PE1.29 Protein Expression During Myogenesis of Hanwoo Satellite Cells 216.00

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***Abstract*—Current study was conducted to understand changes in proteins and proteolytic enzymes during myogenesis and hypoxia cell death of Hanwoo longissimus satellite cell as a model study. Satellite cells from *longgissimus dorsi* tissue of Hanwoo cattle were isolated and purified and samples were taken at near confluence (S1), differentiated and fused (S2), and after hypoxia death (S3). Hypoxia cell death via apoptosis was induced by 1 mM sodium azide. By applying a gel based proteome analysis, 26 spots were identified, which were up or down regulated during myogenesis and hypoxia death. Caspases 8 and 9 were shown only in stages S1 and S2. HSPs 27, 70, and 90 were expressed in all stages, with particular higher intensity for HSP 27 at S 2 stage and HSP 70 at S3 stage.**

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I. Introduction

MOST consumer groups, identified beef tenderness as the prime attribute of eating quality. Furthermore unfortunately the greatest variation in eating quality traits between carcass and between muscles within a carcass was tenderness [1]. There has been apparent that biological understanding and solution(s) for tender meat are priority of beef industry. The larger variations in meat toughness/tenderness are in part due to the heterogeneity of fibers within muscle [2] and geological location of individual muscle. These together determine the cause of rigor mortis and consequently toughness and tenderization. Calpains system [3] and/or 20S proteasome [4] have been considered as the most possible candidate responsible for postmortem meat tenderization. Ouali et al in 2006 hypothesized that muscle tissue becomes meat through apoptosis process and thus (if it is) caspases are likely involved in rigor mortis process and consequently meat tenderization process. The current study was conducted to understand changes in proteins and proteolytic enzymes during hypoxia cell death of Hanwoo longissimus satellite cell as a model study.

II. Materials and Methods

A. *Cell preparation and culture*

Satellite cells were isolated from 30-month-old Hanwoo cattle according to the method originally designed by Dodson et al.[5], followed by some modification[6]. 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 ^{o}C for 90 min, 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^{TM}) 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 ^{o}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 ^{o}C .

B. *Cell death*

Differentiation of the cells was induced by transfer to fresh DMEM medium (fusion medium) with 2% horse serum (HS,GIBCO^{TM}) before they

reached nearly confluence, and culture at 37 ^{o}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.

C. Groups of sample

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 until they reached nearly confluence; stage 2: Differentiation and fusion were induced by fusion medium for 8 days; stage 3: cells death treated with sodium azide 24 hours after 8 d post-confluence.

D. Antibodies

The following antibodies were used in this study: rabbit polyclonal anti-caspase 3 (1:1000, Clone polyclonal, Acris antibodies GmbH), caspase 8 (1:1000, Clone polyclonal, Acris antibodies GmbH), caspase 9 (1:1000, Clone polyclonal, Acris antibodies GmbH), caspase 12 (1:1000, Clone polyclonal, Acris antibodies GmbH), caspase 13 (1:1000, Clone polyclonal, eBioscience), and Monoclonal Antibody to caspase 7 (1:1000, Clone 7CS03, Acris antibodies GmbH), Mouse Anti Hsp27 monoclonal antibody (1:1000, Clone G3.1, stressgen), Hsp 70 (1:2000, Clone C92F3A-5, stressgen), Hsp 90 (1:1000, Clone AC88, stressgen), desmin (1:2500, Clone DE-U-10, sigma), Troponin-T (1:2500, Clone JTL-12, sigma)

E. Western blot

Samples were taken from cells and extracted protein by lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X100, 10 mM Tris-HCl, pH7.4) and stored in liquid nitrogen until analysis**,** was for SDS-PAGE according to the method of Laemmli[7]. Samples were separated on resolving gels of 12.5% acrylamide with 4% acrylamide stacking gels. Gels for western blotting were transferred to Hybond-P ployvinylidin fluoride for 1hr at 200 mA. Membranes were blocked for 1 h with TTBS(20 mM Tris, 137 mM NaCl, 5 mM KCl, 0.05% Tween 20) containing 2% ECL advance blocking agent and then incubated with primary for 60 min. The bound primary antibodies were than labeled (60 min at room temperature) with rabbit x-mouse IgG or HRP -conjugated**.** The bound antibodies were visualized by incubating membranes with BCIP/NBT substrate (Bio-Rad laboratories, CA) or ECL kit (Amersham, UK), according to the method described by Hwang [8].

F. 2-D gel electrophoresis and analysis

Samples (3 x 10^6cells/T75 flask 4 plate) were taken from cells and extracted protein by lysis buffer (7 M urea, 2 Mthiourea, 2% CHAPS, 1% DTT, and 0.8% E-lyte pH 4-7) and then taken 20 ul lysis buffer (each tube 10 ul) for calculate the concentration of this protein (using the 2DE Quant Kit). Protein expression was assessed according to procedure described by Hwang et al.[9]. Briefly, Duplicate gels for each sample were analyzed using 2DE image analysis software (PDQuest, Bio-Rad, USA) according to the manufacturer's instruction. Spots appearing in more than two gels within 50% of variation in optical density were accepted as analytical spots, and an average of normal volume was used for the final analysis. MS/MS spectra were generated by nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The data were processed using a Mass Lynx Windows NT PC system. Peptide masses from MALDI-ToF MS were matched with the theoretical peptides of proteins in the NCBI database using MASCOT and/or Profound software. Also, all MS/MS spectra recorded on tryptic peptides were searched against protein sequences from NCBInr and EST databases using the MASCOT search program.

III. RESULTS AND DISCUSSION

26 protein spots which were up or down regulated during myogenesis and death were identified by 2DE-LC/MS/MS methods. Table 1 shows the results of the expression pattern of proteins for satellite cell of beef *m. longissimus* during incubation from growth of cells to their death. Three protein spots were detected only in the pre-confluence stage, 19 spots were observed only in the post-confluence and 4 protein spots were seen only in the death cells for S1, S2, and S3, respectively.

The proteins identified during S1 stage play important roles during cell growth. The microtubule-associated protein RP/EB family member 1 functions in microtubule polymerization, and spindle function by stabilizing microtubules and anchoring them at centrosomes. The elongation initiation factor 4E is involved in the initiation of

protein synthesis [11].

A greater number of proteins were identified in stage 2 indicating that there are more biochemical processes that are occurring in the cells. For instance, ribosomal protein P2 plays an important role in the elongation step of protein synthesis [11]. Vimentins play a role in muscle growth. The presence of AHA1 and HSP 27 indicates that the defense mechanisms of the cells are actively working. AHA1 is an activator of HSP 90 which together with HSP 27 blocks apoptotic pathways. Both the ATP synthase subunit delta and Cytochrome b-c1 complex play important roles during ATP synthesis.

The proteins in S3 are typical in cells subjected to stress. Small Ubiquitin-like Modifier or SUMO1 activating enzyme subunit 1 a family of small proteins that are covalently attached to and detached from other proteins in cells to modify their function during cellular processes like apoptosis and response to stress. Reticulocalbin 1 regulate calcium-dependent activities in the endoplasmic reticulum lumen or post-ER compartment

We examined caspases 3, 7, 8, 9, 12, 13(Figure 1 A), HSP 27, 70, 90, troponin-T, and desmin(Figure 1 B) during proliferation, fusion and death. Caspase 8 was present during S1 and S2 stages. The dark bands of caspase 8 in S1 indicate that it is in its active form whereas the two lighter bands in S2 are indicative that caspase 8 is inactive. The separate bands suggest that the larger and smaller subunits have separated. Caspase 9 was expressed in S2 as revealed by the dark bands. The results of the dramatic decrease in the expression of caspases 8 and 9 during the death process were also confirmed by our other study at mRNA level [10]. Caspase 3 was detected in S2 while caspase 7 was present in S3. HSP 27, 70 and 90 were consistently present in S1, S2, and S3. This results indicate that the defense mechanism of the cell remain active even during the process of death.

IV. Conclusion

The current data are preliminary results of our long term experiment. By the satellite cell model study, we identified 26 proteins and other proteolytic enzymes up/down regulated proteins during myogenesis and cell death of Hanwoo longissimus muscle. Caspases 8 and 9 were shown only in stages S1 and S2. HSPs 27, 70, and 90 were expressed in all stages, with particular higher intensity for HSP 27 at S 2 stage and HSP 70 at S3 stage.

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Table1. List of spot identifications (ID), consensus protein identity, NCBI gi number (NCBI gi No.), percentage of coverage/number of matched query for MALDI-TOF analysis (C/Q), molecular weight and estimated pI (MW/pI) for the steps of proliferation (S1), fusion and differentiation(S2), chemical hypoxia (S3)induced by sodium azide

ID	Identified fragment	NCBI gi No.	C/Q	MW/pI	S1	S2	S3
4219	PREDICTED: similar to Microtubule-associated protein RP/EB family member 1	gi|73945697	5/1	40835/5.27	1842	0	0
6113	Elongation initiation factor 4E	gi|119416776	23/7	21446/5.44	1038.5	0	0
6219	Chain A, Purine Nucleoside Phosphorylase	gi|4558113	6/1	31862/5.84	2174.5	0	0
1033	Ribosomal protein P2	gi|4506671	28/1	11658/4.42	0	1534.6	0
1039	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	P05630	Q-TOF		0	1811.5	0
2116	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha II polypeptide isoform 2	gi|6754970	3/1	61306/5.55	0	1521.4	0
2225	Vimentin	gi|138535	3/1	53754/5.06	0	1299.9	0
3109	Protein disulfide-isomerase precursor (PDI)	gi|129729	1/1	57507/4.79	0	1420.4	0
3208	Protein kinase C substrate 80K-H [Bos taurus]	gi|41386727	5/1	61082/4.36	0	1534.6	0
3302	Nucleolar protein B23.2	gi|203078	8/1	28482/4.55	0	1383.4	0
4008	ATPD_BOVIN ATP synthase delta chain,	P05630		17612	0	1497.6	0
5327	Mutant beta-actin (beta'-actin)	gi|28336	4/1	42128/5.22	0	820.9	0
5408	AHA1, activator of heat shock 90kDa protein ATPase homolog 1 [Bos taurus]	gi|77736277	3/1	38391/5.43	0	1497.6	0
5725	P4H2_MOUSE Prolyl 4-hydroxylase alpha-2 subunit precursor	Q60716|		61002	0	1383.4	0
6114	Heat shock 27kDa protein 1 [Bos taurus]	gi|61553385	9/1	17602/6.49	0	1811.5	0
6501	Chain A, Crystal Structure Of Bovine Mitochondrial Cytochrome Bc1 Complex	gi|3891848	5/1	49852/5.46	0	2092.8	0
7013	Calreticulin	gi|27806723	10/5	48180/4.31	0	1284.3	0
7101	GrpE-like 1, mitochondrial	gi|77735951	42/6	24519/8.26	0	4614.9	0
7230	Protein disulfide-isomerase A3 precursor (Disulfide isomerase ER-60) (ERP60)	gi|729433	4/1	57293/6.23	0	1104.4	0
7310	Protein kinase C, delta binding protein	gi|134085797	24/16	27470/6.14	0	4614.9	0
7430	CaBP1 [Rattus norvegicus]	gi|488838	3/1	47590/4.95	0	2092.8	0
7442	Protein disulfide-isomerase A3 precursor (Disulfide isomerase ER-60) (ERP60)	gi|729433	18/10	57293/6.23	0	1420.4	0
1115	Vimentin	gi|289450	4/1	53701/5.20	0	0	46107.6
1411	Reticulocalbin 1, EF-hand calcium binding domain	gi|157073966	5/1	38728/4.70	0	0	8962.6
4309	SUMO1 activating enzyme subunit 1	gi|126165258	29/8	38738/5.15	0	0	1605.8
5326	Beta tubulin [Cricetulus griseus]	gi|49481	3/1	50109/4.85	0	0	4627.6

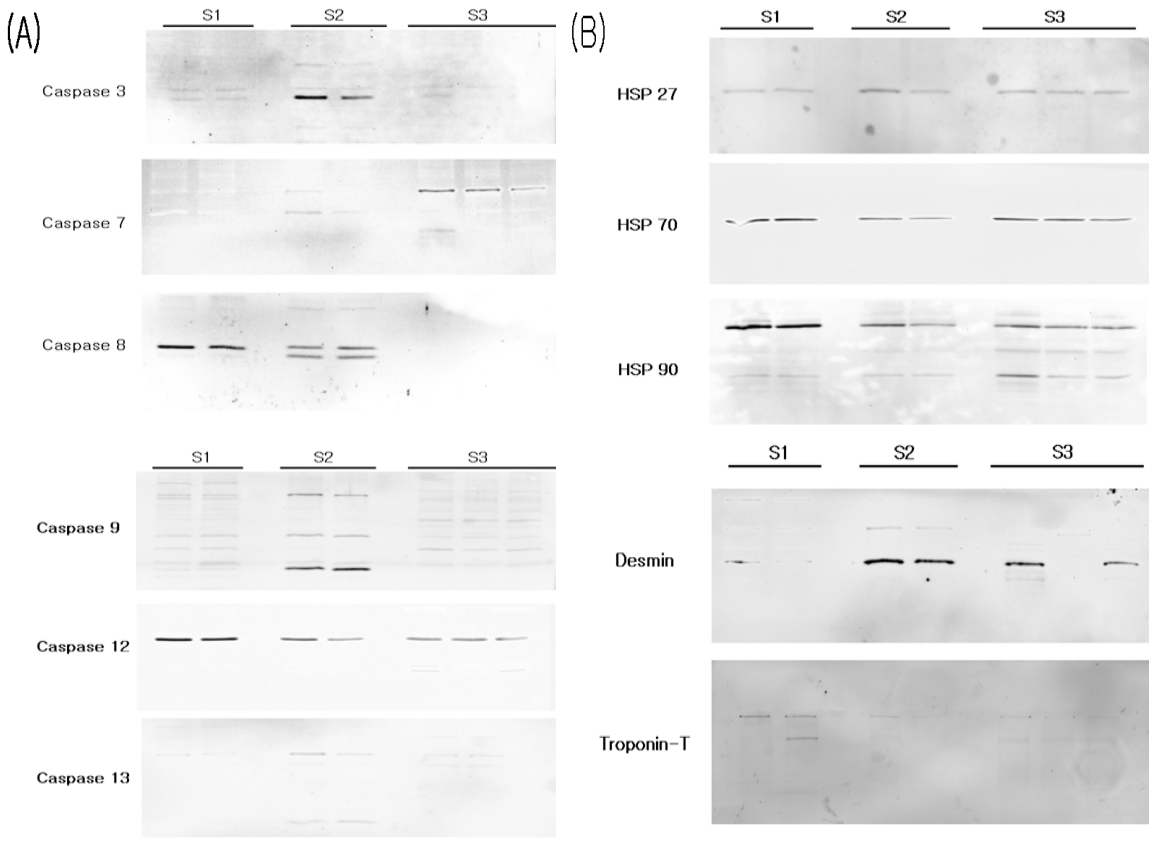

Figure 1. Western blot of (A) caspase 3, 7, 9, 12, 13, (B) HSP 27, 70, 90, desmin, and troponin-T for the steps of proliferation (S1), fusion and differentiation(S2), chemical hypoxia (S3)induced by sodium azide.