Protective effect of protein transduction domain-fusion alpha B crystallin on the cellular aging of human dermal fibroblast cells

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감사의 글

연구자에게 신념이란 무엇인가 보여주신 박희남교수님, 학문에 대한 끊임없는 열정을 보여주신 정혜경교수님, 실험에 있어 무지했던 저에게 새로운 길을 열어주시고, 넓은 아량으로 지켜 봐주신 정지형교수님, 사람이 살면서 인생의 전환점이 한 번쯤은 있다고들 하는데 저에게는 정지형교수님을 만나고 지도 받았던 지금이 아닐까 싶습니다. 한없이 부족한 제가 미진하지만 이렇게 논문이라는 결과물을 배출할 수 있도록 지도해주신 교수님들께 진심어린 존경을 표합니다.

스스로 생각하면서 실험하는 법을 가르쳐주신 김수혁 박사님, 실험실 생활도 즐기면서 할 수 있다는 것을 알려주신 진태원 박사님, 실험의 멘토이자 최고의 친구 최은영 박사님, 어려움이 닥쳤을 때 슈퍼맨처럼 도와주시던 전현주 선배님, 실험에 대한 집중과 열정을 보여주신 안수진 선배님, 선배님들과 함께 하면서 실험실 생활에 대해 많이 배웠습니다.

바쁜 일과 중에도 느린 제가 낙오되지 않도록 손 내밀어주신 이승권님, 조직 순응력을 보여주신 이정민님, 긍정과 성실이 무엇인지 보여주신 동기 최순호님, 실험실 세계의 맥아더 전장우님, 학문에 대한 열정과 세대를 뛰어넘는 친화력을 보여주신 문기혁님, 선생님들께서 함께 있어주셔서 이런 날이 온 것 같습니다. 지면으로나마 감사의 마음을 전합니다.

인생의 갈림길에서 흔들리고 있을 때 자신의 신념을 따를 수 있도록 힘을 주신 이향규교수님, 실험과 논문 속에서 헤매고 있을 때 방향을 잡을 수 있도록 도와주신 박승만님, 저에게 스스로가 강인한 사람이며 실험을 끝까지 완수할 수 있을 것이라는 믿음과 아낌없는 지원을 해주신 윤동규님, 감사의 글로는 감사한 마음의 깊이를 모두 전달할 수 없을 만큼 감사합니다.

매일 기도를 해주신 김차순님. 제가 스스로 선택한 길을 흔들림 없이 걸어 갈 수 있도록 버팀목이 되어주신 이승환님과 방해영님, 존재만으로도 위로가 되는 이재민님. 저의 삶에 있어 최고의 조력자이자 후원자 이화정님, 진심으로 감사합니다.

이 외에 지면이 모자라 나열하지 못한 제가 아는 모든 분들 감사합니다.

2013 년 12 월

이성숙 드림

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ABSTRACT

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(Directed by Professor Hui-Nam Pak)

In the skin aging process, wrinkle is generally formed by genetic and environmental elements. Active oxygen is known to occur in photoaging, typical case of externally caused aging, not to mention in internally caused aging. Active oxygen is known to reduce and transform collagen of dermis, and collagen synthesis, and express enzymes

which break down substrate protein. Recently, there have been researches demonstrating that heat shock proteins have various effects on photoaging process. Alpha B crystallin, smaller type of heat shock proteins, is known to regulate lens epithelial cell death of man exposed to UVA, it is not known what effect it has on skin fibroblasts. This study, using protein transduction domain (PTD) which can transport proteins whose molecular weight is big into the cell efficiently in a short time, transported alpha B crystallin protein into human skin fibroblasts, and observed biological performance with hydrogen peroxide treatment. The observation revealed that, in the cell where PTD-alpha B crystallin is transported, expression of MMP-1 known as collagenase, MMP-2 known as gelatinase, and MMP-9 was inhibited, and collagen increased, and MEK-ERK signal channel for enzyme that break up collagen, were blocked. In conclusion, this study showed that alpha B crystallin protein will be used as emollient for skin aging through protein transduction technology.

Key Words: Alpha B crystallin, Protein transduction domain, Collagen, Matrix Metalloprotease-1, Matrix Metalloprotease-2, Matrix Metalloprotease-9.

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I. INTRODUCTION

The reactive oxygen species (ROS) which are the cause of aging is derived from oxygen. It is created by the reaction in which the stable molecular triplet oxygen ($^3O_2$) conducts with body enzyme system, reduced metabolism, chemicals, contaminants and photochemicals due to many physical, chemical and environmental factors.[1,2,3,4] The excess reactive oxygen species fragment DNA, inactivate the protein and attack the unsaturated fatty acids which are the component of biological cell membranes, thus resulting in not only the lowered physiological function or aging but also many

diseases such as rheumatoid arthritis, diabetes, heart diseases, arteriosclerosis, cancer and others.[5]

It is known that as aging process goes on, the human skin is degraded not only by the reduction of secretion of various hormones, inactivation of immune cell and skin cell and the resulting reduced biosynthesis of protein and body component protein as the inner elements of aging but also by the increased wrinkles, loss of elasticity and dryness of skin due to the contaminants and UV light, as well as the creation of spots, freckles and age spots as the external elements of aging.[6,7]

The collagen is the main component of the dermis of the skin and takes up 75% of dry weight of skin and gives the tensile strength to the skin. The biosynthesis of collagen is conducted at the fibroblasts where three pro-alpha polypeptide chains are twisted each other to form the pro-collagen molecules. Pro-collagen is discharged out of the cell and then the collagen metalloproteinase enzyme is disassembled into amino terminal and carboxy terminal to make the collagen molecules in various arrays.[8]

The collagen molecules are the polypeptide chains with the triple helixes twisted each other with 300 nm long and 1.5 nm wide. Each polypeptide is composed of 1,000 amino acids and includes a lot of glycines, hydroxyprolines and hydroxylysines. The collagen of polypeptide is made of the arrays of amino acids which repeat the (Gly-X-Y)n. The location of X and Y is mostly made of proline and hydroxyproline.[9]

Collagen molecule tends to link each other not through end to end but through the crosslink such as side to side connection that the structure helps the collagen to get the tensile strength helped by the amino acidic structure. The cross link between collagen

molecules are made outside of the cell and forms the collagen fiber through fine fiber of collagen (Fig. 1). The collagen fibers forms the meshwork around the papillary dermis, skin appendages and blood vessels while it forms the thick bundles around the reticular dermis, which are arranged parallel to the surface of skin.[10,11]

The synthesis and cross-linking of collagen fibers plays a great role in healing cut wounds and forming the scars. The principal types of collagen in the human skin are Type I and Type III. 80% of the collagen located in the human dermis is Type I while 15% is Type III. Type I, which is thick in its fiber and located in reticular dermis, is mostly found in the collagen fiber while Type III is thin in its fiber and rarely found dispersed around the papillary dermis and skin appendages.[12]

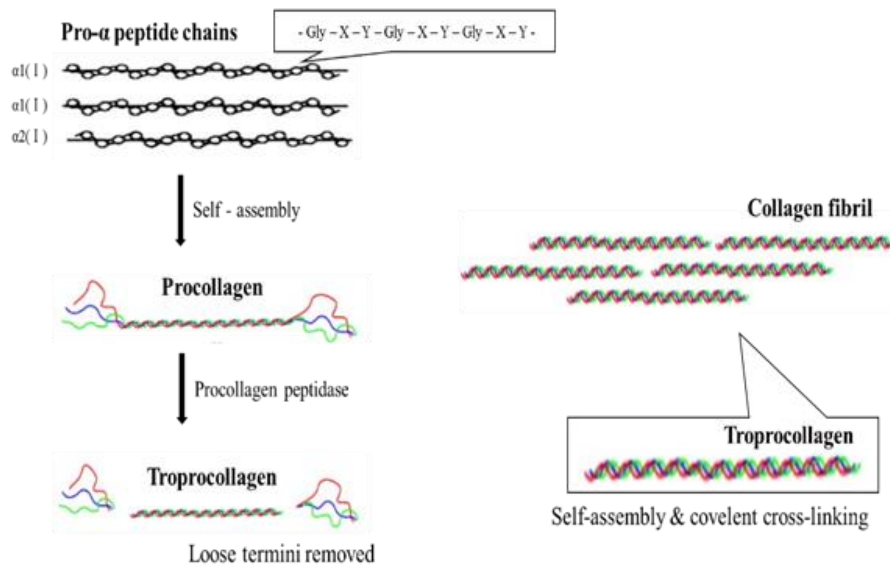


Fig. 1. Collagen synthesis. Large collagen fibril aggregates as a structural unit of collagen molecules (collagen molecule) is called. Collagen molecule is a triple helix of three polypeptide chains shape (triple helix, super helix) as a twisted structure. Amino acid poly peptide is a peptide bond (peptide bond) was created through a chain of line shape[9,10,11].

The matrix metalloproteinases(MMPs) are a large family of zinc-dependent endopeptidases that are collectively capable of proteolyzing all components of the extracellular matrix(ECM)[13,14].

All MMPs with some modifications, the basic structure of a domain can share a core set. The prototype MMP consists of a prodomain, a catalytic domain, a hinge region, and a hemopexin-like domain[13, 15]. As secreted proteins, MMPs are synthesized with a signal peptide responsible for directing secretion out of the cell. The prodomain consists of a conserved cysteine residue that prevents binding and cleavage of the substrate, while the catalytic domain contains a binding site comprised of three histidines that coordinate with a single zinc and is responsible for the enzymatic activity of the protease. MMP-2 and -9 have a series of fibronectin repeats in this domain as well. A flexible hinge region then separates the catalytic domain from a four-bladed propellershaped hemopexin domain. It is felt that substrate specificity as well as endogenous inhibitor binding are influenced by specificities of each MMP's catalytic and hemopexin domains[16,17].

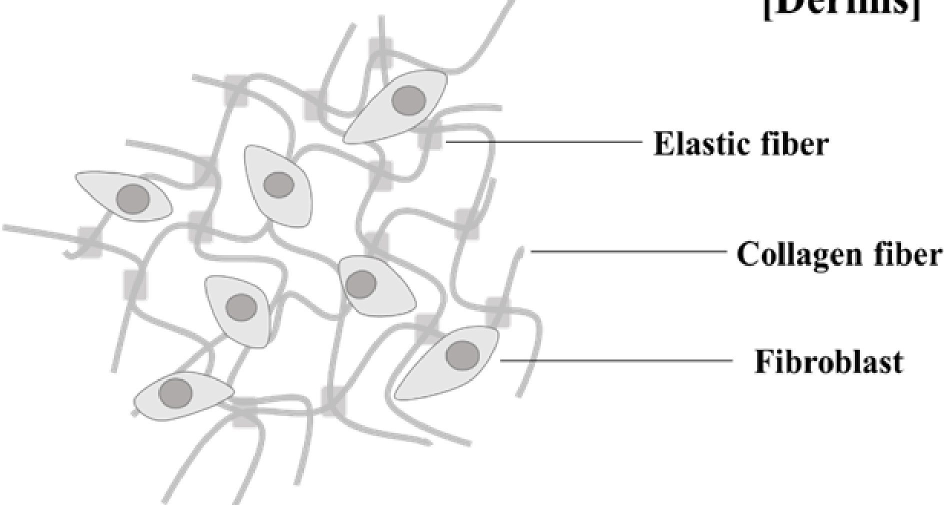
MMPs are traditionally classified according to their structure and/or ECM substrate specificity. The collagenases (MMP-1, MMP-8, and MMP-13) can degrade native collagen and are often hypothesized to be antifibrotic. MMP-1 is the only enzyme being able, after activation, to initiate cleavage of collagen triple helix, which consists of the two major fibril-forming molecules in the skin, type I and III collagens. Stromelysins (MMP-3, MMP-10, and MMP-11) share structural similarity with the collagenases but are unable to degrade native collagen. As their name implies, the

gelatinases (MMP-2, MMP-9) cleave denatured collagen (gelatin) as well as type IV collagen in basement membranes.

The MMPs are synthesized as inactive zymogens maintained by a conserved cysteine residue that interacts with the zinc in the active site and prevents a catalytic water molecule from associating with the metal ion, rendering the protease inactive[15]. Activation requires disruption of this so-called "cysteine switch," which can be accomplished through proteolytic cleavage of the propeptide by trypsin, plasmin, or even other MMPs[18]. In addition, several MMPs contain a furin-sensitive cleavage site for the propeptide, indicating intracellular activation of these enzymes[15].

An interesting story of MMPs with nitrosative and oxidative stress has also emerged. The cysteine switch contains a thiol residue that can be altered by reactive oxygen species (ROS) and reactive nitrogen species (RNS), causing dissociation from the catalytic site, leading to enzyme activation[19, 18].

The oxidative stress is reported to increase the expression of MMP[20,21,22]. Confirm the activated MMP induced by H_2O_2 and the resulting destruction of collagen protein (Fig. 2), this study directly applied the H_2O_2 to the cells.


[Dermis]
Elastic fiber
Collagen fiber
Fibroblast

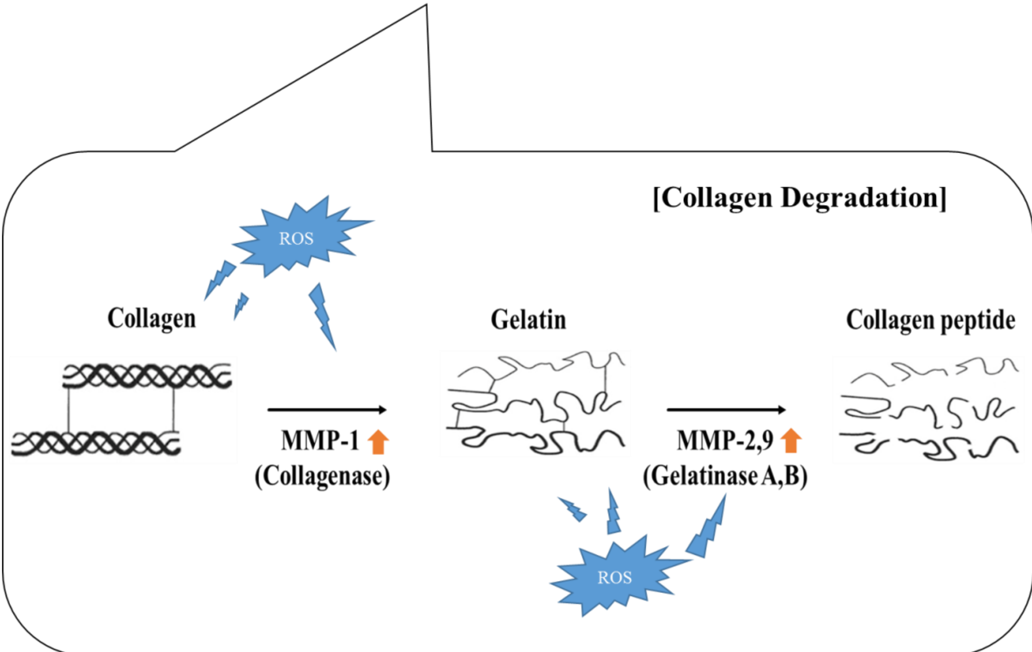

[Collagen Degradation]
ROS
Collagen
Gelatin
Collagen peptide
MMP-1
(Collagenase)
MMP-2,9
(Gelatinase A,B)
ROS

Fig. 2. Components of the dermis and collagen degradation. The dermis is the second layer of skin, beneath the epidermis. The dermis is mainly made up of extracellular matrix (ECM), an extracellular part of human tissue that is largely composed of collagen, elastin, and hyaluronic acid. The dermal ECM is produced mainly by dermal fibroblasts. Collagen fibers form vast and tough networks that provide the dermis with strength, tension, and elasticity. Elastic fibers are also key to keeping the dermis elastic and resilient. Aged dermal fibroblasts produce increased amounts of enzymes called matrix metalloprotienases (MMPs), which degrade collagen and elastin. To make matters worse, the amount of collagen and elastin produced by dermal fibroblasts decreases as the cells age. Thus, the overall loss of collagen and elastin results in skin laxity and fragility, which is visible in the form of wrinkles. The ability of a compound to reduce senescence (cell aging) or stimulate aged fibroblasts to produce increased levels of Collagen and elastin, as well as inhibit the production of MMPs would lead to an effective decrease, if not reversal, of the aging process.

Small heat shock proteins (sHSPs) are widely expressed 20-28 kDa proteins, which are subjected to dynamic regulation in response to cell stresses[23]. sHSPs are characterized by a conserved alpha B crystallin domain of approximately 90 amino acid residues. sHSPs function as molecular chaperones either by preventing the aggregation of unfolded proteins under stressed condition or by facilitating protein refolding in cooperation with other factors, e.g. HSP70 and ATP, after stress removal[22]. In addition, sHSPs associate with cytoskeletal structures and stabilize these elements, which result in increasing stress tolerance[24,25]. The protective effects of sHSPs have been documented in various in vitro conditions after exposure to several noxious stresses, e.g., hyperthermia, oxidative stress, and inflammatory cytokines[26,27,28] and the involvement of sHSPs in various human diseases has also been reported[29]. The sHSPs function as cytoprotective molecular chaperones, preventing stress-induced aggregation of denaturing proteins.

Crystallins constitute the major proteins of vertebrate eye lens and maintain the transparency and refractive index of the lens[30] Mammalian lens crystallins are divided into alpha, beta, and gamma families. Alpha and beta families are further divided into acidic and basic groups. alpha crystallins are composed of two gene products: alpha-A and alpha-B respectively. alpha B crystallin together with alpha A crystallin comprise as much as 40% of the cytoplasmic proteins in lens cells of the eye and are thought to play an essential role. Alpha crystallin can be induced by heat shock similar to other members of the sHSP[31]. Moreover alpha B crystallin shares considerable sequence and structural similarity with HSP27[32]. Thus, alpha B crystallin is considered to be a major

member of sHSP. Both of these proteins have been shown to have anti-apoptotic functions by interfering with the activity of various apoptotic proteins[33]. Indeed, a list of reports indicated that alpha B crystallin is important in the pathology of cancer, as expression of alpha B crystallin has been observed in gliomas[34], renal carcinomas[35], breast carcinomas[36], and ductal carcinoma[37]. The importance of alpha B crystallin in cancer has been vaguely attributed to its anti-apoptotic functions. Also, it has been demonstrated that alpha B crystallin functions under a wide range of stress conditions[38].

Numerous studies have shown that alpha B crystallin participates in the pathological processes of diseases related to the central nervous system. For example, alpha B crystallin is induced in the reactive astrocytes and microglia found in or near senile plaques and in the neurofibrillary tangles of Alzheimer's disease[39]. In addition, high levels of alpha B crystallin and Hsp27 protein were found in tissues, especially in astrocytes, from patients with pathological conditions such as Alexander's disease and Parkinson's disease[40,41]. However, there have been no studies published relating to the wrinkles yet.

In recent years, people are transmitted through the cell membrane and accumulate in the biopolymer to guide the type of protein found in the functional domain, so they are protein carrier (PTD) was called. PTD fusion proteins, such as systems using a powerful new PTD protein drugs are used. They directly affect the specific target of drug development period, reducing the target gene or by using a chemical substance may further have high safety because the protein drug is expected to be effective and powerful drug.

The fusion protein is PTD-cells affected by rapid and occurred in the properties of the target protein. Cells carrying molecules are held in the own activity. They base their operations. PTD is a character, so it is very attractive. PTD cytoplasmic protein or polypeptide polymer because it is possible through the membrane. PTD protein engineering and protein drugs are to accelerate the development. At present, many kinds of protein was performed with different cells or animals, they hold their own activities. PTD is a new treatment and how to find the new features of the protein will be applied. In this study, the protein delivery system was used for PTD. New therapeutic delivery systems in terms of protein (PTD) to the target protein directly to the cells to provide introduced. One of the PTD included human immunodeficiency virus (HIV-1) transactivated TAT protein. By using the TAT protein, a number of target proteins can be delivered into the cells. The interaction of TAT-PTD with cell surface causes the internalization of TAT-fusion proteins.

Protein transduction is the technology to transmit the small peptide having the array of specific amino acids with large size of molecular protein into the cells in a short period of time in an efficient way[42].

There have not been sufficient studies made on the inhibition of skin aging using the PTD and human peptide combined substance.

In this study, using protein delivery system, we studied the potential skin protective effect of PTD-alpha B crystallin as a therapeutic protein in human dermal fibroblasts.

Ⅱ. Materials and Methods

1. Materials

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, CA). phosphate-buffered saline (PBS) was obtained from PAA (PAA Laboratories GmbH, Austria). The antibodies against human Pro-COL1A1, MMP-1 and GAPDH were obtained from Santa cruz Biotechnology (Santa Cruz, CA, USA). Procollagen Type Ⅰ C-Peptide (PIP) EIA Kit was purchased from TAKARA (Takara Bio Inc Japan). Antibodies to human phospho-extracellular signal-regulated kinase 1/2 (pERK1/2), human extracellular signal-regulated kinase 1/2 (ERK1/2) were obtained from Santa cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to MEK, phosphorylated MEK were from Cell Signaling Technology Inc. (Beverly, MA). All inhibitors [U0126, a MEK-specific inhibitor; PD98059, a ERK1/2-specific inhibitor] were purchased from Calbiochem (San Diego, CA). Horseradise peroxidase-conjugated secondary antibodies and enhanced cheminescense (ECL) Western blotting detection system were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2. Expression and purification of alpha B crystallin fusion proteins

Escherichia coli BL21(DE3) pLysS (Novagen, Madison, WI, USA) was transformed with the pHis/TAT-alpha B crystallin plasmid, and then grown for 24 h at 37℃ in LB broth supplied with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and while shaking 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℃. The pHis/TAT-alpha B crystallin fusion protein were then isolated using a urea-denaturing protein purification protocol. The bacterial pallet was isolated by centrifugation at 3000 rpm, resuspended in buffer Z (8 M urea, 100nM NaCl and 20 mM HEPES, pH 8.0), and sonicated 6 times with 15 second pulse continually adding 1 mM phenylmethanesulphonylfluoride (PMSF).

The sample was then clarified by centrifugation at 14,000 rpm 4℃ for 1 h. The clarified lysate was loaded to Ni-IDA column at 1ml/min and then the column was washed using buffer A (washing buffer) (50 mM NaH_2PO_4, 300 mM NaCl) for 1 h at 1 ml/min. The eluted alpha B crystallin fusion protein, the column was loaded by using buffer B (elution buffer) contained increasing concentrations of imidazole (10-500 mM) at 1 ml/min. The protein concentrations in each fraction were quantified by the Bradford assay (BioRad, Hercules, CA, USA), using bovine serum albumin (BSA) as the standard. The purity of the fusion proteins was assessed by SDS-PAGE and Coomassie Brilliant blue staining. The purified fusion proteins dissolved in PBS containing 10% glycerol were aliquoted and stored at -80℃.

3. Cell culture

Human skin fibroblast HDF cells (CEFO, CELL BIO, SEOUL, KOREA) were cultured in DMEM supplemented with antibiotics (100U/ml of penicillin and 100U/ml of streptomycin) and 10% FBS at 37℃ in a humidified atmosphere containing 5% CO2. Sub-cultured HDFs from passage 6 to 14 were used in these experiments.

4. MTT assay

HDF cells were washed with PBS two times and incubated with serum free DMEM containing 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, MO, USA) at 37℃ in humidified atmosphere of 95% air and 5% CO_2 for one to two hours. After the reaction was completed, mix of medium was discarded and 200 µl dimethyl sulfoxide (Amresco, OH, USA) was added to each cell culture wells. Formazan was eluted and 150 µl was transferred to 96-well plate. Samples were read on 570 nm wave length using ELISA reader.

5. Immunoblot analysis

At the termination of culture, the lysate were scraped into microcentrifuge tubes and centrifuged at 14,000 rpm for 15 min at 4℃. The supernatant were washing 2 times with PBS. And then, 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, protease inhibitor cocktail 1ea (Roche). Protein concentration was measured using a 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-Tween 20 (TBS-t 0.1 % tween 20) containing 10% non-fat skim milk for 1 h at room temperature, washed with TBS-t at 4 times for each 7 min and incubated with primary antibodies for overnight at 4℃. The membranes were washed 4 times with TBS-T for each 7 min and HRP-conjugated secondary antibodies for 1 h at room temperature. After washing 4 times for each 7 min, the object size of proteins was detected using ECL Kits. (Millipore Co, Bedford, MA, USA and Santa Cruz Biotechnology).

6. Gelatin Zymography Assay

The cells were transfected as described in the above section. The HDF conditioned medium was harvested 24 h post-treatment and analyzed by gelatin zymography. Twenty microliters of the conditioned medium were electrophoresed on a 10.0% sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 0.1% gelatin at a constant voltage of 120 V for 1 h. the gel was rinsed with 2.5% Triton X-100 and incubated in developing buffer (50.0 mM Tris (pH 7.2), 0.2 mM NaCl and 5.0 mM $CaCl_2$) overnight at 37℃. Depending on the time period for gel development, there

could be subtle differences in the intensity of gelatin lysis bands between each batch experiment. The gel was then stained with 0.25% Coomassie brilliant blue solution, followed by destaining in methanol: acetic acid: 3'DW(40: 10 : 50).

7. 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 Inc Japan).

Transfer 100 µl of antibody-POD conjugate solution into per well, and subsequently add 20 µl sample or standard. Mix and seal the plate and stand for 3 hours at 37℃. Remove contents and wash the wells 4 times with 400 µl PBS. Add 100 µl of Substrate solution into each well and incubate at room temperature for 15min. Add 100 µl of stop solution into each well and tap plate gently to mix. Measure the absorbance at 450 nm with a plate reader.

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

Total cellular RNA was isolated using Ribospin™ (GeneAll Biotechnology, GeneAll, CO, Korea). Total RNA from the cells was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) to produce cDNA. RT-generated cDNA encoding MMP’s and type I procollagen were amplified by PCR using the

following specific primers:

Table 1. Primers used for the 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-1	S 5’ - AAG GCC AGT ATG CAG CTT - 3’	480
	A 5’ - TGC TTG ACC CTC AGA GAC 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[43], type I collagen alpha 1; MMP-1, matrix metalloproteinase 1; MMP-2, matrix metalloproteinase 2; MMP-9, matrix metalloproteinase 9; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; S, sense; A, antisense.

9. Measurement of elastase activity

Fibroblast enzyme solution was prepared as previously described[44]. Elastase activity using the synthetic substrate N-succinyl-tri-alanyl-p-nitroaniline (STANA) (Sigma-Aldrich, MO, USA) was measured as previously described[45]. The release of p-

nitroanline was measured by absorbance at 405 nm and enzymatic activity is expressed as nmol p-nitroanline / h / μg protein. Finally a comparison was performed by expressing activity as relative to elastase activity.

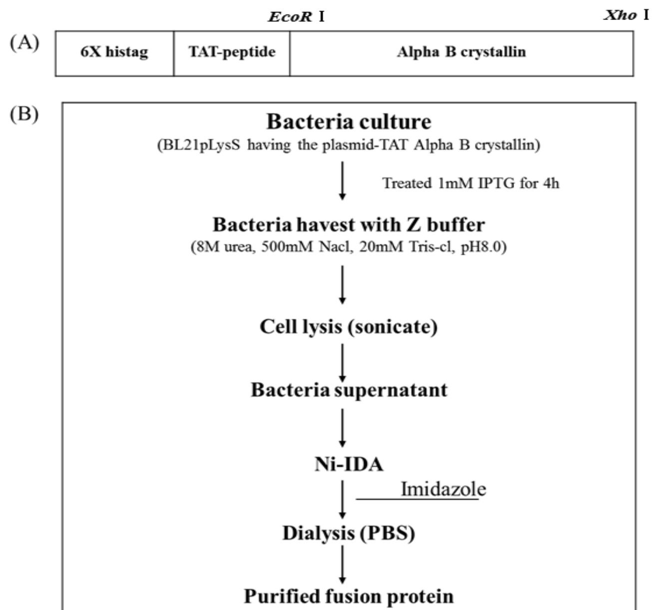
10. Statistical analysis

All statistical parameters were calculated using Graphpad Prism 5.0 software. Values were expressed as the mean ± standard error of the mean (S.E.M.). The results were analyzed by one-way analysis of variance. Statistical analyses were performed using the Dunnett's Multiple Comparison Test and *p*-values of less than 0.05 were considered statistically significant.

Ⅲ. RESULTS

1. Construction and purification of PTD-alpha B crystallin fusion protein

To prepare cell-permeable alpha B crystallin fusion protein, the plasmid pHis-TAT-alpha B crystallin was constructed as described in Materials and Methods (Fig. 3A). Constructed plasmid was transformed to Competent *Escherichia coli* BL21 plysS. The bacteria were cultured in LB medium with shaking at 37℃. 1 mM IPTG was added (O.D600 = 0.6) and incubated for additional 4 h in order to induce the expression of the recombinant proteins. Recombinant pHis/TAT- alpha B crystallin protein was purified by using Ni-IDA affinity column chromatography (Fig. 3B). This protein was analyzed by 12.5% SDS-PAGE and stained with Coomassie brilliant blue (Fig. 3C). And then the protein was dialyzed by using PBS. The purified protein was confirmed by immunoblot analysis with anti-His prove antibody.


(A)
EcoR I
Xho I
6X histag
TAT-peptide
Alpha B crystallin
(B)
Bacteria culture
(BL21pLysS having the plasmid-TAT Alpha B crystallin)
Treated 1mM IPTG for 4h
Bacteria havest with Z buffer
(8M urea, 500mM Nacl, 20mM Tris-cl, pH8.0)
Cell lysis (sonicate)
Bacteria supernatant
Ni-IDA
Imidazole
Dialysis (PBS)
Purified fusion protein

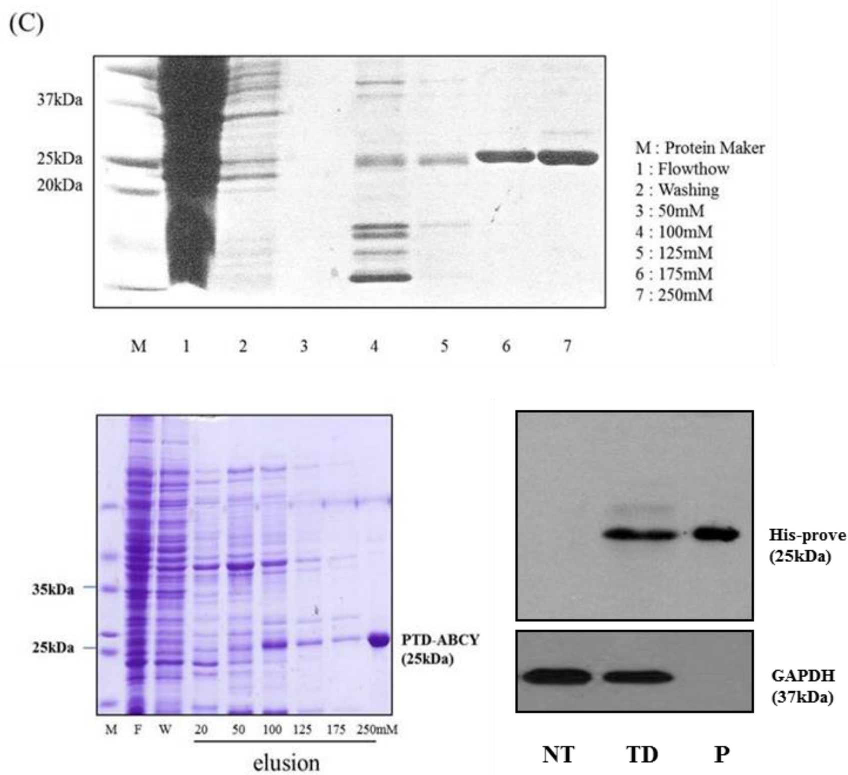

(C)
37kDa
25kDa
20kDa
M : Protein Maker
1 : Flowthow
2 : Washing
3 : 50mM
4 : 100mM
5 : 125mM
6 : 175mM
7 : 250mM
M 1 2 3 4 5 6 7
35kDa
25kDa
PTD-ABCY
(25kDa)
M F W 20 50 100 125 175 250mM
elusion
His-prove
(25kDa)
GAPDH
(37kDa)
NT TD P

Fig. 3. Construction and purification of PTD-alpha B crystallin fusion protein. (A) TAT domain and - alpha B crystallin coding sequence were fused. (B) Diagrammatic representation of protein purification. (C) PTD-alpha B crystallin was purified using Ni-IDA affinity chromatography. Coomassie brilliant blue stain was followed by purification of PTD-alpha B crystallin. HDFs were incubated in DMEM with PTD-alpha B crystallin. Immunoblot analysis was performed using anti-His prove antibody.

2. Effects of PTD-alpha B crystallin on cell viability of human dermal fibroblasts under hydrogen peroxide treatment

In order to know HDF cell survival rate per concentration level when it is treated with hydrogen peroxide, performed MTT Assay. The experiment with the hydrogen peroxide treatment time fixed as 24 h, and its density being 0 – 1000 μM. And, it was identified that, at the 500 μM concentration of hydrogen peroxide, cell survival rate decreases by 60% compared with the cells which are not treated with anything. Based on such results, in subsequent experiments where hydrogen peroxide treatment is used, its concentration was fixed on 500 μM.

When cell is treated with PTD-alpha B crystallin, the experiment was performed with the protein concentration being 0 – 1000 μM range in order to know whether it is toxic or not. In this research, alpha B crystallin was not toxic up to the concentration of 1 μM, the maximum experimental concentration this research set.

In order to identify the survival rate of the cell treated with PTD-alpha B crystallin when it is treated with hydrogen peroxide, performed additional experiment. One hour after Human dermal fibroblasts cells were treated with PTD-alpha B crystallin per concentration, the cells were treated with hydrogen peroxide with its concentration of 500 μM. The experiment proved that at the alpha B crystallin 0.5 μM concentration, cell survival rate is enhanced by up to 120%. In other levels of alpha B crystallin, cell survival rate was higher than the case where the cell is treated with only hydrogen peroxide 500 μM concentration.

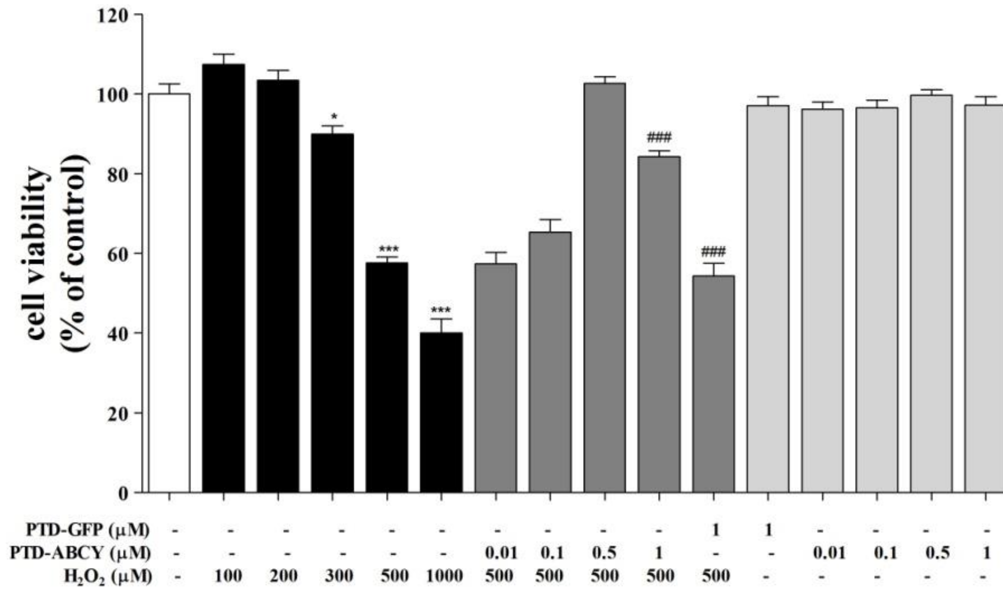


Fig. 4. Effects of PTD-alpha B crystallin on cell viability of human dermal fibroblasts under hydrogen peroxide treatment. The fibroblasts were stimulated with H_2O_2 (0 – 1000 μM), followed by treatment with various concentrations (0.01 μM – 1 μM) of PTD-alpha B crystallin for 24 h. Cell viability was measured by MTT assays. Cell viability is expressed as percentage of control. Values are the mean ± S.E.M. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. the control group; $^{###}p < 0.001$ vs. the H_2O_2 (500 μM) alone group.

3. The mRNA level of MMPs and collagen by hydrogen peroxide in HDF cells

To examine the effect of hydrogen peroxide on the gene expression of Human dermal fibroblasts cells, the result was identified with RT-PCR. MMP-1, MMP-2, MMP-9 and gene expression of collagen were identified per set concentration of hydrogen peroxide.

In both the group where MMP-1 is not treated with hydrogen peroxide and the group where MMP-1 is treated with concentration 100 μM hydrogen peroxide, mRNA was not expressed.

In the group where MMP-2 is not treated with hydrogen peroxide, mRNA was not expressed. And, the higher the stimulating concentration level got, the higher the mRNA expression rate got.

Like the case of MMP-2, in the group where MMP-9 is not treated with hydrogen peroxide, mRNA was not expressed. As the level of concentration of hydrogen peroxide went up from 100 μM to 300 μM, MMP-9 mRNA expression also increased. But, at 500 μM concentration of hydrogen peroxide, even if mRNA was expressed, its expression level was not higher than those in lower concentration levels of hydrogen peroxide.

In the case of COL1A1, when the mRNA expression ratio is set as 1.00 for the group where COL1A1 is not treated with hydrogen peroxide, it was found that the higher the concentration level of hydrogen peroxide got, the lower its mRNA level got. When the concentration level of hydrogen peroxide was 500 μM, COL1A1 mRNA ratio was found to be expressed 50% lower than the case where COL1A1 is not treated with hydrogen peroxide.

When Human dermal fibroblasts cells were treated with hydrogen peroxide 500 μM, the expression amount of MMP-1 increased by 153.98% compared with that of the control group.

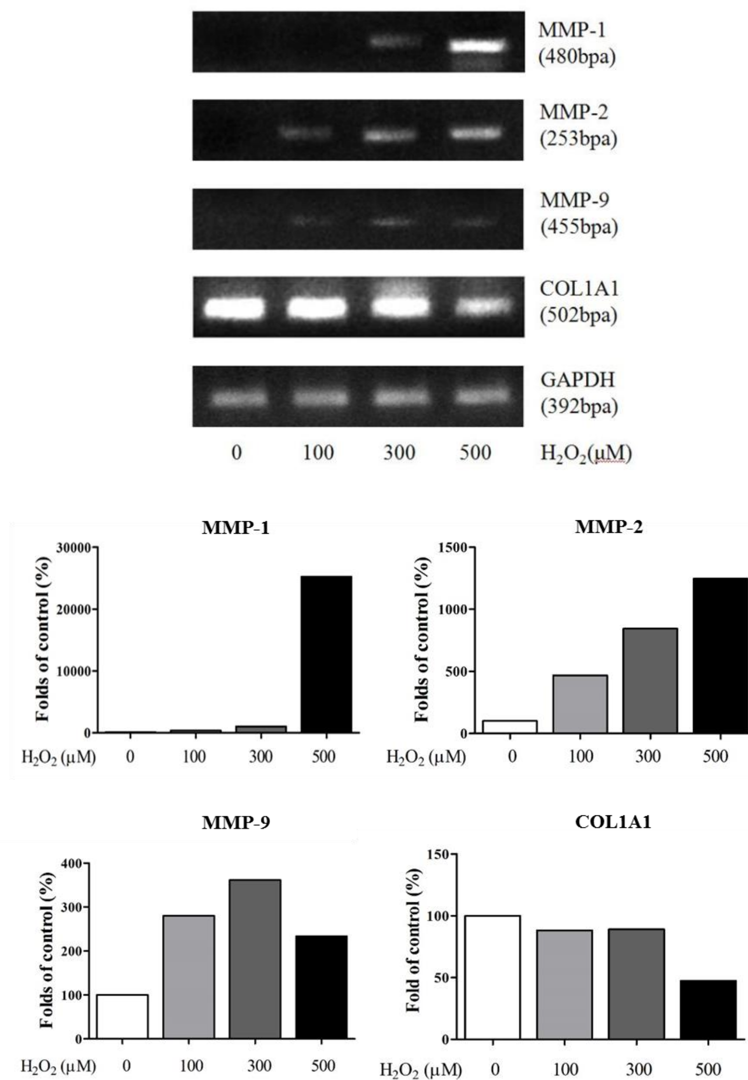
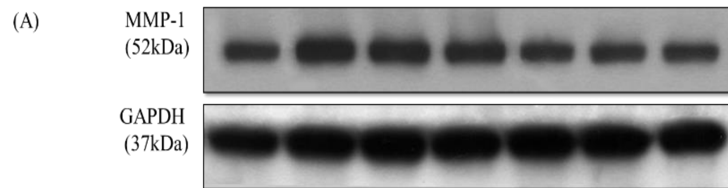
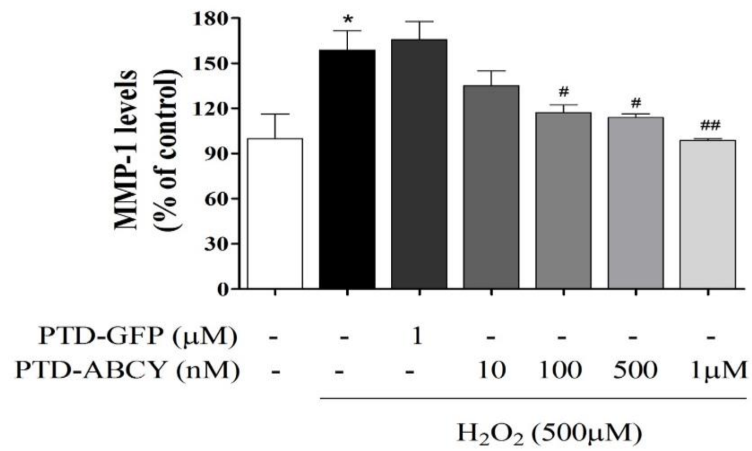


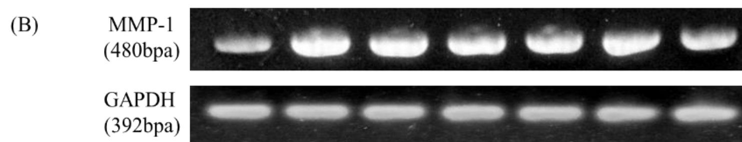
Fig. 5. The mRNA level of MMPs and collagen by hydrogen peroxide in HDF cells. The fibroblasts were washed with PBS and stimulated with various intensities of H_2O_2 (0–500 μM). The H_2O_2 exposed cells were cultured for 24 h, and MMP-1, 2, 9 expression and COL1A1 levels were determined in the culture media by RT-PCR analysis.

4. The effect of PTD-alpha B crystallin on the MMP-1 expression

In order to verify the effect of MMP-1's manifestation in cells by treating the PTD-alpha B crystallin fused cell with hydrogen peroxide in 500 μM concentration, western blot was used to measure the protein manifestation aspect. When HDF cells were treat with hydrogen peroxide 500 μM, manifestation volume of MMP-1 increased by 153.98% in comparison with the control group. The MMP-1 enzyme activity concentration in PTD-alpha B crystallin treated group, from 0.1 μM to 1 μM (111.28±12.68%-98.77±14.67%) was confirmed to gradually decrease, but per each individual (n=3), there existed differences (Fig. 6). In addition, in order to find out the effect of PTD-alpha B crystallin treated the fused cell with hydrogen peroxide, on the MMP-1 genetic manifestation, PTD-alpha B crystallin was verified per each concentration using RT-PCR. From the hydrogen peroxide separate treatment group there was an increase in manifestation of 168.63±6.2% compared to that of untreated group. When PTD-alpha B crystallin was treated at 0.01 μM-0.5 μM concentration, the result was 133.88±7.46%-131.86±5.66%, thus the width of decrease was not significant. However, when PTD-alpha B crystallin was treated at 1 μM concentration, the result was 107.53±5.12% which was similar in manifestation level to that of the unstimulated group.


(A)
MMP-1
(52kDa)
GAPDH
(37kDa)


MMP-1 levels
(% of control)
180
150
120
90
60
30
0
*
#
#
##
PTD-GFP (μM) - - 1 - - - -
PTD-ABCY (nM) - - - 10 100 500 1μM
H2O2 (500μM)


(B)
MMP-1
(480bpa)
GAPDH
(392bpa)

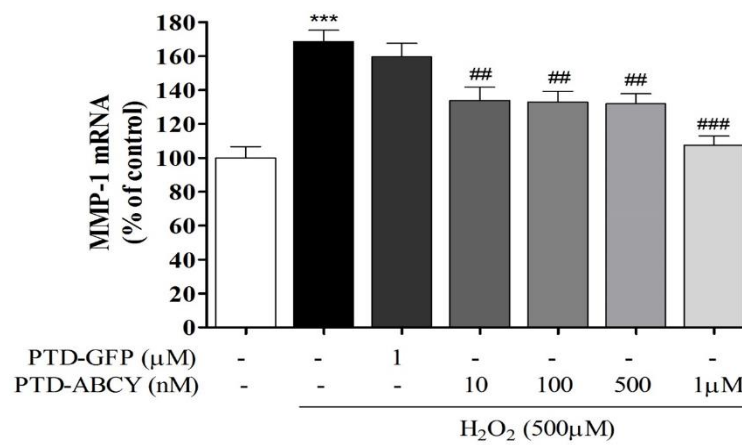
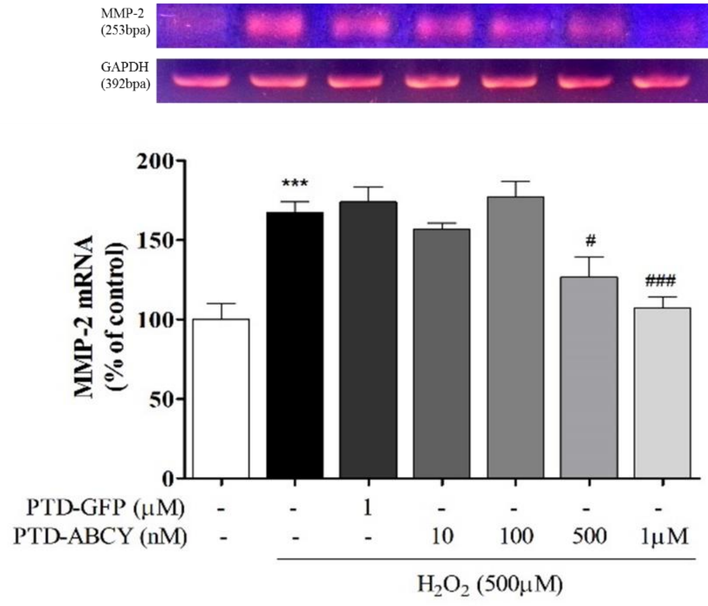

MMP-1 mRNA
(% of control)
180
160
140
120
100
80
60
40
20
0
***
##
##
##
###
PTD-GFP (μM) - - 1 - - - -
PTD-ABCY (nM) - - - 10 100 500 1μM
H2O2 (500μM)

Fig. 6. Effects of PTD-alpha B crystallin on H_2O_2-induced MMP-1 expression in human dermal fibroblasts. Human dermal fibroblasts were treated with the indicated concentration of PTD-alpha B crystallin for 24 h, followed by stimulation with 500 μM H_2O_2 (A) MMP-1 expression levels were determined by Western blotting. (B) The H_2O_2 exposed cells were cultured for 24 h. Levels of MMP-1 mRNA were determined by RT-PCR analysis. GAPDH mRNA was used as an internal control. The MMP-1 band intensities were quantified by densitometry, normalized to the level of GAPDH mRNA, and calculated as a percentage of the basal response. Values are the mean ± S.E.M. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. the control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p < 0.001$ vs. the H_2O_2 (500 μM) alone group.

5. The mRNA level of MMP-2 and MMP-9 by PTD-alpha B crystallin in HDF cells

In order to look for the effect of PTD-alpha B crystallin on the MMP-2, MMP-9 genetic manifestation, known as Gelatinase in HDF cells, we used RT-PCR for verification. As a result of separately treating the hydrogen peroxide, the amount of manifestation in mRNA of MMP-2, based on the untreated group, was shown to be higher by 167.36±5.66%. When PTD-alpha B crystallin was treated at 0.01 μM, 0.5 μM, 1 μM concentration, each manifestation was around 156.55±3.52%, 126.62±11.0%, 107.18±5.85%. However, when PTD-alpha B crystallin was treated at 0.1 μM concentration, the manifestation amount of MMP-2 was 177.44±8.03% which was confirmed to be manifestating at the level of hydrogen peroxide separate treatment group.

In the case of MMP-9, 184.67±6.25% mRNA manifestation was seen in the hydrogen peroxide separate treatment group. When PTD-alpha B crystallin was treated at 0.01 μM – 0.5 μM concentration, the level was 149.88±3.37% - 144.61±12.56% which indicated that the concentration was not significantly affected. When PTD-alpha B crystallin was treated at 1 μM, the result was 114.25±6.23% which confirmed that the amount of mRNA manifestation of MMP-9 was similar to the level of standard manifestation amount.


MMP-2
(253bpa)
GAPDH
(392bpa)
MMP-2 mRNA
(% of control)
200
150
100
50
0
***
#
###
PTD-GFP (μM) - - 1 - - - -
PTD-ABCY (nM) - - - 10 100 500 1μM
H2O2 (500μM)

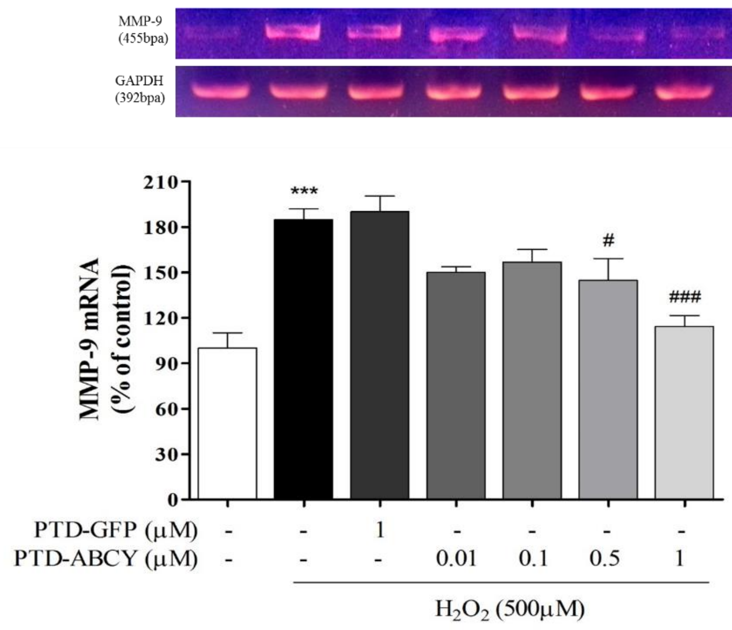
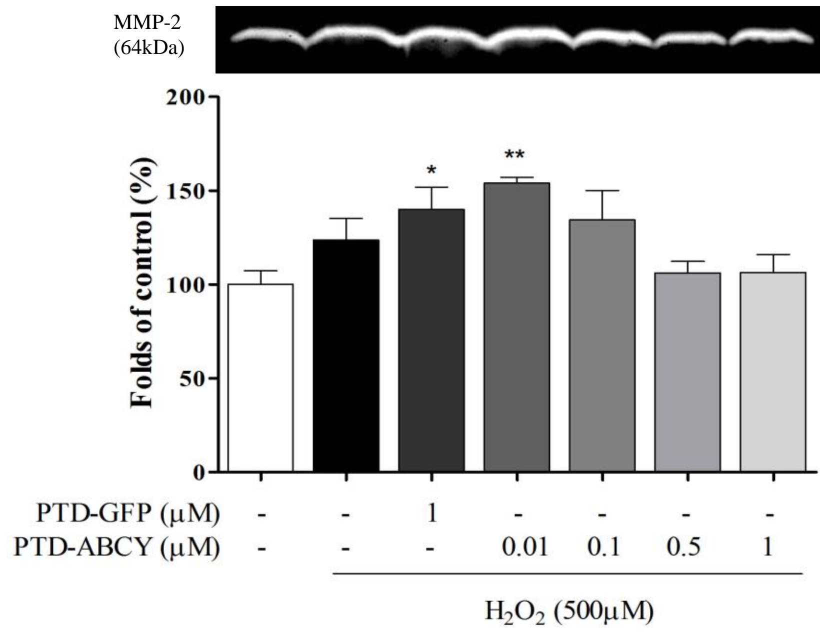

MMP-9
(455bpa)
GAPDH
(392bpa)
MMP-9 mRNA
(% of control)
210
180
150
120
90
60
30
0
***
#
###
PTD-GFP (μM) - - 1 - - - -
PTD-ABCY (μM) - - - 0.01 0.1 0.5 1
H2O2 (500μM)

Fig. 7. Effects of PTD-alpha B crystallin on H_2O_2-induced expression MMP-2 and 9 in human dermal fibroblasts. Human dermal fibroblasts were treated with the indicated concentration of PTD-alpha B crystallin for 24 h, followed by stimulation with 500 μM H_2O_2. The H_2O_2 exposed cells were cultured for 24 h. Levels of MMP-2, 9 mRNA were determined by RT-PCR analysis. GAPDH mRNA was used as an internal control. The MMP-2, 9 band intensities were quantified by densitometry, normalized to the level of GAPDH mRNA, and calculated as a percentage of the basal response. Values are the mean ± S.E.M. $^{***}p < 0.001$ vs. the control group; $^{\#}p<0.05$, $^{\#\#\#}p < 0.001$ vs. the H_2O_2 (500 μM) alone group.

6. The effect of PTD-alpha B crystallin on secretion of MMP-2 and MMP-9.

Using Gelatin Zymography, confirmed the MMP-2, MMP-9 manifestation aspect secreting out of HDF cells. In MMP-2 enzyme activity, it was difficult to expect meaningful result from culture ground treated with PTD-alpha B crystallin and hydrogen peroxide separate treatment group. In culture ground for group treated with hydrogen peroxide, the MMP-9 enzyme activity was increased by 208.52±4.35% compared to the untreated culture ground. Among the PTD-alpha B crystallin treated groups, from concentration level of 0.1 μM to 1 μM (172.33±4.90%-110.59±6.15%), decrease in manifestation depending on the concentration was verified.


MMP-2
(64kDa)
200
150
100
50
0
Folds of control (%)
*
**
PTD-GFP (μM) - - 1 - - - -
PTD-ABCY (μM) - - - 0.01 0.1 0.5 1
H2O2 (500μM)

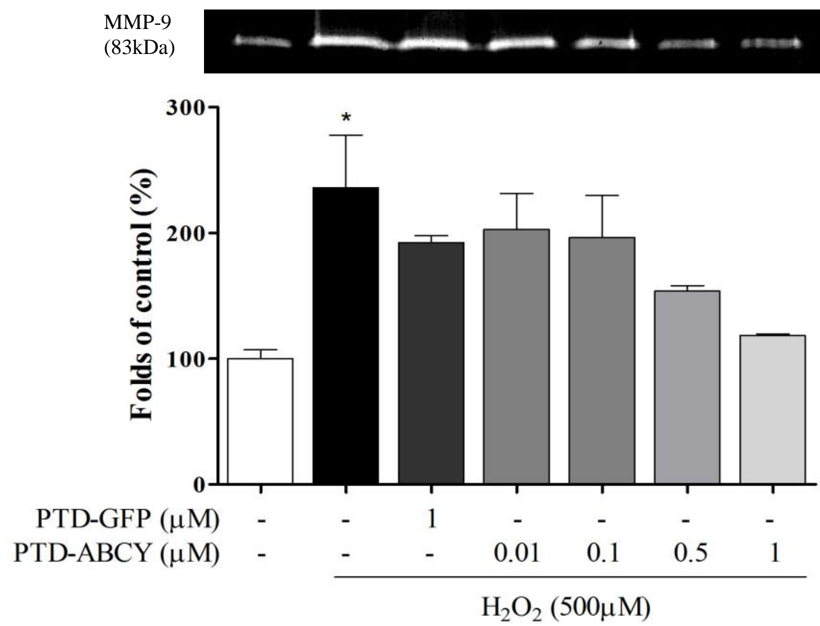

MMP-9
(83kDa)
300
200
100
0
Folds of control (%)
*
PTD-GFP (μM) - - 1 - - - -
PTD-ABCY (μM) - - - 0.01 0.1 0.5 1
H2O2 (500μM)

Fig. 8. The effect of PTD-alpha B crystallin on secretion of MMP-2 and MMP-9 in human dermal fibroblast. Cells were treated with PTD-alpha B crystallin at 0.01, 0.1, 0.5 μM and 1 μM conditions for 24 h, followed by stimulation with 500 μM H_2O_2. Activities of MMP-2 and MMP-9 in conditioned media were determined by gelatin. Lower panel represents respective relative enzyme activities as percent of control group. Values are the mean ± S.E.M. $^{*}p < 0.05$, $^{**}p<0.01$ vs. the control group; $^{\#}p<0.05$, $^{\#\#\#}p < 0.001$ vs. the H_2O_2 (500 μM) alone group.

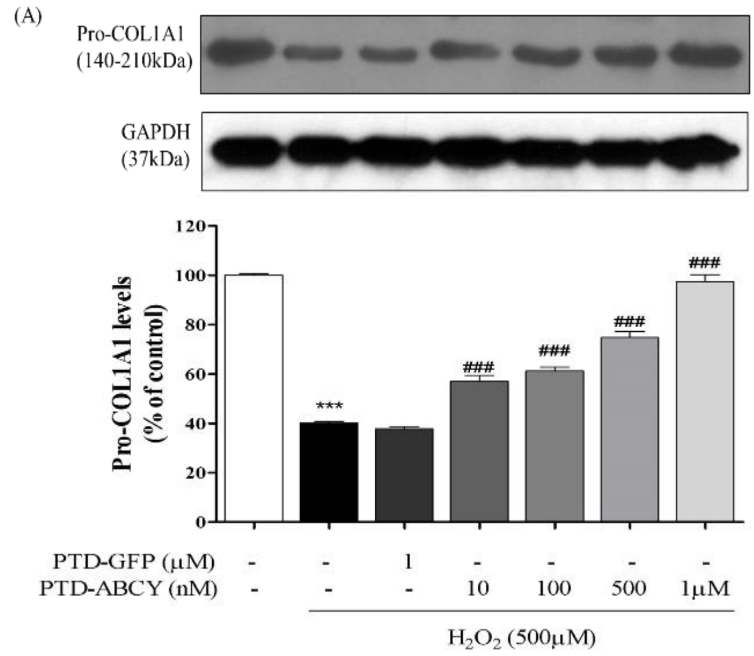
7. The effect of PTD-alpha B crystallin on the production collagen

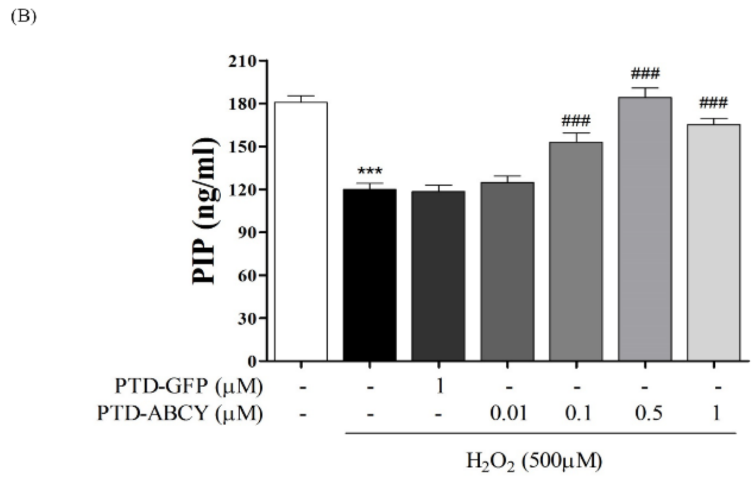
The assessed the protein level of type I procollagen in H_2O_2-induced human dermal fibroblasts at 24 h using Western blot analysis. When HDFs were treated with 500 μM H_2O_2, the level of type I procollagen protein decreased (29.01±5.75%). Processed PTD-alpha B crystallin protein was found to increase in a concentration-dependent manner (53.37±3.45 % - 91.75±8.06 %).

ELISA was used to verify the effect of PTD-alpha B crystallin on the collagen manifestation that secretes out of the cell. In the group untreated with hydrogen peroxide, there was manifestation of procollagen type I and carboxy-terminal peptide (PIP) 180.66±4.38 ng/ml. On the other hand, in the group treated with hydrogen peroxide, the manifestation level was low in which the PIP was around 119.86±4.00 ng/ml. The group in which the PTD-alpha B crystallin was treated at 0.01 μM concentration, the level of manifestation was 124.73±4.16 ng/ml which did not show significant difference against hydrogen peroxide separate treatment group. However, when PTD-alpha B crystallin was treated at 0.1 μM, 0.5 μM, 1 μM concentration levels, each resulted in 152.81±6.20 ng/ml, 184.19±6.13 ng/ml, 165.21±4.10 ng/ml where PIP was confirmed to have recovered to the level of untreated group.

And in order to find out the effect of PTD-alpha B crystallin fused cell treated with hydrogen peroxide, on COL1A1 genetic manifestation, RT-PCR was used to check the PTD-alpha B crystallin per concentration. In case of COL1A1, in the hydrogen peroxide separate treatment group, there was decrease in manifestation of 38.48±7.60% compared to the untreated group. When PTD-alpha B crystallin was treated at 0.01 μM

– 1 μM concentration, mRNA manifestation amount also increased to 54.00±6.86%-98.33±7.58% in accordance with the increase in concentration.


(A)
Pro-COL1A1
(140-210kDa)
GAPDH
(37kDa)
Pro-COL1A1 levels
(% of control)
120
100
80
60
40
20
0
***
###
###
###
###
PTD-GFP (μM) - - 1 - - - -
PTD-ABCY (nM) - - - 10 100 500 1μM
H2O2 (500μM)


(B)
PIP (ng/ml)
210
180
150
120
90
60
30
0
***
###
###
###
PTD-GFP (μM) - - 1 - - - -
PTD-ABCY (μM) - - - 0.01 0.1 0.5 1
H2O2 (500μM)

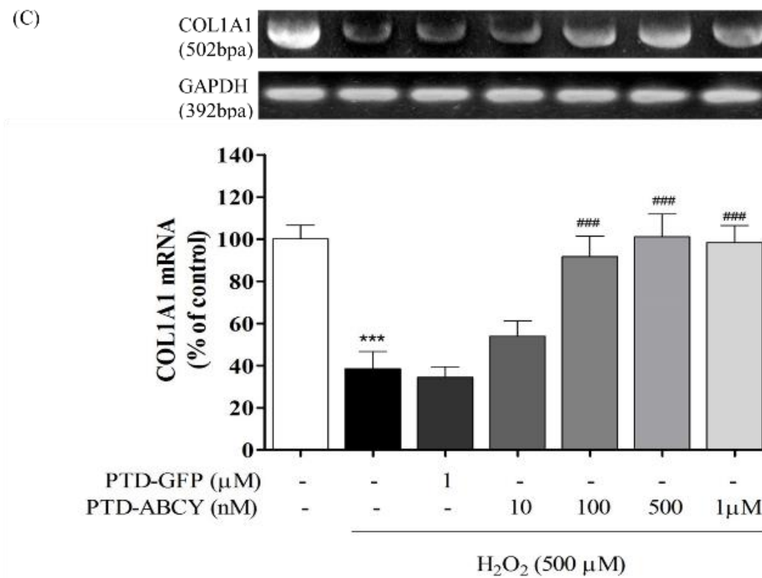


Fig. 9. Effects of PTD-alpha B crystallin on the production of collagen in human dermal fibroblasts. Human dermal fibroblasts were treated with the indicated concentration of PTD-alpha B crystallin for 24 h, followed by stimulation with 500 μM H_2O_2. (A) Type I procollagen expression was determined in cell by Western blotting. (B) The culture supernatants were harvested, and type I procollagen levels were determined by ELISA. (C) The H_2O_2 stimulated cells were cultured for 24 h. Levels of COL1A1 mRNA were determined by RT-PCR analysis. GAPDH mRNA was used as an internal control. The COL1A1 band intensities were quantified by densitometry, normalized to the level of GAPDH mRNA, and calculated as a percentage of the basal response. Values are the mean ± S.E.M. $^{***}p < 0.001$ vs. the control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p < 0.001$ vs. the H_2O_2 (500 μM) alone group.

8. Inhibitory effect of PTD-alpha B crystallin on elastase activity

In order to confirm the effect of PTD-alpha B crystallin on HDF cells, we conducted Elastase inhibition assay. After collecting and homogenizing the HDF cells that have not been treated, the experiment were carried out by treating it with official sample fluid and PTD-alpha B crystallin, Phosphoramidon (cultivated control group).

With the untreated official sample fluid as the standard, PTD-alpha B crystallin (0.01 μM – 1 μM), in accordance with the increase in concentration, was confirmed to notably decrease elastase activity level to 75.85±3.2% - 42.3±6.8%. Cultivated control group Phosphoramidon was confirmed to decrease the elastase activity level to 25.54±1.38%.

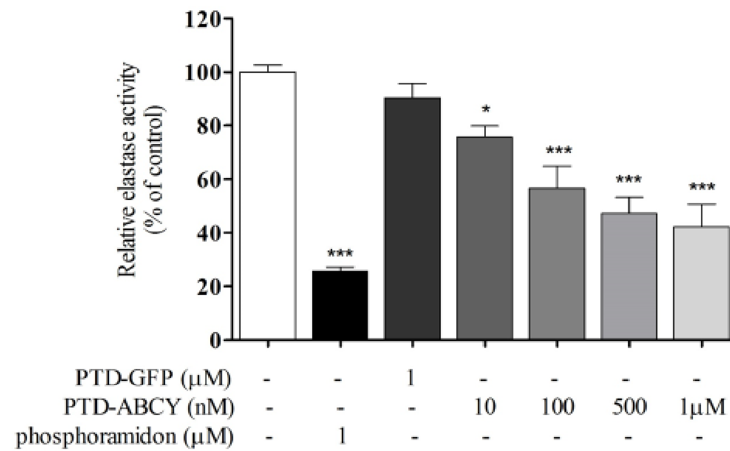
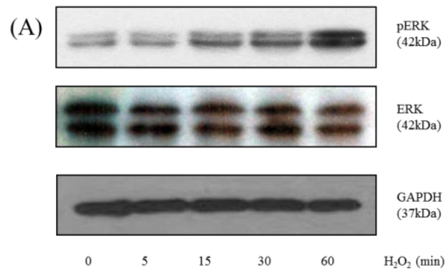


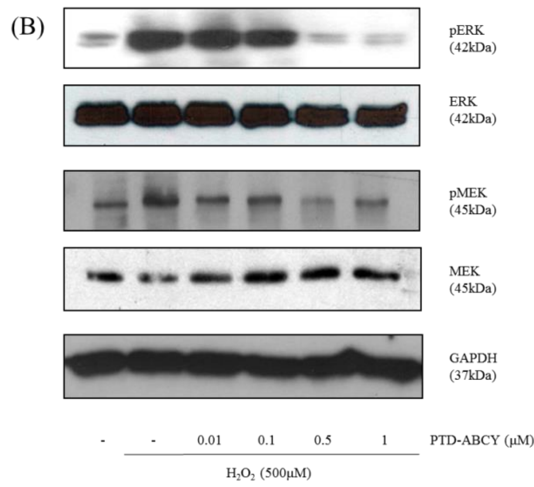
Fig. 10. Inhibitory effect of PTD-alpha B crystallin on elastase activity. Human dermal fibroblasts were treated with the indicated concentration of PTD-Alpha B crystallin (0.01 μM, 0.1 μM, 0.5 μM, 1 μM) for 24 h. Values are the mean ± S.E.M. *$p < 0.05$, ***$p < 0.001$ vs. the control group.

9. The effect of PTD-alpha b crystallin on MEK-ERK signaling pathway

To identify what effect PTD-alpha B crystallin has on ERK, MEK signal in the MMP generating process, we first treated the cell with fixed concentration 500 μM hydrogen peroxide, and checked the time of ERK activation. Protein expressions were measured with western blot.

By treating the cell with different concentration levels of PTD-alpha B crystallin, we identified MEK-ERK phosphorylation. Because MMP-1, 2, and 9 are primarily regulated by ERK activation[45], we examined the effect of PTD-alpha B crystallin on H_2O_2-induced ERK phosphorylation in human dermal fibroblasts. Western blot analysis showed that H_2O_2 exposure leads to phosphorylation of MEK, ERK in human dermal fibroblasts. At concentration 0.5 μM and 1 μM, PTD-alpha B crystallin, phosphorylation of MEK, ERK was inhibited. In another set of experiments, we tested whether the activation of the MEK-ERK1/2 pathway by PTD-alpha B crystallin is associated with PTD-alpha B crystallin stimulating MMPs (MMP-1,2,9) mRNA expression in dermal fibroblasts. To do this, we examined the effects of inhibitors of either MEK or ERK1/2 phosphorylation on PTD-alpha B crystallin stimulating MMPs mRNA expression. These results suggest that MEK-ERK1/2 signaling pathway is the activation of fibroblasts in the skin which is induced by hydrogen peroxide, the expression of MMP-1 which contributed to the upregulation respectively. And the PTD-alpha B crystallin is MEK-ERK1 / 2 signaling pathway by inhibiting the activation of MMPs contribute to the adjustment, respectively.


(A)
pERK
(42kDa)
ERK
(42kDa)
GAPDH
(37kDa)
0
5
15
30
60
H_2O_2 (min)


(B)
pERK
(42kDa)
ERK
(42kDa)
pMEK
(45kDa)
MEK
(45kDa)
GAPDH
(37kDa)
- - 0.01 0.1 0.5 1 PTD-ABCY (μM)
H_2O_2 (500μM)

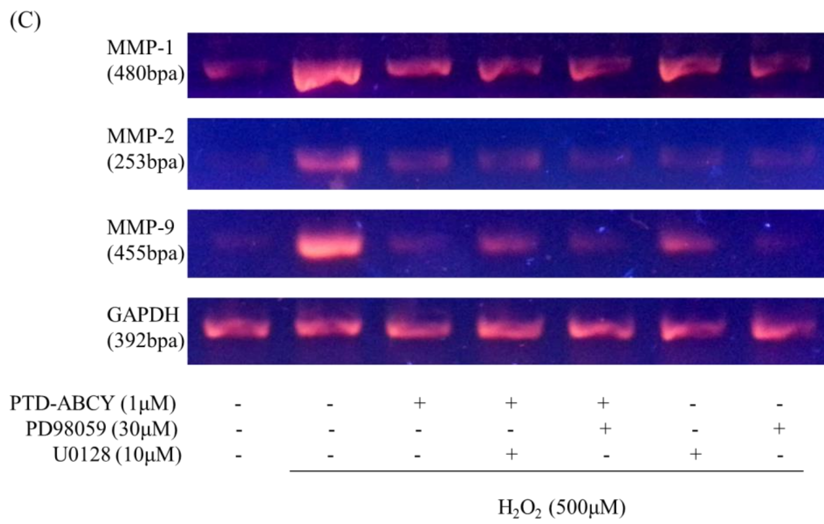

(C)
MMP-1
(480bpa)
MMP-2
(253bpa)
MMP-9
(455bpa)
GAPDH
(392bpa)
PTD-ABCY (1μM) - - + + + - -
PD98059 (30μM) - - - - + - +
U0128 (10μM) - - - + - + -
H_2O_2 (500μM)

Fig. 11. The effect of PTD-alpha B crystallin on MEK-ERK signaling pathway. PTD-alpha B crystallin inhibits H_2O_2-induced MMP expression by inhibiting extracellular signal-regulated kinase (ERK) activation (A) Cells were stimulated with H_2O_2 (500 μM) and harvested at the indicated times. Total cell lysates were prepared and subjected to Western blotting using phospho-specific (p-) ERK antibody. (B) HDF cells were pretreated with the indicated concentrations of PTD-alpha B crystallin for 1 h and then stimulated with H_2O_2 (500 μM). After incubation for 15 min, total cell lysates were prepared. The phosphorylation of the ERK, MEK were detected by Western blotting. (C) Dermal fibroblasts were cultured in the absence or presence of 1 μM of PTD-alpha B crystallin with or without 30 μM of PD98059 (ERK1/2 inhibitor), 10 μM of U0126 (MEK inhibitor). MMPs mRNA were detected by RT-PCR. GAPDH was used as a loading control for all experiments.

Ⅳ. DISCUSSION

In this study, using protein delivery system, we studied the potential skin protective effect of PTD-alpha B crystallin as a therapeutic protein in human dermal fibroblasts.

The fusion protein was produced as TAT-fusion alpha B crystallin protein containing the 11-amino acid transduction domain of TAT. It was constructed by recombinant technology. The pHis-TAT/ alpha B crystallin fusion protein could enter efficiently Human dermal fibroblasts.

The small heat shock protein (sHSP) has the molecule volume of 20-29kDa and its activation increases depending on the various environmental conditions. alpha B crystallin, one of sHSP act as a molecular saffron and through the mutual action with the saffron protein and ATP molecule, it protects the cell in various stressful environments. In addition, it helps the cells to grow by stabilizing the skeletal structure of the cell.

alpha B crystallin has the structure similar to the HSP27 and is known to be have the protecting function against hyperthermia, oxidative stress, and inflammatory cytokines. The protective effect of alpha B crystallin has been researched for central nervous system-related diseases. alpha B crystallin is known to be related to the degenerative process of the central nervous system such as Alzheimer's disease. However, there have been no studies published relating to the wrinkles yet.

The dermal collagen which is the main component of the skin cell accounts for 70-80% of dry weight of corium. Among them, type 1 collagen takes up 80% of the

collagen while type III collagen takes up 15%. In general, the synthesis of type 1 collagen and the activation of MMP-1 keep the proper balance and keep the skin elastic[41]. But once this balance is broken down by the aging and other external elements, the skin wrinkles are created.

Matrix metalloproteinases (MMP) is a group of enzymes which plays a critical role in disassembling the components of extracellular matrix. In both extrinsic and intrinsic ageing, elevated levels and activities of cutaneous MMPs have been demonstrated. MMP-1 is the only enzyme being able, after activation, to initiate cleavage of collagen triple helix, which consists of the two major fibril-forming molecules in the skin, type I and III collagens. MMP-3 and -9 can cleave dermal fibrillar collagens (type I, III, and V) only after initiation by MMP-1[46,47]. This is why the activity of MMP-1 remains under tight control even after UV irradiation[48]. Despite elevated MMP-1 levels, only a small proportion of MMP-1 becomes active, whereas the majority of MMP-1 remains in its inactive proform after acute UV irradiation[48]. Consequently, the active MMP enzymes may degrade collagen only partially, which can remain crosslinked within the insoluble collagen matrix. These insoluble fragments are, however, susceptible to proteolytic cleavage by many other proteases as well. In the absence of perfect repair, collagen damage might accumulate upon repeated UV exposure. A similar scenario might be behind the degradation of elastic fibers. Both MMP-3 and -9 are known to degrade elastin, the stretchable component of elastic fibers[46,47]. It is known that MMP-1 dissolves the native fibrillar Type-1 collagen while Gelatinase A (MMP-2) and Gelatinase B (MMP-9) dissolve the basic membrane. The oxidative stress is reported to

increase the expression of MMP[49,50,51]. Confirm the activated MMP induced by H_2O_2 and the resulting destruction of collagen protein, this study directly applied the H_2O_2 to the cells.

Ultimately, in accordance with increase in MMP's manifestation, breakdown of collagen and synthesis are decreased, thus forming wrinkles.

Accordingly, research regarding skin aging including formation of wrinkles, are being conducted using a method of protecting skin cells via impeding activation of or suppressing biosynthesis of collagen breakdown enzyme and the amount of collagen[52].

In accordance with structural and functional characteristics of various enzyme family that participates in breakdown of extracellular substrate and basilar membrane, MMPs are divided into four subfamily including interstitial collagenase, stromlysin, gelatinase, membrane-type MMP and etc. Majority of cells including fibroblast, which is the skin’s keratinocyte, secrete MMPs.

In skin ageing, ROS stimulation is closely related with collagen degradation of skin dermis by activating the matrix degrading enzymes. Among MMPs, many studies have been focused on the gelatinases and collagenases which are considered to be involved in relation to skin aging[53].

MMP-1 is enzyme which directly acts on collagen[54]. Examination of the MMP-1 expression amount and gene expression amount in the cell revealed that MMP-1 has inhibition effect at 0.5 μM and 1 μM concentration levels of PTD-alpha B crystallin.

As a result of verifying the level of manifestation in MMP-2, MMP-9 mRNA, starting from PTD-alpha B crystallin 500 μM, showed notable result value in comparison with

hydrogen peroxide separate treatment group. Furthermore, MMP-2 in which the processing of PTD-alpha B crystallin is secreted from HDF, and the effect it has on MMP-9 was assessed using zymography. In the case of MMP-2, in comparison with untreated group, hydrogen peroxide treated group's rate of increase was not high, and even when PTD-alpha B crystallin was treated, the effect was shown to be generally inadequate. In the case of MMP-9, in comparison with the controlled group, the rate of increase in the hydrogen peroxide treated group was close to 200%. In the group treated with PTD-alpha B crystallin, when compared with hydrogen peroxide treated group, it was evident that the enzyme manifestation had decreased. PTD-alpha B crystallin can be thought to take on the role of effectively impeded the MMP-9 enzyme secretion activity.

Collagen is a protein that makes up the skin throughout the entire body and is formed as procollagen, which is a precursor. Procollagen is composed of mature collagen and excess peptide, and this peptide breaks down via unique protease before the collagen squeezes into growing collagen fibril. This PICP (Procollagen type I C-Peptide), which is broken down peptide, appears isolated outside of the cell and becomes synthesis indicator[55].

Using ELISA, secreted collagen was measured with culture media. The collagen amount measured in the group where the cell is treated with hydrogen peroxide was lower than that of collagen measured in the group where the cell is treated with PTD-alpha B crystallin. When collagen expression degree was identified with western blotting, collagen in the group where the cell is treated with PTD-alpha B crystallin

was expressed with the similar amount to the collagen in the groups where the cell is not stimulated. The case where COL1A1 gene was identified showed similar results. Such findings demonstrate that PTD-alpha B crystallin inhibits collagen reduction caused by hydrogen peroxide stimulus.

Elastase is an enzyme that breaks down elastin, which is protein, and is able to break down collagen which is another important stroma and thus it is a nonspecific hydrolysis enzyme. In the dermal tissue of the skin, elastin related to collagen and elasticity of skin form mesh structure. As elastin is broken down by elastase, the bond of the skin's mesh structure is cut, thus elastase is known to be the enzyme responsible for being the main cause in the formation of wrinkles. Elastase inhibitor was shown to be effective in improving skin wrinkles[56], and ursolic acid among others are being used as elastase inhibitors[57]. And as one of the white blood cell granule enzyme that breaks down elastin in the body, in an heteroplasm, as enzyme activity is extremely high, it becomes the direct cause of tissue destruction, causing wrinkles and loss of elasticity in the skin[58,59]. As a result of measuring the elastase inhibitory activity related to wrinkle formation, based on official sample fluid, PTD-alpha B crystallin was confirmed to function as elastase inhibitor. This result shows that PTD-alpha B crystallin can be considered to be effective in preventing formation of wrinkles by protecting the structural binding of elastin directly related to the elasticity of the skin.

Through experiments, we identified PTD-alpha B crystallin properly regulates the expressed amounts of MMP, collagen, and elastin, mentioned as direct causes of wrinkle formation. To identify whether PTD-alpha B crystallin is involved in upper

regulating elements of MMP mRNA expression, we also identified degrees of phosphorylation of MEK, ERK. It was found that PTD-alpha B crystallin inhibits activity of MAP (mitogen-activated protein) kinase signal induced by ROS.

Consequently, even if it is cell culture level, PTD-alpha B crystallin is effective in inhibiting wrinkle caused by ROS which occurs in both externally and internally caused aging. It can be clinically used for the medicine for inhibiting skin aging in the future.

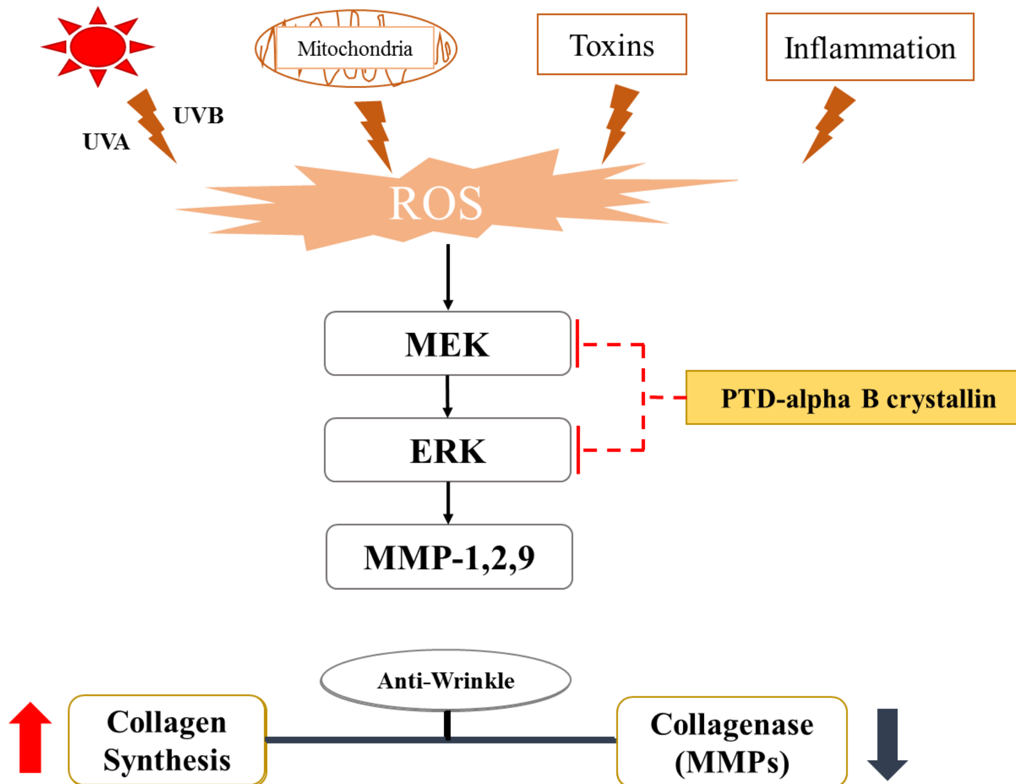


Fig. 12. Schematic diagram summarizing the mechanism for protective effect of PTD-alpha B crystallin against skin aging. In human dermal fibroblasts, PTD-alpha B crystallin proved to inhibit activity of MEK/ERK MAP (mitogen-activated protein) kinase signal induced by ROS.

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ABSTRACT (KOREAN)

단백질 전달도메인 융합 알파비 크리스탈린 단백질의

피부 섬유아세포 보호 효과

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이성숙

피부의 노화 진행과정에서 주름은 유전적인 요소와 환경적인 요소들로 생성된다. 내인성 노화 및 외인성 노화를 촉진하는 자극에 의해 활성산소가 발생하며, 이런 활성산소는 진피의 콜라겐 합성 감소 및 변형과 다양한 단백질 분해효소들의 발현을 증가시키는 것으로 알려져 있다. 본 연구에서 사용한 알파비 크리스탈린은 상피세포 사멸 조절 등의 기능을 하는 것으로

알려져 있지만, 피부 섬유아세포에 대한 생물학적 영향은 연구된 바 없다. 본 연구에서는 사람 진피 섬유아세포에서 과산화수소 자극에 대해 단백질 전달 도메인 융합 알파비 크리스탈린 단백질의 보호 효과 및 기전을 규명하였다. 알파비 크리스탈린 단백질이 전달된 세포에서 MMP-1, MMP-2, MMP-9 등의 단백질 발현이 억제된 반면, 콜라겐 합성은 증가되었다. 이러한 기전은 전달된 알파비 단백질이 MEK-ERK 신호 전달 경로를 차단함으로써 이루어지는 것으로 나타났다. 결론적으로, 본 연구는 피부 노화 및 관련 질환에 주요한 약물 타겟에 대한 자료를 제공할 것으로 사료된다.

핵심 되는 말: 알파-비 크리스탈린 단백질, 단백질 전달 도메인, 교원질, 기질단백질 분해효소-1, 기질단백질 분해효소-2, 기질단백질 분해효소-9.