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Effect of silk fibroin peptide derived from silkworm *Bombyx mori* on the anti-inflammatory effect of Tat-SOD in a mice edema model

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We investigated whether silk fibroin peptide derived from the silkworm, *Bombyx mori*, could inhibit inflammation and enhance the anti-inflammatory activity of Tat-superoxide dismutase (Tat-SOD), which was previously reported to effectively penetrate various cells and tissues and exert anti-oxidative activity in a mouse model of inflammation. Inflammation was induced by topical treatment of mouse ears with 12-O-tetradecanoylphorbol-13-acetate (TPA). Histological, Western blot, and reverse transcription-polymerase chain reaction data demonstrated that silk fibroin peptide or Tat-SOD alone could suppress elevated levels of cyclooxygenase-2, interleukin-6, interleukin-1beta, and tumor necrosis factor-alpha induced by TPA. Moreover, silk fibroin peptide significantly enhanced the anti-inflammatory activity of Tat-SOD, although it had no influence on *in vitro* and *in vivo* transduction of Tat-SOD. Silk fibroin peptide exhibited anti-inflammatory activity in a mice model of inflammation. Therefore, silk fibroin peptide alone or in combination with Tat-SOD might be used as a therapeutic agent for various inflammatory diseases. [BMB reports 2011; 44(12): 787-792]

INTRODUCTION

In oriental medicine, the silkworm, *Bombyx mori*, has been traditionally used to treat flatulence, spasms, and phlegm (1). Recently, silk fibroin protein derived from silkworms has been utilized as a surgical suture and tissue engineering scaffold, because of its properties to resist harsh conditions such as high temperature, moisture, and mechanical stress (2, 3). There is also evidence to show other functions of silk fibroin. Silk fibroin upregulates the expression of several osteoblastogenic genes, such as alkaline phosphatase, collagen type-I alpha-1, and transforming growth factor-beta 1 in MG63 cells (2). Additionally, fibroin peptide has been suggested to ameliorate diabetic hyperglycemia in 3T3-L1 adipocytes (4). Furthermore, silk fibroin peptide has the potential to reduce blood pressure, as evidenced by animal experiments using hypertensive rats (5).

Anti-oxidant enzymes have therapeutic capacity to eliminate oxidative stress, but their hydrophilic properties and large molecular size prevent them from entering target cells. So, protein transduction domains (PTD) were developed as one of several ways to deliver numerous therapeutic molecules to living cells (6, 7). Particularly, superoxide dismutase (SOD) catalyzes peroxidation of numerous harmful free radicals; thus, providing ample cellular protection against oxidative damage (8). We previously reported that anti-oxidant enzymes such as SOD, ribosomal protein S3, and sensitive to apoptosis gene fused with PTD and the fusion proteins are effectively delivered to cells and tissues and exert protection against inflammation, ischemia, and Parkinson's disease (9-11) Additionally, bog blueberry anthocyanins and levosulpiride enhance transduction efficiency of PTD-fused proteins into cells and have the ability to significantly improve the therapeutic potential of PTD-fused proteins (12, 13).

In the present study, we investigated whether silk fibroin peptide could affect the *in vitro* and *in vivo* transduction efficiency of Tat-SOD into HaCaT cells and mice skin. We also compared the anti-inflammatory activities in samples treated with silk fibroin peptide, Tat-SOD alone, and both silk fibroin peptide and Tat-SOD, to search for a possibility of utilizing silk fibroin peptide and/or Tat-SOD as a therapeutic agent for various inflammatory diseases.

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RESULTS

Anti-inflammatory activity of silk peptide and Tat-SOD in a mice ear edema model

We previously reported that Tat-SOD has the potential to be transduced into cells and animal tissues, and that it exhibits anti-oxidant activity and reduces oxidative stress (14). In the present study, we identified the property that allows silk peptide to inhibit inflammation in a mice edema model. As shown in Fig. 1, topical treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA), which generates reactive oxygen species (ROS) and induced inflammation, increased ear thickness approximately three-fold, compared to that in control samples. SOD-treated samples showed swelling similar to TPA-treated samples. However, treatment with Tat-SOD or silk alone prevented edema formation on ear tissues induced by TPA. Additionally, a combined treatment with silk and Tat-SOD suppressed TPA-induced inflammation. These data collectively demonstrate that silk and Tat-SOD protect skin tissues from TPA-induced inflammation in an ear edema model. Moreover, our results also suggest the possibility that co-treatment with silk and Tat-SOD could be a potent therapy against induced inflammation.

Effect of silk and Tat-SOD on pro-inflammatory mediators and cytokines in TPA-induced ear edema

Next, we confirmed the histological observation presented above by assessing elevated expression levels of pro-inflammatory genes in ear tissues using Western blot and reverse transcription-polymerase chain reaction (RT-PCR). As shown in Fig. 2A-C, application of TPA to mice ears upregulated levels of pro-inflammatory mediators and cytokines such as cyclooxygenase-2 (COX-2), interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α. Although SOD-treated samples alone did not reduce upregulation of these genes, Tat-SOD or silk slightly suppressed COX-2, IL-6, and IL-1β expression. Particularly, a combined treatment with Tat-SOD and silk remarkably suppressed the increased expression of these genes. These results were in agreement with our histological analysis. Therefore, it seems likely that co-treatment with silk and Tat-SOD may be useful therapy for various in-

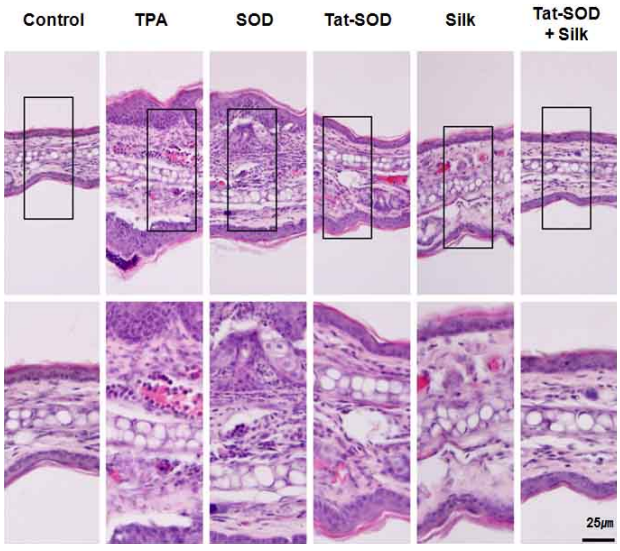

Fig. 1. Anti-inflammatory effects of SOD, Tat-SOD and silk peptide in TPA-induced ear edema model. The ears of mice were pretreated with TPA (1.0 μg/ear) once per day for 3 days. Then, silk (3.0 μg/ear) was topically applied to ears followed by treatment with SOD or Tat-SOD (2.0 μg/ear). Ear biopsies were obtained on the fourth day from the beginning of treatment. Histochemical analysis of ear biopsies from each group was performed using rabbit anti-histidine IgG and biotinylated goat anti-rabbit IgG.

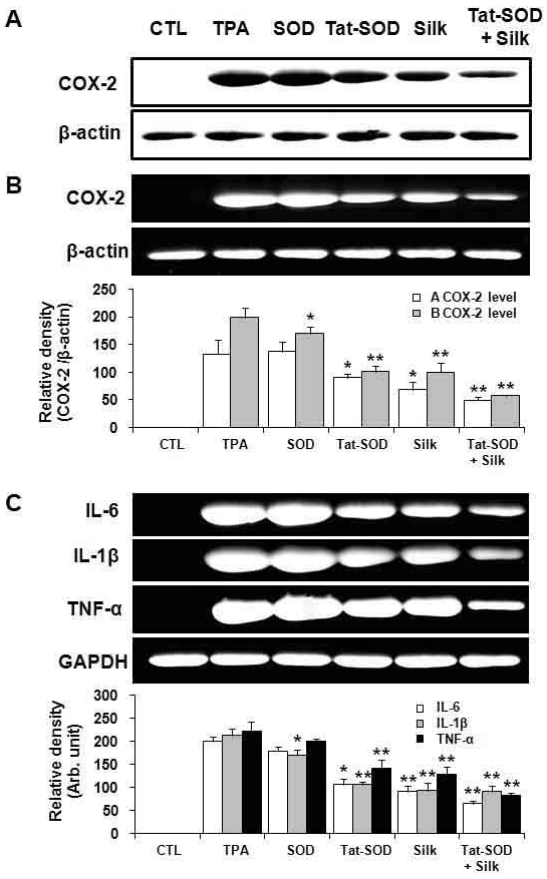

Fig. 2. Effect of silk and Tat-SOD on levels of pro-inflammatory mediators and cytokines in a TPA-induced inflammation mice model. The ears of mice were treated with TPA (1.0 μg/ear), silk (3.0 μg/ear), and SOD or Tat-SOD (2.0 μg/ear) at intervals of 1 h for 3 consecutive days. The expression levels of COX-2, β-actin, IL-6, IL-1β, TNF-α and GAPDH in ear samples were analyzed by (A) Western blotting or (B) and (C) RT-PCR. Lanes are as follows: CTL, untreated control; TPA, TPA-treated skin; SOD, SOD treated skin after TPA treatment; Tat-SOD, Tat-SOD treated skin after TPA treatment; Silk, silk treated skin after TPA treatment; Tat-SOD + silk, Silk and Tat-SOD treated skin after TPA treatment. Relative density of each band is expressed as mean ± SD (n = 5). *P < 0.05 and **P < 0.01, compared with TPA-treated group.

flammation-related diseases.

Delivery of SOD and Tat-SOD to HaCaT cells and mice ears in the absence or presence of silk peptide

Co-treatment with silk and Tat-SOD exhibited enhanced anti-inflammatory activity against TPA-induced inflammation, compared to samples treated with silk or Tat-SOD alone. Therefore, we examined whether silk peptide enhances the transduction of Tat-SOD into HaCaT keratinocytes, thereby leading to more enhanced anti-inflammatory effects. Our results (Fig. 3A and B) demonstrated that SOD, possessing no PTD, completely failed to be transduced into cells irrespective of silk treatment. Although Tat-SOD showed efficient transduction into cells in a dose-dependent manner, silk (0.2 μg/ml) did not enhance the transduction of Tat-SOD into cells. The cells were incubated with various concentrations of silk fibroin peptide (0.5-5 μg/ml) 1 h prior to treatment with Tat-SOD to investigate the possibility that a high silk dose would have an effect on the transduction of Tat-SOD into cells. Fig. 3C shows that both high and low concentrations of silk had no influence on the transduction of Tat-SOD into cells. The amount of Tat-SOD transduced into cells did not change, although the incubation times (10-60 min) did change (Fig. 3D). Next, we examined the effect of silk peptide on *in vivo* transduction of Tat-SOD into mice skin. As evidenced by the *in vitro* transduction data (Fig. 3), SOD alone had no ability to be transduced into mice skin tissues regardless of the presence of the silk peptide (Fig. 4). In addition, the absence or presence of silk was of little or no benefit during *in vivo* and *in vitro* transduction of Tat-SOD. Therefore, these findings suggest that enhanced anti-inflammatory activities by co-treatment with silk and Tat-SOD are likely to be caused by the anti-inflammatory effect of silk, itself, without affecting the transduction of Tat-SOD.

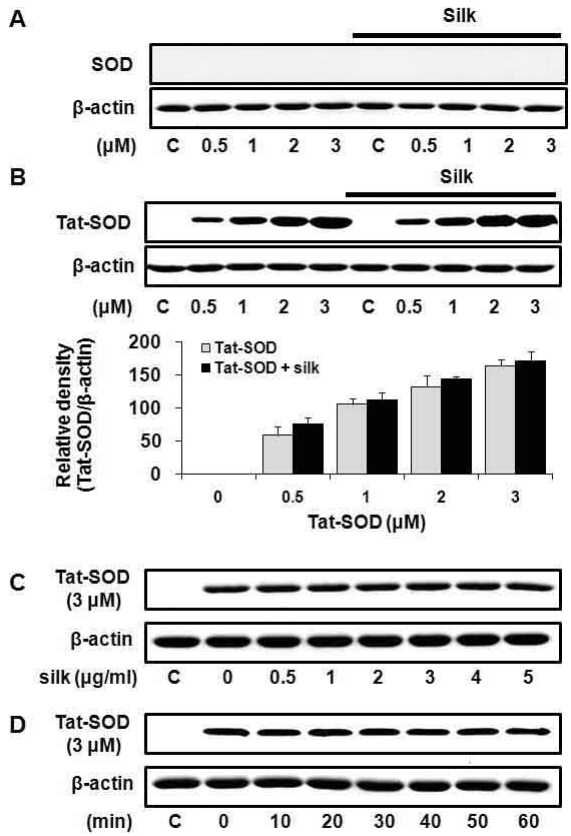

Fig. 3. Transduction levels of SOD and Tat-SOD into HaCaT cells in the presence or absence of the silk fibroin peptide. HaCaT cells were incubated with or without silk fibroin (0.2 μg/ml) followed by treatment with various concentrations (0.5-3.0 μM) of SOD and Tat-SOD for 1 h to assess transduction of (A) SOD and (B) Tat-SOD into HaCaT cells in the presence of silk fibroin peptide. The transduction of Tat-SOD (3.0 μM) into HaCaT cells was assessed following (C) treatment with various concentrations (0.5-5.0 μg/ml) of silk peptide for 1 h or (D) treatment with silk peptide (0.3 μg/ml) for various incubation times (10-60 min). The transduction level of each protein was analyzed by Western blot and band intensities were measured with a densitometer. C, untreated control.

DISCUSSION

ROS, which naturally occur due to various cellular responses and exposure to heavy metals and oxidants, lead to DNA damage, cell death, and various diseases such as inflammation, cancer, and ischemia (15). Treatment with lipopolysaccharides or TPA triggers inflammation and carcinogenesis in animal models (16). Thus, ROS induce the expression of various pro-inflammatory genes and activate numerous signal transduction pathways. For example, keratinocytes synthesize a wide variety of pro-inflammatory cytokines, chemokines, and growth factors such as TNF-α, IL-1, IL-6, and prostaglandins (PGEs). These factors bind to receptors of target cells and activate signal transduction pathways including mitogen activated protein kinase, nuclear factor-kappaB (NF-κB), and PI3K/AKT, thereby modulating cell growth, differentiation, and activation through autocrine and paracrine mechanisms (17, 18). Furthermore, NF-κB activation is a crucial event in ROS-induced signal transduction pathways. Following phosphorylation, the phosphorylated NF-κB translocates to the nucleus and subsequently stimulates transcription of a number of genes related to immune and inflammatory responses (19). Furthermore, COX-2, an inducible enzyme, is upregulated with increased inflammation and carcinogenic states

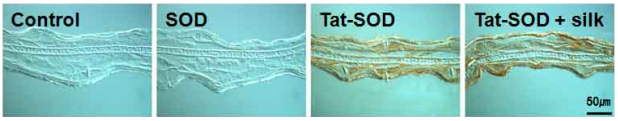

Fig. 4. Transduction of Tat-SOD in mouse skin tissues. Mice in the Tat-SOD + silk treated group were pretreated with silk peptide (3.0 μg/ear) 1 h prior to treatment with Tat-SOD (3.0 μg/ear). SOD or Tat-SOD protein (3.0 μg/ear) was topically applied onto shaved skin of mice in the SOD and Tat-SOD groups, respectively. Mice skin tissues were collected, and SOD and Tat-SOD were detected using rabbit anti-histidine IgG and biotinylated goat anti-rabbit IgG.

and is responsible for the first step in PGE synthesis (20). Consequently, in response to various forms of oxidative stress, cells attempt to diminish increased ROS levels by upregulating anti-oxidant enzymes including SOD, catalase, and glutathione peroxidase (21, 22). Therefore, a number of research groups have proposed various ways to increase levels of therapeutic proteins, such as anti-oxidant enzymes, or suppress elevated expression of inflammatory mediators in target cells as therapy for ROS-related diseases (6, 7).

Several drugs and agents for treating inflammation-related disorders have been studied and are currently undergoing clinical trials (23). Receptor antagonists and antibodies against pro-inflammatory cytokines, such as TNF-α and IL-1, are being developed because of their potency for blocking signal transduction of pro-inflammatory cytokines (23-25). Many chemicals exert anti-inflammatory effects by inhibiting nuclear translocation and activating NF-κB, modulating downstream signaling of Akt, and blocking the COX-2/PGE2 pathway (23, 26, 27).

In the present study, we demonstrated that silk fibroin peptide alone suppresses inflammation in a similar manner as the anti-inflammatory activity of Tat-SOD by evaluating ear tissue thickness in a mouse model of ear edema (Fig. 1). Silk fibroin peptide suppressed elevated levels of the pro-inflammatory cytokines COX-2, IL-6, IL-1β, and TNF-α (Fig. 2). Moreover, treatment with silk fibroin peptide enhanced the anti-inflammatory activity of Tat-SOD (Fig. 1 and 2). As shown in Fig. 3 and 4, silk fibroin peptide failed to increase transduction of Tat-SOD into HaCaT cells or ear tissues. Accordingly, the enhanced anti-inflammatory activity in the sample co-treated with Tat-SOD and silk fibroin peptide might be considered to originate from the silk fibroin peptide itself, rather than from increased levels of Tat-SOD in ear tissues. Therefore, we demonstrated that silk fibroin peptide has the potential to effectively suppress several pro-inflammatory cytokines overexpressed by TPA induction.

Although many questions remain about the exact mechanism by which silk fibroin peptide exerts its anti-inflammatory activity or enhances anti-inflammatory activity of Tat-SOD, it is clear that silk fibroin peptide has the potential to reduce inflammation. Inflammation is a very complicated process, as it is under strict control of various signaling pathways and numerous factors. Additionally, there is a possibility that different experimental conditions, such as use of different cell lines, analytical techniques, and a different silk fibroin preparation process, may produce very different results. Due to the wide variety of biological activities of silk fibroin peptide, many groups are conducting research about its therapeutic efficacy and detailed mechanisms. Therefore, it is necessary to demonstrate the diverse usefulness of silk fibroin peptides through additional studies.

In conclusion, irrespective of treatment type, whether alone or in combination with other anti-inflammatory drugs, silk fibroin peptide may be useful as a therapeutic agent against inflammatory diseases.

MATERIALS AND METHODS

Materials and cell culture

HaCaT human keratinocytes were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) and maintained at 37°C under a humidified atmosphere of 95% air and 5% CO_2. All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of silk peptide

The low molecular weight silk peptides were prepared according to the Rural Development Administration, as described previously (2). Silk cocoons were harvested from the Rural Development Administration (Suwon, Korea). The sliced silk cocoons were degummed twice with Marseilles soap (0.5% of fiber weight) and sodium carbonate solution (0.3% of fiber weight) at 100°C for 1 h and then washed with distilled water to remove silk sericin. Silk fibroin was digested with 6 M HCl for 5 h and the digestion was stopped by adding NaOH. Salt was removed from the silk peptide using an electrodialysis system. After removal of the salt, silk fibroin powder was obtained by freeze-drying. The molecular weight of the silk peptide was approximately 3,000 Da.

Transduction of SOD and Tat-SOD into HaCaT human keratinocytes

Tat-SOD and SOD were purified as described previously (10, 11). Cells seeded in 6-well plates were grown to confluence and treated with or without silk peptide (0.2 μg/ml) for 1 h to assess the effect of silk peptide on the concentration-dependent transduction of Tat-SOD and SOD into HaCaT cells. The cells were then exposed to various concentrations (0.5-3.0 μM) of each protein for an additional 1 h. Next, cells were exposed to various concentrations (0.5-5.0 μg/ml) of silk for 1 h prior to treatment with Tat-SOD (3.0 μM) for 1 h to examine whether silk peptide at a higher concentration influences transduction of Tat-SOD (3.0 μM) into cells. The cells were then harvested, and cell extracts were prepared for Western blot analysis.

Western blot analysis

Proteins in cell lysates and ear biopsy homogenates were resolved by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in PBS and incubated with rabbit anti-histidine, COX-2, and β-actin polyclonal antibodies (1 : 1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibody (1 : 10,000; Sigma- Aldrich). The membrane was visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Piscataway, NJ, USA).

Animal study

Male ICR mice (6-8-weeks-old) were purchased from the Hallym University Experimental Animal Center and maintained at 23°C and a relative humidity of 60% with alternating 12 h cycles of light and dark. They were provided with food and water *ad libitum*. All experimental procedures involving animals and their care conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research and Quarantine Service of Korea and were approved by the Hallym Medical Center Institutional Animal Care and Use Committee.

In vivo transduction of SOD and Tat-SOD into mice ears

Silk peptide (3.0 μg/ear) was topically applied to the ear surface and then the same surface was exposed to Tat-SOD (3.0 μg/ear) for 1 h to investigate whether silk peptide affects Tat-SOD and SOD transduction into mice tissues. Ear biopsy samples were obtained and fixed in 4% paraformaldehyde. The biopsy sample sections were immunostained using a rabbit anti-histidine polyclonal antibody (1 : 400) and biotinylated goat anti-rabbit secondary antibody (1 : 200). The sections were visualized with 3, 3′-diaminobenzidine and observed microscopically.

Anti-inflammatory activity of Tat-SOD in the TPA-induced inflammation mouse model

The right ear of each mouse (n = 5) was topically treated with TPA (1.0 μg) dissolved in 20 μl of acetone, as previously described, to induce skin inflammation (9). Silk fibroin peptide (3.0 μg/ear) was applied to the same surfaces, and Tat-SOD or SOD (2.0 μg/ear) was topically applied 1 h later. The treatments were repeated for 3 consecutive days. On the fourth day, 5 mm diameter ear punches were taken from each mouse. Ear biopsy samples were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin for histological analysis.

RT-PCR

Total RNA from ear biopsy samples was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized from total RNA (2 μg) using reverse transcriptase (1,000 U) and 0.5 μg/μl of oligo-(dT) primer. The cDNA were PCR-amplified with the following specific primers: COX-2 antisense, 5′-TGGACGAGGTTTTTCCACCAG-3′; COX-2 sense, 5′-CAAAGGCCTCCATTGACCAGA-3′; IL-6 antisense, 5′-TGGATGGTCTTGGTCCTTAGCC-3′; IL-6 sense, 5′-CAAGAAAGACAAAGCCAGAGTCCTT-3′; IL-1β antisense, 5′-GTGCTGCCTAATGTCCCCTTGAATC-3′; IL-1β sense, 5′-TGCAGAGTTCCCCAACTGGTACATC-3′; TNF-α antisense, 5′-TGGCACCACTAGTTGGTTGTCTTT-3′; TNF-α sense, 5′-AAGTTCCCAAATGGCCTCCC-3′; β-actin antisense, 5′-GGACAGTGAGGCCAGGATGG-3′; β-actin sense, 5′-AGTGTGACGTTGA CATCCGTAAAGA-3′ and GAPDH antisense, 5′-CTCGCTCCTGGAAGATGGTG-3′; GAPDH sense, 5′-GGTGAAGGTCGGTGTGAACG-3′. PCR products were resolved on 1% agarose gel and visualized with UV light after ethidium bromide staining.

Statistical analysis

Data are expressed as mean ± SD. Comparisons between groups were performed with the Student's *t*-test. $P < 0.01$ and $P < 0.05$ were considered statistically significant.

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