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Effect of Thermal Stimulation on Gene Expression Related to Skeletal Muscle-derived Cell Density

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Authors' contributions

This work was carried out in collaboration between both authors. Author MN designed the study, performed the experiment and analysis and wrote the first draft of the manuscript. Author HK designed the study, performed the experiment and analysis and wrote the manuscript, instruction and management of research. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Our previous study demonstrated favorable changes in plasma protein levels such as adiponectin by fomentation in healthy people. We also reported that the thermal stimulation caused changes of mRNA levels to prevent atherosclerosis in human skeletal muscle-derived cell (SMDC). However, cell number decreased to 74.6% by heat stimulation. In order to clarify this mechanism, we investigated whether the heat stimulation affects the levels of mRNA related to cell density or number of SMDC.
Study Design: Experimental study comparing transcriptome between cells cultured at higher temperature and control cells.
Place and Duration of Study: From September 2015 to March 2017, Division of Physiology and Metabolism, University of Hyogo.
Methodology: SMDC was cultured at 42°C and 37°C and its gene expression was analyzed by using microarray technique.
Results: Thermal stimulation of SMDC significantly altered the expression of 10 genes related to apoptosis, 1 gene related to cell division and 1 gene related to cell adhesion. mRNA expression of

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apoptosis promoting gene, such as THAP2 (THAP domain containing, apoptosis associated protein 2), PDCD6 (programmed cell death 6), BCL2L13 (BCL2-like 13), LOC728613 (programmed cell death 6 pseudogene), CASP4 (caspase 4), and FAS (Fas cell surface death, receptor) was up-regulated. On the other hand, PAWR (PRKC, apoptosis, WT1, regulator) was downregulated, and mRNA expression of anti-apoptotic genes, such as NOL3 (nucleolar protein 3), CIAPIN1 (cytokine-induced apoptosis inhibitor 1) and NAIF1 (nuclear apoptosis inducing factor1), was up-regulated, Gene Ontology analysis showed alterations in the expression of genes that promote apoptosis and cell growth inhibition. Pathway analysis demonstrated the pathways that promote apoptosis, stimulate cell growth and negatively or positively regulate cell adhesion.

Conclusion: The present study suggested that thermal stimulation of SMDC might predominantly promote apoptosis from consistent changes in related gene expression by any analysis.

Keywords: Thermal stimulation; heat; fomentation; cell growth; cell division; apoptosis; skeletal muscle-derived cells; transcriptom.

1. INTRODUCTION

Our previous study [1] demonstrated an increase in the serum adiponectin level and decrease in soluble urokinase-type plasminogen activator receptor by fomentation for skeletal muscle in healthy people. We also reported that the thermal stimulation caused changes of mRNA levels to prevent atherosclerosis in human skeletal muscle-derived cell (SMDC) [2]. From these results, it was considered that thermal stimulation of skeletal muscle is useful for preventing arteriosclerosis.

However, cell number decreased to 74.6% by heat stimulation. Cell density or number is changed by various mechanism such as cell detachment, cell growth suppression by slower cell division, necrotic cell death or programmed cell death (apoptosis). In this in vitro experiment, neither cell morphological change nor cell detachment was observed 20 hours after the start of thermal stimulation. The number of cells seeded at the start of subculture was considered to be comparable.

It is known that cell apoptosis is induced by thermal stimulation at 43°C or higher. In cancer cells, which are more vulnerable to heat than normal cells, apoptosis is induced by thermal stimulation above 42.5°C, and cytotoxicity is rarely observed below that [3,4]. It has also been reported that thermal stimulation below 42.5°C causes minimal damage to cells and hardly induces cell death [5]. Even in vivo, a heating temperature of 40 ± 2°C is considered to be safe with a low risk of low-temperature burns [6].

In order to clarify this mechanism, we investigated whether the heat stimulation affects the levels of mRNA related to cell density or number of SMDC. It is important to verify the safety when utilizing thermal stimulation for the purpose of preventing atherosclerotic cardiovascular disease (ASCVD) in medical and nursing practice.

2. MATERIALS AND METHODS

Methods for cell culture and gene expression analysis have already been reported [2].

SMDC (SkMC, Lonza, Japan) is embryonic human cells derived from quadriceps muscle. SMDC used for microarray analysis was analyzed using 5 replicates. Changes in mRNA expression due to heat stimulation were compared by using microarray analysis between heat-exposed SMDC cultured at 42°C and control cells cultured at 37°C. SMDC were cultured in SkGM-2 BulletKit (Lonza, Japan). A thermal load was initiated when growth saturation densities of 50% to 70% were confirmed in SMDC cultures. Total RNA was extracted after thermal loading for 20 hours by using Total RNA Purification Maxi Kit (Norgen Biotek Corporation, Canada). The electrophoresis pattern of the total RNA was confirmed with an Agilent 2100 Bioanalyzer, and quantitative evaluation by NanoDropND 1000 was performed. The microarray analysis was done by using Gene Chip Human Genome U133 Plus 20 Array (Affymetrix). Detected signals were subjected to the scatter plot, gene ontology (GO), cluster and pathway analyses by using the Gene Spring GX software.

“Significant change in magnification” in this study was set to 2-fold or more or 0.5-fold or less which is common in microarray analysis. All factors whose expression changed to 2-fold or more or

0.5-fold or less were those whose expression changed with a significant probability of p <0.04.

Gene functions were confirmed using genes such as 'PubMed' and 'NCBI gene' on genes whose expression fluctuation was confirmed to be 2-fold or more and 0.5-fold or less. We also confirmed the relationship between each factor and skeletal muscle and its association with ASCVD using 'PubMed'.

GO analysis was performed using the gene list whose expression change more than 2-fold and less than 0.5-fold. The probe list detected by GO analysis was classified by confirming each function of GO using the database AmiGO 2 (http: // amigo.geneontology.org/amigo).

The pathway analysis was carried out using a list in which the gene expression varied more than twice or less than 0.5-fold by thermal stimulation in the 42°C. group as compared with the 37°C group. Analysis method is Single Experiment Analysis, WikiPathways database (http: // www. Wikipathways.org / index.php / WikiPathways) was used for analysis.

3. RESULTS

Thermal stimulation to SMDC resulted in a significant increase in expression of 1,072 genes and significant inhibition of expression of 1,123 genes as described previously [2]. Among them, there were significant changes of 10 genes related to apoptosis, 1 gene related to cell division and 1 gene related to cell adhesion (Table.1). The upper panel of Table 1 demonstrated upregulation of 6 apoptosis related genes such as THAP2 (THAP domain containing, apoptosis associated protein 2), PDCD6 (programmed cell death 6), BCL2L13 (BCL2-like 13), LOC728613 (programmed cell death 6 pseudogene), CASP4 (caspase 4), and FAS (Fas cell surface death receptor). The upregulation of CDC14B (cell division cycle 14B: regulator of anti-oncogene p53) was also shown. On the other hand, 3 genes related to anti-apoptosis such as NOL3 (nucleolar protein 3), CIAPIN1 (cytokine-induced apoptosis inhibitor 1) and NAIF1 (nuclear apoptosis inducing factor1) were upregulated and 1 gene related to apoptosis such as PAWR (PRKC, apoptosis, WT1, regulator) was downregulated as shown in the middle panel of Table 1.

GO analysis was performed on 1072 genes whose expression was more than doubled in the 42°C group compared to the 37°C group. 152 genes were significantly (p <0.001) consistent with the GO classification gene list. Among the significantly upregulated genes, 6 GO term classification were associated with cell proliferation and apoptosis (Table 2). Heat stimulation of SMDC upregulated 4 GO terms associated with apoptosis: 1) "regulation of apoptotic process", 2) "regulation of programmed cell death", 3) "granzyme-mediated apoptotic signaling pathway", and 4) "positive regulation of programmed cell death". Heat stimulation of SMDC upregulated 2 GO terms associated with cell death: 1) "regulation of cell death" and 2) "positive regulation of cell death". In GO analysis, no significant changes of mRNA related to cell adhesion could be detected.

As shown in the upper panel of Table 3, heat stimulation of SMDC upregulated 2 pathways that were involved in apoptosis 1) "TNFα Signaling Pathway" and 2) "Type II interferon signaling (IFNG)". As shown in the middle panel of Table 3, heat stimulation of SMDC upregulated 2 pathways that were involved in cell proliferation, 1) "MAPK Signaling Pathway" and 2) "Oncostatin M Signaling Pathway". Heat stimulation of SMDC downregulated 1 pathway that is involved in cell growth inhibition, "TGFβ Signaling Pathway". Also, heat stimulation of SMDC upregulated and downregulated "Integrin mediated Cell Adhesion" as shown in the lower panel of Table 3.

Effect of heat stimulation on SMDC mRNA expression, GO and pathway related to cell density were summarized in Table 4.

4. DISCUSSION

We have previously observed slight decrease in cell density of SMDC cultured at 42°C for 20 hours. The direct factors regulating cell density is related to cell proliferation or cell death including apoptosis, and cell detachment or attachment. Microarray analysis of SMDC cultured at this condition demonstrated various changes in the expression of genes related to cell density such as apoptosis, cell growth inhibition and cell detachment.

Table 1. Cell density-related mRNA expression changed by heat stimulation in SMDC

Gene symbol	Gene title	Fold change	Function
Changes to promote apoptosis or inhibit cell growth			
Upregulated			
THAP2	THAP domain containing, apoptosis associated protein 2	3.63	apoptosis
PDCD6	programmed cell death 6	3.306	programmed cell death
* CDC14B	cell division cycle 14B	2.865	regulation of p53
BCL2L13	BCL2-like 13 (apoptosis facilitator)	2.244	apoptosis
LOC728613	programmed cell death 6 pseudogene	2.189	apoptosis
CASP4	caspase 4, apoptosis-related cysteine peptidase	2.132	apoptosis
FAS	Fas cell surface death receptor	2.028	programmed cell death
Changes to inhibit apoptosis			
Upregulated			
NOL3	nucleolar protein 3 (apoptosis repressor with CARD domain)	4.518	anti-apoptosis
CIAPIN1	cytokine induced apoptosis inhibitor 1	2.28	anti-apoptosis
NAIF1	nuclear apoptosis inducing factor1	2.03	anti-apoptosis
Downregulated			
PAWR	PRKC, apoptosis, WT1, regulator	0.474	apoptosis
Changes to inhibit cell adhesion			
Downregulated			
ISLR	immunoglobulin superfamily containing leucine-rich repeat	0.304	Cell adhesion

** cell growth inhibition-related gene*

Table 2. GO analysis upregulated by heat stimulation in SMDC

Ontology	GO ACCESSION	GO term	Corrected p-value
Biological process	GO: 0042981	regulation of apoptotic process	0.020
	*GO: 0010941	regulation of cell death	0.020
	*GO: 0010942	positive regulation of cell death	0.026
	GO: 0043067| GO: 0043070	regulation of programmed cell death	0.028
	GO: 0008626	granzyme-mediated apoptotic signaling pathway	0.035
	GO: 0043068| GO: 0043071	positive regulation of programmed cell death	0.045

**: cell death-related GO*

Corrected P-value: The probability that the significantly varied expression probe would match the GO-classified gene list was calculated (p <0.001)

Table 3. Cell density-related pathways changed by heat stimulation in SMDC

	Pathway	*p*-value	Matched Entities	Pathway Entities	Function
Pathways that promote apoptosis					
Upregulated	TNF α Signaling Pathway_WP231_72093	3.08E-04	8	87	Apoptosis, chronic inflammation
	Type II interferon signaling (IFNG)_WP619_71168	7.69E-04	5	37	Apoptosis
Pathways that promote cell growth					
Upregulated	MAPK Signaling Pathway_WP382_72103	7.13E-06	14	168	Regulation of cell proliferation and differentiation
	Oncostatin M Signaling Pathway_WP2374_72050	9.27E-03	5	65	Cell proliferation
Downregulated	TGFβ Signaling Pathway_WP560_68944	1.02E-03	6	55	Cell growth inhibition
Pathways that promote cell adhesion or detachment					
Upregulated	Integrin-mediated Cell Adhesion_WP185_71391	3.56E-03	7	99	Cell adhesion
Downregulated	Integrin-mediated Cell Adhesion_WP185_71391	5.46E-03	7	99	Cell adhesion

Table 4. Summary effect of heat stimulation on SMDC mRNA expression, gene ontology and pathway related to cell density

	Apoptosis				Cell growth inhibition				Cell detachment			
	promotion: Changes to decrease cell density		suppression: Changes to increase cell density		promotion: Changes to decrease cell density		suppression: Changes to increase cell density		promotion: Changes to decrease cell density		suppression: Changes to increase cell density	
mRNA expression	6	THAP2 PDCD6 BCL2L13 LOC728613 CASP4 FAS	4	NOL3 CIAPIN1 NAIF1 *PAWR	1	CDC14B			1	*ISLR		
GO analysis	4	GO: 0042981 GO: 0043067| GO: 0043070 GO: 0008626 GO: 0043068| GO: 0043071			2	GO: 0010941 GO: 0010942						
Pathway analysis	2	TNFα Signaling Pathway Type II interferon signaling (IFNG)					3	MAPK Signaling Pathway Oncostatin M Signaling Pathway *TGFβ Signaling Pathway	1	*Integrin-mediated Cell Adhesion		**Integrin-mediated Cell Adhesion

**: downregulated*
***: upregulated*

It has been reported that heat treatment at 42°C slightly increases apoptosis in HL-60 human leukemia cells [7] at 42°C. In HeLa cells, apoptosis was increased by 1 hour of heat treatment, and was enhanced by inhibition of HSP72 and HSP27 [8]. In H9c2 cells, it has been suggested that heat stimulation causes temperature-dependent (40-44°C) and time-dependent cytotoxicity and apoptosis [9]. It has also been reported that heat stimulation at 42°C did not cause apoptosis in 16HBE140 cells [10].

As shown in Table 4, increased apoptosis-related gene expression in SMDC at this condition was consistently observed by individual mRNA expression, GO analysis and pathway analysis. On the other hand, decreased apoptosis, increased and decreased cell growth inhibition as well as cell detachment were inconsistently observed by each analysis. Thus, the most important direct factor causing cell density reduction was considered to be increased apoptosis related gene expression.

The individual mRNA causing increased apoptosis were THAP2, PDCD6, BCL2L13, LOC728613, CASP4, and FAS. The GO term causing increased apoptosis were “regulation of apoptotic process”, “regulation of programmed cell death”, “granzyme-mediated apoptotic signaling pathway” and “positive regulation of programmed cell death”. Pathway causing increased apoptosis were TNFα signaling pathway and type II interferon signaling. The upregulated apoptosis genes were variable according to the method of analysis.

GO is a conceptual arrangement of features such as gene functions for the purpose of cross-species information utilization [11]. The GO resource is the world's most comprehensive source of information about the function of genes and gene products (proteins and non-coding RNAs). This information is not only human-readable but also machine-readable and therefore plays a critical role in the computational analysis of genomic and biomedical data [12]. The term defined by GO is called the GO term. GO analysis roughly captures how genes with significant expression fluctuations behave as a group of genes rather than a single function [13,14]. In this study, analysis was performed using a probe list in which gene expression was significantly increased or decreased. Therefore, the results of this study suggest that the genes with significant expression fluctuations acted as a group of genes to promote apoptosis. It is considered that it tended to promote apoptosis as a biological system rather than individual gene expression. Additionally, the analysis target in GO is limited to the case where GO term is given. The GO term is not given to all genes, and one gene may have multiple GO terms. Many of the gene lists included in microarray probes have not been clarified in function. It is also important to confirm individual gene expression in order to investigate whether genes whose expression has increased or decreased are associated with apoptosis.

Genes perform their functions by interacting with various molecules to form networks. Pathway analysis was an analysis of the relationships between a series of biomolecules that work together for a function. Relationship analysis has not been performed in individual gene expression analysis or GO analysis. Pathway analysis includes relationships between genes [15]. Pathway analysis in this study suggested that multiple genes worked in collaboration with various factors in the regulation of apoptosis and cell proliferation. In vivo study, increased activity of the apoptotic pathway in skeletal muscle is also associated with sepsis, disuse syndrome, cancer-induced skeletal muscle atrophy, and age-related sarcopenia [16]. Also, studies on changes in gene expression after burns [17] have been conducted, and changes in the expression of apoptosis-related genes have also been confirmed. In addition, in previous studies [17] that confirmed gene expression after severe burns, GO such as leukocyte aggregation, T cell aggregation, and lymphocyte activation was included, but this could not be confirmed in this study. Severe burns include processes related to inflammation / immune system and cell activity, which are thought to be related to post-burn pathological processes [17]. A previous study [18] that examined gene expression in elderly burn patients reported that the expression of immune system signaling pathways was suppressed during the acute post-burn phase in pathway analysis. In this study, it is possible that no damage such as burns occurred to the cells.

There are indirect factors regulating cell density such as heat shock proteins (HSPs). Expression of HSPs is increased in SMDC by the same condition of heat stimulation as shown in our previous report [2]. Increased expression of HSP72 by thermal stimulation has the effect of suppressing muscle atrophy and promoting protein synthesis and muscle hypertrophy [19]. HSP70 suppresses the apoptotic pathway by its

chaperone function [20]. It has also been reported that induction of HSP70 suppresses apoptosis [9]. In this study, increased expression of HSP70 by thermal stimulation that may act to suppress apoptosis. However, the relationship between the induction of HSP70 expression and the acquisition of resistance of cells to heat has not been clarified [4]. It is necessary to verify whether the effective induction of HSP70 may protect cells from damage and enhance skeletal muscle function. HSPs are also associated with unfolded protein response (UPR) [21]. However, the expression of UPR stress markers activating transcription factor 6 (ATF6) and HSPA5 was reduced in this study. It is considered that the thermal stimulation improved the chaperone function such as HSPs and activated the enzymes involved in folding, and it is possible that the endoplasmic reticulum stress caused by the thermal stimulation was alleviated. From these facts, the possibility of apoptosis due to endoplasmic reticulum stress is low. However, it is considered that the expression of apoptosis-related factors was induced by the stress caused by thermal stimulation.

In this study, we used the microarray method. With microarrays, it is possible to comprehensively confirm gene expression. Therefore, the expression of various genes related to SMDC cell proliferation was observed, and the factors that influenced it were inferred. However, only the gene expression status can be confirmed by microarray, not the protein level. Since complex metabolism is involved in humans, thorough verification is required.

Many genes negatively and positively regulate cell density by heat stimulation. Taken together with our present and previous study, promotion of direct apoptosis-related genes might play a predominant role in decreased cell density.

The practical application of the hot compresses on muscle to prevent atherosclerosis particularly in patients with sarcopenia requires further validation of safe temperature and duration.

5. CONCLUSION

Although we have previously reported that heating SMDC at 42°C for 20 hours caused effective changes of mRNA expression for prevention of atherosclerosis, the present study suggested that slight reduction of cell number in heating SMDC at this condition observed in our previous study might involve several genes, especially genes promoting apoptosis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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