PE1.32 mRNA Expression of u-Calpain, Calpastatin and Caspases During Fusion and Death of Hanwoo Muscle Satellite Cells 230.00

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***Abstract*: Calpains and calpastatin have been implicated in muscle tissue differentiation and development, cell cycle progression, and apoptosis. Effector caspases catalyze wholesale destruction of cellular proteins which is a major cause of cellular death. Here we have investigated the levels of u-calpain gene, calpastatin gene and caspases gene mRNA expression in different stages of life of cultured Hanwoo muscle satellite cells. Primarily, satellite cells were isolated from *longissimus dorsi* tissue of Hanwoo cattle and cultured in the laboratory. Chemical hypoxia induced by sodium azide triggered apoptosis of cell death. The samples of cells were divided into three stages: satellite cells cultured before they reached nearly confluence; cells differentiation were induced by fusion medium for 8 days; cells death treated with 1 mM sodium azide 24 hours after 8 d post-confluence. Semi-quantitative RT-PCR showed that satellite cells after 8 d post-confluence had increased u-calpain, calpastatin and caspase 9 mRNA expression compared to pre-confluence cells. And sodium azide treated cells had decreased caspase 8 and caspase 9 mRNA expression compared to untreated cells. However there was no significant difference in the u-calpain, caspatatin, caspase 3, and caspase 7 mRNA expressions between the treated and untreated cells. Further studies are underway for a better understanding the intricate biochemical mechanisms governing muscle cell death processes after slaughter.**

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I.INTRODUCTION

URING the harvesting process of meat there is a Dgeneralized cell death often referred to as necrosis or apoptosis[1]. This process of cell death has been well studied with respect to various physiological functions in *vivo* but its role in post-mortem meat quality has received little attention.

In a post-mortem muscle, the proposed pathway for apoptosis is possible by interaction of calpains, caspases and calpastatin though other enzymatic systems may be involved. When there is cease of aerobic conditions in the cell; the cell uses anaerobic glycolytic pathway for its survival. This results in accumulation of lactic acid leading to decrease in pH, which is deterimental to organelle membranes leading to release of calcium ions from sarcoplasmic reticulum and mitochondria into the cytoplasm of the cell [2]. Sodium azide (NaN3) as a mitochondrial respiratory chain complex IV inhibitor be used to induce myocyte cell death (apoptosis) in many experiment[3,4,5]. Here we previously created a chemical hypoxia model of satellite cells mediated by 1 mM azide as a cellular model to investigate the intricate biochemical mechanisms governing muscle cell death processes after slaughter.

II. MATERIALS AND METHODS

Drugs used and laboratory wares

Unless specified otherwise, all chemicals and laboratory wares were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, Nj, USA), respectively.

Cell preparation and culture

Satellite cells were isolated from 30-month-old Hanwoo cattle according to the method originally designed by Dodson et al. [6], followed by some modification [7]. In brief, *longissimus dorsi* muscle was excised Hanwoo cattle within a few minutes after slaughter. The epimysium and most of fat was trimmed off and discarded. Muscle strips were

ground in a small, sterile meat grinder. After enzymatic digestion with 1 mg/ml pronase at 37°C for 90 minutes, the cell-pronase solution was centrifuged at 1500 g for 6 min. The supernatant was discarded and the pellet was resuspended, single cells were separated and incubated in a DMEM (growth medium) containing 15% fetal bovine serum (FBS, GIBCO™) and antibiotics. When the cells reached confluence, they were collected and resuspended in phosphate-buffered saline (PBS) supplemented with 0.25% trypsin and 0.02% EDTA. After centrifugation, the pellet was resuspended in 100 ul PBS containing 10 ug anti-M-cadherin antibodies and then incubated with 20 ul of anti-mouse IgG1 microbeads at 4°C for 1 h. Finally, cell suspensions (10^7 cells in 500 ul PBS) were loaded into a magnetic cell sorting system. AutoMACS (Milteny Biotec, Germany) to isolate the satellite cells, which were maintained in a humidified environment of 95% air and 5% CO_2 at 37°C.

Cell induction of differentiation and chemical hypoxia induced cell death

Differentiation of the cells was induced by transfer to fresh DMEM medium (fusion medium) with 2% horse serum (HS,GIBCO™) before they reached nearly confluence, and culture at 37°C in a gas mixture containing 5% CO_2/95% air for 8 days. The medium was changed every 2 days. Sodium azide (1 mM) was prepared fresh in the culture medium (serum free medium) for each experiment, and fusion cells were treated with NaN_3 24 h after 8 d post-confluence.

Groups

Culture of bovine satellite cells through the steps of proliferation, fusion and differentiation, chemical hypoxia induced by sodium azide triggered apoptosis of isolated Hanwoo skeletal muscle satellite cell. The samples of cells were divided into three stages: stage 1: satellite cells cultured before they reached nearly confluence; stage 2: cells were induced by fusion medium for 8 days; stage 3: cells death treated with sodium azide 24 hours after 8 d post-confluence.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from the cells at each stage using the phenol-guanidine-isothiocyanate method with Trizol according to the manufacturer's protocol. More than 5 x 10^5 cells were used for one total RNA sample. The purity of the total RNA was assessed by the ratio of optical density 260 nm to 280 nm (acceptable values being between 1.6 and 2.1). First strand cDNA was synthesized from 2 ug of the total RNA using the M-MLV Reverse transcriptase with the anchored oligo $d(T)_{12-18}$ primer. Gene mRNA expression was measured by RT-PCR. The PCR program initially started with a 94°C denaturation for 4 min, followed by 20 to 42 cycles of 94°C/45 sec, Ta °C /45 sec, 72°C/1 min. Linear amplification range for each gene was tested on the adjusted cDNA. Primer sequences, optimal PCR annealing temperatures (Ta) and cycle number are listed in Table 1.

After electrophoresis of polymerase chain reaction products, the images were scanned and converted to electronic picture files. The relative intensities of each gene bands in the electrophoresis gels were calculated by Quantity One program (Bio-Rad, v4.62).

Statistical analysis

mRNA levels were expressed as transcript number. Mean and standard deviation of all experiments performed were calculated after normalization to beta actin. For analysis of differences between stages, One-Way ANOVA was used (SPSS, v16.0). P values < 0.05 are considered statistically significant.

III. RESULTS AND DISCUSSION

Behavior of satellite cell

Proliferation of Hanwoo satellite cells was observed by day 20 of culture (Fig. 1). All satellite cells are mononucleate cells at day 3 (Fig. 1a). At day 7, when the cells had nearly reached confluence (Fig. 1b), they were induced into differentiating by fusion medium, which resulted in myotube formation at day 15(Fig. 1c). These results showed that adult Hanwoo muscle satellite cells have the potential to differentiate into multinucleated cells.

Analysis of gene expression during satellite cell differentiation and cell death

In these satellite cell cultures, either untreated or treated with sodium azide, we evaluated the levels of mRNA for caspase 3, caspase 7, caspase 8, caspase 9, u-calpain and calpastatin using semi quantitative RT-PCR method described above.

Semi-quantitative RT-PCR showed that satellite cells after 8 d post-confluence had increased u-calpain, calpastatin and caspase 9 mRNA expression compared to pre-confluence cells.

And sodium azide treated cells had decreased caspase 8 and caspase 9 mRNA expression compared to untreated cells. However there was no significant difference in the u-calpain, caspatatin, caspase 3, and caspase 7 mRNA expressions between the treated and untreated cells (Fig. 2).

Differentiation and fusion of myoblast into myotubes are associated with protein turnover and structural adaptation through mechanisms that are not yet well understood. Several studies either in fetal myoblast primary culture [8, 9, 10] or *in vivo* during muscle regeneration [11, 12] have suggested that calpains might play a role in the fusion process. We showed by semi quantitative RT-PCR analysis that suggest u-calpain, calpastatin and caspase 9 might be involved in myoblast fusion during myogenic differentiation. Temporal changes in the activity of calpains and calpastatin during the first 24 of postmortem aging were similar in the muscles studied: u-calpain and calpastatin declined significantly and m-calpain remained relatively unchanged [13]. And one chemical hypoxia model of neonatal cardiomyocytes mediated by 1 mM azide that exhibits features of prominent internucleosomal DNA fragmentation, cell membrane leakage, mitochondrial dysfunction, and increased calpain mRNA[5]. Effector caspases catalyze wholesale destruction of cellular proteins which is a major cause of cellular death. Caspases also target caspase-activated DNase. In normal cells, activity of a caspase-3-activated deoxyribonuclease (CAD) is suppressed by binding to its inhibitor ICAD. Catalytically active caspase-3 cleaves ICAD, permitting CAD to enter the nucleus and cause the degradation of chromosal DNA into nucleosomal units characteristic of apoptosis [14, 15]. And our results showed that sodium azide treated cells had decreased caspase 8 and caspase 9 mRNA expression compared to untreated cells. Our results obtained with adult Hanwoo satellite cells, which were different from those obtained with fetal myoblast by others, possibly illustrated another difference between fetal and adult myoblast. Similarly, differences might also exist between the *in vitro* behaviors of satellite cells isolated from adult animals and cell lines established from myoblasts isolated from neonate animals (such as L8 myoblast).

IV. Conclusion

This study demonstrates that satellite cells can be isolated from adult Hanwoo muscle, and that these cells being able to proliferate and to differentiate into myotubes, and we created a chemical hypoxia model of fusion satellite cells mediated by sodium azide that can be evaluated the biochemcal changes in muscles after slaughter the cattle.

References

[1] Fidzianska, A., Kaminska, A. and Glinka. Z. (1991). Muscle cell death. Ultrastructural difference between muscle cell necrosis and apoptosis. Neuropatologia Polska. 29(1-2), 19-28.

[2] Vignon, X., Beaulaton, J. and Ouali, A. (1989). Ultrastructural localization of calcium in post-mortem bovine muscle: A cytochemical and X-ray microanalytical study. Histochem. J. 21, 403-411.

[3] Chen S.J., Bradley M.E., Lee T.C. (1998). Chemical hypoxia triggers apoptosis of cultured neonatal rat cardiac myocytes: modulation by calcium-regulated proteases and protein kinases. Molecular and Cell Biochemistry. 178,141-149

[4] Inomata K. & Tanaka H. (2003). Protective effect of benidipin against sodium azide-induced cell death in cultured neonatal rat cardiac myocytes. Journal of Pharmacological Sciences, 93,163-170.

[5] Kositprapa C., Zhang B.C., Berger S., Canty Jr JM., Lee T.C. (2000). Calpain-mediated proteolytic cleavage of troponin I induced by hypoxia or metabolic inhibition in cultured neonatal cardiomyocytes. Molecular and Cellular Biochemistry, 214, 47-55.

[6] Dodson M.V., Martin E.L., Brannon M.A., Mathison B.A. and Mcfarland D.C. (1987). Optimization of bovine satellite cell derived myotube formation in vitro. Tissue & Cell 19,159-166.

[7] Sung H. K., Ki C. C., Young O. S. (2006). Satellite cells isolated from adult Hanwoo muscle can proliferate and differentitate into myoblasts and adipose-like cells. Molecular cells 22, 239-245.

[8] Brustis, J. J., Elamrani, N., Balcerzak, D., Safwate, A., Soriano, M., Poussard, S., Cottin, P. and Ducastaing, A. (1994). Rat myoblast fusion requires exteriorized m-calpain activity. Eur. J. Cell. Biol. 64(2), 320-327.

[9] Kwak, K. B., Chung, S. S., Kim, O. M., Kang, M. S., Ha, D. B., and Chung, C. H. (1993). Increase in the level of m-calpain correlates with the elevated cleavage of filamin during myogenic differentiation of embryonic muscle cells. Biochim. Biophys. Acta 1175(3), 243–249.

[10] Cottin, P., Brustis, J. J., Poussard, S., Elamrani, N., Broncard, S., and Ducastaing, A. (1994). Ca^{2+}-dependent proteinases (calpains) and muscle cell differentiation. *Biochim. Biophys. Acta* 1223(2), 170–178.

[11] Spencer, M. J., Croall, D. E., and Tidball, J. G. (1995). Calpains are activated in necrotic fibers from mdx dystrophic mice. *J. Biol. Chem.* 270(18), 10909–10914.

[12] Spencer, M. J., and Tidball, J. G. (1996). Calpain translocation during muscle fiber necrosis and regeneration in dystrophindeficient mice. *Exp. Cell Res.* 226(2), 264–272.

[13] Ilian, M. J., Morton, J. D., Kent, M. P., Le Couteur, C. E., Hickford, J., Cowley, R. and Bickerstaffe, R. (2001). Intermuscular variation in tenderness: Association with the ubiquitous and muscle-specific calpains. J. Anim. Sci. 79: 122-132.

[14] Enari, M., Sakahira, H., Yokoyana, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998). A caspases-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature. 391: 43-50.

[15] Sakhira, H., Enari, M. and Nagata, S. (1998). Cleavage of CAD activation and DNA degradation during apoptosis. Nature. 391: 96-99.

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Table 1: Sequence, annealing temperature (Ta) of primers and cycle number used in the semi-quantitative RT-PCR analysis of mRNA levels of the genes expressed in bovine satellite cells.

Gene	Sequence	Ta(^{o}C)	Cycle number
u-Calpain	CGCAACTACCCAGCCACT GAGAAGAAACGCAGCACAAA	57	30
Calpastatin	CCTACCCAAGCACTCATC GCGATCCCTTCTTCTTTA	55	32
Caspase 3	CCAAGATTTAGTGCCGATGC TCCTCAACCCGTCTCCCT	57	30
Caspase 7	CCCGCAAACAATGATACAG TTGGCACAAGAGCAGTCG	55	28
Caspase 8	AGTGCCCTTCCCTTATTG CCCATTCCAGATGTTTCG	54	28
Caspase 9	CGCCACCATCTTCTCCCTG GCTCGTCCACCTTCTCACTCA	60	38
Beta actin	AGGACCTCTACGCCAACA CCTTCACCGTTCCAGTTT	56	24

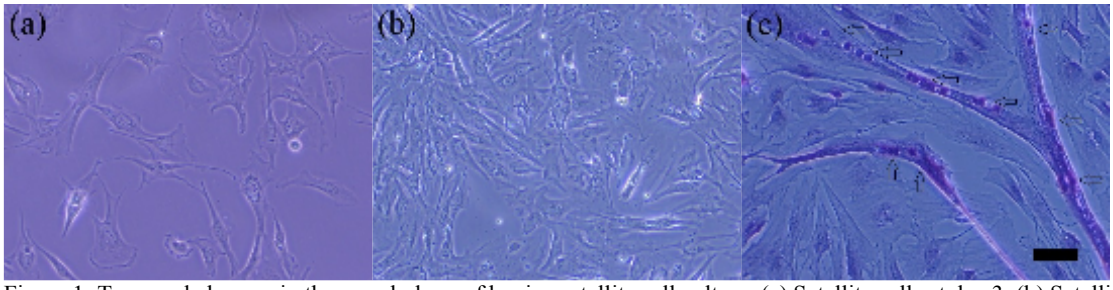

Figure 1: Temporal changes in the morphology of bovine satellite cell culture. (a) Satellite cells at day 3. (b) Satellite cells reached confluence at day 7. (c) Myotubes at day 15 and stained with hematoxylin & Eosin: the arrows indicate the fused cells. Bar = 100 um.

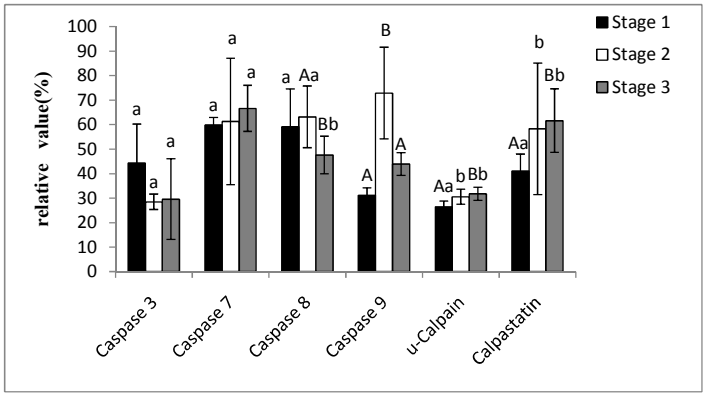

Figure 2: mRNA levels of caspase3, capase 7, capase 8, caspase 9, u-calpain and calpastatin during fusion and death of muscle satellite cells satellite cells. Means ± standard deviations (average of 9 band intensity) with each stage followed by different lower superscripts are significantly different at $P \leq 0.05$ and larger are $P \leq 0.01$. ^{AaBb} Means bearing the same letter did not significantly ($P > 0.05$).