Effect of Heat shock protein 90 on TGF-β Induced Collagen Synthesis of Human Dermal Fibroblast

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Effect of Heat shock protein 90 on TGF-β Induced Collagen Synthesis of Human Dermal Fibroblast

Directed by Professor Dong Kyun Rah

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Sae Bin Lee

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This certifies that the Doctoral Dissertation of Sae Bin Lee is approved.

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My admission to Yonsei university college of medicine in 1997 seems as though it was just a couple of days ago, but 20 years have passed and I was able to complete the doctoral course in medicine. I experienced a number of difficulties and hard times as I went through the doctoral course after completing my residency training at the plastic surgery department. However, I would like to express my sincere gratitude to academic advisor Rha, Dong Kyun for guiding me to take a step closer to the field of learning known as medicine. In addition, I would like to express my deep gratitude to professor Kim, Hoguen, professor Rhie, Jong Won, professor Chung, Kee-Yang and professor Lee, Won Jai for devoting their precious time to my dissertation, and I would also like to express my deep gratitude to professor Lew, Dae Hyun, the head professor of the plastic surgery department, for looking out for me from the years of my residency training at the plastic surgery department. I would like to express my gratitude to professor Min, Hyun Jin who is my wife as well as to professor Song, Seung Yong for providing practical pieces of advice on performing unfamiliar experiments.

I would like to express my endless gratitude and love to my beloved wife Min, Hyun Jin and my son Lee, Chae Woon for supporting me throughout my hospital management and academic learning as well as to my father, my mother, my father-in-law and my mother-in-law for providing their endless encouragement and support.

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Heat shock proteins (Hsps) interact with non-native proteins under various stressful conditions, prevent abnormal protein folding, and aid in proper protein folding. Recently, it was shown that various Hsps are involved in the wound healing process. Hsp 90 is one of several stress proteins, and its expression and activity are upregulated under stressful conditions. It has been shown that Hsp 90 is involved in the fibrosis pathway through stabilization of TGF-β receptors and Src kinases, which are important in the intracellular fibrosis process. The aim of present study was to investigate the role of Hsp 90 in TGF-β induced collagen synthesis of human dermal fibroblasts.

Human dermal fibroblast cells were cultured, and the effects of TGF-β, 17-N-allylamino-17 demethoxygeldanamycin (17AAG), and transfection of Hsp

90 plasmids on cell viability were evaluated. Real-time PCR, western blot, and immunofluorescence assay were performed to evaluate the influence of Hsp 90 level on TGF-β induced collagen synthesis. Western blot analysis was also used to investigate the Smad 2/3 and Akt pathways, and to identify the signal pathways involved in collagen synthesis. Finally, the expression of Hsp 90 and collagen were compared in keloid and control tissue by immunohistochemical analysis.

The expression of collagen was significantly increased after treatment with TGF-β, both at the RNA and protein levels. Pretreatment with 17AAG inhibited TGF-β induced collagen synthesis. Overexpression of Hsp 90 increased TGF-β induced collagen synthesis. Pretreatment with 17AAG and overexpression of Hsp 90 affected the Smad pathway through modulation of Smad 2/3 phosphorylation. However, involvement of the Akt pathway was not obvious. Using human skin tissue, we found that expression of Hsp 90 was increased in keloid compared with control tissue.

We found that inhibition or overexpression of Hsp 90 in human dermal fibroblast influenced TGF-β induced collagen synthesis through modulation of Smad 2/3 phosphorylation. Furthermore, the expression of Hsp 90 was increased in keloid tissue compared with control tissue, supporting the results of our in vitro experiments showing that Hsp 90 is involved in the TGF-β induced collagen synthesis.

Keywords: Hsp 90, 17AAG, Hsp 90 overexpression, collagen synthesis

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I. INTRODUCTION

Fibrosis is a key process in wound healing. Pro-fibrotic stimuli lead to activation of surrounding fibroblasts and induce the progression of fibrosis.[1] Activated fibroblasts increase the release of collagen and other extracellular matrix components (ECM).[2] Transforming growth factor β (TGF-β) is an important mediator in fibroblast activation and the fibrosis process.[2]

TGF-β is a 25 kDa protein consisting of two identical 12.5 kDa subunits that are linked by a disulfide bond. TGF-β expression and activity are increased in all kinds of skin injuries such as acidic or alkali conditions, heat denaturation,

shear stress or surgical incision. TGF-β binding induces the activation of kinases.[1] These activated receptors phosphorylate Smad 2/3, which translocates to the nucleus and functions as a transcriptional regulator.[3] Other signals in addition to Smad, such as Akt, are also activated by TGF-β and TGF-β receptor interaction.[4] It has been reported that both Smad and non-Smad pathways are important in TGF-β induced fibrosis.[5]

Heat shock proteins (Hsps) are a family of molecular chaperones. Hsps account for up to 2-3% of total cellular proteins, and play an important role in proper protein folding.[1] According to molecular weight, Hsps are categorized as either Hsp 100, Hsp 90, Hsp 70, Hsp 60, Hsp 40, or small heat shock proteins.[6] Hsps also participate in cytokine production, inflammation, and the pathogenesis of cytoskeleton-related diseases.[1] Among chaperones, Hsp 90 family proteins are the most abundant proteins in eukaryotic cells, and are composed of four main types: Hsp 90α, Hsp 90β, GP96, and TRAP1. Various stressful conditions induce the activation of Hsp 90 proteins, which require ATP for their proper function.[1,7]

17-N-Allylamino-17-demethoxygeldanamycin(17AAG) is a geldanamycin derivative able to bind the ATP binding site of Hsp 90, which inhibits its activity.[8] Using kidney cell lines and a mouse renal fibrosis model, Noh et al. demonstrated that 17AAG inhibits the fibrotic pathway.[5] In that study, 17AAG repressed ECM production, upregulated the epithelial marker E-cadherin. In liver tissue, inhibition of Hsp 90 reduced the activation of hepatic

stellate cells, which participate in hepatic fibrosis. They demonstrated that treatment with 17AAG downregulates collagen synthesis in hepatic stellate cells, suggesting that it could be useful in the treatment of liver fibrotic diseases.[9]

Although the minimizing external skin wound is one of the main concerns to surgeons, the role of Hsp 90 and its inhibitors in dermal fibrosis is poorly understood. In this study, we hypothesized that Hsp 90 is involved in dermal collagen synthesis, and sought to investigate the signaling pathways through which collagen synthesis is mediated. We inhibited the action of Hsp 90 with the Hsp 90 inhibitor 17AAG, and enhanced the expression of Hsp 90 through transfection of Hsp 90 plasmids. We also investigated the expression level of Hsp 90 in keloid tissue, which is characterized by increased synthesis of ECM constituents such as collagen, and compared this level with that in normal skin tissue.

II. MATERIALS AND METHODS

1. Cell culture and human tissue

A human dermal fibroblast cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA), supplemented with heat-inactivated 10% fetal bovine serum, penicillin (30 U/ml), and streptomycin (300 μg/ml).

Recombinant human TGF-β (PeproTech, Rocky Hill, NJ, USA) was used to treat culture media for 4 hours. The dose of TGF-β was determined referring to previous researches.[4,5] For TGF-β treatment, commercial Hemagglutinin (HA)-tagged Hsp 90 plasmid (Addgene, Cambridge, MA, USA) was used for transient transfection, which was performed with the FuGene HD transfection reagent (Promega, Fitchburg, WI, USA). 2 μg of Hsp 90 plasmid was mixed with 6 μl of FuGene HD and applied to 6 well culture plate for 6 hours before 5 ng/mL TGF-β treatment. 17AAG (Sigma-Aldrich, St. Louis, MO, USA) was used to treat culture media (up to 5 μM) for 12 hours before 5 ng/mL TGF-β treatment. We determined the dose of 17AAG which did not influence the cell viability using CCK-8 assay.

Keloid tissues were obtained from active-stage keloid patients (n=4), and normal tissues were obtained from nonkeloid patients (n=3). Experiments were approved by the institutional review board of the Yonsei University

College of Medicine, and were performed in accordance with the Declaration of Helsinki.

2. Cell viability measurement

The viability of dermal fibroblast was determined using the CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's protocol. Briefly, cells (2×10^4 per well) were cultured in 96-well plates and grown overnight. 10 μl of CCK-8 reagent was applied to each well and further incubated for 30 minutes at 37°C. Absorbance at 450 nm was detected, and the value was used to compare the viability of cells in each condition.

3. Western blot analysis

Cells were harvested using RIPA buffer with a mixture of protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA) 4 hours after TGF-β treatment. The BCA assay (Thermo, Rockford, IL, USA) was performed to quantify the amount of protein extracts. Protein samples were separated with SDS-PAGE and then transferred to nitrocellulose membranes. Primary antibodies against collagen (Abcam, Cambridge, UK), GAPDH (SantaCruz Biotechnology, Santa Cruz, CA, USA), Hsp 90 (Abcam, Cambridge, UK), phospho-Smad 2/3 (Cell Signaling Technology, Danvers, MA, USA), total Smad 2/3 (Cell Signaling Technology, Danvers, MA, USA), phospho-Akt (Abcam, Cambridge, UK), and total Akt (Abcam, Cambridge, UK) were applied

overnight at 4°C. After washing three times, membranes were incubated with HRP-labeled goat anti-mouse/rabbit Ig (Jackson Labs, Bar Harbor, ME, USA). ECL reagent (Amersham, Buckinghamshire, UK) was used to detect signals.

4. Real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Waltham, MA, USA) 4 hours after TGF-β treatment. The first strand cDNA was synthesized using the Super-Script System (Life Technologies, Carlsbad, CA, USA) with Oligo(dT) as the primer. The unique primer sequences were as follows:

β2M: 5′-CTATCCAGCGTACTCCAA-3′,

5′-CAAAGTCACATGGTTCAC-3′;

Collagen: 5′-CTAAAGGCGAACCTGGTGAT-3′,

5′-TCCAGGAGCACCAACATTAC-3′.

Primer pairs for real-time PCR were designed and manufactured by Bioneer (Bioneer, Daejeon, Korea). The SYBR Green qPCR kit (Finnzymes, Vantaa, Finland) was used to compare gene expression levels.

5. Immunofluorescence and confocal imaging

Immunofluorescence assay was performed following the protocol used in our previous work.[10,11] Briefly, cells were cultured in a four-chamber slide (Thermo, Waltham, MA, USA). Fixation was performed with 4%

paraformaldehyde for 20 minutes at room temperature (RT) 4 hours after TGF-β treatment. Permeabilization was performed using 0.2% Triton X-100 for 10 minutes, and blocking was followed by treatment with 1% BSA-PBS for 1 hour at RT. Primary antibody diluted in 1% BSA-PBS was applied to culture media and incubated overnight at 4°C. Alexa 488-conjugated secondary antibody (Thermo) was added for 1 hour at RT. The cells were mounted with Vectashield mounting solution (Vector Laboratories, Burlingame, CA, USA) and observed under a confocal microscope (Olympus, Tokyo, Japan).

6. Immunohistochemistry

Paraffin-embedded block samples were prepared. Deparaffinization and rehydration were performed in graded alcohol solutions. Methanol containing 1% hydrogen peroxide was applied to the slide to inhibit the endogenous peroxidase activity. Slides were incubated with anti-collagen (Abcam, Cambridge, UK), and anti-Hsp 90 (Abcam, Cambridge, UK) at 4°C overnight. Slides were then incubated for 30 minutes with the diluted secondary antibody against rabbit immunoglobulin (Dako, Carpinteria, CA, USA). Chromogen 3,3'-diaminobenzidine tetrahydrochloride was applied to visualize the antibody-bound immune complexes. The negative control was stained with an isotype-matched antibody.

7. Statistical analysis

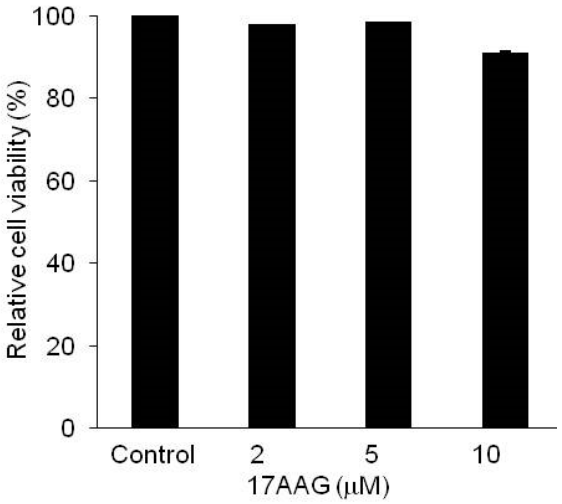
All statistical analyses were carried out using the SPSS Statistics 20 software package. Comparison among groups was performed using ANOVA with subsequent Fisher's least significant difference method. P-values <0.05 were considered significant.

III. RESULTS

1. The effects of 17AAG, Hsp 90 overexpression, and TGF-β on cell viability

We evaluated the effects of treatment with TGF-β, both with and without 17AAG, and Hsp 90 overexpression on the viability of human dermal fibroblasts. Results from the CCK-8 assay showed that treatment with 17AAG did not influence the viability of cells, up to treatment with 10 μM 17AAG for 12 hours. Overexpression of Hsp 90 also did not influence cell viability in our transfection system. Further experiments with a combination of TGF-β and either 17AAG or Hsp 90 overexpression confirmed that our experimental conditions did not influence cell viability (Fig. 1).

A



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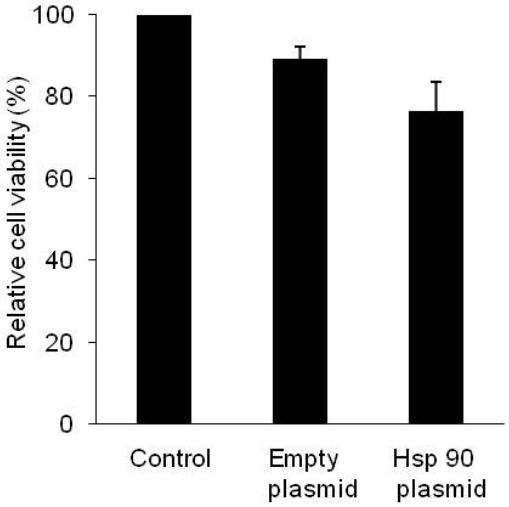


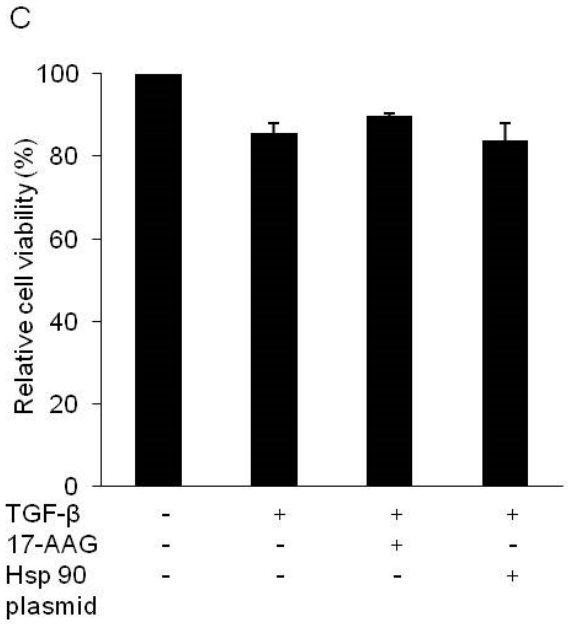


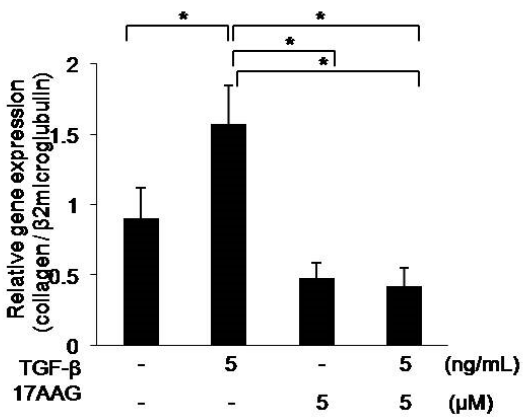
Figure 1. The effects of 17AAG, Hsp 90 overexpression, and TGF-β on cell viability

Human dermal fibroblasts were cultured and the CCK-8 assay was performed to evaluate the effects of 17AAG (A), overexpression of Hsp 90 (B), and a combination of treatment with TGF-β and either 17AAG or Hsp 90 overexpression (C), on viability of cells. (N=3) Treatment with TGF-β, treatment with 17AAG, and overexpression of Hsp 90 did not influence the viability of human dermal fibroblast.

2. 17AAG inhibits TGF-β induced collagen synthesis

We set our experimental condition as 5 ng/mL of TGF-β, and up to 10μM of 17AAG. We treated human dermal fibroblasts with TGF-β, both with and without pretreatment with 17AAG. We found that TGF-β induced upregulation of collagen synthesis both at the RNA and protein levels. With 17AAG pretreatment, the increase of collagen synthesis was abolished at the RNA level (Fig. 2A). By Western blot assay, we found that 17AAG inhibited TGF-β induced collagen synthesis dose-dependently (Fig. 2B). We performed these experiments three times, and found that the effects of TGF-β and 17AAG on collagen synthesis were reproducible and statistically significant (Fig. 2C). We also performed an immunofluorescence assay, and evaluated the effects of TGF-β and 17AAG on the amount and expression pattern of collagen synthesis. As in the Western blot assay, TGF-β induced the expression of collagen, and 17AAG decreased collagen synthesis induced by TGF-β, although it was not statistically significant. (Fig. 2D, E).

A



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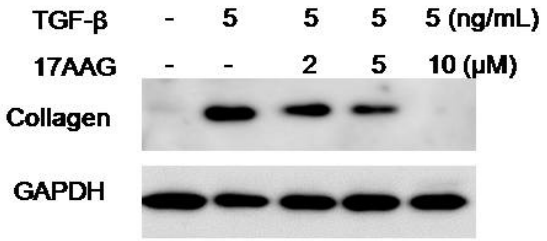

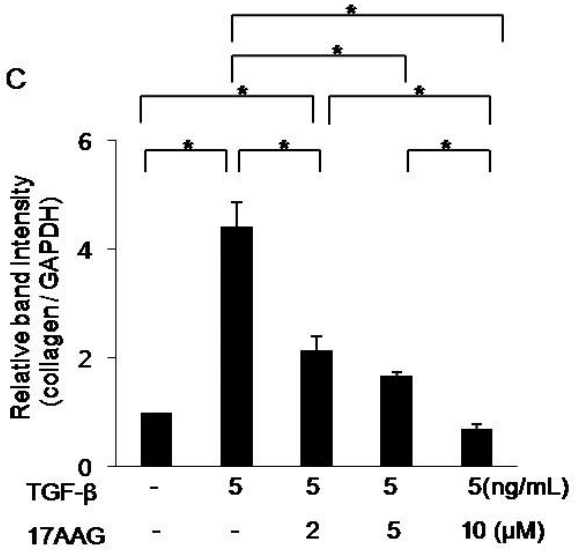

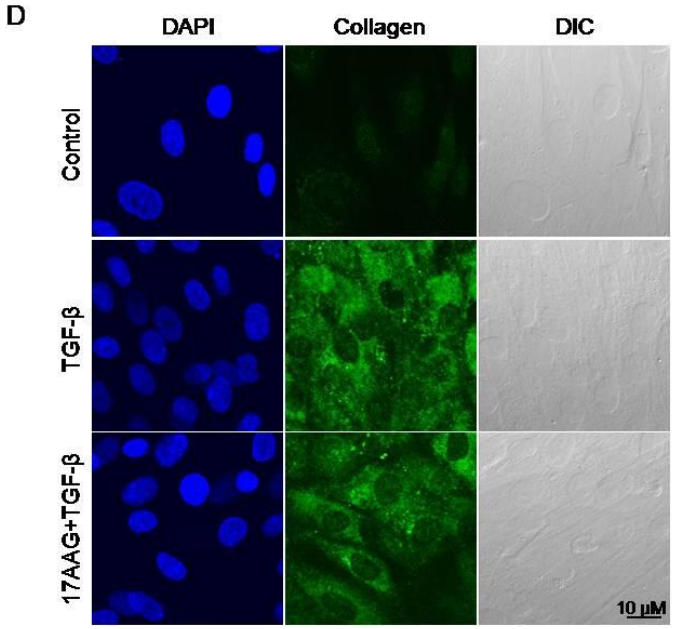

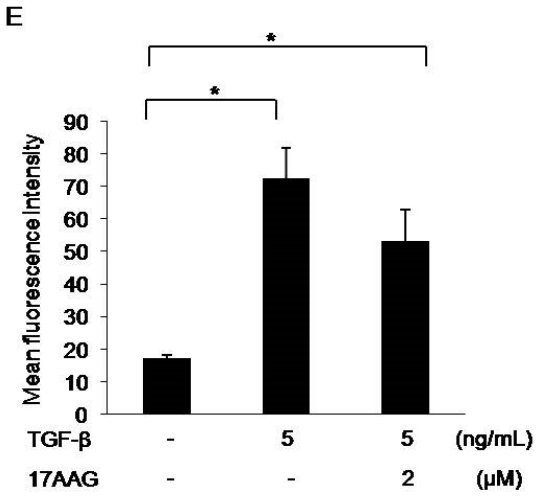

Figure 2. The effect of Hsp 90 inhibition on TGF-β induced collagen synthesis

(A) Real-time PCR assay was performed to evaluate the effect of 17AAG on TGF-β induced collagen synthesis in human dermal fibroblasts. The amount of collagen transcription was compared after TGF-β treatment (5 ng/mL), with and without 17AAG (up to 10μM) pretreatment. Independent experiments were replicated three times. 17AAG inhibited TGF-β induced collagen synthesis at RNA level. (B) The amount of collagen protein was compared after TGF-β (5 ng/mL) treatment, with and without 17AAG (up to 10μM) pretreatment, by Western blot assay. 17AAG inhibited TGF-β induced collagen synthesis at protein level. (C) The relative band intensity of collagen was calculated after three independent experiments. (D) An immunofluorescence assay was performed to evaluate the expression of collagen after TGF-β (5 ng/mL) treatment, with and without 17AAG (2μM) pretreatment. 17AAG inhibited TGF-β induced collagen synthesis. (E) Three independent experiments were performed and fluorescence was compared.(*$p<0.05$)

3. Overexpression of Hsp 90 upregulates TGF-β induced collagen synthesis

As inhibition of Hsp 90 influenced the expression of TGF-β induced collagen synthesis, we sought to upregulate the level of Hsp 90 and evaluate its effect on collagen synthesis. When we transfected HA-tagged Hsp 90 plasmids into human dermal fibroblast, overexpression of Hsp 90 itself caused upregulation of collagen synthesis at RNA and protein levels, and it was statistically significant by Real-time PCR and Western blot assay (Fig. 3A, B). And overexpression of Hsp 90 further increased TGF-β induced collagen synthesis both at the RNA and protein levels (Fig. 3A, B). We performed these experiments three times, and found that the effect of overexpression of Hsp 90 on TGF-β induced collagen synthesis was statistically significant (Fig. 3C). An immunofluorescence assay also showed that overexpression of Hsp 90 further increased TGF-β induced collagen synthesis (Fig. 3D), although we could not find statistical significance after three times of independent experiments (Fig. 3E).

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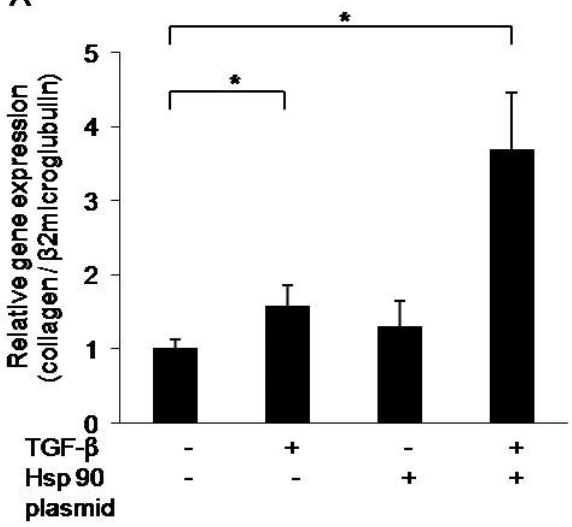

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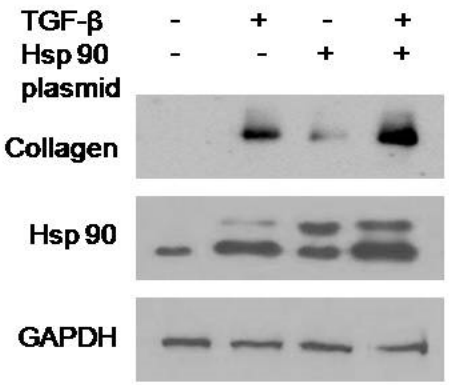

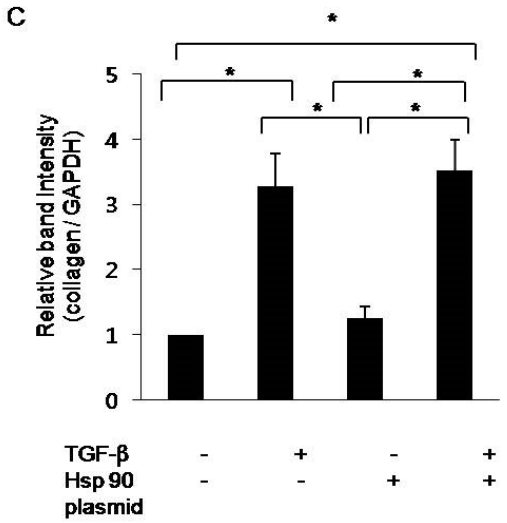

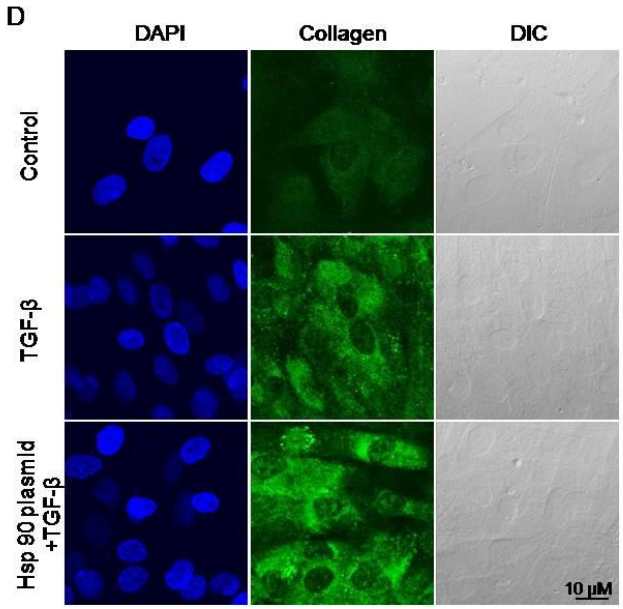

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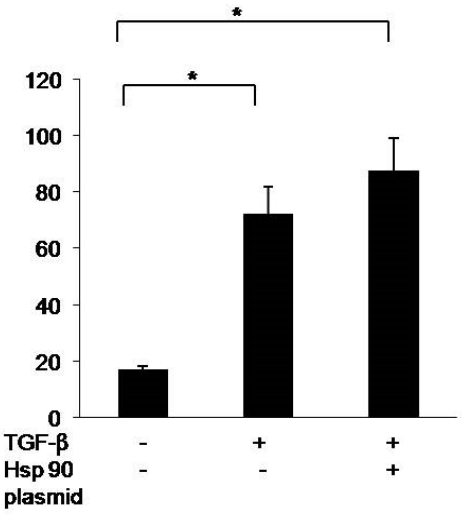


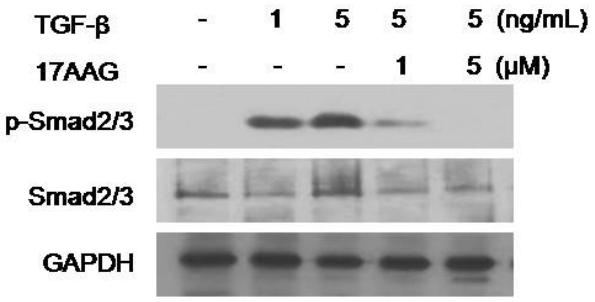
Figure 3. The effect of Hsp 90 overexpression on TGF-β induced collagen synthesis

(A) Human dermal fibroblasts were transfected with HA-tagged Hsp 90 plasmids and a real-time PCR assay was performed. The amount of collagen transcription was compared after TGF-β (5 ng/mL) treatment, with and without overexpression of Hsp 90. Independent experiments were performed three times. Overexpression of Hsp 90 upregulated TGF-β induced collagen synthesis at RNA level. (B) The amount of collagen protein was compared after TGF-β (5 ng/mL) treatment, with and without overexpression of Hsp 90, by Western blot assay. Overexpression of Hsp 90 upregulated TGF-β induced collagen synthesis at protein level. (C) The relative band intensity of collagen was calculated after three independent experiments. (D) An immunofluorescence assay was performed to evaluate the expression of collagen after TGF-β (5 ng/mL) treatment, with and without overexpression of Hsp 90. Overexpression of Hsp 90 upregulated TGF-β induced collagen synthesis. (E) Three independent experiments were performed and fluorescence was compared. (*$p<0.05$)

4. 17AAG inhibits activation of TGF-β induced Smad pathway

We next examined the effect of 17AAG on the Smad pathway, which is known to be involved in TGF-β induced collagen synthesis. As shown in Fig. 4, treatment with TGF-β induced phosphorylation of Smad 2/3, and pretreatment with 17AAG inhibited TGF-β induced phosphorylation of Smad 2/3 in a dose-dependent and statistically significant manner (Fig. 4A, B). In addition to the Smad pathway, the Akt pathway was also activated by treatment with TGF-β, as shown by increased phospho-Akt expression (Fig. 4C, D). 17AAG inhibited TGF-β induced phosphorylation of Akt, however, pretreatment with 17AAG also decreased the total amount of Akt (Fig. 4C).

A



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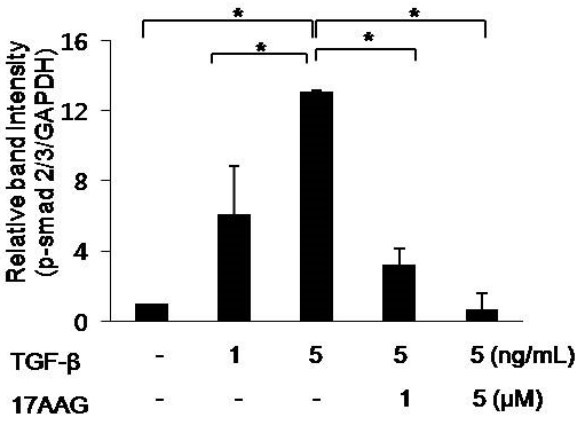

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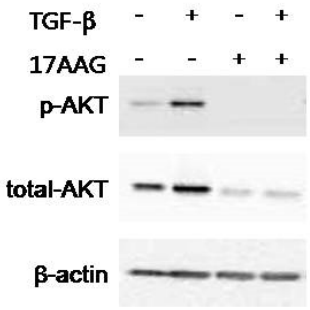

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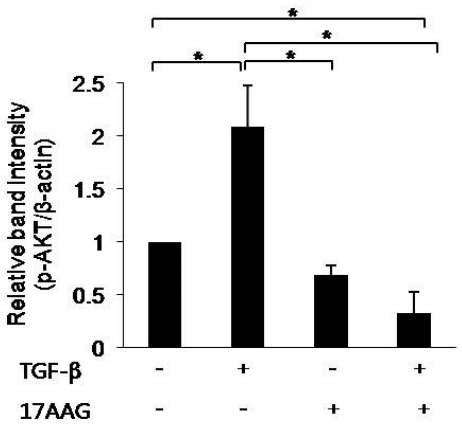


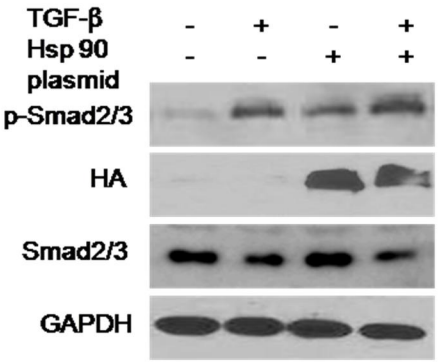
Figure 4. The effect of Hsp 90 inhibition on TGF-β induced Smad pathway

(A) Human dermal fibroblasts were pre-treated with 17AAG (2 μM and 5 μM) and a Western blot assay was performed. Expression of phospho-Smad 2/3 and total Smad 2/3 were measured after treatment with TGF-β (2 ng/mL and 5 ng/mL), with and without 17AAG pretreatment. 17AAG inhibited TGF-β induced Smad 2/3 phosphorylation. GAPDH was used as a loading control. (B) The relative band intensity of phospho-Smad 2/3 was calculated after three independent experiments. (C) Expression of phospho-Akt and total Akt were measured after treatment with TGF-β (5 ng/mL), with and without 17AAG (5 ng/mL) pretreatment. 17AAG inhibited TGF-β induced phosphorylation of Akt, however, pretreatment with 17AAG also decreased the total amount of Akt. β-actin was used as a loading control. (D) The relative band intensity of phospho-Akt was calculated after three independent experiments. (*$p<0.05$)

5. Overexpression of Hsp 90 induces activation of TGF-β induced Smad pathway

We also evaluated the effect of Hsp 90 overexpression on the Smad pathway. As shown in Fig.5, transfection of Hsp 90 induced phosphorylation of Smad 2/3, and the combination of TGF-β with overexpression of Hsp 90 further increased the phosphorylation of Smad 2/3. Both these effects were statistically significant (Fig. 5A, B).

A



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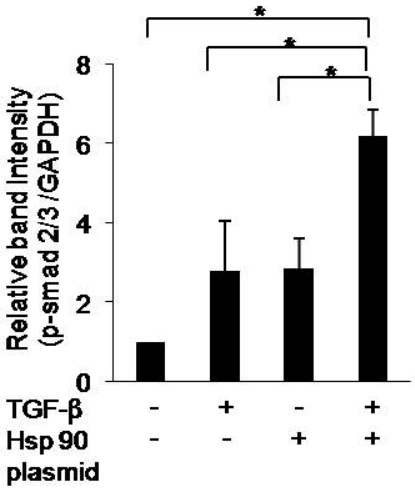

Figure 5. The effect of Hsp 90 overexpression on TGF-β induced Smad pathway

(A) Human dermal fibroblasts were transfected with HA-tagged Hsp 90 plasmids and a Western blot assay was performed. Expression of phospho-Smad 2/3, total Smad 2/3 and HA, after treatment with TGF- β (5 ng/mL), with and without overexpression of Hsp 90, was compared. Overexpression of Hsp 90 increases TGF-β induced Smad 2/3 phosphorylation. GAPDH was used as a loading control. (B) The relative band intensity of phospho-Smad 2/3 was calculated after three independent experiments. (*$p<0.05$)

6. Hsp 90 expression and collagen deposition are increased in human keloid tissues

Finally, we measured the expression level of Hsp 90 and collagen deposition in keloid tissues, which are characterized by abnormal fibroblast proliferation and excessive ECM synthesis. By immunohistochemical staining, we found that expression of Hsp 90 was increased in keloid tissues compared with normal tissues, as was collagen deposition (Fig. 6A, B).

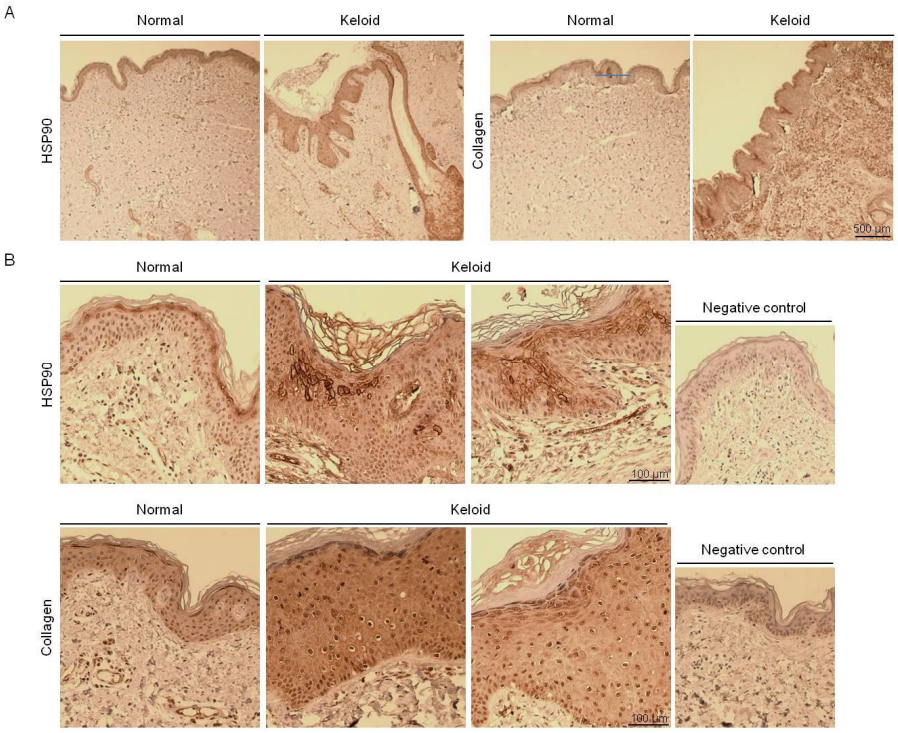

Figure 6. Histologic analysis of keloid and normal tissues

An immunohistochemistry assay was performed to evaluate the expression of Hsp 90 and collagen in keloid and normal skin tissues. The expression of both Hsp 90 and collagen was increased in keloid tissues compared with normal tissues. Images were taken under light microscopy (A: 4× magnification, B:20× magnification).

IV. DISCUSSION

Hsps interact with non-native proteins under various stressful conditions, prevent abnormal protein folding, and aid in proper protein folding.[10,11,12] Hsps are also involved in modulation of antigen presentation and regulation of signal molecules such as kinases, and can act as transcription factors.[13,14] Recently, it was shown that various Hsps are involved in the wound healing process.[1] For example, Hsp 47 is an ER-resident molecular chaperone that specifically binds to procollagen, preventing its aggregation and the formation of procollagen bundles, which promotes wound healing.[15] In contrast with Hsp 47, Hsp 70 attenuates the epithelial-to-mesenchymal transition, and inhibits Smad 3 activation, thereby suppressing wound healing.[15] It has been shown that Hsp 90 is involved in the fibrosis pathway through stabilization of TGF-β receptors and Src kinases, which are important in the intracellular fibrosis process.[16] As with previous studies, we found that Hsp 90 was involved in fibrogenesis through modulation of TGF-β induced collagen synthesis through another mechanism.

TGF-β expression and activity are increased in all kinds of skin injuries such as acidic or alkali conditions, heat denaturation, shear stress or surgical incision. TGF-β plays a critical role in regulating multiple cellular responses that occur in all three phases of skin wound healing, inflammation, proliferation, and remodeling. In the proliferation phase, TGF-β promotes wound closure and resolution by making fibroblast transdifferentiate into

myofibroblast and produce ECM including collagen. Manipulations of TGF-β have been used to improve wound healing and reduce scarring.[1,17] This is why we evaluated the effect of Hsp90 on TGF-β induced collagen synthesis.

Hsp 90 is one of several stress proteins, and its expression and activity are upregulated under stressful conditions.[7] However, we found that pretreatment with 17AAG alone slightly decreased the expression of collagen (Fig. 2), suggesting that endogenous Hsp 90 could play a role in collagen synthesis even in stress-free conditions. We also found that unidentified finding that overexpression of Hsp 90 itself increased the synthesis of collagen (Fig. 3). We also have found that overexpression of Hsp 90 itself activated Smad pathway increasing phospho-Smad 2/3 (Fig. 4), and to our knowledge, this is the first study to report the effects of overexpressed Hsp 90 level on collagen synthesis, and Smad pathway. Our finding further supports that modulation of Hsp 90 is a key process in dermal fibrosis.

Unexpectedly, western blot analysis showed that treatment with TGF-β mildly increased expression of Hsp 90 (Fig. 3B). This suggests that Hsp90 and TGF-β act synergistically, via interactive mechanisms, and further study of the effects of TGF-β on Hsp 90 is an interesting avenue for future research.

Similarly to previous studies that proved the involvement of Smad pathway in TGF-β induced collagen synthesis, we found that inhibition of Hsp 90 function abolished TGF-β induced phosphorylation of Smad 2/3 and affected collagen synthesis. In addition, we found that increased Hsp 90 expression

affects TGF-β induced phosphorylation of Smad 2/3, which has not previously been reported to our knowledge. Previous work has shown that Hsp 90 also modulates non-Smad pathways such as the Akt and glycogen synthase kinase pathways.[18] In our study, inhibition of Hsp 90 after pretreatment with 17AAG decreased total Akt protein expression, although Hsp 90's effect on phospho-Akt, which represents the activated Akt signal pathway, was not clear. We are aware of another study reporting, in accordance with our results, that treatment with 17AAG decreased Akt protein expression, although that report did not distinguish between phosphorylated and total Akt.[19] As in that study, we are unable to explain the fact that 17AAG decreased the expression of total Akt, and further studies are required to investigate this issue.

Although Hsps are usually located intracellularly, they also can be found extracellularly, and their functions in these regions can differ. For example, it has been found that the fibrogenesis-related functions of extracellular Hsp 70 are different from those of intracellular Hsp 70.[20] In our study, we evaluated the intracellular function of Hsp 90 in fibrogenesis and did not investigate the corresponding extracellular functions of Hsp 90. There is a possibility that extracellular Hsp 90 might affect dermal fibroblasts in a different manner.

There is a previous study reporting that treatment with 17AAG induces apoptosis.[19] However, 17AAG did not influence the viability of normal human dermal fibroblasts at 5 μM for12 hours in our study, and an important

difference between the conditions in the present study and that study is the duration of treatment. Interestingly, another study found that a low dose of 17AAG protects neural progenitor cells from ischemia-induced cell death.[21] These confounding data suggest that the function of 17AAG depends on concentration, treatment duration, and type of target cells.

Pharmacologic inhibition of Hsp 90 usually interferes with the adenosine triphosphate-dependent association with co-chaperones, which facilitate client protein stabilization.[22] 17AAG, a synthetic analogue of geldanamycin, which is a naturally occurring Hsp 90 inhibitor, also inhibits Hsp 90 function by this mechanism and is under clinical trials. Other second generation Hsp90 inhibitors, such as alvespimycin, have also been evaluated for the management of various tumors, and more than 10 Hsp 90 inhibitors are currently under evaluation for clinical cancer treatment. Several Hsp 90 inhibitors are very close to use in the clinic for treatment of fibrotic diseases.[23]

A previous study on the overexpression of Hsps indicates that both a proliferative (Hsp 70) and a matrix synthesis (Hsp 47, Hsp 27) component are present in keloid tissue.[24] In that study, the expression of Hsp 90 was not different between keloid and normal tissue samples. However, in our immunohistochemical staining assay, we found that Hsp 90 was highly expressed in keloid tissue, both in epithelial and subepithelial areas. In our in vitro experiments, we found that overexpression of Hsp 90 induced collagen synthesis in human dermal fibroblasts. These findings suggest that

overexpression of Hsp 90 might be one of the mechanisms involved in keloid pathogenesis, and further studies should be performed on the role of Hsp 90 in keloid formation.

V. CONCLUSION

In this study, we demonstrated that TGF-β induced collagen synthesis in human dermal fibroblasts is inhibited by 17AAG and increased by overexpression of Hsp 90, and that these changes occur through modulation of Smad 2/3 phosphorylation (Fig. 7). Furthermore, we found that the expression of Hsp 90 was greatly increased in keloid tissue compared with normal tissue. These findings suggest that Hsp 90 plays a role in skin wound healing and fibrosis in the dermis.

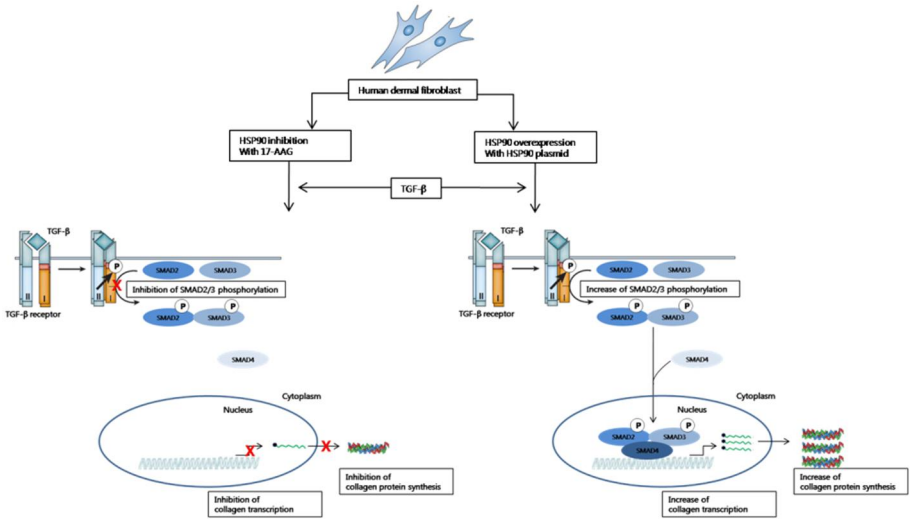

Figure 7. The effect of Hsp 90 on TGF-β induced collagen synthesis

Hsp 90 regulates TGF-β induced collagen synthesis through modulation of Smad 2/3 phosphorylation. Hsp 90 inhibition decreases TGF-β induced collagen synthesis through inhibition of Smad 2/3 phosphorylation. Hsp 90 overexpresion increases TGF-β induced collagen synthesis through induction of Smad 2/3 phosphorylation.

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ABSTRACT (IN KOREAN)

Heat shock protein 90이 인간 진피 섬유모세포의 TGF-β 유도 콜라겐 합성에 미치는 영향

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Heat shock protein (Hsp)은 다양한 스트레스 상황에서 비고유 단백질과 상호작용하여 비정상적인 단백질 folding (protein folding)을 방지하고 정상적인 단백질 folding을 도와준다. 최근에 다양한 Hsp가 창상 치유 과정에 관련되어 있다고 알려졌다. Hsp 90은 스트레스 상황에서 발현과 활성도가 증가되는 스트레스 단백질의 하나이다. Hsp 90은 세포내 섬유화 과정에서 중요한 역학을 하는 TGF-β

수용체와 Src 키나아제를 안정화 함으로써 섬유화 경로에 관련되어 있음이 밝혀졌다. 본 연구에서는 인간 진피 섬유모세포의 TGF-β 유도 콜라겐 합성에 있어서 Hsp 90의 역할을 밝히고자 하였다.

인간 진피 섬유 모세포를 배양한 후 TGF-β 처치, 17AAG 전처치를 통한 Hsp 90 억제, Hsp90 플라스미드 트랜스펙션을 통한 Hsp 90 과발현이 세포 생존력 (cell viability)에 미치는 영향을 평가하였다. Hsp 90의 억제와 과발현이 콜라겐 합성에 미치는 영향을 평가하기 위해 Real-time PCR, Western blot, 면역 형광측정을 시행하였다. Western blot을 시행하여 Smad 2/3, Akt 신호 전달 경로를 조사하여 어떤 신호 전달 경로를 통해 Hsp 90이 TGF-β 유도 콜라겐 합성에 영향을 주는지 확인하였다.

결과에서 TGF-β 처치는 RNA 수준과 단백질 수준 모두에서 콜라젠 발현을 증가시켰다. 17AAG 전처치로 인한 Hsp 90의 억제는 TGF-β 유도 콜라젠 합성을 억제시켰다. Hsp 90 플라스미드 트랜스펙션을 통한 Hsp 90의 과발현은 TGF-β 유도 콜라젠 합성을 증가시켰다. 17AAG 전처치로 인한 Hsp

90 억제는 Smad 2/3 인산화를 억제시켰고 Hsp 90의 과발현은 Smad 2/3 인산화를 증가 시켰다. 면역조직화학염색을 통해 Hsp 90의 발현이 정상 피부 조직보다 켈로이드 조직에서 증가되어 있음을 확인하였다.

결론적으로 본 연구에서 TGF-β 유도 콜라젠 합성은 Hsp 90의 억제에 의해서 감소되고 Hsp 90의 과발현에 의해 증가되며 이러한 변화는 Smad 2/3 인산화의 조정(modulation)을 통해서 일어남을 밝혔다. 또한 Hsp 90은 켈로이드 조직에서 발현이 증가되어 있음을 확인했다. 이는 Hsp 90이 진피에서 창상 치유와 섬유화에 중요한 역할을 하고 있음을 제시해 준다.

핵심되는 말: Hsp 90, 17AAG, Hsp 90 과발현, 콜라겐 합성