Biological effects of the Alpha B crystallin core domain on dermal fibroblasts and keratinocytes

Jung Min Lee

The Graduate School Yonsei University

Graduate Program in Science for Aging

Molecular Cell Biology

Biological effects of the Alpha B crystallin core domain on dermal fibroblasts and keratinocytes

Directed by Professor Sang-Hak Lee

Doctoral Dissertation
Submitted to the Department of Graduate Program in Science for Aging at the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Science for Aging

Jung Min Lee

December 2016

This certifies that the Doctoral Dissertation of
Jung Min Lee is approved.

Thesis Supervisor: Sang Hak Lee

Thesis Committee Member: Jae Woo Song

Thesis Committee Member: Ji Hyung Chung

Thesis Committee Member: Kyung Hye Lee

Thesis Committee Member: Se Hoon Kim

The Graduate School
Yonsei University
June 2016

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Biological effects of the Alpha B crystallin core domain on dermal fibroblasts and keratinocytes

Jung Min Lee

Graduate Program in Science for Aging

The Graduate School, Yonsei University

(Directed by Professor Sang-Hak Lee)

Alpha B crystallin, which is as smaller type of heat shock protein, is generated by the heart on an ongoing basis. Alpha B crystallin consists of the crystallin of the eyes and is one of the proteins that protect the eyes from UVA rays. Alpha B crystallin is also a chemical that is released in response to stress, such as extreme

temperatures or anoxia. In addition, Alpha B crystallin helps to prevent protein aggregation or protein folding by making proteins, which aggregate or fold in the wrong form in a cell-soluble state.

For this reason, Alpha B crystallin is currently being studied in relation to various medical conditions, such as dementia, stroke, and breast cancer. However, no study has been conducted on its biological effects on human dermal fibroblasts and human epidermal keratinocytes.

In this study, a biochemical analysis was conducted with the core domain of Alpha B crystallin to examine its effects on the collagenesis of human dermal fibroblasts, MMP expression, and cell-cell interactions among human epidermal keratinocytes, as well as to identify the mechanism. Among Alpha B Crystallin proteins, Alpha B crystallin core domain plays an important role in protecting fibroblasts from an increase in MMPs and a decrease in Collagen due to active oxygen. We believe the mechanism for this is attributed to the reduction of MMP expression by disrupting the MAP kinase to inhibit the activation of the transcription factor. It is believed that the core domain of Alpha B crystallin facilitates the cell-cell interactions of human epidermal keratinocytes, inhibiting paracellular efflux by increasing junction protein, and thereby helping to protect skin tissue and maintain skin moisture.

These findings were still effective without the C-terminal extended domain, which is required for the N-terminal domain and assembly of alpha B crystallin proteins. This means that protein assembly is not needed and therefore the size of the protein could be smaller. For this reason, it appears that it could be used for cosmetic and medical applications in the future.

Key Words: Alpha B crystallin core domain, human dermal fibroblasts, human epidermal keratinocytes, cell-cell interaction

Biological Effects of the Core Domain of Alpha B crystallin on Dermal Fibroblasts and Keratinocytes

Jung Min Lee

Graduate Program in Science for Aging

The Graduate School, Yonsei University

(Directed by Professor Sang-Hak Lee)

I. INTRODUCTION

Skin aging goes hand-in-hand with intrinsic aging over time and is also caused by photo-aging due to ultraviolet (UV) rays[1,2,3]. Intrinsic aging is characterized by a decreased number of human dermal fibroblasts (HDFs) and a reduced amount of collagen and elastin as well as extracellular matrix [4,5].

Continuous exposure of the skin to UV rays leads to photo aging[6]. UV rays increase active oxygen[7], which reduces and transforms collagenesis in the dermal layers[8], increases the expression of various protein breakdown enzymes[9,10], and causes elastin denaturalization[11]. All of these factors contribute to a reduction in skin moisture and elasticity, thus creating wrinkles[12,13]. To prevent the reduction and transformation of the number of HDFs, collagen, and elastin due to intrinsic aging and photo-aging, studies need to be conducted on how to strengthen one's epidermal skin barrier and reproduce dermal skin.

Alpha B crystallin (aBC), which is also known as a smaller type of heat shock protein[14,15], is generated by the heart on an ongoing basis[16]. aBC is an important protein consisting of the crystallin of the eyes[17] and is one of the proteins expressed to protect the eyes from UVA rays[18]. aBC is also a chemical that responds to stress, such as extreme temperatures or anoxia[19,20]. In addition, aBC helps to prevent protein aggregation or protein folding by making proteins, which aggregate or fold in the wrong form in a cell a soluble state. For this reason, aBC is currently being investigated in relation to various medical conditions, such as dementia[21], stroke[22], and breast cancer[23]. However, no study has been conducted on its biological effects on HDFs and human epidermal keratinocyte (HEKs).

aBC is composed of three domains – N-terminal domain (NTD), alpha B core domain (ACD), and C-terminal domain (CTD)[24]. While the biochemical functions

of each domain in the structural asset of protein are known, nothing is known regarding the functions of these proteins in skin cells and which domain plays an important role among each domain. For this reason, this study was conducted to compare and analyze NTD and ACD and observe the effect of a certain domain on the collagenesis of HDFs, the expression of the matrix metalloproteinases (MMPs), and cell-cell interactions among HEKs, as well as to identify the mechanisms.

We believe that the results of this study can provide basic data on how to control skin aging, reduce wrinkles, protect the skin cell barrier, treat related skin disease, and develop materials.

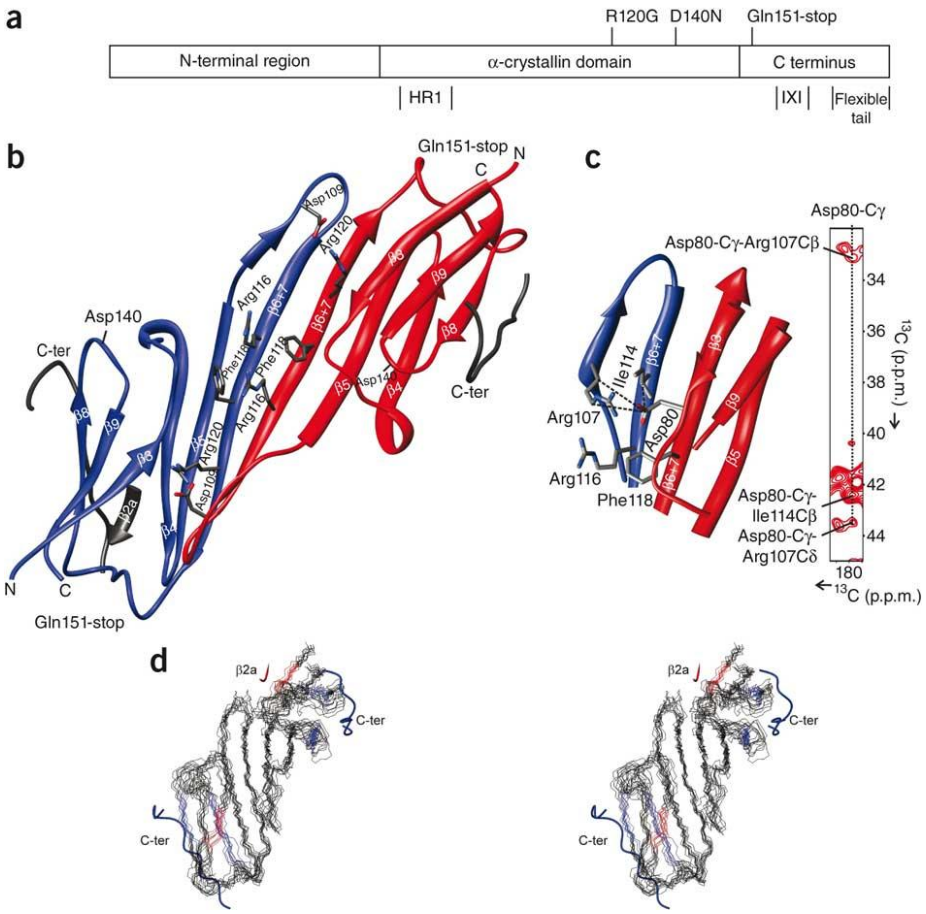


Figure 1. Structure of the aBC dimer and its intermolecular interactions[25].

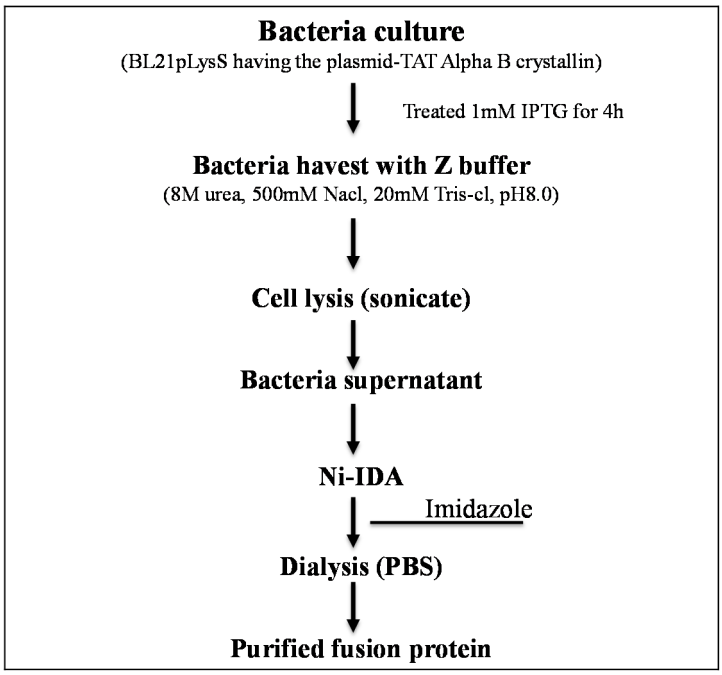
II. MATERIALS AND METHODS

1. Materials

For this study, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), high-glucose–containing Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Gibco-Invitrogen (Carlsbad, CA, USA). The antibodies for pro-COL1A1, MMP-1, and GAPDH and the antibodies for phospho-ERK1/2, ERK1/2, phosphor-p38, and p38 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All specific inhibitors of the protein kinases were purchased from Calbiochem (San Diego, CA, USA). Horseradish peroxidase–conjugated secondary antibodies and the enhanced chemiluminescence (ECL) Western blotting detection system were also obtained from Santa Cruz Biotechnology.

2. Expression and purification of aBC fusion proteins

Escherichia coli BL21(DE3) pLysS (Novagen, Madison, WI, USA) was transformed with the pHis/TAT-aBC plasmid (TAT-NTD and TAT-ACD) and then grown for 24 h at 37°C in LB broth supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol while being shaken at 200 rpm. Protein expression was induced by the addition of 1 mM β-D-1-thiogalactopyranoside (IPTG) for 4 h while shaking at 37°C. The pHis/TAT-aBC fusion proteins (TAT-NTD and TAT-ACD) were then isolated using a urea-denaturing protein purification protocol. The bacterial pallet was isolated by centrifugation at 3,000 rpm, resuspended in buffer Z (8 M urea, 100 nM NaCl, and 20 mM HEPES; pH 8.0), and sonicated 6 times with a 15-sec pulse, while 1 mM phenylmethanesulphonylfluoride (PMSF) was continuously added. The sample was then clarified by centrifugation at 14,000 rpm at 4°C for 1 h. The clarified lysate was loaded to a Ni-IDA column at 1 ml/min, and then the column was washed using buffer A (50 mM NaH_2PO_4, 300 mM NaCl) for 1 h. For the eluted fusion protein, the column was loaded by using buffer B (elution buffer) containing increasing concentrations of imidazole (10 to 500 mM) at 1 ml/min. The protein concentrations in each fraction were quantified by the Bradford assay (BioRad), using bovine serum albumin (BSA) as the standard. The purity of the fusion proteins was assessed by SDS-PAGE and Coomassie Brilliant blue staining.



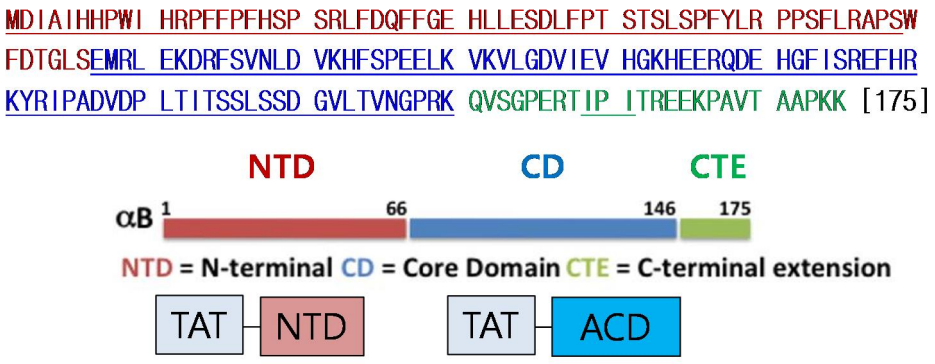


Figure 2. Purification step of TAT-aBC proteins (TAT-NTD and TAT- ACD).

3. Cell culture

Human dermal fibroblasts (HDFs) (Cascade Biologics, city, Portland, OR, USA) were cultured in DMEM supplemented with antibiotics (100 U/ml of penicillin and 100 U/ml of streptomycin) and 10% FBS at 37°C in a humidified atmosphere containing 5% CO2. Subcultured HDFs from passages 6 to 14 were used in these experiments. Human epidermal keratinocytes (HEKs) purchased from Lonza (Walkersville, MD, USA) were grown in keratinocyte culture media (Lonza). HEKs cells were grown in an atmosphere containing 95% air and 5% CO2 at 37°C. Unless otherwise indicated, confluent fifth-passage keratinocytes were incubated in complete medium for 24 h before treatment with protein. Where multiple treatments are indicated, all subsequent treatments took place in the continued presence of the supplement.

4. MTT assay

Cells were washed twice with PBS and incubated with serum-free DMEM containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 1 to 2 h. After the reaction was completed, the medium mix was discarded and 200 μl DMSO was added to each cell-culture well. Formazan was eluted and 150 μl was transferred

to 96-well plates. Samples were read at the 570 nm wavelength using an ELISA reader.

5. Immunoblot analysis

At the termination of the culture, the lysates were scraped into microtubes and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was washed twice with PBS, and then the harvested cells were solubilized in cell lysis buffer containing 1 M HEPES (pH 7.5), 5 M NaCl, 0.5 M EDTA, Triton-X 100, and a protease inhibitor cocktail (Roche, Basel, Switzerland). The protein concentration was measured using BSA. The same amounts of proteins from whole-cell lysates were loaded to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore Co, Bedford, MA, USA). After blocking the membrane with Tris-buffered saline with Tween® 20 (TBST, 0.1% Tween® 20) containing 10% non-fat skim milk for 1 h at room temperature, the membranes were washed 4 times for 7 min each with TBST and incubated overnight with primary antibodies at 4°C. The membranes were again washed 4 times for 7 min each with TBST and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. After washing the membranes again 4 times for 7 min each, the size of the proteins was measured using ECL kits.

6. Cell-cell interaction assay

HEKs were cultured in 24-well plates as feeder cells. After 24 h, they were incubated with peptides in serum-free medium for 1 h. Other HEKs cultured in 175 T flasks as donor cells were trypsinized and collected and then labeled using Vybrant DiO cell-labeling solutions from Molecular Probes (Eugene, OR, USA) according to the manufacturer's protocol. After 1 h, feeder cells were washed with PBS, and the donor cells were treated on this plate and incubated for 10 min at 37°C. Next, the cells were detached from the culture plate using a nonenzymatic cell dissociation solution (Corning, NY, USA) and then transferred to a black 96-well plate for measurement of the fluorescence wavelength. The fluorescence intensity was measured in a fluorescence microplate reader at Ex 482 nm and Em 501 nm.

7. Cell permeability assay

HEKs grown on 0.6-cm^2 transwell filters were stimulated with proteins for 24 h, and then a cell permeability assay was performed using 4-kDa FITC-dextran (Sigma-Aldrich). In HEK layers stimulated with peptides, the medium in the apical and basal compartments was replaced with P buffer containing 10 mg/ml of FITC–dextran and 500 ul of P buffer, respectively. After 5 min, the medium from

the basal compartment was collected, and fluorescence was measured using a microplate reader (Bio-Rad, CA, USA).

8. Enzyme-linked immunosorbent assay (ELISA)

The culture medium was collected after stimulation and used to assess collagen synthesis. The collagen content was determined by an enzyme-linked immunosorbent assay (ELISA) using anti-type I collagen antibody (Takara Bio, Otsu, Shiga, Japan) according to the manufacturer's protocol.

9. Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using Ribospin^{TM} (GeneAll Biotechnology, Seoul, Korea). Total RNA from the cells was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to produce cDNA. RT-generated cDNA encoding each gene was amplified by PCR using the primers specified in Table 1.

Table 1. Primers Used for PCR

Gene	PCR primer sequence	Product size (bp)
COL1A1	S 5' - CTG GCA AAG GCG GCA AA - 3'	502
	A 5' - CTC ACC ACG ATC ACC ACT CT - 3'	
MMP-2	S 5' - TTT CCA TTC CGC TTC CAG GGC AC - 3'	253
	A 5' - TCG CAC ACC ACA TTT CCG TCA CT - 3'	
MMP-9	S 5' - CCT GCC AGT TTC CAT TCA TC - 3'	455
	A 5' - GCC ATT CAC GTC GTC CTT AT - 3'	
GAPDH	S 5' - GCC AAA GGG TCA TCA TCT - 3'	392
	A 5'- GTA GAG GCA GGG ATG ATG TT - 3'	

*COL1A1, type I collagen alpha 1; MMP-2, matrix metalloproteinase 2; MMP-9, matrix metalloproteinase 9; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; S, sense; A, antisense.

10. Statistical analysis

All statistical parameters were calculated using Graphpad Prism 5.0 software. Values were expressed as the mean ± standard error of the mean (SEM). The results were analyzed by a one-way analysis of variance. Statistical analyses were performed using Dunnett's multiple comparison test, and p-values of <0.05 were considered statistically significant.

III. RESULTS

1. Effect on the cell viability of HDFs

To determine the HDFs cell survival rate per concentration level when treated with TAT-fusion proteins (TAT-aBC, TAT-NTD, and TAT-ACD), an MTT assay was performed. The purpose of this assay was to identify the survival rate of the cells treated with wild-type aBC and its two deletion mutants (TAT-NTD and TAT-ACD) at several different concentrations. However, these proteins produced no effect on the cell death of HDFs.

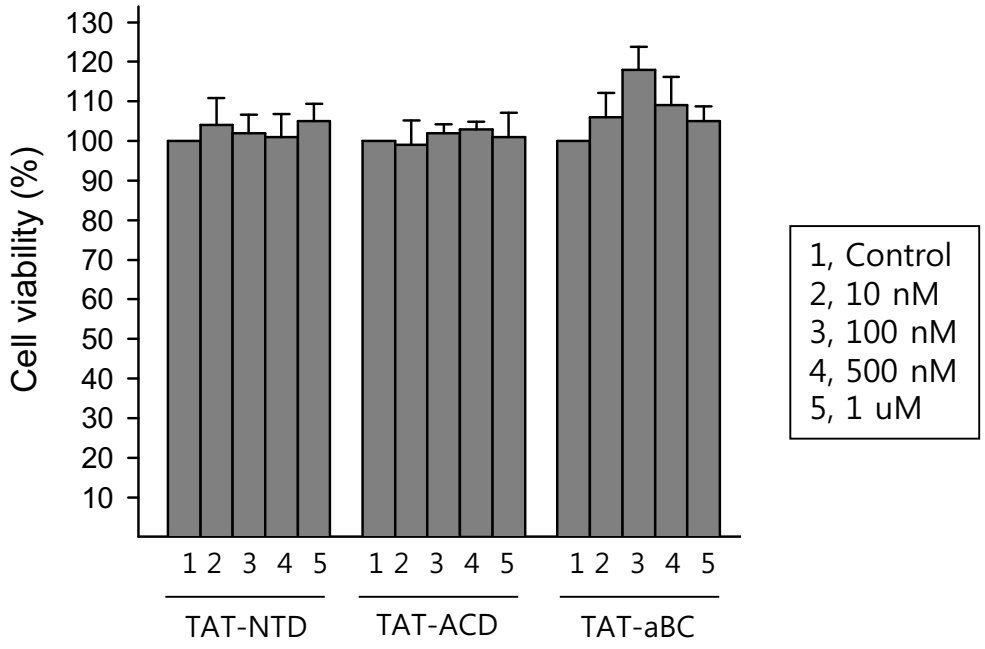


Figure 3. Effect of TAT-fusion proteins on the cell viability of HDFs.

Cell viability was measured using a MTT assay on cells grown in 96-well plate to 80% confluence, cells were treated with each protein for 24 h, and cell viability was expressed as the relative absorbance compared with the control. Error bars represent the SEM of the amount of cell viability. Mean ± SEM from 3 independent experiments.

2. TAT-ACD increases collagen expression in HDFs

The purpose of these experiments was to determine which part of aBC protein was related to HDFs. As shown in Figure 4 below, aBC protein is composed of three domains—NTD, ACD, and CTD. CTD, which is composed of short sequences in each domain, has no specific role except protein assembly. For this reason, two domains of the TAT-NTD and TAT-ACD were compared with a wild-type protein. After assessing the effects of HDFs on collagenesis, TAT-NTD turned out to be ineffective, whereas TAT-ACD induced the highest expression. Therefore, further experiments were conducted with TAT-ACD.

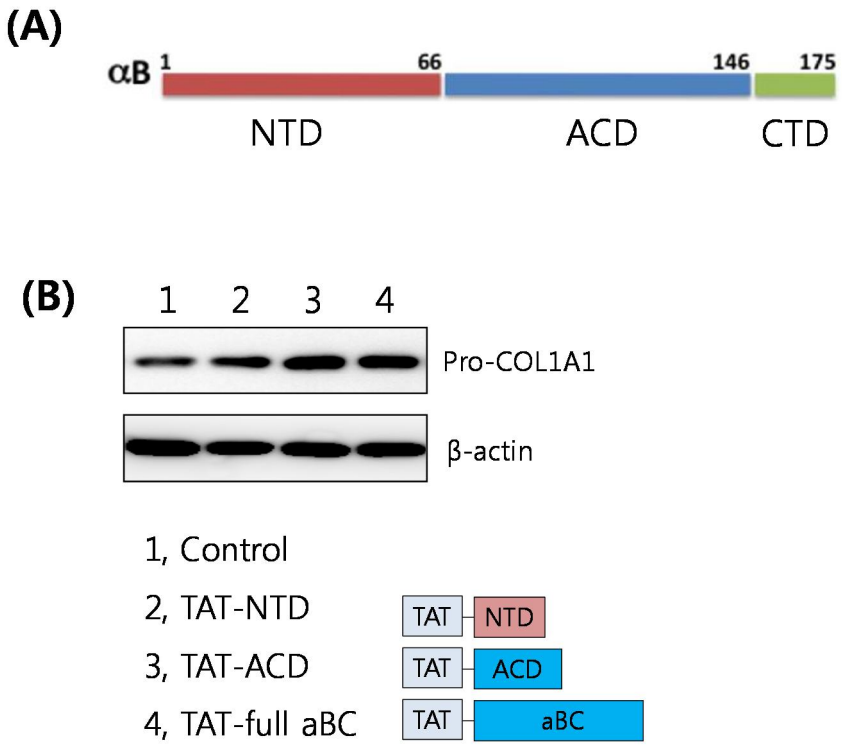


Figure 4. Effect of TAT-aBC and it mutant proteins on the collagen expression in HDFs.

(A) Domain of Alpha B crystallin protein.

NTD, N-terminal domain; ACD, alpha B core domain; CTD, C-terminal domain.

(B) HDFs were treated with 500 nM of each protein for 24 h. The levels of collagen in cell lysates were determined by immunoblot analysis.

3. Expression of MMPs by hydrogen peroxide in HDFs

The hydrogen peroxide(H_2O_2), which is a representative stimulation to skin cells, was processed to confirm the effects of TAT-ACD on HDFs. First of all, the H_2O_2 was processed for 24 h by concentration to determine in which concentration the H_2O_2 facilitated the expression of MMPs, the protein breakdown enzymes of HDFs. Then, its expression level was checked using RT-PCR. It turned out that the expression of MMP-2 and MMP-9 increased in the concentration of H_2O_2 500 uM, as shown in Figure 5 below.

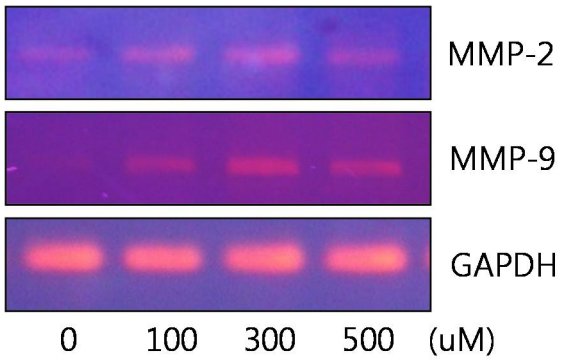


Figure 5. Expression of MMPs by hydrogen peroxide in HDFs.

HDFs were treated with various concentration of H_2O_2, and MMP-2 and 9 expression levels were determined with RT-PCR analysis.

4. Effect of TAT-ACD on MMP expression in HDFs.

The following experiments were conducted to see what effects TAT-ACD had on MMP expression regarding H_2O_2 stimulation in HDFs. To this end, TAT-ACD was preprocessed for 1 h in HDFs, the H_2O_2 was processed, and immunoblot analysis was conducted 24 h later to observe the impression of MMPs. It turned out that the level of MMP-2 and 9, which was increased by the H_2O_2, significantly decreased due to the TAT-ACD preprocessing.

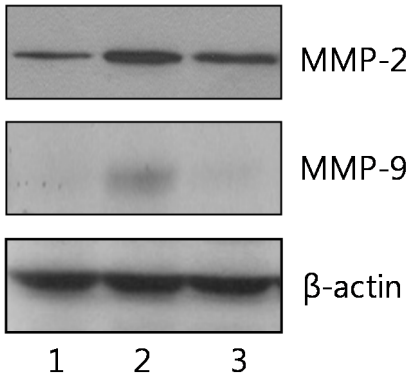


Figure 6. Effects of TAT-ACD on H_2O_2-induced MMPs expression in HDFs.

HDFs were pretreated with 500nM of TAT-ACD for 1 h and incubated with 500 μM H_2O_2 for 24 h. MMP-2 and 9 expression levels were determined with immunoblot analysis. (1) Control; (2) treatment of 500 μM H_2O_2; (3) 500μM H_2O_2 + 500nM of TAT-ACD.

5. Change of MMPs mRNA levels by TAT-ACD

To confirm the results from the previous experiments, TAT-ACD was experimented in the same way for the H_2O_2 stimulation in HDFs in order to determine changes in MMPs mRNA. After checking with RT-PCR, it turned out that the expression of the MMPs, whose levels had increased to the same level as that of protein by the H_2O_2, was significantly reduced. This shows that TAT-ACD was effective in inhibiting the increase of MMPs due to active oxygen.

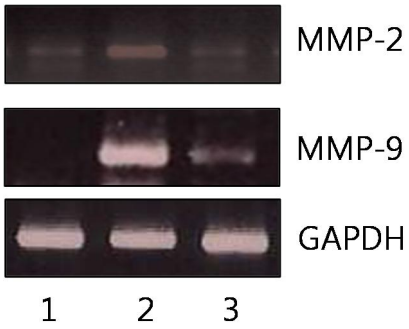


Figure 7. Change in the MMPs' mRNA levels by TAT-ACD in HDFs.

HDFs were pretreated with 500 nM TAT-ACD for 1 h and incubated with 500 μM H_2O_2 for 24 h, and MMP-2 and 9 expression levels were determined with RT-PCR. (1) control; (2) treatment of 500 μM H_2O_2; (3) 500 μM H_2O_2 plus 500 nM of TAT-ACD.

6. Effect of TAT-ACD on the production of collagen

After confirming that TAT-ACD reduced MMPs, the same experiment was conducted again to see whether the protein recovered the collagen level as well. Immunoblot analysis was then conducted to check the collagen level. It turned out that the TAT-ACD significantly recovered the collagen level that had been reduced by H_2O_2 stimulation.

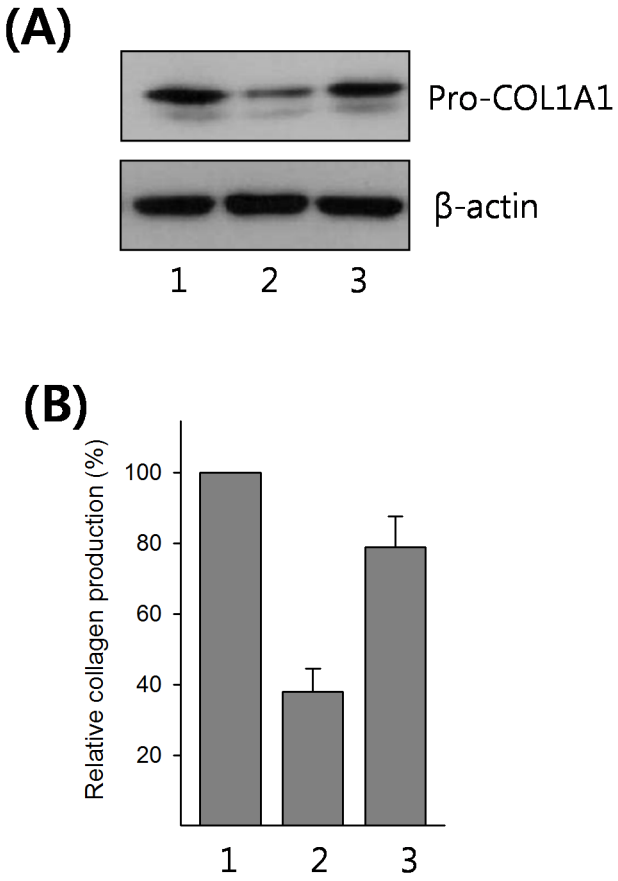


Figure 8. Effect of TAT-ACD on the production of collagen in HDFs.

HDFs were pretreated with 500 nM of TAT-ACD for 1 h and incubated with 500 μM H_2O_2 for 24 h. Collagen levels were determined with immunoblot analysis (A) and ELISA (B). (1) control; (2) 500 μM H_2O_2; (3) 500 μM H_2O_2 plus 500 nM of TAT-ACD.

7. Effect of TAT-ACD on the MAP kinase signaling pathway

The activation mechanism of several MAP kinases was checked to determine which stimulation in a cell such effects on HDFs of TAT-ACD were delivered. The ERK1/2 of HDFs and the phosphorylation of p38 were observed with each specific antibody, and it turned out that the TAT-ACD inhibited the activation of two MAP kinases.

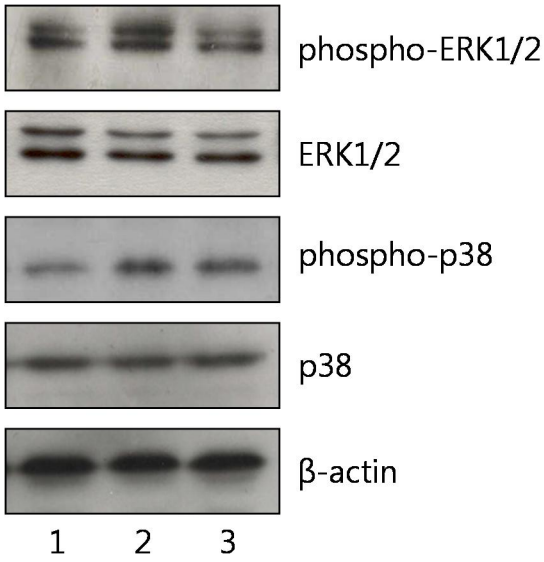


Figure 9. Effect of TAT-ACD on MAP kinase signaling pathway.

HDFs were pretreated with 500 nM of TAT-ACD for 1 h and incubated with 500 μM H_2O_2 for 24 h. Collagen levels were determined with immunoblot analysis using MAP kinase specific antibodies. (1) Control; (2) treatment of 500 μM H_2O_2; (3) 500 μM H_2O_2 + 500 nM of TAT-ACD.

8. Effect of TAT-ACD on cell-cell interactions in HEKs

Three proteins (TAT-aBC, TAT-NTD, and TAT-ACD) were used to conduct a cell-cell interaction assay to determine the effects of TAT-ACD on HDFs and whether it influenced the cell-cell interactions of HEKs. The cell junction of HEKs holds great biological significance in that it serves as a primary defense for the skin barrier. It turned out that TAT-NTD was ineffective and that the wild-type protein and TAT-ACD significantly facilitated the cell junction. In addition, TAT-ACD had a slightly higher cell junction effect compared with the wild-type protein.

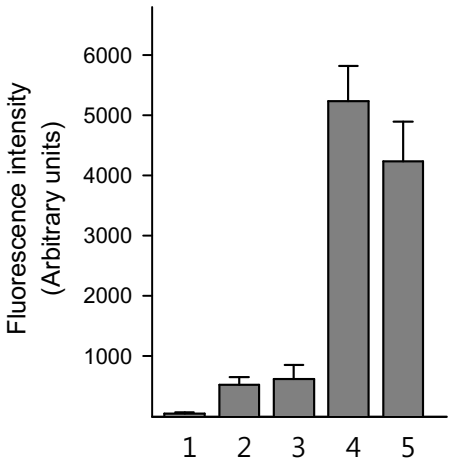


Figure 10. Effect of TAT-ACD on cell-cell interactions in HDFs.

By overnight seeding in 24-well plates with feeder cells, aBC proteins (TAT-NTD, TAT-ACD, TAT-full aBC) (500 nM) were pretreated 1 h, and then donor keratinocytes labeled with fluorescent dye were added on feeder cells for 10 min. After cells were detached, the fluorescent value was detected in a fluorescence microplate reader at Extension 482 nm and Emission 501 nm. (1) blank (only seeding cells); (2) control (seeding cells plus donor cells); (3) seeding cells plus TAT-NTD treated-donor cells; (4) seeding cells plus TAT-ACD treated donor cells; (5) seeding cells plus TAT-full aBC-treated donor cells.

9. Effect of TAT-ACD on cell permeability in HEKs

A cell permeability assay was conducted to see whether the effects of TAT-ACD on the cell junction of the above-mentioned HEKs inhibited paracellular flux. It turned out that TAT-ACD inhibited the paracellular flux of HEKs in a similar pattern to the above cell-cell interaction results.

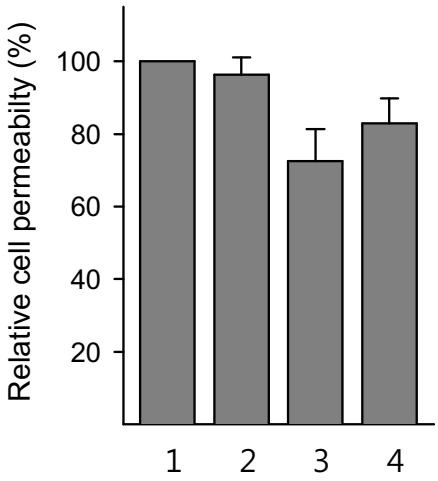


Figure 11. Effect of TAT-ACD on cell permeability in HEKs.

HEKs monolayers were stimulated with aBC proteins (500 nM) for 24 h. After treatment with 4-kDa FITC-dextran, the fluorescence was determined by a fluorimeter. (1) control; (2) TAT-NTD treatment; (3) TAT-ACD treatment; (4) TAT–full aBC treatment.

10. The effect of TAT-ACD on the junction protein expression

Junction protein levels are known to be important to adjust the paracellular flux of HEKs. For this reason, ACD proteins were processed by concentration to check whether they facilitated the expression of junction protein in HEKs. And then, immunoblot analysis was conducted to observe the expression of junction protein. It turned out that ACD proteins facilitated the expression of claudin-1, occludin proteins in a concentration-dependent manner.

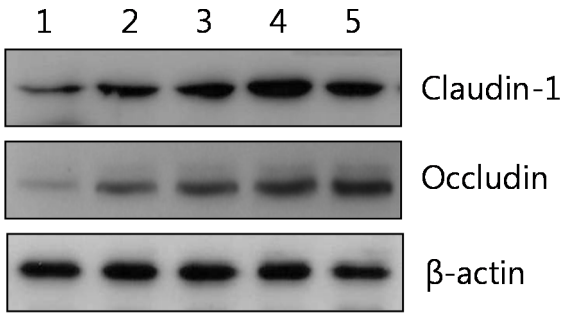


Figure 12. Effect of TAT-ACD on the junction protein expression in HEKs.

HEKs were treated with various concentration of TAT-ACD protein for 24 h. The expression levels of junction proteins were determined by immunoblot analysis. (1) control; (2) TAT-ACD (10 nM); (3) TAT-ACD (100 nM); (4) TAT-ACD (500 nM); (5) TAT-ACD (1 uM).

Ⅳ. DISCUSSION

Skin aging is mostly caused by two processes: Intrinsic aging by active oxygen generated by the body's metabolism without any specific environmental elements and photo-aging, a type of aging on the face, neck, and hands when they are exposed to environmental elements such as UV rays[26]. Physiological aging of the skin begins to appear at the age of about 20 and increases with age. As aging progresses, the surface of the skin shows pigmentation like freckles[27] and loose folds or wrinkles due to decreasing elasticity[28,29]. One of the fundamental causes of such skin aging is the hypoactivity of fibroblasts in the dermal layer of the skin. HDFs are the cells responsible for the synthesis and degradation of fibrous connective tissues, the expression of collagen and elastin[30,31], and the retention of healthy skin.

With advancing years, however, HDFs activity gradually slows down the production of collagen and elastin[32,33,34] and to enhance MMPs, a protease by active oxygen, causing wrinkles[35,36]. MMP-1 breaks down the interstitial collagens (types I, II, and III); MMP-2 and MMP-9, also known as gelatinase A and B, degrade gelatin and type IV and V collagens in the skin's extracellular matrix (ECM)[37,38]. Thus, in order to alleviate skin aging, the fundamental causes need to be addressed, namely collagen increase in HDFs and MMP decrease.

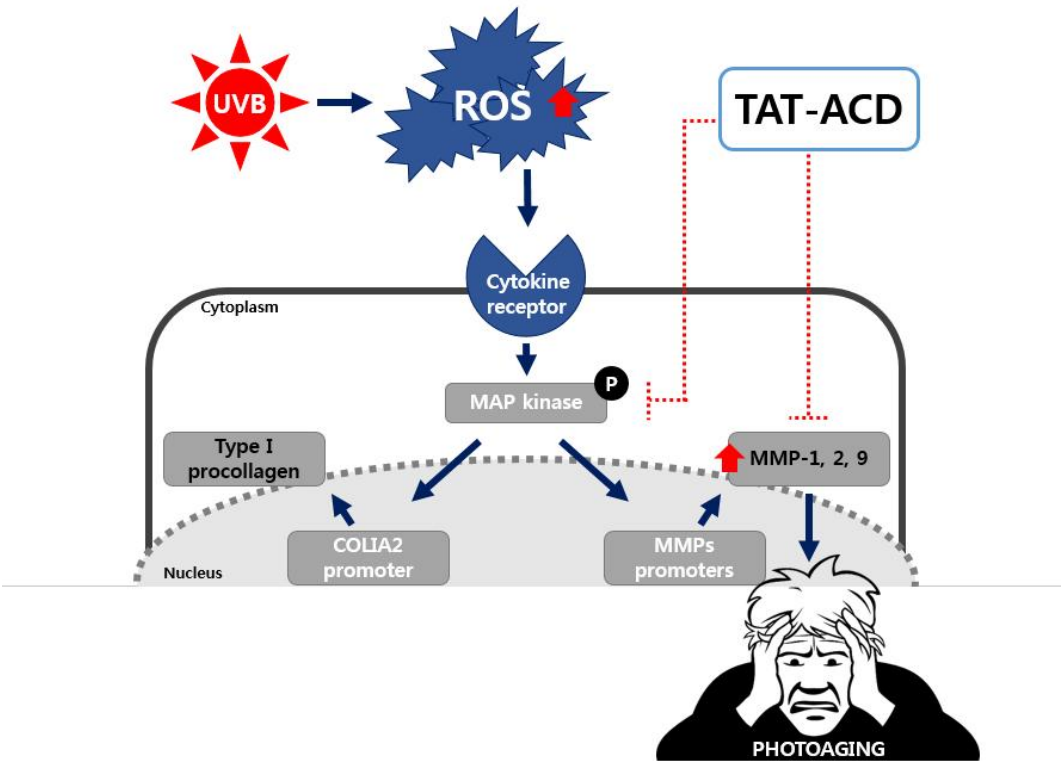


Figure 13. A simplified depiction of the proposed antiphotoaging mechanism of ACD.

Another cause of skin aging is the malfunction of the skin barrier in the epidermal layer of the skin [39,40,41]. The skin barrier prevents body water loss[42,43] and protects the skin from external stimuli[44,45]. The epidermal layer consists of HEKs and keratinocyte intercellular lipids[46,47], the latter playing a key role in maintaining homeostasis of the skin[48,49] because they prevent water loss from the body[50]. When these skin barriers are damaged, percutaneous water loss increases and enhances roughness and dryness of the skin. This leads to rapid skin aging. This is why intercellular binding and expression of junctional proteins are important for paracellular flux, which functions as a skin barrier that protects the skin and prevents skin aging.

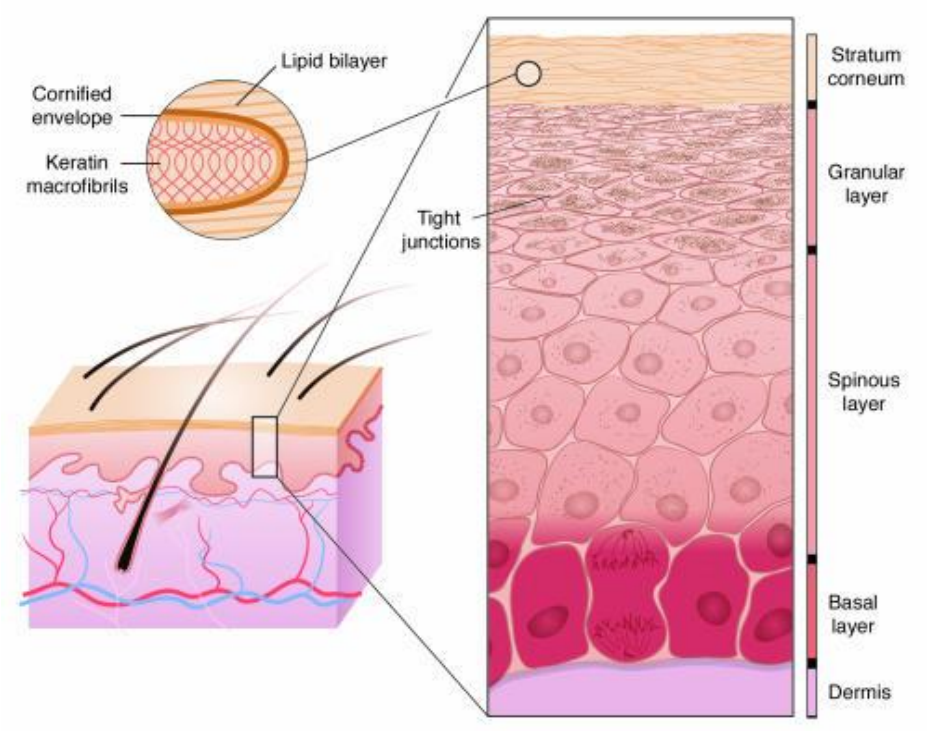


Figure 14. Schematic diagram of the stages of epidermal differentiation resulting in a permeability barrier[51].

This study has investigated the functions of aBC on HDFs and HEKs, which have not previously been the focus of biological research on mutant proteins, such as the NTD and ACD.

The results of this study show that ACD is aBC protein that contributes to collagen synthesis in HDFs. TAT-ACD inhibited an increase in MMPs in HDFs in reaction to active oxygen stimulation through H_2O_2 treatment; it also recovered the collagen level and suppressed MAP kinases (e.g., ERK1/2 and p38) of the HDFs. Those results may indicate that HDFs protective effects of aBC are due to TAT-ACD, which has effect on inhibiting MAP kinases and may lead to the suppression of transcription factors such as AP-1 and a decrease in MMP expression to maintain collagen.

Meanwhile, TAT-ACD significantly promoted cell-cell interactions of HEKs, indicating that the TAT-ACD may inhibit paracellular flux by increasing the concentration of the junction protein in a dose-dependent manner, which contributes to protection of the skin tissue and maintenance of its moisture and sebum. In conclusion, ACD may function without CTD that is required for protein assembly of aBC protein. When protein assembly becomes unnecessaryt and the proteins can be reduced in size, this condition may be favorable for application as a type of material for cosmetics and medical science.

V. REFERENCE

1. Yaar M, Gilchrest BA. Photoageing: mechanism, prevention and therapy. British Journal of Dermatology 2007;157:874-887.

2. Naylor EC, Watson RE, Sherratt MJ. Molecular aspects of skin ageing. Maturitas 2011;69:249-256.

3. Guinot C, Malvy DJM, Ambroisine L, Latreille J, Mauger E, Tenenhaus M, et al. Relative contribution of intrinsic vs extrinsic factors to skin aging as determined by a validated skin age score. Archives of dermatology 2002;138:1454-1460.

4. Varani J, Dame MK, Rittie L, Fligiel SE, Kang S, Fisher GJ, Voorhees JJ. (2006). Decreased collagen production in chronologically aged skin: roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. The American journal of pathology 2006;168:1861-1868.

5. Fisher GJ, Quan T, Purohit T, Shao Y, Cho MK, He T, et al. (2009). Collagen fragmentation promotes oxidative stress and elevates matrix metalloproteinase-1 in fibroblasts in aged human skin. The American journal of pathology 2006;174:101-114.

6. Ma W, Wlaschek M, Tantcheva-Poór I, Schneider LA, Naderi L, Razi-Wolf Z, et al. Chronological ageing and photoageing of the fibroblasts and the dermal connective tissue. Clinical and experimental dermatology 2001;26:592-599.

7. Panich U, Sittithumcharee G, Rathviboon N, Jirawatnotai S. Ultraviolet Radiation-Induced Skin Aging: The Role of DNA Damage and Oxidative Stress in Epidermal Stem Cell Damage Mediated Skin Aging. Stem cells international 2016.

8. Varani J, Spearman D, Perone P, Fligiel SE, Datta SC, Wang ZQ, et al. Inhibition of type I procollagen synthesis by damaged collagen in photoaged skin and by collagenase-degraded collagen in vitro. The American journal of pathology 2001;158:931-942.

9. Quan T, Qin Z, Xia W, Shao Y, Voorhees JJ, Fisher GJ. Matrix-degrading metalloproteinases in photoaging. In Journal of Investigative Dermatology Symposium Proceedings; 2009 Aug. Elsevier; Vol. 14, No. 1, p.20-24.

10. Philips N, Keller T, Hendrix C, Hamilton S, Arena R, Tuason M, Gonzalez S. Regulation of the extracellular matrix remodeling by lutein in dermal fibroblasts, melanoma cells, and ultraviolet radiation exposed fibroblasts. Archives of dermatological research 2007;299:373-379.

11. Kawaguchi Y, Tanaka H, Okada T, Konishi H, Takahashi M, Ito M, Asai J. Effect of reactive oxygen species on the elastin mRNA expression in cultured human dermal fibroblasts. Free Radical Biology and Medicine 1997;23:162-165.

12. Fujimura T, Haketa K, Hotta M, Kitahara T. Loss of skin elasticity precedes to rapid increase of wrinkle levels. Journal of dermatological science 2007;47:233-239.

13. Tsuji N, Moriwaki S, Suzuki Y, Takema Y, Imokawa G. The Role of Elastases Secreted by Fibroblasts in Wrinkle Formation: Implication Through Selective Inhibition of Elastase Activity¶. Photochemistry and Photobiology 2001;74:283-290.

14. Asomugha CO, Gupta R, Srivastava OP. Structural and functional properties of NH2-terminal domain, core domain, and COOH-terminal extension of αA-and αB-crystallins. Molecular Vision 2011.

15. Horwitz J. Alpha-crystallin can function as a molecular chaperone. Proceedings of the National Academy of Sciences 1992;89:10449-10453.

16. Bennardini F, Wrzosek A, Chiesi M. Alpha B-crystallin in cardiac tissue. Association with actin and desmin filaments. Circulation Research 1992;71:288-294.

17. Kannan R, Sreekumar PG, Hinton DR. Novel roles for α-crystallins in retinal function and disease. Progress in retinal and eye research 2012;31:576-604.

18. Christopher KL, Pedler MG, Shieh B, Ammar DA, Petrash JM, Mueller NH. Alpha-crystallin-mediated protection of lens cells against heat and oxidative stress-induced cell death. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 2014;1843:309-315.

19. Iwaki T, Iwaki A, Tateishi J, Sakaki Y, Goldman JE. Alpha B-crystallin and 27-kd heat shock protein are regulated by stress conditions in the central nervous system and accumulate in Rosenthal fibers. The American journal of pathology 1993;143:487.

20. Tamm ER, Russell P, Johnson DH, Piatigorsky J. Human and monkey trabecular meshwork accumulate alpha B-crystallin in response to heat shock and oxidative stress. Investigative ophthalmology & visual science 1996;37:2402-2413.

21. Yoo BC, Kim SH, Cairns N, Fountoulakis M, Lubec G. Deranged expression of molecular chaperones in brains of patients with Alzheimer's disease. Biochemical and biophysical research communications 2001;280:249-258.

22. Aoyama A, Steiger RH, Fröhli E, Schäper R, Deimling A, Wiestler OD, Klemenz R. Expression of αB-crystallin in human brain tumors. International journal of cancer 1993;55:760-764.

23. Kim HS, Lee Y, Lim YA, Kang HJ, Kim LS. αB-Crystallin is a novel oncoprotein associated with poor prognosis in breast cancer. Journal of breast cancer 2011;14:14-19.

24. Slingsby C, Wistow GJ. Functions of crystallins in and out of lens: roles in elongated and post-mitotic cells. Progress in biophysics and molecular biology 2014;115:52-67.

25. Jehle S, Rajagopal P, Bardiaux B, Markovic S, Kühne R, Stout JR, et al. Solid-state NMR and SAXS studies provide a structural basis for the activation of [alpha] B-crystallin oligomers. Nature structural & molecular biology 2010;17:1037-1042.

26. Fisher GJ, Kang S, Varani J, Bata-Csorgo Z, Wan Y, Datta S, Voorhees JJ. Mechanisms of photoaging and chronological skin aging. Archives of dermatology 2002;138:1462-1470.

27. Costin GE, Hearing VJ. Human skin pigmentation: melanocytes modulate skin color in response to stress. The FASEB Journal 2007;21:976-994.

28. Wu Y, Kalra P, Moccozet L, Magnenat-Thalmann N. Simulating wrinkles and skin aging. The visual computer 1999;15:183-198.

29. Kambayashi H, Yamashita M, Odake Y, Takada K, Funasaka Y, Ichihashi M. Epidermal changes caused by chronic low-dose UV irradiation induce wrinkle formation in hairless mouse. Journal of dermatological science 2001;27:19-25.

30. Sephel GC, Davidson JM. Elastin production in human skin fibroblast cultures and its decline with age. Journal of investigative dermatology 1986;86:279-285.

31. Berthod F, Hayek D, Damour O, Collombel C. Collagen synthesis by fibroblasts cultured within a collagen sponge. Biomaterials 1993;14:749-754.

32. Takeda K, Gosiewska A, Peterkofsky B. Similar, but not identical, modulation of expression of extracellular matrix components during in vitro and in vivo aging of human skin fibroblasts. Journal of cellular physiology 1992;153:450-459.

33. Fenske NA, Lober CW. Structural and functional changes of normal aging skin. Journal of the American Academy of Dermatology 1986;15:571-585.

34. Varani J, Fisher GJ, Kang S, Voorhees JJ. Molecular mechanisms of intrinsic skin aging and retinoid-induced repair and reversal. In J Invest Dermatol Symp Proc; 1998 Aug. Vol. 3, No. 57, p. 0

35. Majeed M, Bhat B, Anand S, Sivakumar A, Paliwal P, Geetha KG. Inhibition of UV-induced ROS and collagen damage by Phyllanthus emblica extract in normal human dermal fibroblasts. Journal of cosmetic science 2011;62:49.

36. Zaw KK, Yokoyama Y, Abe M, Ishikawa O. Catalase restores the altered mRNA expression of collagen and matrix metalloproteinases by dermal fibroblasts exposed to reactive oxygen species. European Journal of Dermatology 2006;16:375-379.

37. Fisher GJ, Datta SC, Talwar HS, Wang ZQ, Varani J, Kang S, Voorhees JJ. Molecular basis of sun-induced premature skin ageing and retinoid antagonism. Nature 1996;379:335-339.

38. So SH, Lee SK, Hwang EI, Koo BS, Han GH, Chung JH, et al. Mechanisms of Korean red ginseng and herb extracts (KTNG0345) for anti-wrinkle activity. Journal of Ginseng Research 2008;32:39-47.

39. Roskos KV, Guy RH. Assessment of skin barrier function using transepidermal water loss: effect of age. Pharmaceutical research 1989;6:949-953.

40. Biniek K, Levi K, Dauskardt RH. Solar UV radiation reduces the barrier function of human skin. Proceedings of the National Academy of Sciences 2012;109:17111-17116.

41. Luebberding S, Krueger N, Kerscher M. Age-related changes in skin barrier function–Quantitative evaluation of 150 female subjects. International journal of cosmetic science 2013;35:183-190.

42. Rawlings AV, Harding CR. Moisturization and skin barrier function. Dermatologic therapy 2004;17:43-48.

43. Niessen CM. Tight junctions/adherens junctions: basic structure and function. Journal of Investigative Dermatology 2007;127:2525-2532.

44. Elias PM. The skin barrier as an innate immune element. In Seminars in immunopathology; 2007 Apr. Springer-Verlag; Vol. 29, No. 1, p.3-14.

45. Turksen K, Troy TC. Barriers built on claudins. Journal of cell science 2004;117:2435-2447.

46. Lee SH, Jeong SK, Ahn SK. An update of the defensive barrier function of skin. Yonsei medical journal 2006;47:293-306.

47. Proksch E, Brandner JM, Jensen JM. The skin: an indispensable barrier. Experimental dermatology 2008;17:1063-1072.

48. Feingold KR. Thematic review series: skin lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis. Journal of lipid research 2007;48:2531-2546.

49. Harding CR, Moore AE, Rogers SJ, Meldrum H, Scott AE, McGlone FP. Dandruff: a condition characterized by decreased levels of intercellular lipids in scalp stratum corneum and impaired barrier function. Archives of dermatological research 2002;294:221-230.

50. Imokawa G, Akasaki S, Minematsu Y, Kawai M. Importance of intercellular lipids in water-retention properties of the stratum corneum: induction and recovery study of surfactant dry skin. Archives of dermatological research 1989;281:45-51.

51. Segre JA. Epidermal barrier formation and recovery in skin disorders. The Journal of clinical investigation 2006;116:1150-1158.

ABSTRACT (KOREAN)

알파 비 크리스탈린 코어 도메인이 피부 섬유아세포와 표피 세포에 미치는 생물학적 영향

<지도교수 이상학>

연세대학교 대학원 노화과학협동과정

이정민

알파 비 크리스탈린은 심장에서 끊임없이 만들어지는 소분자 열 충격 단백질로 알려져 있다. 눈의 수정체에 중요한 구성성분으로써, 자외선 UVA 로부터 눈을 보호하기 위해 발현되는 단백질 중 하나이다. 극한 온도나 산소결핍 등의 스트레스에 반응하는 화학물질이기도 하며, 세포 안의 다른 단백질들이 잘못된 형태로 접히거나 응집하려고 할 때 가용성의 상태로 만들어 이를 차단하는 데 도움을 준다. 이러한 결과들로 인해 현재 알파 비 크리스탈린은 치매 및 뇌종양, 유방암 등

다양한 분야에서 연구되고 있으나 피부 섬유아세포와 표피 세포에 대한 생물학적 영향은 연구된 바 없다. 따라서 본 연구에서는 알파 비 크리스탈린 코어 도메인을 이용한 생화학적 분석을 통해 섬유아세포의 콜라겐 합성 및 MMPs 발현에 대한 영향, 그리고 표피 세포간 결합에 대한 효과를 관찰하고 그 메커니즘을 규명하였다.

섬유아세포에서 활성산소를 통한 MMPs 증가와 콜라겐 감소에 대한 알파 비 크리스탈린의 보호 효과는 코어 도메인이 주요 기능을 하며, 그 메커니즘은 MAP 인산화효소를 저해함으로써 전사인자의 활성 억제를 통한 MMPs 발현 감소가 콜라겐 유지에 기인하는 것으로 사료된다. 알파비 크리스탈린 코어 도메인은 표피 세포간 결합을 촉진하며, 연접 단백질을 증가시킴으로써 세포 간극 유출을 억제하여 피부조직 보호 및 유·수분 유지에 기여할 것으로 사료된다. 또한, 상기의 결과는 알파비 크리스탈린 코어 도메인이 N 말단 도메인 및 단백질 구축에 필요한 C 말단 도메인이 없어도 기능을 하는 것으로 나타나, 단백질의 크기를 작게 조절 가능함으로써, 향후 화장품 및 의료 소재로서의 응용에 유리할 것으로 판단된다.

핵심 되는 말: 알파 비 크리스탈린 코어 도메인, 섬유아세포, 표피 세포, 세포간 결합