The role of heat shock protein 70 in mouse contact hypersensitivity model

Jin Mo Park

Department of Medicine

The Graduate School, Yonsei University

The role of heat shock protein 70 in mouse contact hypersensitivity model

Directed by Professor Min-Geol Lee

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Jin Mo Park

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This certifies that the Doctoral Dissertation of Jin Mo Park is approved.

Thesis Supervisor: Min-Geol Lee

Thesis Committee Member: In Hong Choi

Thesis Committee Member: Jung-Won Park

Thesis Committee Member: Hyunah Lee

Thesis Committee Member: Hyoung-Pyo Kim

The Graduate School

Yonsei University

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ABSTRACT

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Allergic contact dermatitis (ACD) is one of the most common skin diseases through contact hypersensitivity (CHS). In CHS, multiple cells, inflammatory mediators, and cytokines are known to be involved to regulate the immune response. In previous study, I revealed the reactive oxygen species (ROS) generation by 2, 4, 6-trinitrobenzene sulfomic acid (TNBS) in mouse dendritic cells, XS-106 cells, followed by ATP synthase carbonylation. Also I identified the carbonylation of heat shock protein 70 (Hsp70) and exogenous antioxidant role of protein transduction domain fused Hsp70 (PTD-Hsp70) which lowered ROS and IL-12 generation in TNBS treated XS-106 cells. In this study, I investigated the role of Hsp70 in contact hypersensitivity (CHS) using PTD-Hsp70 in BALB/c mice. PTD-Hsp70 pretreatment: (i) suppressed ear swelling; (ii) down-regulated phosphorylated p38 but up-regulated phosphorylated extracellular signal regulated kinase; (iii) increased the populations of $CD4^+CD25^+$ T cells and $CD4^+CD25^+Foxp3^+$ T cells; (iv) decreased the secretion of tumor necrosis factor-α (TNF-α), interlukin (IL)-12, interferon-γ and IL-2 but up-regulated IL-4, IL-17, IL-23 and transforming growth factor beta (TGF-β) at challenge phase in drainage lymph node (dLN); (v) down-regulated IL-6 and IL-12 but up-regulated IL-4, IL-23 and TGF-β at challenge phase in ear tissue. This experiment suggests that PTD-Hsp70 attenuates contact hypersensitivity. In summary, PTD-Hsp70 (i) regulates the mitogen activated

protein kinase (MAPK) pathway which in turn prevents inflammation and apoptosis; (ii) down-regulates primary inflammatory cytokines and Th1 cell differentiation; (iii) regulates through Th2 mediated cytokines; (iv) increases TGF-β and regulatory T cells; and (v) may deprived pathognomic role of Th17 related to inflammation.

In conclusion, topical application of PTD-Hsp70 attenuates CHS through MAPK pathway and regulating Th1 cytokines, Th2 cytokines, and regulatory T cells.

Key words: allergic contact dermatitis, BALB/c, contact hypersensitivity, heat shock protein 70, reactive oxygen species, Th17, TNBS

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I. INTRODUCTION

Allergic contact dermatitis (ACD) is one of the common skin diseases, which results in great socioeconomic consequences for patients[1,2]. Although the pathogenesis of ACD has not been fully elucidated, it is regarded as contact hypersensitivity (CHS) by Th1 cells[3]. The mechanism of CHS is hapten specific T cell mediated inflammation, divided into a sensitization phase and an elicitation phase[4].

Recently, a few reports have revealed the generation of reactive oxygen species (ROS) in CHS by dendritic cells (DCs) and interferon gamma (IFN-γ) during antigen presentation[5, 6]. Previously, the ROS generation by 2, 4, 6-trinitrobenzene sulfomic acid (TNBS) in XS-106 DCs followed by ATP synthase carbonylation was demostrated[7]. Furthermore, I verified heat shock protein 70 (Hsp70) carbonylation and an antioxidant effect of protein transduction domain fused Hsp70 (PTD-Hsp70), which lowered ROS in TNBS-treated XS-106 DCs.

PTDs are short cationic peptides that endow the transduction of large molecules into eukaryotic cells[8]. Human polyhomeotic homology-1 (Hph-1)-Hsp70 was engineered for PTD-Hsp70, and PTD-Hsp70 showed its therapeutic effects without immunogenicity in various cells and tissues[9, 10].

Heat shock protein (Hsp) is a highly conserved protein that is expressed under the conditions of various physiological and environmental insults. Hsps are ubiquitously expressed in all organisms, and they are classified into subgroups by their molecular

weight (table 1)[11].

Table. 1. Cellular localization and function of the major HSP families in mammals.

Family	HSP	Location	Function
HSP10	Ubiquitin	Cytosol / nucleus	Tag protein for degradation
	HSP10	Mitochondria	Cofactor of HSP60, tolerance of ischemia
HSP40	HSP47	ER	Heat inducible, procollagen chaperone
	Hdj1 Hdj2	Cytosol / nucleus	Cofactor of HSP70, increase ATPase activity and substrate release
HSP60	HSP56	Cytosol	Stress inducible, bind the steroid hormone receptor complex and FK506, rotamase function
	HSP60	Mitochondria	Constitutive/inducible, weak ATPase activity, binding and folding of imported proteins, tolerance of hyperthermia and ischemia
	HSP65		Antitumorigenic action
	TCP-1	Cytosol/nucleus	Constitutive, weak ATPase activity, folding of actin and tubulin
HSP70	HSP70/HSP72	Cytosol/nucleus	Stress inducible, ATPase activity, tolerance of hyperthermia, ischemia/hypoxia, resistance to oxidative, UV and TNF stresses, protection against protein aggregation, regulation of HS response, protection of transcription/translation, tumorigenicity, antiapoptotic
	HSP73/HSC70	Cytosol/nucleus	Constitutive, ATPase activity, folding, trafficking, tolerance of hyperthermia, promote lysosomal degradation
	Grp75	Mitochondria	Constitutive/inducible, ATPase activity, transport and folding of polypeptides into matrix
	Grp78 (BiP)	ER	Constitutive/inducible, ATPase activity, protein secretion and translocation and degradation into ER
HSP90	HSP90 α/β	Cytosol/nucleus	Partly inducible, ATPase activity, autophosphorylation, tolerance of hyperthermia, ischemia, apoptosis, role in cell cycle and proliferation and in signal transduction, prevents aggregation
	Grp94	ER	Constitutive/inducible, ATPase activity, protein folding and secretion

In stress conditions, Hsp acts as a molecular chaperone by assisting with correct folding of nascent and misfolded proteins, preventing protein aggregation or promoting of selective degradation of misfolded or denatured proteins[12, 13]. Hsp70 with a size ranging from 68 to 75 kDa, is mostly induced under a variety of stress conditions[14]. This highly conserved protein affects cell survival by interacting with various components both upstream and downstream of mitochondrial events[15]. As a chaperone, Hsp70 serves an important housekeeping role in various diseases. Hsp70 involves in anti-inflammation by inhibiting apoptosis pathways. Recently, the anti-inflammatory effects of Hsp70 were verified in numerous diseases, including rheumatoid arthritis, cerebral ischemia, and gastric mucosa disease[16-18]. Another interesting function of Hsp70 is related to immune stimulation. Hsp70 up-regulates regulatory T cells (Treg) for specific antigens and helps immature DCs to

differentiate into mature DCs[19, 20]. However, inflammation control by Hsp70 is still controversial.

A recent theory on Th17 cells, which secrete interlukin (IL)-17, suggests an association with many inflammatory dermatoses. These include allergic contact dermatitis, atopic dermatitis, psoriasis, scleroderma, and so on[21]. Some reports have described the importance of IL-17 both in the sensitization and elicitation phases of CHS[22, 23].

So far, only a limited numbers of paper have presented the association between Hsp70 and CHS in which Hsp70 augments the CHS[24]. However, in this study, I observed the regression of CHS induced by 2,4,6-trinitro-1-chlorobenzene (TNCB) in BALB/c mice when PTD-Hsp70 was used, which is in contrast with other previous studies.

Herein, I report this newly discovered effects of Hsp70 on CHS and describe how Hsp70 regulates CHS associated cytokines, including Th17 cell cytokines.

II. Materials and Methods

1. Animals and reagents

A. Mice

Four-week-old female BALB/c mice were purchased from an animal center (Laboratory Animal Center Inc, Shizuoka, Japan). All animals were healthy and housed in an air conditioned animal room at 23℃ and a relative humidity of 55%, and fed a laboratory diet and water. All animal experiments were approved by the Animal Study Committee of the Yonsei University, Severance Hospital.

The experimental groups were divided as follows:

Group 1 (Control group): No pretreatment with TNCB or PTD-Hsp70 (*n=15*)

Group 2 (TNCB only): Sensitization and elicitation with TNCB (*n=15*)

Group 3 (S. Hsp70): PTD-Hsp70 pretreatment before sensitizing with TNCB (*n=15*)

Group 4 (C. Hsp70): PTD-Hsp70 pretreatment before challenging with TNCB (*n=15*)

B. Reagents

TNCB as a hapten was purchased from Sigma (Deisenhofen, Germany). PTD-Hsp70, Hph-1-Hsp70 fusion protein, was provided by Professor Sang-Kyou Lee (Yonsei University, Seoul, Korea). In brief, BL21 (DE3) *Escherichia coli* (Molecular Probes Inc, Eugene, OR, USA) transformed with plasmids encoding the Hph-1-Hsp70 and Hsp70 were induced for 4 hrs at 37℃ with 1 mM isopropyl-β-D-thiogalactopyranoside and sonicated in lysis buffer (pH 8.0) containing 50 mM NaH_2PO_4, 10 mM imidazole, and 300 mM NaCl. Cellular lysates were resolved by centrifugation at 12,000 rpm for 20 mins at 4℃ and loaded on Ni-NTA columns (Qiagen**,** GmbH, Germany). Bound proteins were washed and eluted with 50, 100, 250 mM, or 3 M imidazole with > 95% purity. The eluted proteins were desalted using PD-10 Sephadex G-25 (Amersham Pharmacia, Piscataway, NJ, USA), supplemented with 10% glycerol, aliquoted, and flash-frozen at -80℃.

2. Stimulation with TNCB and measurement of ear swelling

Abdominal hair was removed from the mice before the sensitization phase. In brief, I painted the shaved abdomen of naïve mice with a mixture of 100 μl of 3% TNCB in acetone and olive oil in a ratio of 4:1 at day 0. To elicit CHS, one ear of each mouse was painted with 10 μl of 1% TNCB on both sides 6 days after sensitization. After 24 hrs, each two researchers measured the swelling of TNCB-exposed ears using a dial thickness gauge (Mitutoyo Corporation, Tokyo, Japan), and compared the results with other groups to evaluate systemic CHS.

3. Application of PTD-Hsp70

1 hr before sensitizing with TNCB, 2 μM of Hph-1-Hsp70 was epicutaneously applied on the shaved abdomen, and the spot was covered for 2 hrs with Tegaderm (3M, St. Paul, MN, USA) in group 2. In group 3, 2 μM of Hph-1-Hsp70 was epicutaneously applied on the right ear 1 hr before challenging with TNCB, and the spot was covered for 2 hrs with Tegaderm.

4. Immunohistochemistry of ear tissue

I confirmed local administration of PTD-Hsp70 on mice ear tissue using the HistoMouse MAX Kit (Invitorgen, Carlsbad, CA, USA). Before the staining, I applied 2 μl of PTD-Hsp70 on the ear tissue of BALB/c mice for 30 mins, 1 hr, 2 hrs, 4 hrs, 8 hrs and 16 hrs. Acetone-fixed 6 μm cryostat section were incubated in 3% H_2O_2 buffer for 10 mins before incubation in 10% normal goat serum to block non-specific binding. After blocking, the sections were incubated in anti-mouse Hsp70 antibody at 4^oC overnight. Biotinylated secondary antibody was then used to detect the primary antibodies, and the sections were incubated in peroxidase-conjugated streptavidin for 1 hr at room temperature. Peroxidase activation was visualized with 3-amino-9-ehtylcarbazole (AEC) solution. As negative controls, the primary antibodies were substituted with PBS.

5. Western blot analysis of P-p38 and P-ERK in ear tissue

Western blotting was carried out with 30 μg of protein obtained from whole cell lysates of ear tissues. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA, USA) and detected using ECL (Amersham Biosciences, Piscataway, NJ, USA). The anti-DNP (Cell Signaling, Beverley, MA, USA), anti-Hsp70, and phospholyated-p38(P-p38) mitogen activated protein kinase (MAPK) antibodies (Sigma-Aldrich, St. Louis, MO, USA) were used at a dilution of 1:5000. An anti-actin antibody (Sigma-Aldrich) was used as a loading control at 1:5000. The secondary antibodies for immunoblot analysis were anti-mouse IgG–horseradish peroxidase (HRP) and anti-rabbit IgG–HRP conjugates. Proteins were visualized by enhanced chemiluminescence, according to the manufacturer's instructions (Amersham Biosciences).

6. Flow cytometric analysis of drainage lymph node

I analyzed $CD4^+CD25^+$ T cells and $CD4^+CD25^+Foxp3^+$ T cells in the lymph node (LN) by a FACSCalibur (Becton Dickinson, Mountain View, CA USA). For each treatment group, LNs were pooled and single cell suspensions were prepared by gently mashing the nodes on a nylon mesh filter (100 μm pore size; The Spectra Company, Los Angeles, CA, USA). The cell suspensions were washed once with PBS and re-suspended in FACS buffer containing 10 mM HEPES buffer (Sigma-Aldrich), 0.01% sodium azide (Sigma-Aldrich), and 1% heat inactivated fetal bovine serum (Gibco BRL Life Technologies, Grand Island, NY, USA) at 10^7 cells ml^{-1}. The cells were then incubated with antibodies on ice for 30 mins, washed twice, and resuspended in FACS buffer at 10^4 cells ml^{-1}. The antibodies used were rat anti-CD4 (Gibco BRL Life Technologies), rat anti-CD25 (Pharmigen, San Diego, CA, USA) and rat anti Foxp3 (Pharmigen).

7. ELISA study for cytokine detection in supernatants

Mice were sacrificed 24 hrs after sensitization and elicitation. Then Skin drainage LNs from cervical LNs and individual mouse ear was collected. Briefly LNs were gently disrupted by rubbing between the ends of two microscope slides and then

LNs were washed with PBS and re-suspended in complete RPMI 1640 medium. Ear tissue was homogenized in 0.5 mL of phosphate buffered saline with protease inhibitor (Roche Diagnostics, Basel, Switzerland). Homogenized ear tissue was centrifuged for 30 mins at 18770 g at 4℃. Cytokines were measured in the supernatants of lymph nodes and ear tissue by ELISA, using match-paired specific antibodies against IL-4, IL-6, IL-10, IL-12, IL-17, IL-22, IL-23, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and transforming growth factor (TGF)-β according to the manufacturer's protocol (BD Biosciences, San Jose, CA, USA).

8. Statistical analysis

SAS 9.1 (SAS Institute Inc, USA) program was used for statistical analysis of the data. Each group was compared to other groups using Bonferroni's method.

III. RESULTS

1. Transduction of PTD-Hsp70 into ear tissue by topical application

I presented intracellular delivery of PTD-Hsp70 into XS-106 DCs in our previous study[7]. Furthermore, other reports verified transduction of Hph-1-PTD into various tissues[9]. PTD-Hsp70 and Hsp70 were applied on the ear of each mouse for 1 hr. I tested whether Hph-1-PTD could transfer Hsp70 into the mice skin after 30 mins, 1 hr, 2 hrs, 4 hrs, 8 hrs and 16 hrs. PTD-HspP70 was successfully delivered to the dermis of ears treated with PTD-Hsp70 at all time points as compared to the ears of control mice which was treated with Hsp70 (Fig. 1).

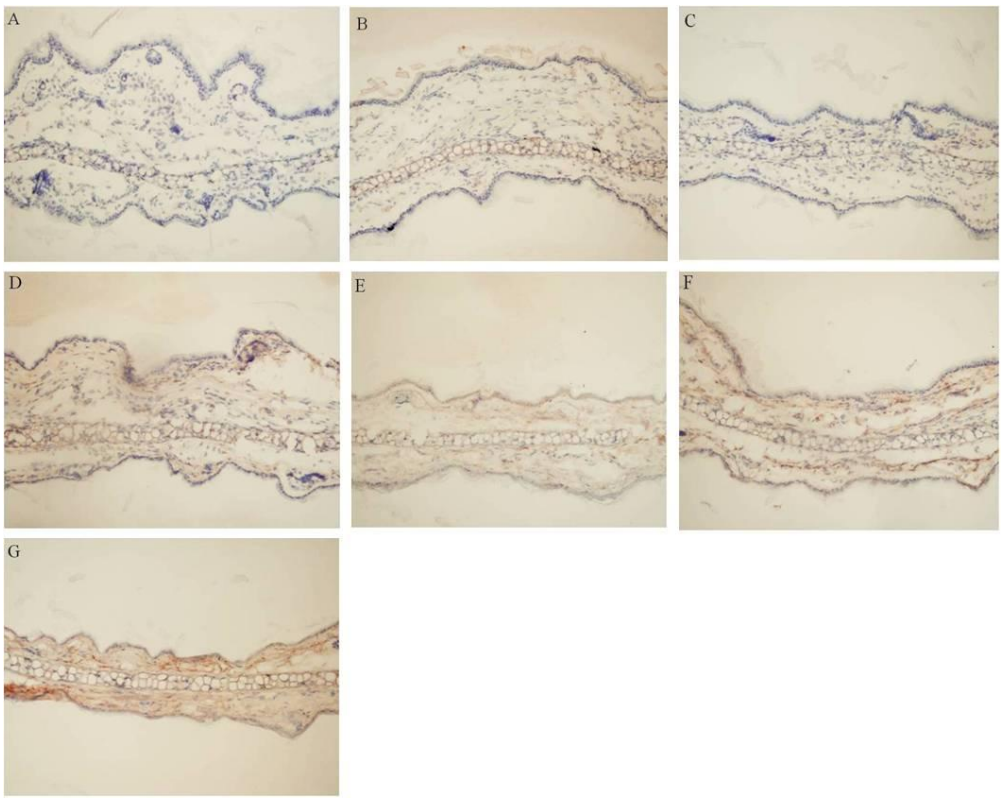


Fig. 1. Penetration of PTD-Hsp70 into ear tissue. Ear tissue of control mice treated with Hsp70 (A), PTD-Hsp70 treated ear tissues 30 mins after treatment (B) 1 hr (C) 2 hrs (D), 4 hrs (E), 8 hrs (F) and 16 hrs (G). PTD-Hsp70 penetrated successfully the dermis (red to brown colored), as compared to control mice.

2. Systemic suppression of CHS by topical application of PTD-Hsp70

I evaluated the suppression of TNCB-induced CHS in PTD-Hsp70 pretreated mice by measuring the ear swelling response. The TNCB only group showed 53.3 mm thickness ear swelling. But PTD-Hsp70 pretreatment decreased the ear swelling. PTD-Hsp70 pretreatment before sensitization (S. Hsp70) decreased ear swelling from 53.3 mm to 44.7 mm compared to the TNCB only group (TNCB). Also PTD-Hsp70 pretreatment before elicitation (C. Hsp70) showed significantly less swelling response form 53.3 mm to 33.8 mm compared to the TNCB only group (Fig. 2). PTD-Hsp70 pretreatment before elicitation more effectively lessen the ear swelling than that of sensitization (Table. 2, n=15, $p<0.001$).

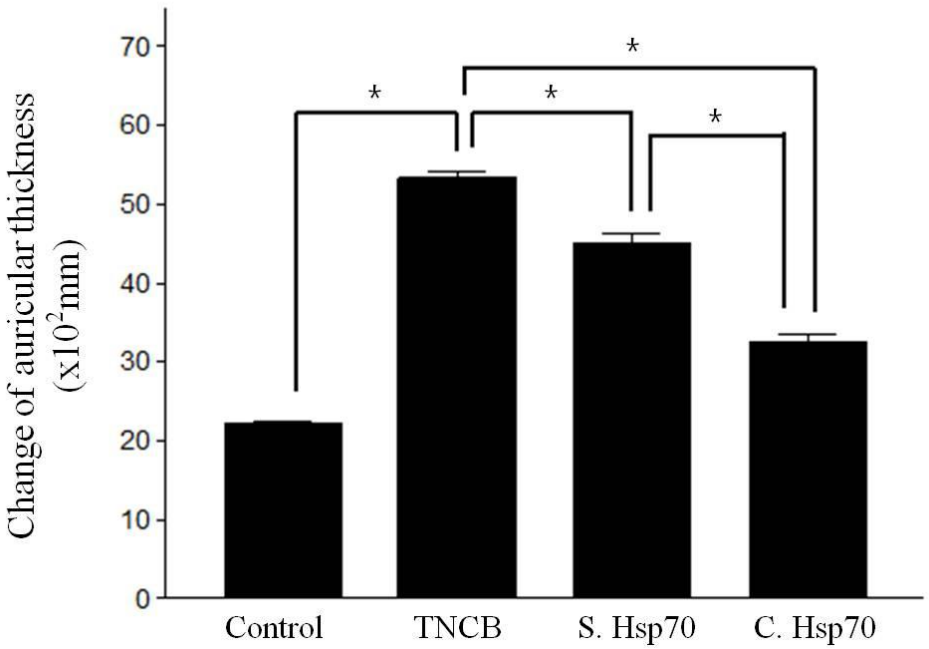


Fig. 2. Suppression of ear swelling by PTD-Hsp70 pretreatment. Ear swelling was measured 24 hrs after sensitization and challenge by TNCB. PTD-Hsp70 pretreatment was performed on the abdomen of the mice 1 hr before sensitization and elicitation. Both PTD-Hsp70 pretreated groups showed significantly low level of ear swelling (n=15, *, $p<0.001$, compared with the auricular thickness in control, TNCB only, S. Hsp70 and C. Hsp70). Results indicate the change of auricular thickness ± SEM.

Table. 2. Comparison of ear swelling response between the control and PTD-Hsp70 pretreated groups. Both PTD-Hsp70 pretreated groups decreased ear swelling compared to the TNCB only group (n=15, $p<0.001$). The C. Hsp70 group presented more strong inhibition than the S. Hsp70 group (n=15, $p<0.001$).

Effect group	Compared group	Estimate (mm)
C. Hsp70	TNCB	-19.5
S. Hsp70	TNCB	-8.6
C.Hsp70	S. Hsp70	-10.9

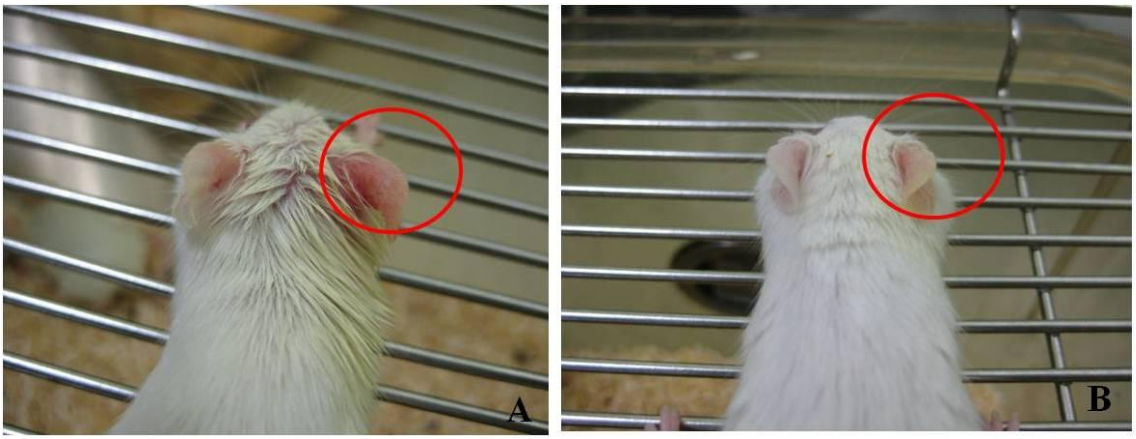


Fig. 3. Reduced ear swelling response by PTD-Hsp70 pretreatment. Mice CHS model, ear swelling, by TNCB (A). PTD-Hsp70 was applied on mice ear before challenging by TNCB and ear swelling was significantly decreased in the PTD-Hsp70 pretreated mice (B).

3. Down-regulation of P-p38 and up regulation of P-ERK in ear tissue by PTD-Hsp70

Hsp70 is known to have anti-inflammatory, anti-apoptosis, and antioxidant effects as a chaperone. In previous studies, I confirmed the anti-inflammatory and anti-oxidant effects of PTD-Hsp70 by demonstrating decreases in IL-12 and ROS production. To examine the effects of Hsp70, I evaluated the expression of phosphorylated extracellular signal regulated kinase (P-ERK) and phosphorylated p38 (P-p38) using PTD-Hsp70. P-p38, which is an active form of p38 was down-regulated in the PTD-HSP70 pretreated group than in the TNCB only group (Fig. 4A). However, P-ERK expression was further increased in the PTD-Hsp70 pretreated group than in the TNCB only group (Fig. 4B).

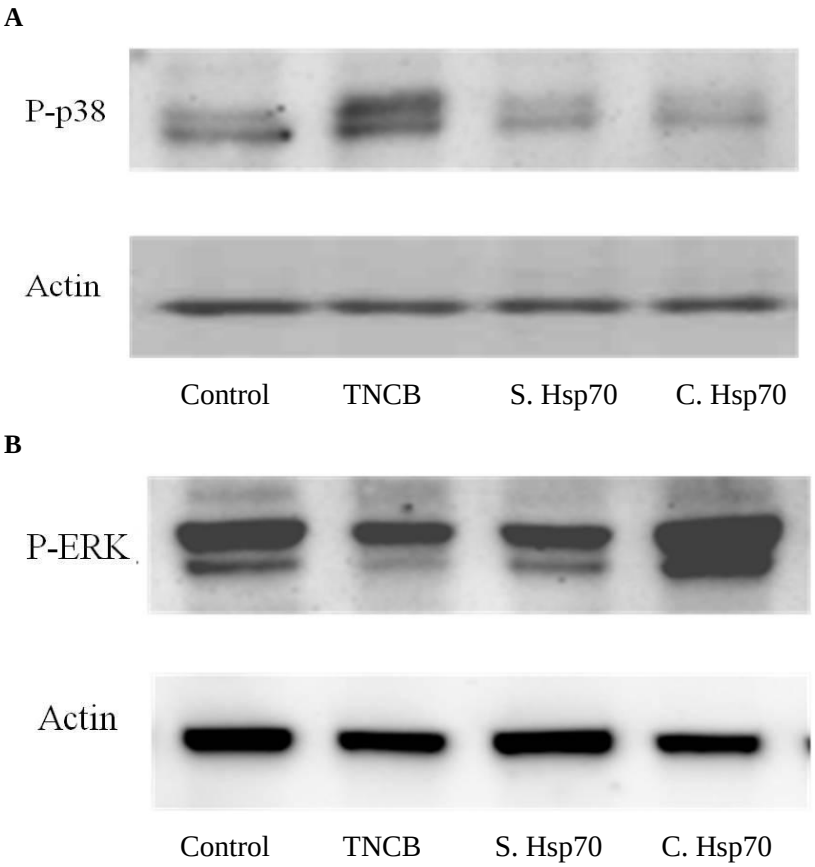
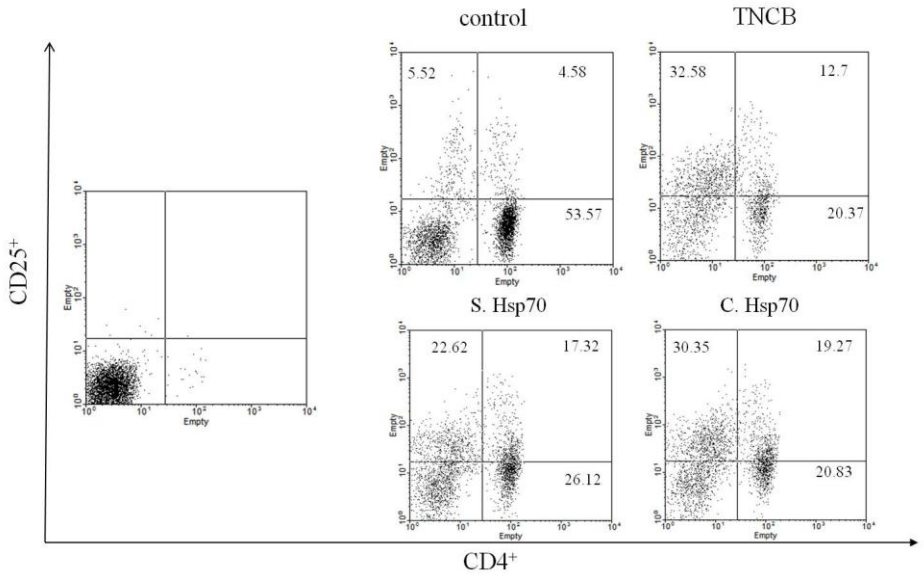


Fig. 4. Expression of P-p38 and P-ERK in ear tissue. PTD-Hsp70 down-regulated P-p38 (an active form of p38) in both S. Hsp70 and C. Hsp70 groups more than in the TNCB only group (A). PTD-Hsp70 increased expression of P-ERK in both S. Hsp70 and C. Hsp70 groups more than in the TNCB only group (B).

4. Increased populations of $CD4^+CD25^+$ T cells and $CD4^+CD25^+Foxp3^+$ T cells by PTD-Hsp70 in lymph node

I investigated whether PTD-Hsp70 increase the populations of Tregs or not. Treg is known to suppress CHS by regulating effector T cells. Percentages of $CD4^+CD25^+$ T cells and $CD4^+CD25^+Foxp3^+$ T cells in LN were measured by flow cytometry. The numbers of $CD4^+CD25^+$ T cells and of $CD4^+CD25^+Foxp3^+$ T cells were increased in PTD-Hsp70 pretreated groups compared to the TNCB only group. In the case of $CD4^+CD25^+$ T cells, the percentage was increased by 36.37% in S. Hsp70 and 51.73% in C. Hsp70 compared to the TNCB only group (Fig. 5A). Also $CD4^+CD25^+Foxp3^+$ T cell population was increased by 49.02% in S. Hsp70 and 65.88% in the C. Hsp70 compared to the TNCB only group (Fig. 5B).

A



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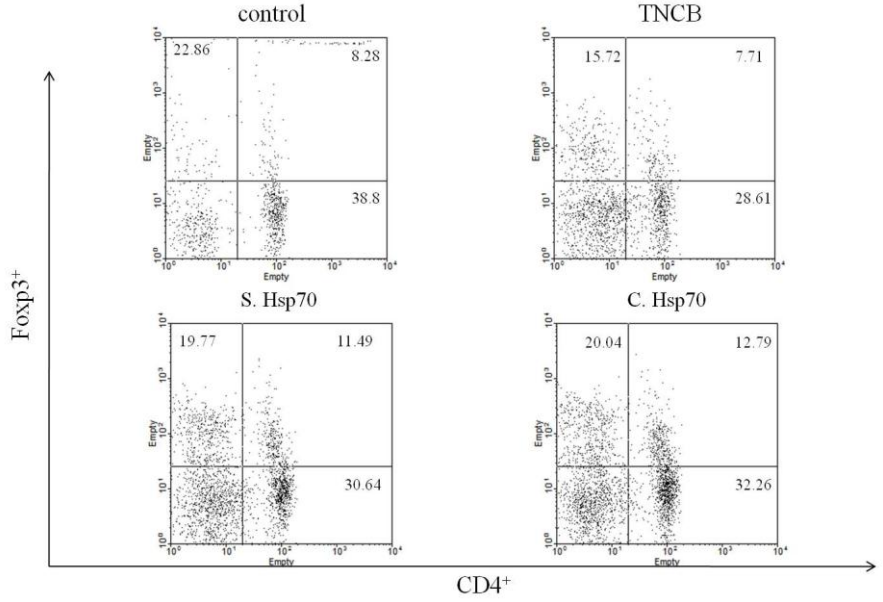
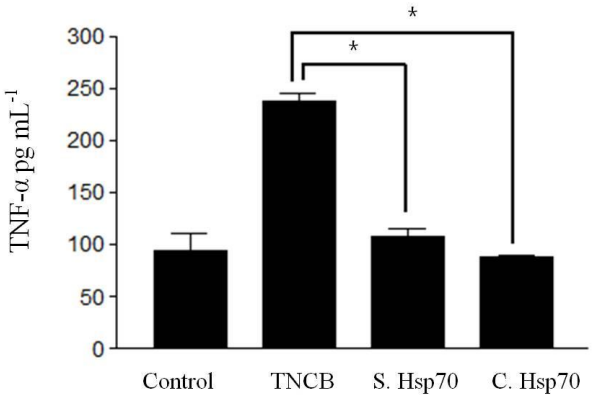


Fig. 5. Increased populations of $CD4^+CD25^+$ T cell and $CD4^+CD25^+Foxp3^+$ T cell by PTD-Hsp70. PTD-Hsp70 pretreatment was performed before sensitization (S. Hsp70) and elicitation (C. Hsp70), and T regulatory cells in LN were analyzed by FACS. The percentages of $CD4^+CD25^+$ T cells (A) and $CD4^+CD25^+Foxp3^+$ T cells (B) were markedly higher in S. Hsp70 and C. Hsp70 groups.

5. Down-regulation of pro-inflammatory cytokines (TNF-α, IL-6) by PTD-Hsp70 in ear tissue and lymph node

Various cytokines were investigated in this study through ELISA in CHS. TNF-α and IL-6 are primary and also pro-inflammatory cytokines secreted by T lymphocyte and keratinocyte (KC). Each cytokine was evaluated in LNs and ear tissues. TNF-α level in LNs of the TNCB only group was 238.9 pg mL^{-1}. PTD-Hsp70 pretreatment significantly down-regulated TNF-α in the S. Hsp70 group to 107.4 pg mL^{-1} and much more in the C. Hsp70 group to 88.1 pg mL^{-1} (Fig. 6A). In ear tissues, both TNF-α and IL-6 were down-regulated in the S. Hsp70 group and the C. Hsp70 group than the TNCB only group. Although TNF-α level presented down-regulated pattern, from 140.4 pg mL^{-1} to 116.7 pg mL^{-1} in the C. Hsp70 group compared to the TNCB only group, it was not statistically significant. Only IL-6 was significantly down- regulated form 84.4 pg mL^{-1} to 57.2 pg mL^{-1} in the C. Hsp70 group compared to the TNCB only group (Fig. 6B).

A



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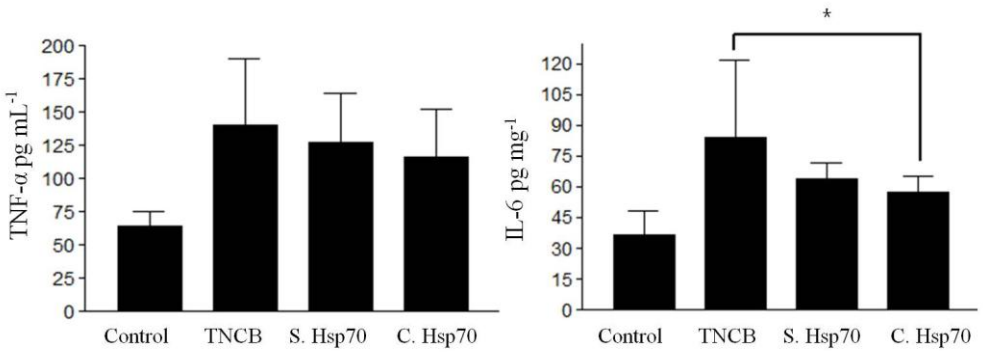
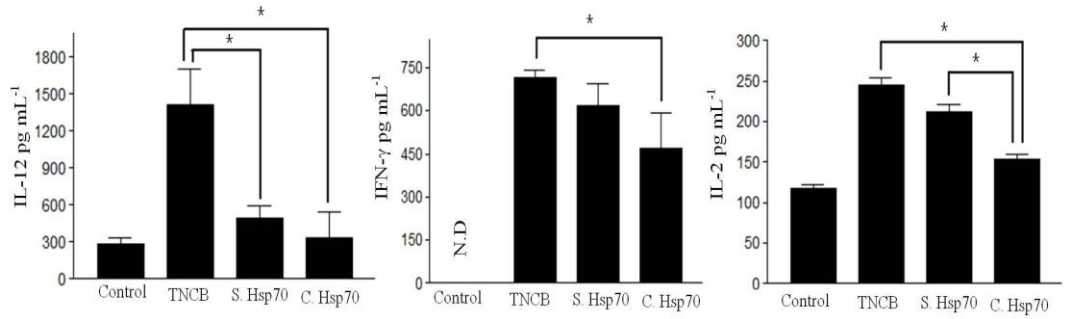


Fig. 6. Down-regulation of pro-inflammatory cytokines. TNF-α and IL-6 protein levels were measured in LNs (A) and ear tissues (B) 24 hrs after sensitization and elicitation. PTD-Hsp70 pretreatment down-regulated TNF-α level both in the sensitization and the elicitation phase in LNs. However, in ear tissues, only IL-6 was down-regulated by PTD-Hsp70 in the elicitation phase (*, $p<0.05$). Results indicate the change of cytokines ± SEM.

6. Down regulation of Th1 cell associated cytokines (IL-12, IFN-γ, IL-2) by PTD-Hsp70 in ear tissue and lymph node

As CHS favors a Th1 cell mediated response, I evaluated the effects of PTD-Hsp70 on Th1 cell associated cytokines. In LNs, PTD-Hsp70 pretreatment in the sensitization and elicitation phase significantly down-regulated IL-12, which promotes Th1 cell differentiation. The TNCB only group secreted 1419.8 pg mL^{-1} of IL-12. However, PTD-Hsp70 pretreatment lowered IL-12 secretion to 495.6 pg mL^{-1} in the S. Hsp70 group and to 338.1 pg mL^{-1} in the C. Hsp70 group. Although IFN-γ and IL-2 were decreased, a significant reduction was observed only in the elicitation phase by PTD-Hsp70 compared to the TNCB only group. (Fig. 7A). IFN-γ was decreased from 716.54 pg mL^{-1} in the TNCB only group to 467.76 pg mL^{-1} in the C. Hsp70 group. IL-2 was decreased from 244.73 pg mL^{-1} in the TNCB only group to 154.188 pg mL^{-1} in the C. Hsp70 group. In ear tissues, PTD-Hsp70 pretreatment decreased IL-12 level both in the sensitization and elicitation phases. However, the level of IFN-γ and IL-2 were not significantly changed (Fig. 7B).

A



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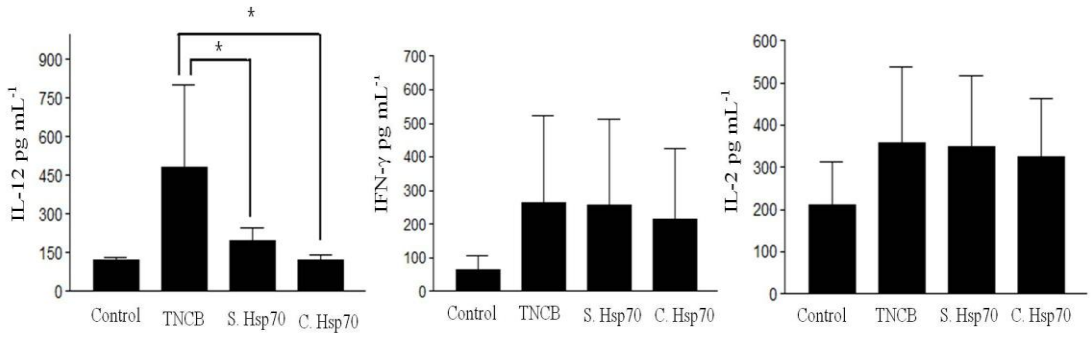
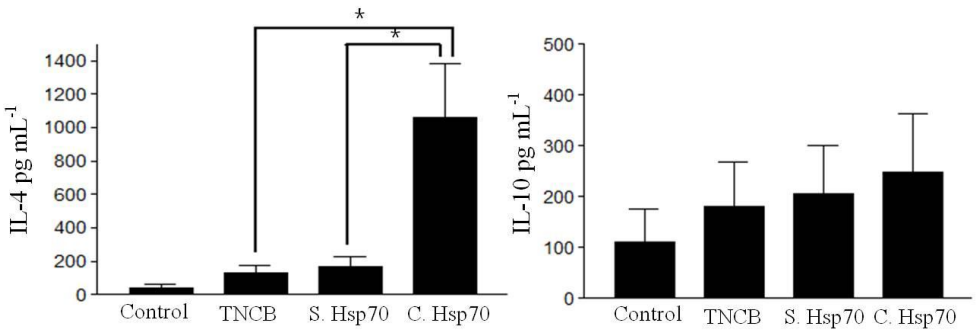


Fig. 7. Down-regulation of Th1 associated cytokines. IL-12, IFN-γ, and IL-2 levels were measured in LNs (A) and ear tissues (B) 24 hours after sensitization and elicitation. PTD-Hsp70 pretreatment significantly down-regulated IL-12 in both the sensitization and elicitation phases, but IFN-γ and IL-2 were significantly down-regulated by PTD-Hsp70 pretreatment only in the elicitation phase. However, in ear tissues, only IL-12 was significantly down-regulated by PTD-Hsp70 in both phases of CHS (*, $p<0.05$). Results indicate the change of cytokines ± SEM. N.D; non detected

7. Up-regulation of Th2 cell associated cytokines (IL-4, IL-10) by PTD-Hsp70 in ear tissue and lymph node

I measured the protein level of the Th2 cell cytokines, IL-4 and IL-10 in both LNs and ear tissues. Th2 cells produce IL-4 and IL-10, which are known to down-regulate CHS. In this study, both IL-4 and IL-10 were increased by PTD-Hsp70 pretreatment, but IL-10 was not significantly increased in LNs and ear tissues. In LNs, PTD-Hsp70 pretreatment markedly up-regulated IL-4 secretion in the C. Hsp70 group from 134.5 pg mL^{-1} to 1060.9 pg mL^{-1} compared to the TNCB only group. Also in ear tissues, PTD-Hsp70 pretreatment significantly increased IL-4 from 51.3 pg mL^{-1} to 87.9 pg mL^{-1} in the S. Hsp70 group and to101.8 pg mL^{-1} in C. the Hsp70 group compared to the TNCB only group (Fig. 8).

A



B

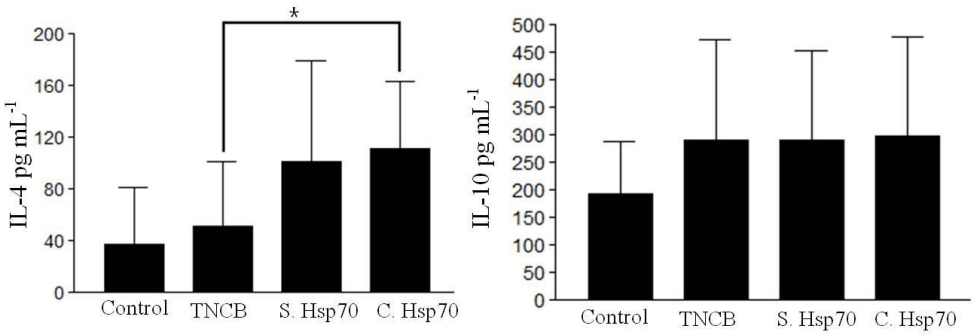
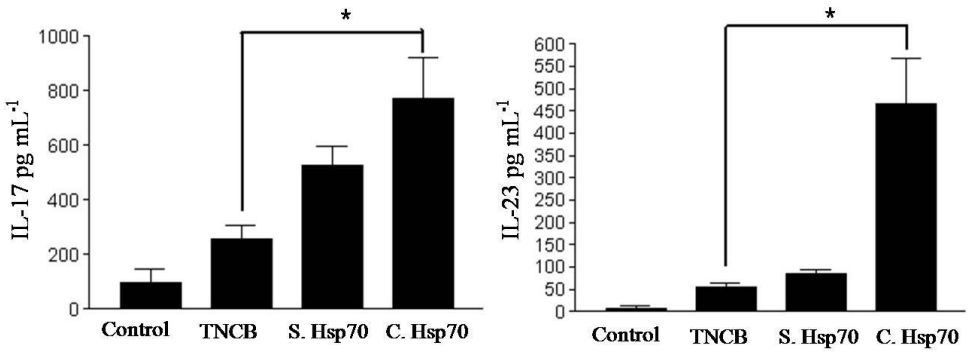


Fig. 8. Up-regulation of Th2 associated cytokines. IL-4 and IL-10 protein levels were measured in LNs (A) and ear tissues (B) 24 hrs after sensitization and elicitation. In LNs, PTD-Hsp70 pretreatment significantly up-regulated IL-4 only in the elicitation phase. IL-4 level was significantly increased in the elicitation phase of ear tissues. Although IL-10 was increased by PTD-Hsp70 pretreatment, it was not significant (*, $p<0.05$). Results indicate the change of cytokines ± SEM.

8. Increased secretion of Th17 specific cytokines (IL-17, IL-23) by PTD-Hsp70 in ear tissue and lymph node

Th17 cells characteristically secrete IL-17, and secretion of IL-17 is up-regulated by IL-23. In this study, PTD-Hsp70 pretreatment before elicitation increased the level of IL-17 from 255.3 pg mL^{-1} to 771.7 pg mL^{-1} in LNs compared to the TNCB only group. Also, IL-23 was significantly up-regulated from 55.9 pg mL^{-1} to 467.1 pg mL^{-1} in the C. Hsp70 group compared to the TNCB only group (Fig. 9A). The level of IL-17 in ear tissues was up-regulated, but this increase was not significant. However, PTD-Hsp70 pretreatment increased the IL-23 level from 98.6 pg mL^{-1} to 278.6 pg mL^{-1} in ear tissues in the C. Hsp70 group compared to the TNCB only group (Fig. 9B).

A



B

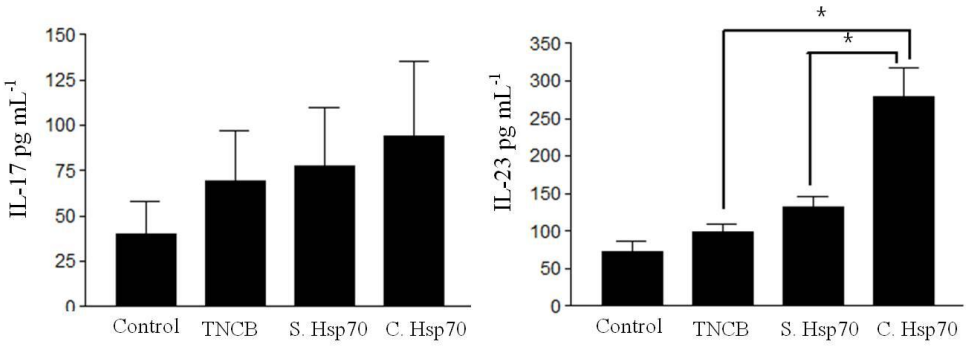


Fig. 9. Up-regulation of Th17 specific cytokines. IL-17 and IL-23 levels were measured in LNs (A) and ear tissues (B) 24 hrs after sensitization and elicitation by TNCB. In LNs, PTD-Hsp70 pretreatment significantly up-regulated IL-17 and IL-23 in the elicitation phase. IL-23 was also increased in the elicitation phase of ear tissues. Although IL-17 showed up-regulated pattern by PTD-Hsp70 pretreatment, it was not significant in ear tissues (*, $p<0.05$). Results indicate the change of cytokines ± SEM.

9. Increased level of regulatory cytokine (TGF-β) by PTD-Hsp70 in ear tissue and lymph node

Previously, I confirmed that PTD-Hsp70 pretreatment increased the population of $CD4^+CD25^+$ T cells and $CD4^+CD25^+Foxp3^+$ T cells (Fig. 5). TGF-β protein level was measured to examine whether PTD-Hsp70 induce the regulatory cytokine in CHS. PTD-Hsp70 pretreatment significantly up-regulated TGF-β level in the elicitation phase of LNs to 261.2 pg mL^{-1} and ear tissues to 167.1 pg mL^{-1} as compared to the level of 119.39 pg mL^{-1} in LNs and 102.9 pg mL^{-1} in ear tissues of the TNCB only group (Fig. 10).

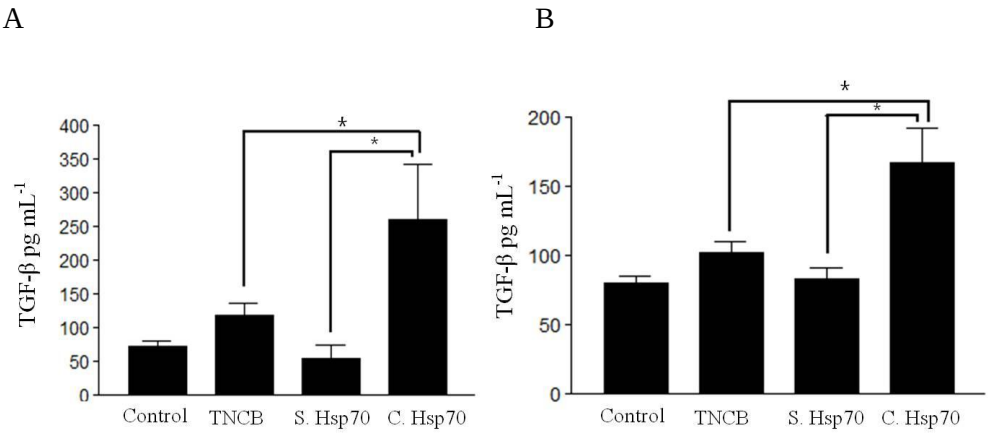


Fig. 10. Up-regulation of regulatory cytokines. PTD-Hsp70 pretreatment significantly elevated the expression of TGF-β in LNs (A) and in ear tissues (B) at the elicitation phase (*, $p<0.05$). Results indicate the change of cytokines ± SEM.

Ⅳ. DISCUSSION

Previously, the generation ROS, carbonylation of ATP synthase and Hsp70 in the TNBS treated XS-106 cells was verified[7]. Also I observed the exogenous antioxidant effect of PTD-Hsp70 which reduced ROS and IL-12 secretion at XS-106 cells. In this study, I investigated the role of Hsp70 in CHS and how Hsp70 suppresses allergic contact dermatitis in BALB/c mice using PTD-Hsp70.

The pathogenesis of ACD is known as CHS, and is mediated by $CD8^+$ T cells primed in lymphoid organs during the sensitization phase and recruited in the skin by secondary application of the same hapten at the elicitation phase[4]. ACD is synonymous with CHS in pathogenesis, progress in the sensitization, and elicitation and resolution phases. While both the sensitization and elicitation phases are required in the pathogenesis of ACD, ACD shows its clinical lesions after the elicitation phase. In brief in the sensitization phase, skin cells release inflammatory mediators (IL-18, IL-1β, TNF-α, ATP, PGE2, LTB4, ROS, histamine) and matured dendritic cells activate effector $CD8^+$ T cells to produce IFN-γ[3] and/or IL-17 in the drainage LN (dLN). In the elicitation phase, similar to the sensitization phase, inflammatory mediators (IL-18, IL-1β, TNF-α, ATP, PGE2, LTB4, histamine, CCL2) are released from skin cells and $CD8^+$ T cells produce IFN-γ, TNF-α and IL-17. Finally at the resolution phase, regulatory T cells produce IL-10, Th2 cell cytokines and $CD4^+CD25^+Foxp3^+$ T cells[4].

Although, I verified that PTD-Hsp70 reduces CHS through its antioxidant effect by lowering TNBS induced generation of ROS *in vitro*[7], transduction of PTD-Hsp70 was verified only *in vitro* and *in vivo* after ocular, intranasal and, intradermal administration without cytotoxic effects or immunogenicity[9, 26]. The transduction of PTD-Hsp70 was not proven by skin application. Normal skin barrier delivers molecules up to 500 dalton, but PTD working as a protein vector, facilitates the transduction of macromolecules across the otherwise impermeable, double-layered cell membrane[26]. I performed immunohistochemical staining to identify the transduction of PTD-Hsp70 which was topically applied onto the mouse ear. Penetration of PTD-Hsp70 into the skin was confirmed through the time serial. Two

hours after application of PTD-Hsp70, it had penetrated almost to the subcutaneous level and lasted for more than 16 hrs in the ear skin. Thus, PTD-Hsp70 can function and deliver Hsp70 through mucosal, intra-dermal administration and topical application.

In this study, I applied 2 μl of PTD-Hsp70 epicutaneously 1 hr before the sensitization and elicitation phases to examine whether PTD-Hsp70 pretreatment suppresses systemic CHS. PTD-Hsp70 pretreatment successfully decreased systemic CHS as the level of ear swelling was significantly less in the elicitation phase as well as in the sensitization phase compared to the TNCB-induced model.

CHS is regulated by Th1 and Th2 cells. Th1 cells whose differentiation from Th0 cells, mediated by IL-12, up-regulate the secretion of IL-2 and IFN-γ which induce CHS[21, 25, 27]. Antigen presenting cells, including Langerhans cells, produce IL-12 to promote T cell development to favor Th1 cells by up-regulating IFN-γ in T cells[28] and produce IL-2 to initiate cutaneous inflammatory response in the elicitation phase[3]. IL-12 also facilitated $CD8^+$ T cell development for CHS[29]. In this study, PTD-Hsp70 down-regulated Th1 associated cytokines (IL-12, IFN-γ, IL-2) from dLN and IL-12 from ear tissues in the elicitation phase. In contrast with Th1 cell associated cytokines, Th2 cell associated cytokines that are produced from $CD4^+$ T cells, including IL-4 and IL-10, negatively regulate CHS[30]. As expected, PTD-Hsp70 pretreatment increased the level of IL-4 in LNs and ear tissues, but IL-10 showed only up-regulating pattern without significance.

TNF-α produced by keratinocyte (KC) maturates DCs to function as antigen presenting cells and send them to LN in CHS[31]. TNF-α is also known to regulate $CD8^+$ T cell for survival[32]. In this study, PTD-Hsp70 pretreatment decreased TNF- α both in LNs and ear tissues. However, it was not significant in ear tissues. IL-6 produced from skin cells, especially secreted from KC, roles as a one of the pro/ /primary-inflammatory cytokine in CHS[33]. I analyzed IL-6 secretion in ear tissues and the secretion of IL-6 was suppressed by PTD-Hsp70 pretreatment at the elicitation phase.

Recently, some studies introduced experimental therapeutic model into CHS pathogenesis[33-35]. During the differentiation process of Th17 cell from Th0 cell,

TGF-β and IL-23 strongly up-regulate the differentiation in mouse[4]. Moreover, a few studies suggested that Th17 cells, secreting cytokine IL-17, mediate ACD[22, 23, 36]. IL-17 produced by Th17 cells acts as a pro-inflammatory cytokine in CHS[5, 23]. However, in this study, PTD-Hsp70 pretreatment increased the level of IL-17, IL-23 and TGF-β in the elicitation phase. TGF-β has multi-functions in inflammation. TGF-β has inhibitory effects on macrophage and DCs and induces the differentiation of Treg cells[37]. In CHS, TGF-β is involved in skin induced tolerance and induces Tregs to inhibit CHS by Smad3 signaling pathway[38].

Regarding Tregs, PTD-Hsp70 pretreatment increased the number of $CD4^+CD25^+$ T cells and $CD4^+CD25^+$ $Foxp3^+$ T cells in the dLN. The progress of CHS is down regulated by $CD4^+CD25^+$ T cells and $CD4^+CD25^+$ $Foxp3^+$ T cells which present essential functional markers for T regulatory cells and block CD8+ effector T cells in CHS[39]. Also $CD4^+CD25^+$ T cells are known to regulate CHS by releasing suppressive cytokines and cell to cell contact[35].

p38 and P-ERK are known to mediate inflammation and apoptosis pathway from oxidative stress. Other studies described that TNF-α initiate the activation of p38 in MAPK, which triggers the activation of apoptotic pathways through the generation of oxidative stress, activation of Bax, cytochrome c release, and activation of caspases[40, 41] but down-regulate ERK 1/2 phosphorylation[42]. In this study, PTD-Hsp70 pretreatment resulted in down-regulation of p38 (P-p38) and up-regulation of P-ERK in ear tissue.

Until now, only limited number of literatures verified the role of Hsp70 in CHS[24, 43]. In those literatures, the expression of Hsp70 was increased in skin. Yusuf et al[24] identified that Hsp27 and Hsp70 in the skin are inflammatory mediators of ACD. Moreover, they showed suppressive effect of anti-Hsp70 on CHS by inhibiting the secretion of IL-17 and IFN-γ. However, in this study exogenous PTD-Hsp70 down-regulated CHS. I suggest that these different results are from intra-cellular effect of PTD-Hsp70 to CHS as a chaperone. Although exogenous Hsp70 produce the inflammatory cytokines[44], endogenous Hsp could induce anti-inflammatory T cell regulation[45]. Also I suspect that the opposite result may from the difference between the mouse specie and hapten.

Ⅴ. SUMMARY OF RESULTS AND CONCLUSION

This study demonstrated that topical application of PTD-Hsp70 attenuates CHS. As mentioned above, PTD-Hsp70 down-regulated Th1 cell associated cytokines but up-regulated a Th2 cell cytokine IL-4 which negatively regulates CHS. Also PTD-Hsp70 down-regulated primary/pro-inflammatory cytokines. Regarding Tregs, PTD-Hsp70 increased populations of $CD4^+CD25^+$ T cells, $CD4^+CD25^+$ $Foxp3^+$ T cells, and TGF-β. In MAPK pathway, PTD-Hsp70 pretreatment decreased systemic CHS through down-regulating P-p38 and up-regulating P-ERK.

Hsp70 is a chaperone protein. Indeed, some studies confirmed that Hsp70 down-regulates other inflammatory diseases by inducing cytokines change[16-18].

In conclusion, I confirmed that topical application of PTD-Hsp70 attenuates CHS by regulating cytokines and other inflammatory pathways.

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Abstract (In Korean)

열충격 단백 70 (Heat shock protein 70) 이 마우스 모델 접촉성 과민반응증에 미치는 영향

<지도교수 이민걸>

연세대학교 대학원 의학과

박진모

알레르기 접촉피부염은 피부과 질환 중에서 비교적 흔한 질환중 하나이다. 알레르기 접촉피부염의 병리 기전은 접촉성 과민반응증 (contact hypersensitivity, CHS) 에 의한 지연성 면역 매개 증상이다. CHS 과정에는 수지상 세포, 림프구, 자연 고사 세포 등의 여러 세포와 면역조절 물질 및 싸이토카인 등이 관여한다고 알려져 있다.

최근 우리 교실에서는 알레르겐인 TNBS 가 마우스의 수지상 세포주인, XS-106 세포에서 활성 산소종(reactive oxygen species, ROS)을 생성하고 ROS 가 ATP synthase 와 열충격 단백 70 (heat shock protein, Hsp70)의 carbonylation 에 영향을 미침을 확인하였다. 또한, CHS 과정에서 Hsp70이 항산화 효과가 있는지 알아보기 위하여 protein transduction domain fused Hsp70 (PTD-Hsp70)을 XS-106 세포주에 처리한 결과 ROS의 발생이 억제되고 IL-12의 분비가 증가되는 것을 확인하여 ROS 가 접촉과민반응의 유발에 관여함을 관찰하였다.

이처럼, 접촉피부염의 발생기전과 관련하여 Hsp70이 중요한 역할을 한다면, Hsp70이 접촉성 과민반응증 관련 싸이토카인에 영향을 미칠 것으로 생각한다. 그래서 이번 연구에서는 PTD-Hsp70 이 BALB/c 마우스 모델 CHS 발생 과정에 어떠한 영향을 미치는지를 싸이토카인을 중심으로 관찰하였다. 실험군의 경우, BALB/c 마우스를 감작기 이전에 PTD-Hsp70을 도포한 군, 유발기 이전에 PTD-Hsp70을 도포한 군, TNCB 로 CHS 를 만든 군으로 나누어 각 군마다 15마리가 사용되었다.

실험 결과, 첫째, PTD-Hsp70을 도포한 두 가지 군에서 CHS 군 보다 귀의 부종 정도가 유의하게 감소하였다. 둘째, PTD-Hsp70을 도포한 군의 귀 조직을 Western blot 한 결과, apoptosis 와 연관된 P-p38의 발현은 감소되고 P-ERK 의 발현은 증가되었다. 셋째, PTD-Hsp70을

도포한 군의 drainage lymph node(dLN) 를 flow cytometry 로 분석한 결과 $CD4^+CD25^+$ T 세포와 $CD4^+CD25^+Foxp3^+$ T 세포가 CHS 군 보다 유의하게 증가함을 확인하였다. 넷째, PTD-Hsp70을 유발기전에 도포한 군의 dLN 에서 시행한 ELISA 검사에서 tumor necrosis factor-α, interlukin(IL)-12, interferon-ɣ, IL-2 가 감소한 반면, IL-4, IL-17, IL-23 및 transforming growth factor (TGF)-β는 증가하였다. 다섯째, PTD-Hsp70을 유발기전에 도포한 군의 귀 조직에서 시행한 ELISA 검사상 IL-6 와 IL-12는 감소한 반면 IL-4, IL-23, TGF-β 증가하였다.

이상을 종합해 볼때, PTD-Hsp70 은 첫째, MAPK 전달경로를 통해서 염증반응 및 고사반응을 조절하고, 둘째, 염증성 싸이토카인과 Th1 세포의 분화를 억제하며, 셋째, Th2 세포 관련 싸이토카인의 분비를 증가시키며, 넷째, 조절 T 세포와 TGF-β 를 증가시켜 CHS 반응을 억제시킨다는 사실을 확인 하였다.

결론적으로 PTD-Hsp70을 CHS 발생전에 전처리를 할 경우, MAPK 전달경로 및 Th1, Th2, 및 조절 T 세포에서 분비되는 싸이토카인을 조절하여 CHS 를 억제한다는 사실을 알 수 있었다.

핵심되는말: 알레르기 접촉피부염, BALB/c, 접촉성 과민반응증, 열충격 단백70, 활성산소종, Th17, TNBS