Protective effect of heat shock protein 27 using protein transduction domain-mediated delivery on ischemic heart injury

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Protective effect of heat shock protein 27 using protein transduction domain-mediated delivery on ischemic heart injury

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Abstract

Protective effect of heat shock protein 27 using protein transduction domain-mediated delivery on ischemic heart injury

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(Directed by Professor Yangsoo Jang)

Heat shock proteins (HSPs) provide one kind of defense against stresses such as high temperature, hypoxia/ischemia and oxidative stress in all mammalian cell types. One such protein which is highly induced during the stress response is hsp27, and its expression is correlated with the cellular survival in response to cytotoxic insults. Recent studies have been established that the overexpression of hsp27 protects against cell death triggered by a variety of stimuli including ischemia/reperfusion, hyperthermia, and various

cytotoxic agents. Previous findings have suggested that augmentation of the heat shock response by increasing the expression of hsp27 represents a potential therapeutic strategy for the treatment of critically ill patients. In this study, using protein delivery approach, we studied the potential cardiac protective effect of hsp27 as a therapeutic protein in rat cardiac myoblast H9c2 cells and rat LAD ligation model of focal ischemia. Human hsp27 cDNA was cloned and fused with a gene fragment encoding the basic domain from HIV-1 Tat protein called the protein transduction domain (PTD). Then, the efficiency of transduction of the resulting Tat-hsp27 fusion protein into H9c2 cells was analyzed. The Tat-hsp27 fusion protein was efficiently delivered to H9c2 cells, and its transduction showed cytoprotective effect against the hypoxic stress. Moreover, transduction of Tat-hsp27 also attenuated hypoxia-induced apoptosis, which was accompanied by reduced caspase-3 activity. In addition, intraperitoneal injection of Tat-hsp27 into rat resulted in efficient protein transduction in heart tissues within 2 hr, and decreased cardiac infarction, as determined at 7 days after focal ischemia. These experimental findings suggest that the PTD-mediated delivery of therapeutic proteins may represent a novel strategy of protein therapy.

Key Words : hsp27, protein transduction domain, hypoxia, ischemia.

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I. INTRODUCTION

Mammalian cells are dependent on its capability to adapt and to mediate the various stressors and metabolic insults it encounters. Many of the stress stimuli that are capable of triggering apoptosis, such as oxidative stress and thermal stress, induce the synthesis of heat shock proteins (HSPs) that confer a protective effect against a wide range of cellular stresses.[1,2] HSPs are highly

conserved molecules that fulfill a range of functions, including cytoprotection, intracellular assembly, folding and translocation of oligomeric proteins.[3,4] The increased expression of HSPs confers protection against a broad array of otherwise lethal cellular injuries.[5,6] For example, *in vitro* experiments have demonstrated that the increased expression of heat shock protein 70 (hsp70) protects cultured human respiratory epithelial cells against hypoxia-mediated cell injury and death.[7] *In vivo* studies have shown that induction of HSPs confers protection in various models of organ injury, including sepsis, acute lung injury, and ischemia reperfusion injury.[8,9]

The HSPs can be divided into two classes, the larger HSP and the smaller HSP, based on their molecular weight. The smaller HSP (sHSP) exhibit a monomeric molecular mass ranging between 9 and 43 kDa. The biological mechanisms proposed to account for the sHSP protective effect include molecular chaperoning of non-native protein.[10] One such protein which is highly induced during the stress response is hsp27. Hsp27 function as molecular chaperones in protein biosynthesis facilitates protein folding and translocation.[11] Protective effects of the hsp27 have been described against several noxious stresses like hyperthermia, hypertonic stress, and various cytotoxic agents, including ischemia-mediated injury in cardiac myocytes.[12] Recent several experimental data showed that expression of hsp27 confers

protective effect against hyperthermia in several cell lines.[13,14] Hsp27 is also associated with cytoskeletal structures, and stabilization of these elements may contribute to the increased stress tolerance. For example, hsp27 acts as an inhibitor of actin filament turnover in smooth muscle cells and seems to stabilize the actin filaments.[15]

Recently, a novel protein delivery system, termed protein transduction domain (PTD) has been introduced to deliver the proteins of interest into cells directly.[16] This has been most markedly observed for the HIV Tat protein,[17] *drosophila* homeoproteins (antennapedia, Antp)[18] and the herpes simplex virus VP22 protein[19]. The mechanisms by these PTDs cross biologic membranes are not well understood, though it appears that transduction mediated by Tat does not occur through classical receptor-, transporter-, or endosome-mediated mechanisms.[20,21] Among them, Tat-PTD (11 amino acid, YGRKKRRQRRR) can efficiently deliver proteins into cells and this system appears not to be limited by size of fusion protein.[22] This Tat-PTD can transduce even large proteins such as β-galactosidase across the blood-brain barrier and into non-dividing tissue after intraperitoneal injection in mice.[23]

Although hsp27 apparently has cardioprotective effects against hypoxic stress in the previous studies, the effect of intracellular protein delivery of hsp27 has not been examined yet. In the current study, human hsp27 cDNA

gene was cloned and fused with a gene fragment encoding the 11-amino acid transduction domain, Tat. Then, the efficiency of transduction of the resulting Tat-hsp27 fusion protein into cardiac myoblast cells was analyzed. Finally, we demonstrated the ability of Tat-mediated transduction of the recombinant hsp27 protein to confer cytoprotection against an ischemic stress *in vitro* and *in vivo*. Taken together, out findings suggest that Tat-hsp27 fusion protein could be a useful tool in protein therapy.

II. MATERIALS AND METHODS

1. Molecular cloning of human hsp27 and construction of expression vectors

The human hsp27 cDNA was generated by PCR using human heart cDNA library (Clonetech, Palo Alto, CA, USA) as a template. The sense primer was 5’- CGCGGCCTCGAGATGACCGAGCGCCGCGTC-3' and the antisense primer was 5-GGCCGCGAATTCTTACTTGGCGGCAGTC-3'. The PCR product was digested with *Xho*I and *Eco*RI and subcloned into the *Xho*I and *Eco*RI sites of the pTAT-HA bacterial expression vector (a gift from Dr. Stephen F. Dowdy, UCSD, USA). The pTAT-HA bacterial expression vector contains an N terminal 6-histidine tagging sequence, followed by the 11-amino acid transduction domain of the Tat protein, and a polylinker region.[22] The correct sequence of the pTAT-HA-hsp27 vector was confirmed by DNA sequencing using the universal T7 primer.

2. Expression and purification of hsp27 fusion proteins

Escherichia coli BL21(DE3)pLysS (Novagen, Madison, WI, USA) transformed with the pTAT-HA-hsp27 plasmid were grown for overnight at 37℃ in LB broth supplemented with 100 μg/ml ampicillin and 34 μg/ml

chrolamphenicol. These cultures were diluted 100-fold with fresh LB media and cultured at 37℃ for 3 hr while shaking at 180 rpm. Protein expression was induced by the addition of 1 M IPTG to a final concentration of 1 mM for 4 hr while shaking at 37℃. The Tat-hsp27 fusion proteins were then isolated using a urea-denaturing protein purification protocol.[22] The bacterial pellet was isolated by centrifugation, washed with PBS, resuspended in buffer Z (8 M urea, 100 mM NaCl, and 20 mM HEPES, pH8.0), and sonicated on ice 3 times with 15 second pulses. The sample was then clarified by centrifugation at 13,000 rpm at 4℃ for 30 min. The clarified lysate was then equilibrated in 20 mM imidazole and applied at room temperature to a preequilibrated 25 ml column packed with 5 ml Ni-NTA resin in buffer Z, including 20 mM imidazole. The column was allowed to proceed by gravity flow, and the flow-through was then reapplied. The column was washed with 50 ml of 20 mM imidazole in buffer. The proteins were eluted by the stepwise addition of binding buffer containing increasing concentrations of imidazole (100-1,000 mM). The proteins were loaded onto a PD-10 Sephadex size exclusion column in order to rapidly exchange the buffer. 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 20% glycerol were aliquoted and stored at -80℃.

3. Cell culture

The rat heart-derived H9c2 cells and rat vascular smooth muscle cells (rVSMCs) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). H9c2 cells were cultured in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) supplement with 10% FBS and 1% penicillin streptomycin (Gibco, Grand Island, NY, USA), at 37℃ in a humidified atmosphere of 5% CO_2 - 95% air. rVSMCs were maintained in DMEM supplement with 10% FBS and 1% penicillin streptomycin (Gibco, Grand Island, NY, USA) at 37℃ in a humidified atmosphere of 5% CO_2-95% air. All experiments were performed using cells between passage numbers 7 and 20. Primary cultures of human umbilical vein endothelial cells (HUVECs) (Clonetics, Walkersville, MD, USA) were maintained in EBM-2 medium (Clonetics) supplemented with EGM-2 kit (complete medium) (Clonetics) containing 2% fetal calf serum (FCS), 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R^3-IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-1000, and 0.1% heparin, according to manufacturer's instructions. For all experiments, cells were used up to the ten passages and harvested at 90%

confluence.

4. Introduction of fusion protein into cultured cells

For the transduction of hsp27 fusion proteins, cells were grown to confluence in six-well plates. The culture medium was replaced with fresh medium containing 10% FBS and was then treated with various concentrations of Tat-hsp27 fusion proteins. The cells were washed with PBS, followed by trypsinization without EDTA (Gibco, Grand Island, NY, USA) for 10 min and then washed with PBS. Cells were prepared for analysis by immunoblot and immunocytochemistry as described below.

5. Immunoblot analysis

Protein-treated cells were washed once in PBS and lysed in a lysis buffer (Cell signaling, Beverly, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM PMSF. Protein concentrations were determined using the Bradford protein assay kit (BioRad, Hercules, CA, USA). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co, Bedford, MA, USA). After blocking the membrane with Tris-buffered

saline-tween 20 (TBS-T, 0.1% tween 20) containing 5% non-fat dried milk for 1 hr at room temperature, membrane was washed twice with TBS-T and incubated with primary antibodies for 1 hr at room temperature or for overnight at 4°C. The membrane was washed three times with TBS-T for 10 min, and then incubated for 1 hr at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities were quantified using Photo-Image System (Molecular Dynamics, Uppsala, Sweden).

6. Immunocytochemistry

Cells were grown on 4-well plastic dishes (SonicSeal Slide, Nalge Nunc, Rochester, NY, USA) and treated with 500 nM Tat-hsp27 or wt-hsp27. Following incubation for 1 hr, the cells were washed twice with PBS and then fixed with 4% paraformaldehyde in 0.5 ml PBS for 30 min at room temperature. The cells were washed again with PBS and then permeabilized for 30 min in PBS containing 0.2% triton. The cells were then blocked in PBS containing 10% goat serum and then incubated for 1 hr with rabbit polyclonal HA antibody. The cells were rewashed three times for 10 min with PBS and

incubated with FITC-conjugated goat anti-rabbit antibody as the secondary antibody for 1 hr. Photographs of cells were taken under fluorescence by immunofluorescence microscopy (Olympus, Melville, NY, USA). For flowcytometry, Cells stained with FITC-conjugated secondary antibody were harvested and analyzed using FACSCalibur (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

7. Cell viability assay

H9c2 cells were plated in triplicate wells of 24 well plates at a density of 2 x 10^3 per well, and treated with 500 nM Tat-hsp27 or wt-hsp27 for 1 hr prior to exposure to hypoxia condition for 24 hr. Culture plates containing H9c2 cells in DMEM/F-12 were subjected to hypoxic stress in an anaerobic chamber (ThermoForma, Marietta, OH, USA) maintained at 37℃ in which ambient oxygen was replaced by a mixture of 5% CO_2, 5% H_2 and 90% N_2. Cell viability was determined by the MTT assay. After the incubation period, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma, St. Louis, MO, USA) was added to each well to a final concentration of 0.5 mg/mL and was incubated at 37℃ for 3 hr to allow MTT reduction. The formazan crystals were dissolved by adding dimethylsulfoxide

(DMSO) and absorbance was measured at the 570 nm with a spectrophotometer.

8. Measurement of caspase-3 activity

The activity of caspase-3 in the H9c2 cells was determined spectrophoto metrically with an Apoalert™ CPP32/caspase-3 assay kit (BD Science, Palo Alto, CA, USA) by measuring the release of the chromophore, pnitroanilide (*p*NA), following hydrolysis of DEVD-*p*NA. Cultured H9c2 cells were incubated in DMEM/F-12 without FBS and treated with 500 nM Tat-hsp27 or wt-hsp27 for 1 hr prior to exposure to hypoxia condition for 24 hr. After then, cells were harvested, resuspended in chilled cell lysis buffer and incubated on ice for 10 min. Cells were centrifuged and supernatants were transferred to a fresh tube. Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). The 50 µl of 2X reaction buffer containing 10 mM dithiothreitol (DTT) was added and each sample was incubated with 5 µl of DEVD-*p*NA (4 mM) at 37°C for 1 hr. Caspase-3 activity was read in a microtiter plate reader at 405 nm, and a standard curve was generated using as standard CPP32 chromogenic substrate.

9. Rat left anterior descending coronary artery (LAD) ligation model

All animal procedures were carried out according to a protocol approved by the Yonsei Animal Care and Use Committee in accordance with NIH guidelines. Male Sprague-Dawley rats weighing 150-200 g were used for all experiments. After induction of anesthesia (Ketamine 80 mg/kg, Xylazine 4 mg/kg body weight intraperitoneally), a tracheostomy was performed and the animal was ventilated on a Harvard Rodent Respirator (Model 683, Harvard Apparatus, Holliston, MA, USA) using room air at a respiratory rate of 60-65/min at a tidal volume of 2.4 ml. Ketamine and Xylazine were maintained throughout the experiment, and the level of consciousness of each animal was monitored by verifying a lack of response to foot pad stimulation. Rats were euthanized by removal of the heart under anesthesia at the conclusion of the experiment. Ligation of the left coronary artery was performed as described previously.[24] In brief, under anesthesia and artificial ventilation, the heart was exposed via left thoracotomy, and the left coronary artery was ligated 2 to 3 mm from its origin between the pulmonary artery conus and the left atrium with a 6-0 Prolene suture. The heart was subjected to regional ischemia for one week. Before ligation of the left coronary artery, Tat-hsp27 (5 mg/kg) or saline was administered for 2 hr by intraperitoneal injection. The chest wall was then closed, and the animal was allowed to recover. Tat-hsp27 or Saline was injected for one week once a day.

10. Determination of infarct size

The heart was removed from the anesthetized rat and quickly cannulated onto the Langendorff perfusion apparatus. The heart was perfused with Krebs-Ringer Bicarbonate Buffer (KRBB : 120 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 20 mM MOPS) for 10 min to wash out the blood and then fixed with 10% formalin. The heart was sliced transversely from the apex to the base in 2.5 mm thicknesses and weighed separately. Within 24 hr after fixation, each section was embedded in paraffin. Serial 5 μm myocardial sections were cut with microtome and mounted on siliconized slides. Paraffin sections from the middle of the infarct region were stained with hematoxylin and eosin (HE) and evaluated with light microscopy to assess the histological effects. Sections were also stained with Masson trichrome (Accustain Trichrome Stain [Masson], Sigma Diagnostics, St Louis, Mo, USA) to distinguish areas of connective tissue. Normal and infarct areas were measured by using NIH images, and the infarct area was determined as described previously.[25]

11. Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis as performed by student's t-test. Relationships were considered statistically significant

when p value was less than 0.05.

III. RESULTS

1. Construction and expression of hsp27 fusion protein

To prepare the cell-permeable hsp27 fusion protein, we constructed the expression vector to produce hsp27 as a fusion protein with Tat-PTD (11 amino acids), the peptide derived from HIV-1 Tat protein transduction domain. The human hsp27 gene was amplified from a cDNA by PCR using two oligonucleotide primers at the amino and carboxyl-terminal ends of the ORF, and the PCR product was inserted into an expression vector, pTAT-HA, which places His-tag, Tat and hemagglutinin (HA) at the 5'-end of the multi-cloning sites (Fig. 1). The control hsp27 protein (wild-type hsp27, wt-hsp27) without a Tat sequence was also constructed. The wt-hsp27 was the same in the structure as Tat-hsp27 except for the lack of Tat domain.

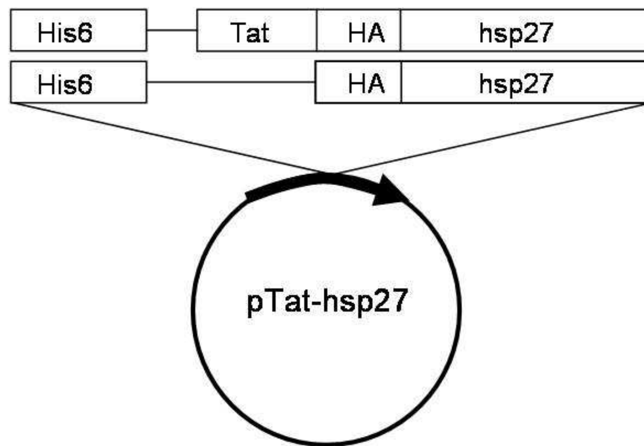


Fig.1. Schematic representation of Tat-hsp27 and wt-hsp27 protein expression vectors.

2. Expression and purification of Tat-hsp27 fusion protein

We devised a denaturing protocol, using either 8 M urea, to purify Tat-hsp27 fusion protein to increase both the transduction potential and the yield by recovering all of the recombinant protein present in bacterial inclusion bodies (Fig. 2). The recombinant pTat-hsp27 plasmid was introduced into *E. coli* BL21 for protein expression and induced to express Tat-hsp27 fusion protein by the addition of IPTG to the growth medium (Fig. 3A). Recombinant Tat-hsp27 protein was purified using the Ni-NTA affinity column chromatography (Fig. 3B). Tat-hsp27 fusion protein was found to be nearly homogeneous and >95% pure, as determined by SDS-PAGE analysis with Coomassie brilliant blue staining (Fig. 3C). The control hsp27 protein (wild-type hsp27, wt-hsp27) without a Tat sequence was also purified by same methods as those for Tat-hsp27. Since the Tat-hsp27 protein was fused on the amino-terminus with 11 amino acids of Tat, the gel mobility of the Tat-hsp27 protein was thus slower than that of wt-hsp27. The purified products were confirmed by immunoblot analysis with anti-hsp27 antibody (Fig. 3D).

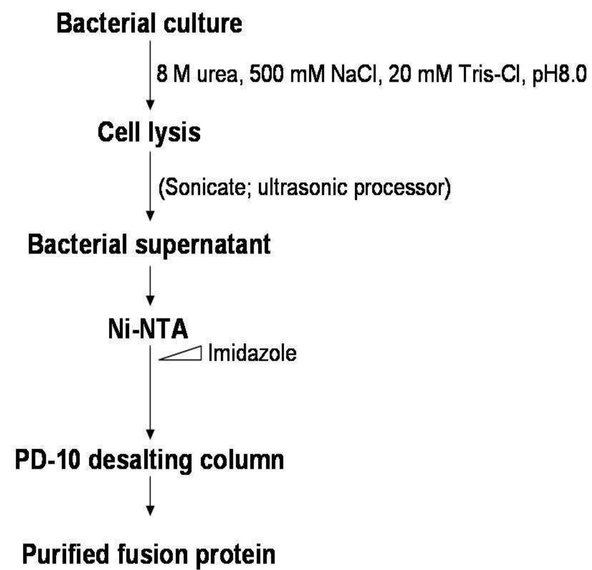


Fig. 2. Purification protocol of Tat-hsp27 fusion protein. Ni-NTA, nickel-nirilotriacetic acid affinity column; PD-10, sephadex PD-10 size exclusion column.

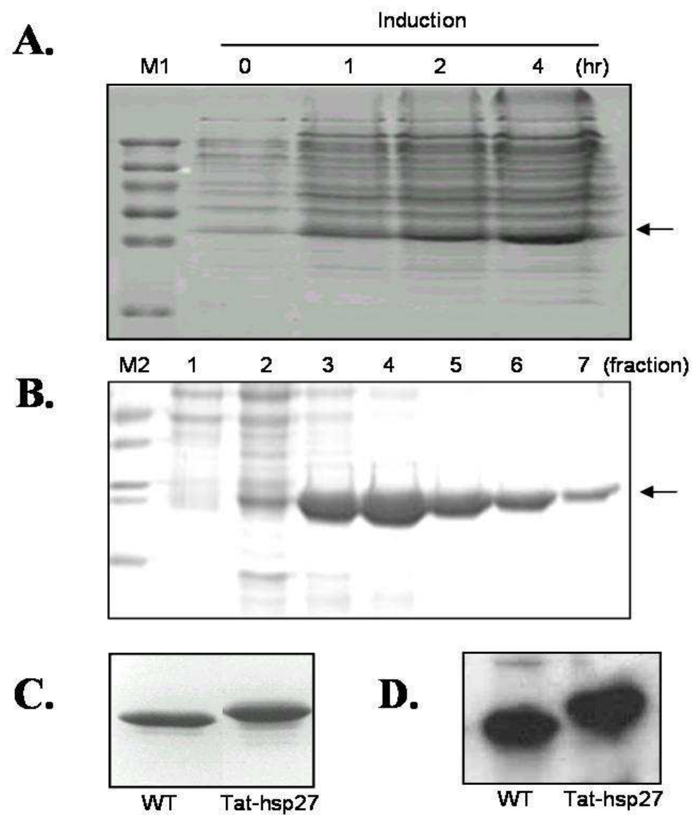


Fig. 3. The purification of recombinant Tat-hsp27 protein. A. Time course of the production of recombinant Tat-hsp27 induced by IPTG. The arrow represents the band of recombinant Tat-hsp27. B. Tat-hsp27 fusion protein purified by Ni-NTA affinity chromatography. C. Tat-hsp27 and wt-hsp27 protein purified by PD-10 column. D. Immunoblot analysis of purified hsp27 fusion proteins using anti-hsp27 antibody. WT, wild-type hsp27; M1 and M2, molecular weight marker (M1: 100, 70, 50, 40, 30, 18 kDa, M2: 66, 45, 36, 29, 24 kDa).

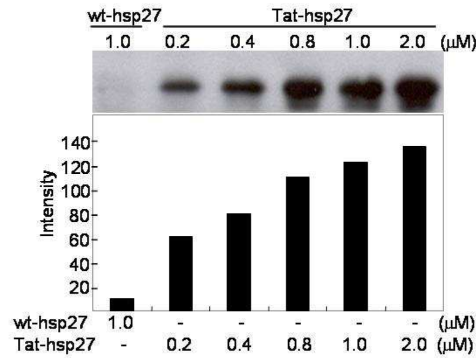
3. Transduction of Tat-hsp27 fusion protein

Tat-hsp27 fusion protein was introduced into cardiac myoblast H9c2 cells efficiently at concentration- and time-dependent manner. Tat-hsp27 fusion protein was added to H9c2 cells at concentrations ranging from 0.2 to 2 μM for 1 h, and the amounts of protein taken up were analyzed by immunoblot using anti-hsp27 antibody. As shown in Fig. 4A, Tat-hsp27 protein entry increased in a concentration-dependent manner, and wt-hsp27 was not taken up. The H9c2 cells were incubated with Tat-hsp27 protein for varying periods of time. Intracellular Tat-hsp27 was detected after 10 min and its level was eventually increased (Fig. 4B). The same results were also obtained by immunoblot analysis using the anti-HA or anti-His antibody (data not shown).

Transduction of the Tat-hsp27 fusion protein was also verified using both immunocytochemistry and flow cytometry. Tat-hsp27 (500 nM) protein was added to the cultured H9c2 cells and analyzed by immunochemistry. Cells treated with Tat-hsp27 showed a significant increase of intracellular staining using primary anti-HA antibody and HRP-conjugated secondary antibody (Fig. 5A) or FITC-conjugated secondary antibody (Fig. 5B). No significant changes of immunofluorescence were observed with wt-hsp27 or control groups (Fig. 5B). The same results were obtained by fluorescence activated cell sorting (FACS) analysis (Fig. 5C). Taken together, these results demonstrate that Tat-

hsp27 protein could be delivered into cardiac myoblast H9c2 cells. The enhanced transduction efficiency was also observed in NIH3T3 cells, HUVECs and rVSMCs (Fig. 6).

A. concentration



B. time

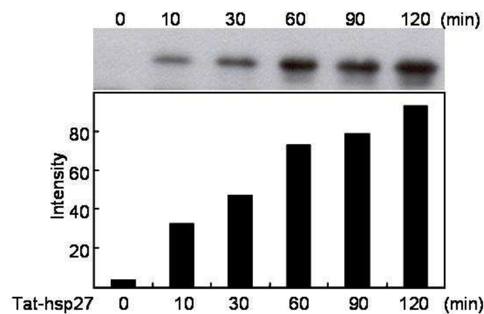


Fig. 4. Transduction of the recombinant Tat-hsp27 fusion protein into H9c2 cells. A. Dose-dependent transduction of Tat-hsp27 into cultured H9c2 cells. The H9c2 cells were treated with various concentrations of Tat-hsp27 fusion proteins for 1 hr. Samples were harvested, lysed and immunoblotted with anti-hsp27 antibody. B. Time-dependent transduction of Tat-hsp27 into cultured H9c2 cells. Tat-hsp27 fusion protein (500 nM) was treated into H9c2 cells for 120 min. At the indicated times, samples were harvested and immunoblotted with anti-hsp27 antibody. Each band was quantified by scanning densitometry.

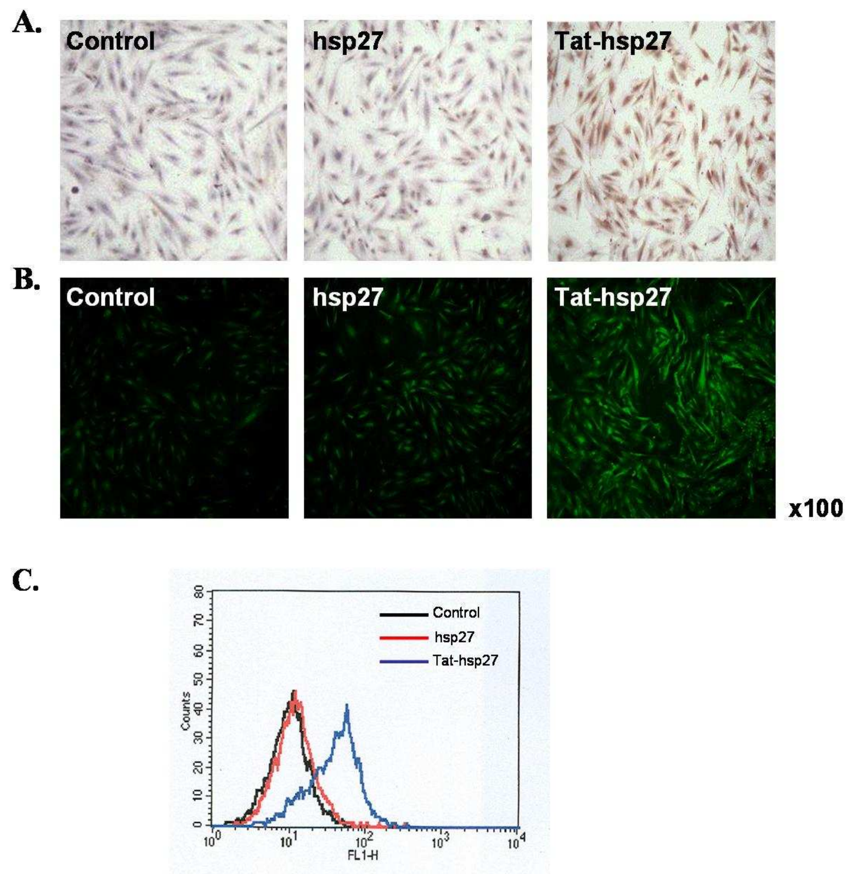


Fig. 5. Immunocytochemistry and FACS analysis of transduced Tat-hsp27 fusion protein in H9c2 cells. A and B. Immunocytochemistry demonstrating transduction of Tat-hsp27 fusion protein in H9c2 cells. H9c2 cells were treated with 500 nM of wt-hsp27 or Tat-hsp27 fusion proteins. Following incubation for 1 hr, the cells were incubated with anti-HA antibody. And then the cells were treated with HRP-conjugated (A) or FITC-conjugated (B) secondary antibody and analyzed by immunofluorescence microscopy. C. FACS analysis of H9c2 cells treated with Tat-hsp27 fusion protein. H9c2 cells were treated with 500 nM of wt-hsp27 or Tat-hsp27 fusion proteins. After 1 hr, the cells were incubated with anti-HA antibody and FITC-conjugated secondary antibody and then FACS peaks were analyzed by FACSCalibur.

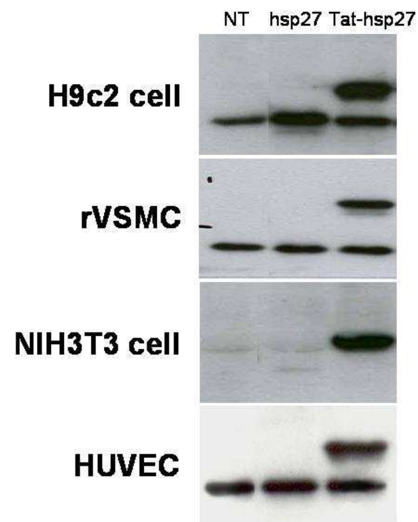


Fig. 6. Transduction of Tat-hsp27 fusion proteins in the various cell types. Cultured cells were treated with either 500 nM wt-hsp27 or Tat-hsp27 for 1 hr. Samples were harvested, lysed and immunoblotted with anti-hsp27 antibody. NT : No Treatment.

4. Protective effect of Tat-hsp27 transduction on hypoxia-induced cell death

We next determined whether the transduction of Tat-hsp27 protein in this manner would protect H9c2 cells against lethal hypoxia stress. H9c2 cells were pretreated with 200 ~ 1,000 nM of Tat-hsp27 for 1 hr and then subjected to hypoxia for 24 hr. Cell viability was determined using MTT assay. The treatment of H9c2 cells with Tat-hsp27 protein led to a significant increase in survival against a hypoxic insult (Fig. 7). The most apparent protective effect was observed when the pretreatment was performed with 500 nM of Tat-hsp27. Thus we used 500 nM of Tat-hsp27 protein in the following experiments. The H9c2 cells were treated with 500 nM of wt-hsp27 or Tat-hsp27 for 1 hr and exposed to hypoxic stress for 24 hr. As shown in Fig. 8, significant protective effect of the Tat-hsp27 against hypoxic stress was observed in the H9c2 cells. After hypoxic stress for 24 h, the viability of H9c2 cells decreased to about 60.2% compared to that in the normoxic condition. However, Tat-hsp27 treatment restored the cell viability to nearly 90% of that of the control (normoxic condition), and the viability in Tat-hsp27 sample was significantly higher than in non-treated control sample or wt-hsp27 sample under hypoxia ($p < 0.05$). A similar result was obtained using the trypan blue exclusion (data not shown). The protective effect of Tat-hsp27 was also

observed when the H9c2 cells were exposed to $CoCl_2$, hypoxia mimic agent (Fig. 9).

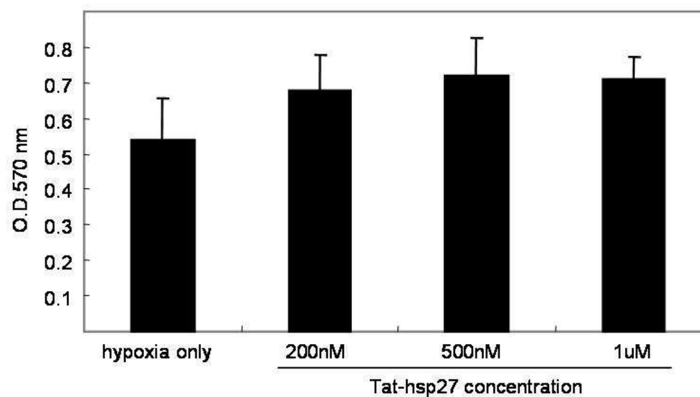


Fig. 7. Viability of H9c2 cells transduced with various concentrations of Tat-hsp27 under hypoxic condition. Cultured H9c2 cells were treated with various concentrations of Tat-hsp27 for 1 hr and followed by subsequent 24 h exposure to hypoxia. Cell viability was assessed by the MTT assay. Data are averages of four independent experiments.

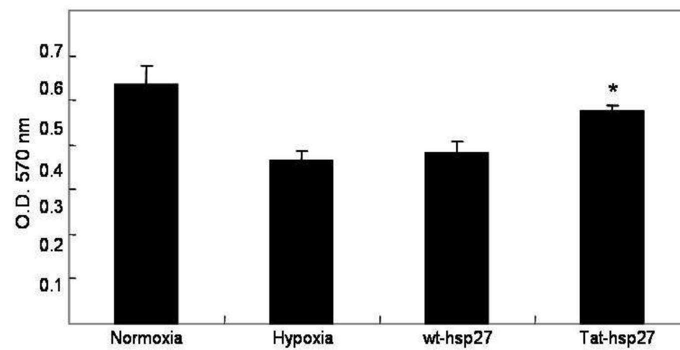


Fig. 8. Protective effect of Tat-hsp27 transduction on hypoxia-induced cell death. The H9c2 cells were treated with 500 nM of wt-hsp27 or Tat-hsp27 for 1 h and followed by subsequent 24 h exposure to hypoxia. Cell viability was assessed by the MTT assay. Data are averages of four independent experiments. * $P < 0.05$ compared to the control and wt-hsp27 treatment group.

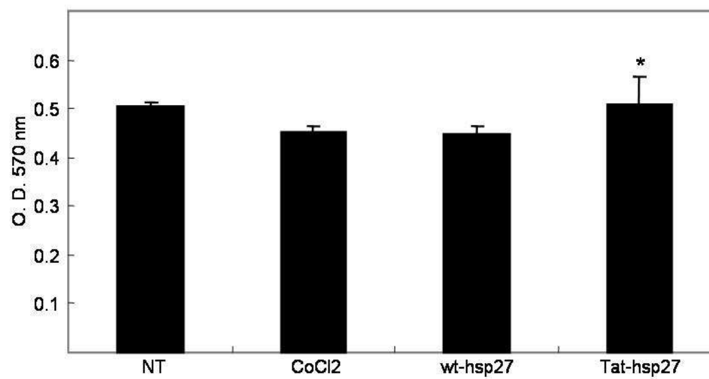


Fig. 9. Protective effect of Tat-hsp27 transduction on the H9c2 cell death by hypoxia mimic agent. Cultured H9c2 cells were treated with either 500 nM wt-hsp27 or Tat-hsp27 for 1 hr and followed by a subsequent 24 hr exposure to 100 μM $CoCl_2$. Cell viability was assessed by the MTT assay. Data are averages of four independent experiments. * $P < 0.05$ compared to the control and wt-hsp27 treatment group.

5. Effect of Tat-hsp27 transduction on hypoxia-induced caspase-3 activation

Hypoxia injury has been reported to induce apoptosis in cardiac cells. So we next studied whether the apoptotic cell death was altered by Tat-hsp27 protein transduction in H9c2 cells. Apoptotic death of H9c2 cells was examined by caspase-3 activity assay. The caspase-3 activity was determined by measuring spectrophotometrically at 405 nm amount of *p*NA cleaved from the substrate DEVD-*p*NA. In an incubation of H9c2 cells under hypoxic condition for 24 hr, apoptotic activity was increased by 3-fold compared to control cells under normoxic condition. There was a dramatic decrease down to 70% of the original level (hypoxic condition) in the caspase-3 activity after hypoxia in Tat-hsp27 transduction sample, whereas no difference between non-treated and wt-hsp27-treated samples was detected (Fig. 10).

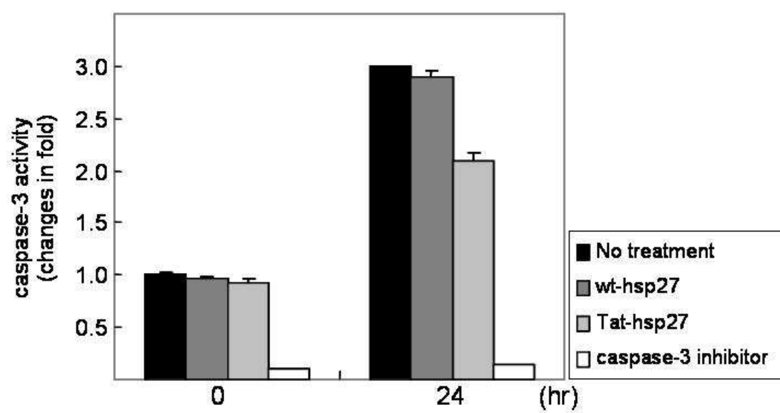


Fig. 10. Effect of Tat-hsp27 transduction on hypoxia-induced caspase-3 activation. The H9c2 cells were incubated with 500 nM of wt-hsp27 or Tat-hsp27 for 1 hr and then subjected to hypoxia. After 24 hr, caspase-3 activity was analyzed. Results are representative of three independent experiments.

6. *In vivo* transduction of Tat-hsp27 fusion protein in rat

To test whether Tat-hsp27 can be delivered into intact heart, Tat-hsp27 protein was first injected intraperitoneal (i.p.) into adult male Sprague-Dawley (SD) rats. Other studies suggested that PTD fusion protein could achieve robust transduction in the heart 2 hr after systemic injection. We performed immunoblot analysis to detect Tat-hsp27 in heart tissues at 2 hr after intraperitoneal injection of the fusion protein. Dose-dependent increases of transduced Tat-hsp27 protein were detected in heart tissues, compared with those of control rat injected with wt-hsp27 protein (Fig. 11). Tat-hsp27 was also detected in the several organs of SD rat (Fig. 12).

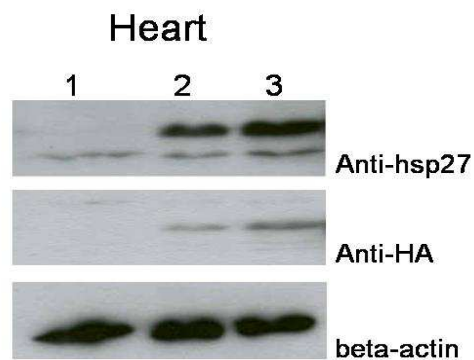


Fig. 11. Transduction of Tat-hsp27 into rat heart. Tat-hsp27 was injected i.p. into SD rats. After 2 hr heart was isolated and transduced Tat-hsp27 was detected by immunoblot analysis using anti-hsp27 or anti-HA antibody. Lane 1, saline control; Lane 2, Tat-hsp27 (3 mg/kg); Lane 3, Tat-hsp27 (5 mg/kg).

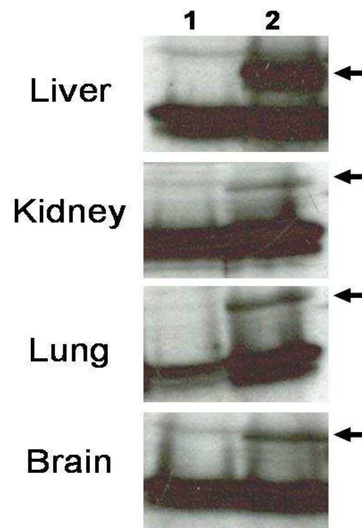


Fig. 12. Transduction of Tat-hsp27 into organs of rat. Tat-hsp27 was injected i.p. into SD rats. After 2 hr each organ was isolated and transduced Tat-hsp27 was detected by immunoblot analysis using anti-hsp27 or anti-HA antibody. Lane 1, saline control; Lane 2, Tat-hsp27 (5 mg/kg).

7. Myocardial ischemic damage reduced by Tat-hsp27 transduction *in vivo*

We also investigated whether *in vivo* injection of Tat-hsp27 could protect myocardial in acute ischemia animals subjected to acute coronary artery ligation. The left anterior descending coronary artery (LAD) was ligated in open-chest pentobarbital-anesthetized SD rats. Rats were randomly assigned to normal group, control ischemia group (saline treatment) and Tat-hsp27 treatment group with ischemia. A central thoracotomy was performed, the heart exposed, and the ligation of the left anterior descending coronary (LAD) artery, just below the first diagonal branch with a 6-0 suture was performed. Tat-hsp27 (5 mg/kg) was administered by i.p. injection during 2 days once a day before ligation. Tat-hsp27 was injected for 7 days once a day, and rats were sacrificed. LV infarct was determined from histological sections stained with Masson trichrome and hematoxylin/eosin to distinguish clearly between fibrous scar tissue and non-infarct tissue (Fig. 13). There was no significant difference in heart weights or ratios of heart weight to body weight (saline control, 6.2±0.2 mg/g versus Tat-hsp27, 6.2±0.3 mg/g) between two groups. Infarcts were observed in Masson trichrome stained myocardial sections after 7 days ischemic injury. Quantitative analysis revealed that administration with Tat-hsp27 before the onset of ischemia reduced the infarct volume compared

with saline control. Both the absolute area of the infarct and the percentage of the whole LV area taken up by the infarct were comparable between the saline- and Tat-hsp27-treated rats (Fig. 13C).

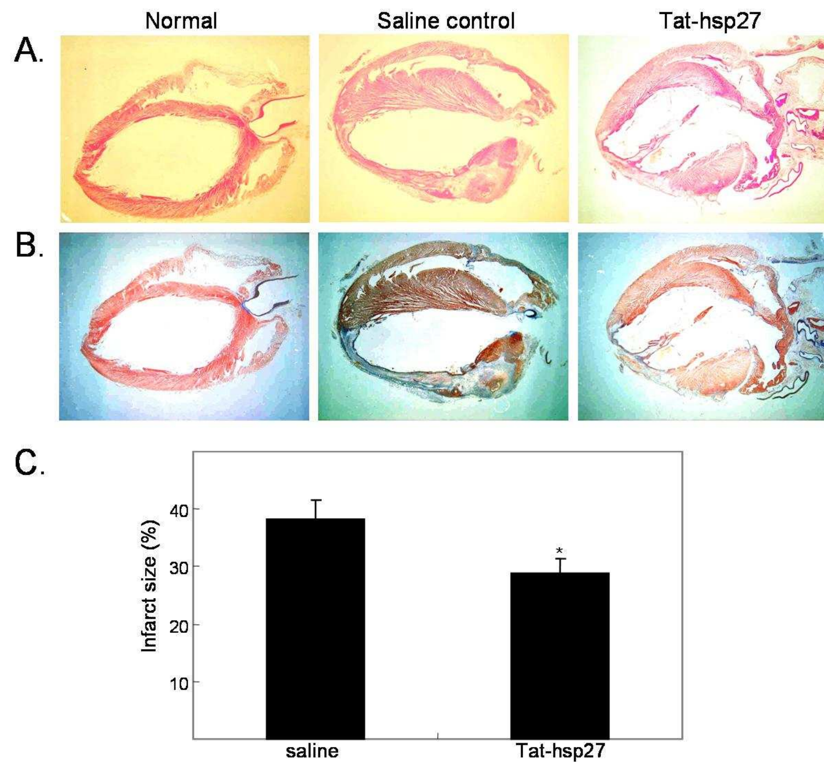


Fig. 13. Effect of Tat-hsp27 on infarct size in myocardial ischemia. A and B. Photomicrographs show representative myocardial sections stained with Hematoxylin/eosin and Masson trichrome in normal, ischemia-saline and ischemia-Tat-hsp27 groups. C. Quantitative analysis demonstrated that Tat-hsp27 treated hearts decreased infarct size after ischemia. Data are mean ± SEM (n=5). * $p<0.05$.

IV. DISCUSSION

This study demonstrates the ability of Tat-mediated transduction of the recombinant hsp27 protein to confer cardiac protection against an ischemic stress. The hsp27 has the cellular function that protects cells from the deleterious effects induced by heat or oxidative injuries.[26] This cytoprotective effect of hsp27 has been demonstrated in hypoxic injury studies with gene delivery using viral vector.[27,28] Recently, the protein transduction domains (PTDs) including Tat represent a potentially valuable tool for the treatment of several diseases.[29] In the present study, the focus was to determine whether direct intracellular delivery of hsp27 using Tat-PTD system could preserve cytoprotective effect after ischemic insult which induces cardiac cell death in the H9c2 cells and rat LAD ligation model. We first produced the Tat-hsp27 fusion protein containing the 11-amino acid transduction domain of Tat using recombinant technology. The resultant purified Tat-hsp27 fusion protein had additional peptides, 6-His-TAT-HA, at its N-terminus. The final product had a single detectable band, which is compatible with the predicted molecular mass by SDS-PAGE and immunoblot analysis with more than 95% purity. The Tat-hsp27 fusion protein could penetrate into H9c2 cells efficiently.

A variety of different roles for hsp27 during lethal stimuli have been

proposed to account for the cytoprotective effects.[30,31] Especially because of its cardiac protective effects,[32,33] we chose to study the effects of a Tat-hsp27 protein transduction in the present study. We observed that Tat-mediated delivery of hsp27 confers cytoprotection against hypoxic stress in H9c2 cells. Although cardiac cells express substantial amounts of hsp27, overexpression with gene delivery in primary culture cells confers additional protection against hypoxic insult.[28,34] Tat-hsp27 delivery significantly increased cardiac cell survival after hypoxic insult, and it did so by protecting the cells from hypoxia-induced cell death associated with caspase-3 activation. The loss of cardiac cells through cell death by hypoxia (ischemia) is a major problem in heart failure.[35,36] Hypoxia induces mitochondrial cytochrome c release and caspase-3 activation, leading to cell death.[37] The hsp27 also plays a role in mediating the inhibition of the cell death effector caspase-3.[38,39] In our results, the level of caspase-3 activity correlated positively with the degree of cell death in the H9c2 cells exposed to hypoxic insult, and this increase of caspase-3 activity was reduced by Tat-hsp27 transduction. The transduction of Tat-hsp27 increased cell viability after hypoxia stress by 30%, as compared to treatment with wt-hsp27, through inhibiting the caspase-3 pathway in H9c2 cells (Fig. 7-10). Recent evidence has shown that hsp27 regulates cell death through the interaction with key components of the apoptotic signaling

pathway, in particular, those involved via caspase cascade activation.[40,41] Our results suggest that intracellularly delivered hsp27 suppresses caspase-associated cell death, at least in part, by causing interaction with cytochrome c or procaspase-3 following preventing its correct function, thereby allowing the cells better tolerate potentially lethal hypoxia.

In our additional experiments, the transduction of Tat-hsp27 also provides for diminished cardiac injury from acute ischemia in rat LAD ligation model. At the dosage of 5 mg/kg, administration of Tat-hsp27 resulted in an up to ~32% reduction in infarct sizes 7 days after ischemia (Fig. 13). Although many genes have the potential to treat cardiac ischemia, target genes or their translated products are often difficult to express *in vivo*. However, PTD-mediated protein transfer can overcome this disadvantage. To date, many protein deliveries have been examined for cardiac ischemia using target proteins such as apoptosis repressor with caspase recruitment domain (ARC) [42] and BH4 domain peptide[43]. Compared with other delivery systems, the PTD transduction system described in this study may have several advantages.[44,45] First, PTD consists of only ten amino acids or thereabouts which does not substantially increase the particle mass of the fusion protein. Second, the transduction of PTD-fusion protein does not require a helper protein. Third, the PTD-mediated protein may be transduced into all mammalian cell types.

Therefore, protein delivery using PTD represents a potentially valuable tool for exploring molecular mechanisms and therapeutic potentials of target proteins *in vitro* and *in vivo*.

In summary, our works demonstrate that direct intracellular delivery of hsp27 using a PTD system is capable of protecting the cardiac cells and tissue from ischemic stress. Based on our results, we suggest that the PTD-mediated delivery of hsp27 may be a useful therapeutic method for ischemic heart diseases.

V. CONCLUSION

This study demonstrates that intracellular delivery of hsp27 protein fused to a protein transduction domain Tat shows significant protective effect against ischemia-induced cell death *in vitro* and *in vivo*. We first produced a Tat-hsp27 fusion protein containing the 11-amino acid transduction domain of TAT using recombinant technology. The Tat-hsp27 fusion protein could enter efficiently H9c2 cells within 1 hr. Tat-hsp27 delivery significantly increased cardiac cell survival after hypoxic insult, and it did so by protecting the cells from hypoxia-induced apoptosis associated with caspase-3 activation. Also rats with MI induced by left anterior descending coronary artery ligation were intraperitoneal injected with Tat-hsp27 or saline everyday. After 1 week treatment with Tat-hsp27, when compared with saline-treated group, myocardial infarction was significantly reduced. Compared with other delivery systems, the PTD transduction system may have several advantages including stability, lack of toxicity, and ability of transduction into all cell types. Gene delivery using plasmid or viral vectors is not feasible, particularly in an acute disease, because of the extended time needed for gene expression after transfection or viral infection. Thus, it will be of important interest to determine whether cytoprotective protein fused to a PTD can protect the

cardiac tissue *in vivo* against the ischemic damage. Especially because the target protein with PTD can be introduced rapidly, it is a potentially valuable tool for managing heart diseases such as acute myocardial infarction.

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ABSTRACT (KOREAN)

단백질 전달 도메인을 이용한 열충격 단백질 27 전달의 허혈성 심장 손상 억제 효과

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권 준 혜

열충격 단백질 (hsp)은 대부분의 생명체에 존재하는 단백질로 세포 내외부의 자극에 대한 보호 기능과 다른 단백질들의 폴딩과 이동에 중요한 역할을 한다고 알려져 있다. 이중 분자량이 27 kDa인 열충격 단백질 27 (hsp27)은 세포사멸을 초래하는 다양한 스트레스에 의해 전달되는 세포사멸 경로를 억제함으로써 세포를 보호하는 대표적인 단백질이다. 동물세포나 조직에서 허혈, 고혈당, 활성산소 등의 세포독성을 초래하는 자극에 대해 hsp27의 유전자 전달 방법에 의한 과발현으로 이들 자극으로 인한 세포사멸의 억제 기능에 대한 많은 연구결과가 보고되어 왔다. 본 연구에서는 생체물질 전달 시스템중의 하나인 단백질 전달 도메인 (protein transduction domain, PTD)

을 이용하여 hsp27 단백질을 심장세포와 조직에 전달하여 그 효능을 관찰하였다. 사람의 hsp27 cDNA을 얻은 후 단백질 전달 도메인 Tat-PTD에 융합하여 재조합 단백질 Tat-hsp27을 제조하였다. Immunoblot, 면역 형광염색법으로 Tat-hsp27의 세포 내 전달 효율을 분석한 결과 대부분의 세포에 빠른 시간 내에 전달되었다. 심근유래 세포인 H9c2 세포 안으로 전달된 Tat-hsp27 단백질에 의해 저산소 자극에 대한 세포 사멸이 억제되었고 세포고사의 주요 경로인 caspase-3의 활성이 현저히 감소됨을 관찰하였다. 설치류 좌하행지 관상동맥 결찰 모델 (rat left anterior descending coronary artery ligation model)을 제작하여 1주일간 Tat-hsp27을 복강 내 주사하고 심장 조직을 조직학적 방법을 통해 분석한 결과, 심근 경색의 크기가 대조군에 비해 현저히 감소되었다. 본 연구결과를 통하여 치료 목적 단백질을 단백질 전달 도메인과의 융합 단백질로 제조하여 생체에 전달함으로써 단백질 치료의 새로운 방법으로 응용할 수 있는 가능성을 제시하였고, 특히 이 방법은 목적 단백질의 전달 효율과 빠른 전달 시간 등의 장점을 갖고 있어 심근경색 등의 급성 질환에 매우 효과적인 치료 방법으로 이용될 수 있을 것으로 사료된다.

핵심되는 말 : 열 충격 단백질 27, 단백질 전달 도메인, 저 산소 자극, 허혈