- to 40-year olds, as changes observed between these age groups are likely to reflect aging-related muscle degeneration. To determine the key

players associated with the aging-related process, we selected markers of muscle proteolysis based on the results of previous studies, as follows: caspase-3, Bax, B-cell leukemia/lymphoma (Bcl-2), AIF and Hsp70.

MATERIALS AND METHODS

Muscle sampling

Samples of the semitendinosus muscles were collected from 10-, 20-, 30- and 40-year old men who were undergoing anterior cruciate ligament reconstruction involving semitendinosus and autograft tendons. Six muscle samples were analyzed for each age group. All of the human subjects were healthy with no muscle-related clinical conditions. The muscle tissue was prepared from the musculotendinosus junction, mounted, immediately frozen in liquid nitrogen and stored at -80ºC for the immunohistochemical and biochemical analyses. Ethical consent was obtained from the Kyung Hee Medical Center Institutional Review Board .

Histology

Human semitendinosus muscle tissue samples (n = 6 for each age group) were fixed in 4% paraforaldehyde, embedded in O.C.T. compound, divided into 15-μm thick sections, and stained with hematoxylin and eosin Y (H&E).

In situ TdT-mediated dUTP nick end-labeling staining

TUNEL assays were performed to detect DNA strand breaks using a commercial kit following the protocol provided by the manufacturer (Chemicon International, Temecula, CA, USA). Briefly, 15-μm thick sections of skeletal muscles (n = 6 for each age group) were mounted onto Silane-coated glass slides. The dehydrated sections were treated first with 20 μg/ml DNase-free Proteinase K (Sigma-Aldrich Corporation, St. Louis, MO, USA) to retrieve antigenic epitopes, then with 3% H_2O_2 to quench endogenous peroxidase activity. Free 3'-OH termini were labeled with digoxigenin-dUTP for 1 h at 37ºC utilizing a terminal deoxynucleotidyl transferase reaction mixture. Incorporated digoxigenin-conjugated nucleotides were detected using a horseradish peroxidase-conjugated anti-digoxigenin antibody and 3,3'-diaminobenzidine. The dehydrated sections were cleared in xylene, mounted with Canada balsam, and enclosed with coverslips.

Immunohistochemistry

Sections of 15-μm thick frozen muscle tissue (n = 6 for each age group) were mounted onto Silane-coated glass slides and fixed in 4% paraformaldehyde (Sigma-Aldrich Corporation) for 1 h at 4ºC. Endogenous peroxidase activity was blocked by immersing the sections in 3% H_2O_2 in 100% methanol for 15 min. All of the samples were incubated in 10% normal donkey serum (NDS) in PBS for 1 h at room temperature, and then incubated with antibodies against caspase-3 for the detection of the pro form and active form of caspase-3 (diluted 1:100;

Cell Signaling Technology, Inc., Danvers, MA, USA) and AIF (diluted 1:100; Cell Signaling Technology, Inc., Danvers, MA, USA). Serial sections were used alternatively for the detection of caspase-3 and AIF. The immunohistochemical procedures used antibodies from several sources to establish antibody specificity and confirm immunostaining and protein expression. The activated caspase-3 immunohistochemistry worked well without requiring any special pretreatment procedures, except routine heat antigen retrieval. Primary antibody binding was visualized using peroxidase-labeled donkey anti-rabbit antibody (diluted 1:100; Jackson ImmunoResearch Inc., West Grove, PA, USA) with 0.05% 3,3-di-aminobenzidine tetrahydrochloride and 0.01% H_2O_2 as the substrate. After staining, the sections were dehydrated, cleared in xylene, and then mounted with Canada balsam mounting medium.

Extraction of total RNA and reverse transcriptase PCR

Frozen semitendinosus muscle was homogenized on ice in 1 ml of ice-cold TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA, USA). The total RNA was solubilized in RNase-free H_2O and quantified twice by measuring the optical density (OD) at 260 nm. cDNA was synthesized from 2 μg of total RNA, and reverse transcription (Promega Corporation, Madison, WI, USA) was performed at 42ºC for 1 h following incubation at 95ºC for 5 min. cDNA amplification was carried out according to the following procedure: at 95ºC for 1 min, at 56ºC (β-actin) or 58ºC (AIF, caspase-3, Bacl-2, and Bax) for 1 min, at 72ºC for 1 min. 26 to 40 cycles were run, and the reaction was prolonged for 10 min at 72ºC. Primers were constructed from the published sequences (Tab. 1). The

Tab. 1. The primers used for semi-quantitative RT-PCR amplification of cDNA.

Product	Sequence	T_A (°C)	Cycle	PCR length (bp)
AIF	F: 5' AGACGATCCCAAATAATGCAG 3' R: 5' TAGCTCTAGGTGAGTCTTGG 3'	58	26	163
Caspase-3	F: 5' CGAAATTCAAAGGATGGCTCCTGGTT 3' R: 5' CGGTTAACCCGGGTAAGAAATGTGCAT 3'	58	40	104
Bcl-2	F: 5' GCACGCTGGGAGAAAGGGTACGAT 3' R: 5' CACATCTCCAGCATCCCACTCGTA 3'	58	40	98
Bax	F: 5' TGCCTCAGGATGCGTCCACCAA 3' R: 5' CGGCAATCATCCTCTGCATGCTCCAT 3'	58	40	96
HSP70	F: 5' CATGGTGCTG ACCAAGATGA AGGAGAT 3' R: 5' GCGCAGCCGC CTCACGGCTC CCAGCCTGTT 3'	63	30	374
β-actin	F: 5' TCATGAGTGTGACGTTGACATCCGT 3' R: 5' CCTAGAAGCATTTGCGGTGCACGATG 3'	56	30	304

T_A, annealing temperature; bp, base pair.

PCR products were separated on 1.5% agarose gels, visualized by ethidium bromide staining using the i-MAX gel image analysis system (CoreBioSystem,

Seoul, Korea), and analyzed using Alpha Ease™ FC software (Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical analysis

The statistical analysis was performed using GraphPrism 4.0.3 software (GraphPad Software, Inc., San Diego, CA). All the data is presented as the mean standard deviation (SD), and a Student's t-test was used to compare group means. The caspase-3, AIF antibody and TUNEL assays were observed with light microscopy at ×200. Images were captured on a Zeiss fluorescent microscope and myofibers were counted and measured using Axiovision 4 software (Zeiss).

RESULTS

Morphological characteristics

We used hematoxylin and eosin (H&E) staining to examine the morphological changes induced by aging-related apoptosis in human male semitendinosus muscle (Fig. 1a-d). The detection of large quantities of nuclei is a distinctive feature of necrosis, so H&E staining was conducted to observe the amount of nuclei produced. None of the tissues showed evidence of necrosis. Nuclear DNA breaks in the skeletal muscle tissue were detected using TUNEL staining to estimate the extent of apoptosis. Apoptotic cells were identified via careful observation of TUNEL-stained sections and serial H&E-stained sections because some necrotic cells could be also TUNEL-positive.

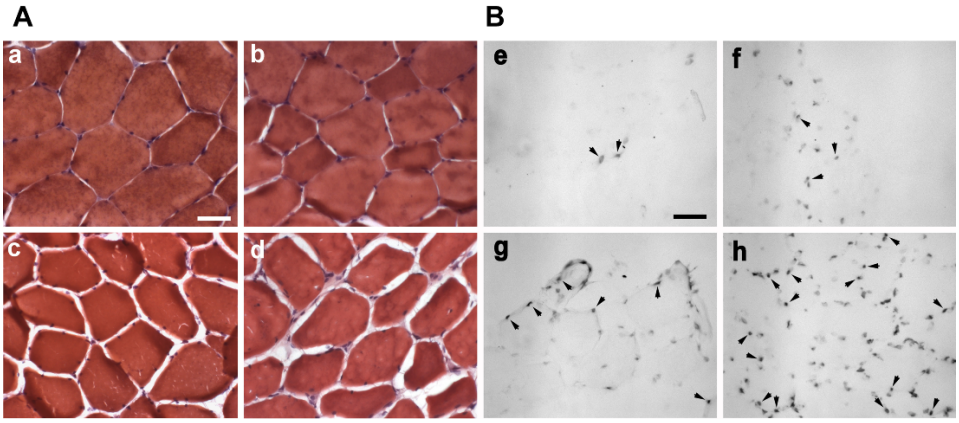

Fig. 1. Morphological changes in human semitendinosus muscle. A – H&E staining. B – Staining of fragmented DNA in frozen sections of skeletal muscle using the TUNEL method. a, e – age 10; b, f – age 20; c, g – age 30; d, h – age 40. Arrows indicate stained nuclei and apoptotic myonuclei. The images were obtained at an objective magnification of ×200. Scale bars = 150 μm.

Apoptosis detection

The results of the TUNEL staining showed higher numbers of muscle fibers which were positively stained for DNA fragmentation in 40-year old individuals (Fig. 1h) than in 10-year old individuals (Fig. 1e). For the assessment of TUNEL staining, the number of positive cells was counted and an average value was calculated for each experimental group (Tab. 2). The size (mm^2) of each area

Tab. 2. Apoptotic cell counts in human semitendinosus muscle.

Age (y)	10	20	30	40
TUNEL-positive cells/HPF (x400)	0.72 ± 0.22	2.29 ± 1.45	5.22 ± 2.15*	12.42 ± 5.78 **

Values are means ± SE, * – 10 versus 30 years old, $p < 0.05$, ** – 10 versus 40 years old, $p < 0.005$

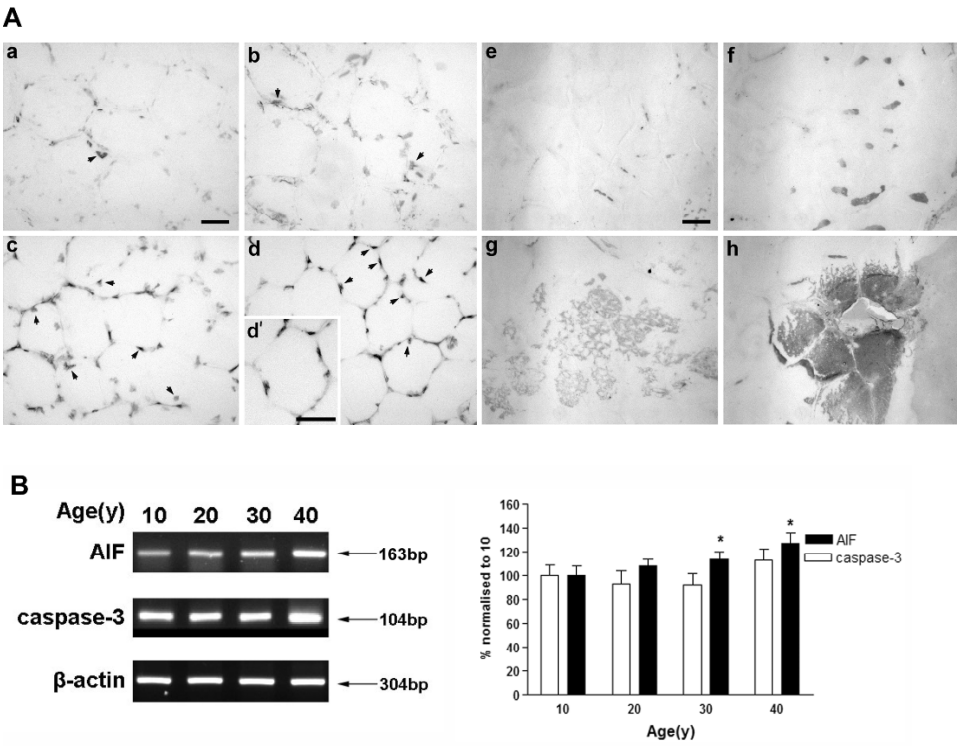

Fig. 2. The expression of the AIF and caspase-3 genes. A – The immunoreactivity and mRNA levels of AIF (a-d) and caspase-3 (e-h) in human semitendinosus muscle. The arrows indicate AIF-positive muscle cells. a, e – age 10; b, f – age 20; c, g – age 30; d, h – age 40; d´ – higher magnifications of images for age 40. The images were obtained at an objective magnification of ×200. Scale bars = a-h – 150 μm and d´ – 100 μm. B – A quantification of PCR signals obtained using a densitometric analysis of the signal product optical density (OD). The bands were quantified by normalization to those from the 10-year old individuals. *$p < 0.05$, normalized to 10-year old muscles.

containing TUNEL-positive cells and the width (μm) from the border of the infarction core were measured with a microruler at a magnification of ×100. The number of apoptotic cells was counted three times at the sectors under a high power (×400) light microscope, and their means were calculated and recorded as the number of cells per high-power field (cells/HPF).

Apoptosis-related factor protein and mRNA expression in muscle

Since caspase-3 and AIF play pivotal roles in apoptosis, we tested whether AIF and caspase-3 are involved in this process (Fig. 2). The results indicated an increase of approximately 127 ± 8% in the AIF mRNA levels in the 40-year old subjects compared to the levels for the 10-year old subjects (Fig. 2A: a-d, 2B). Our results show that expressed caspase-3 is mostly found in the cytosol of apoptotic cells, as they display typical apoptotic morphological changes, although those cells did not show nuclear immunostaining (2A: e-h). Caspase-3 expression measured by IHC did not increase with age, and there was no change in the level of caspase-3 mRNA expression measured by RT-PCR in the 40-year old subjects compared to the 10-year olds (113 ± 9% vs 100 ± 8%). Bcl-2 and Bax expression respectively decreased to 85 ± 11% and 91 ± 12% in the 40-year olds (all $p < 0.05$ vs 10-year olds), while the Bcl-2/Bax ratio did not change between the 10- and 40-year olds (100 ± 11% vs 105 ± 16%; Fig. 3). The expression of Hsp70, which is known to prevent apoptosis, decreased in the skeletal muscle with age (100 ± 11% in 10-year olds, 68 ± 8% in 20-year olds, 72 ± 2% in 30-year olds and 82 ± 12% in 40-year olds).

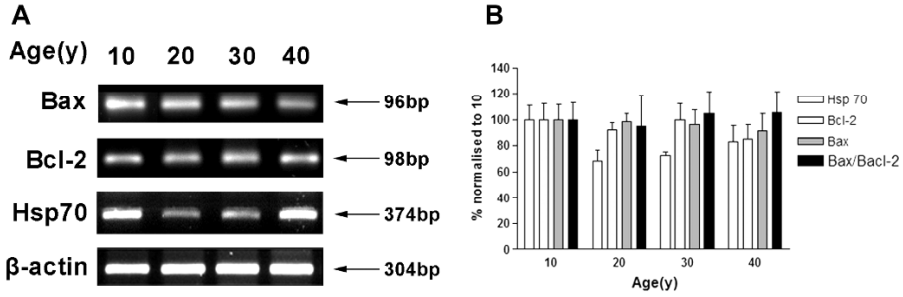

Fig. 3. The expression levels of the mRNAs of apoptotic genes associated with the mitochondrial pathway. Bax, Bcl-2, Bcl-2/Bax ratio and HSP70 mRNA levels were estimated using RT-PCR. A quantification of the PCR signals was performed using a densitometric analysis of the signal product optical density (OD). The bands were quantified by normalization to those from the 10-year old individuals.

DISCUSSION

Here, we report on the induction of apoptosis in human semitendinosus muscles as estimated by the TUNEL assay and assays of apoptotic regulatory factors, including Bcl-2, Bax, caspase-3, AIF and HSP 70. Recent studies have identified some of the apoptotic proteins that may contribute to the induction of apoptosis in aged skeletal muscle [1, 5]. We chose skeletal muscle samples from middle-aged individuals because degeneration begins around middle age, and the organism has no self-renewal activity for the maintenance of skeletal muscle cells. Using H&E staining, we examined the morphological changes induced by aging in human semitendinosus muscles. The detection of large quantities of nuclei is a distinctive feature of necrosis. H&E staining was conducted to observe the amount of nuclei produced, and none of the tissues showed evidence of necrosis. Since DNA fragmentation due to cleavage is one of the most important biochemical hallmarks of programmed cell death [32], our study is important in demonstrating an increase in apoptosis, as determined by apoptotic DNA fragmentation, in the semitendinosus muscle of individuals ranging in age from 10 to 40 years old. These results are similar to those showing an age-related increase in the incidence of apoptosis as measured by the TUNEL assay in human skeletal muscle [33, 34].

Caspase-3 activation can lead to cleavage of the DNA repair protein poly(ADP-ribose) polymerase (PARP), and eventually, DNA fragmentation and cell death [16]. We showed that caspase-3 was not activated in the skeletal muscle (Fig. 2A: e-h, 2B) and that there was no significant change in caspase-3 activity related to aging. This could be because in apoptosis in the skeletal muscles, cytochrome c initiates the caspase-dependent apoptotic pathway, whereas AIF and EndoG induce cell death independently of caspase activation [28]. By contrast, using RT-PCR we found that aged skeletal muscles were associated with increased expression of AIF, indicative of the activation of caspase-independent cell death. AIF is a principal mediator of cell death, because the apoptotic signaling pathways may converge at this point. AIF resides in the mitochondria, and upon stimulation, translocates to the nucleus to induce DNA fragmentation in a caspase-independent manner [22, 25]. We found that AIF activity increased with age, and was especially well characterized in samples from 40-year old individuals. This is similar to the results of Dirks and Leeuwenburgh [28], who demonstrated an increase in AIF in a plantaris homogenate. These results indicate a possible role for the AIF apoptotic-signaling pathway in increasing the apoptotic tendency of aged skeletal muscle. The relevance of caspase-independent apoptosis to age-related muscle was supported by a positive nuclear content of AIF and the extent of apoptotic DNA fragmentation.

The Bcl-2 family of proteins plays a key role in regulating the mitochondrial release of apoptotic mediators, regardless of age, with Bcl-2 being anti-apoptotic, while Bax is pro-apoptotic [12, 26]. In a recent study, anti-apoptotic

Bcl-2 and pro-apoptotic Bax were shown to be involved in apoptosis during the aging of skeletal muscles in rats [34]. We determined whether there were any changes in the anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) proteins with age, and determined the Bcl-2/Bax ratio. Bcl-2 and Bax expression respectively decreased to 85 ± 11% and 91 ± 12% in the 40-year old subjects ($p < 0.05$ vs 10-year olds), while the Bcl-2/Bax ratio did not change between the 10- and 40-year olds (100 ± 11% vs 105 ± 16%). These results are consistent with those from a previous study showing that caspase-3 expression, the Bcl-2/Bax ratio and the level of cytochrome *c* release did not increase in the gastrocnemius muscles of old rats, even though the degree of apoptosis did increase [1].

Heat shock proteins play an important role in helping cells to cope with many stresses [36, 37], and recent studies have identified mechanisms of HSP70-mediated inhibition of AIF-dependent cell death [38], and an increase in the level of HSP70 in aged gastrocnemius muscle in old rats, which might represent a compensatory mechanism in reponse to pro-apoptotic signaling [21, 29]. We proposed that the level of HSP70 would increase in cases of AIF-dependent cell death of semitendinosus muscle. Previous studies showed that a decreased level of HSP70 was observed in aged female Wistar rats compared to young adult rats [39]. In our study, the decrease of HSP70 expression from the 10- to 40-year old skeletal muscle may suggest that it was insufficient to prevent the apoptotic process from damage or various stresses. However, this observation needs to be clarified via further investigation. This result and the discrepancy between the studies may be explained as a result of differences in species, muscle compartment and subject age.

CONCLUSION

Our data shows that the level of apoptosis increased in human semitendinosus muscle with age. The involvement of apoptotic pathways in the aging process is suggested by the selective alterations in the apoptotic regulatory factors Bax, Bcl-2, AIF, caspase3 and HSP70. The increased expression of AIF suggests a relationship between AIF and the aging-related apoptotic process. The involvement of different apoptotic pathways in the aging process is suggested by the selective alterations in the apoptosis regulatory proteins. Molecular mechanisms by which AIF interacts with apoptosis regulatory factors in young and aged skeletal muscle remain to be elucidated in further studies.

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