Enhancement of Anti-Inflammatory Activity of PEP-1-FK506 Binding Protein by Silk Fibroin Peptide

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Silk fibroin (SF) peptide has been traditionally used as a treatment for flatulence, spasms, and phlegm. In this study, we examined whether SF peptide enhanced the anti-inflammatory effect of PEP-1-FK506 binding protein (PEP-1-FK506BP) through comparing the anti-inflammatory activities of SF peptide and/or PEP-1-FK506BP. In the presence or absence of SF peptide, transduction levels of PEP-1-FK506BP into HaCaT cells and mice skin and anti-inflammatory activities of PEP-1-FK506BP were identified by Western blot and histological analyses. SF peptide alone effectively reduced both mice ear edema and the elevated levels of cyclooxygenase-2, interleukin-6 and -1β, and tumor necrosis factor-α, showing similar anti-inflammatory effect to that of PEP-1-FK506BP. Furthermore, co-treatment with SF peptide and PEP-1-FK506BP exhibited more enhanced anti-inflammatory effects than the samples treated with SF peptides or PEP-1-FK506BP alone, suggesting the possibility that SF peptide and PEP-1-FK506BP might interact with each other. Moreover, the transduction data demonstrated that SF peptide did not affect the transduction of PEP-1-FK506BP into HaCaT cells and mice skin, indicating that the improvement of anti-inflammatory effect of PEP-1-FK506BP was not caused by enhanced transduction of PEP-1-FK506BP. Thus, these results suggest the possibility that co-treatment with SF peptide and PEP-1-FK506BP may be exploited as a useful therapy for various inflammation-related diseases.

Keywords: Inflammation, PEP-1-FK506BP, protein transduction, reactive oxygen species, silk fibroin peptide, TPA

During various cellular processes, reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anion, and hydrogen peroxide, are generated and, consequently, can cause lipid peroxidation, DNA damage, cell death, and various diseases, resulting in inflammation, cancer, Parkinson’s disease, and ischemia [1, 5, 19]. Treatment with lipopolysaccharides (LPS) or 12-O-tetradecanoylphorbol-13-actate (TPA), known as inflammation inducers used in animal models [25, 30], can activate macrophages and secrete pro-inflammatory mediators, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), and pro-inflammatory cytokines, including interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-alpha (TNF-α). In addition, nuclear factor-kappaB (NF-κB) and mitogen-activated protein kinase (MAPK) pathway are related to inflammatory responses [7, 8].

FK506 binding proteins (FK506BPs) are members of an immunophilins family, which can bind immunosuppressive drugs such as rapamycin and FK506, used in prevention of graft rejection following organ transplantation [2, 11, 21]. In particular, FK506BP, a small peptide of 12 kDa, was first associated with rapamycin. The resultant FK506BP–rapamycin complex binds to and then disrupts the formation of activated mammalian target of rapamycin complex 1 (mTORC1), which can control protein synthesis, cell proliferation, and cell growth in response to nutrients and growth factors, leading to the suppression of protein synthesis and cell growth [4, 6, 16]. Furthermore, we previously reported that, through fusing FK506BP with a
PEP-1 domain, PEP-1-FK506BP could be delivered to cell lines and tissues and exert anti-inflammatory activity, thereby suggesting that PEP-1-FK506BP may be used as a potential therapeutic drug for inflammatory diseases and especially atopic dermatitis [13, 14].

Silk protein derived from silkworms (Bombyx mori) consists of fibroin and sericin. Although sericin, which naturally coats fibroin proteins, can induce allergic and inflammatory responses, fibroin protein, showing the resistance to temperature, moisture, and mechanical stresses, has been used for surgical suture after the removal of sericin. Moreover, many studies have reported that silk fibroin (SF) peptide can be a good candidate for tissue engineering scaffold applications [18]. In addition, various biological properties of silk fibroin proteins have been widely studied. Low molecular weight SF is shown to up-regulate the osteoblastogenic gene expression, such as alkaline phosphatase, collagen type-I alpha-1, and TGF-beta1, in MG63 cells [12]. Fibroin peptide, when orally administered to hypertensive rats, proved to significantly decrease the blood pressure of hypertensive rats [10]. Hyun et al. [9] reported that fibroin peptide can improve diabetic hyperglycemia in 3T3-L1 adipocytes.

Therefore, the objective of this study was to examine whether SF peptides could exert in vivo anti-inflammatory activity, using reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis, and also whether co-treatment with SF peptide and PEP-1-FK506BP may be exploited as a potential therapy for various inflammatory diseases, compared with treatment with PEP-1-FK506BP or SF peptide alone.

**Materials and Methods**

**Materials and Cell Culture**

Primary antibodies against COX-2 and beta-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents were obtained from Sigma-Aldrich (MO, USA) unless otherwise stated. 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 kept at 37°C under a humidified atmosphere of 95% air and 5% CO2.

**Preparation of SF Peptide**

The preparation of low molecular weight SF peptide was conducted according to the Rural Development Administration (RDA) as previously described [12]. Silk cocoons were harvested from the RDA (Suwon, Korea). The sliced silk cocoons were degummed twice with Marseilles soap (0.5% of the weight of fiber) and sodium carbonate solution (0.3% of the weight of fiber) at 100°C for 1 h, and then washed with distilled water to remove silk sericin. SF was digested by 6 M HCl for 5 h and stopped by the addition of NaOH. Removal of salt from SF peptide was performed using an electrodialysis system, after which SF powder was finally obtained using freeze-drying methods. The molecular mass of the SF peptide was approximately 3,000 Da.

**Transductions of PEP-1-FK506BP and FK506BP into HaCaT Human Keratinocytes Cells**

PEP-1-FK506BP and FK506BP were purified as previously described [13, 14]. To assess the concentration dependence of PEP-1-FK506BP and FK506BP transduction in the absence or presence of SF peptide, cells were grown to confluence in wells of a 6-well plate, pretreated with or without SF peptide (0.3 µg/ml) for 1 h, and then exposed to various concentrations (0.5–3.0 µM) of each protein for an additional 1 h. To investigate the difference in the transduction of PEP-1-FK506BP (3.0 µM) in the presence of various concentrations (0.5–5.0 µg/ml) of SF peptide, cells were exposed to various concentrations of SF peptide for 1 h, prior to treatment with PEP-1-FK506BP (3.0 µM) for 1 h. Then, the cells were 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 (SDS-PAGE) and electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in PBS and incubated with a rabbit anti-histidine polyclonal antibody for detections of FK506BP and PEP-1-FK506BP or the primary antibodies against COX-2 and beta-actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibody (dilution 1:10,000; Sigma-Aldrich, St. Louis, MO, USA). The membrane was visualized by enhanced chemiluminescence (ECL) according to the manufacturer’s instructions (Amersham, Piscataway, NJ, USA). Band intensity was measured using a densitometer and analyzed using Image J software (NIH, Bethesda, MD, USA).

**Animal Study**

Male 6–8-week-old ICR mice were purchased from the Hallym University Experimental Animal Center. The animals were housed at a constant temperature (23°C) and relative humidity (60%) with alternating 12 h cycles of light and dark. They were permitted access to 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 PEP-1-FK506BP and FK506BP into Mice Ear**

To examine the effect of SF peptide on the transduction of PEP-1-FK506BP and FK506BP into mice tissues, SF peptide (3.0 µg/ear) was topically applied on the ear surface and then the same surface of mice ear was exposed to PEP-1-FK506BP or FK506BP (3.0 µg/ear) 1 h later. Ear biopsy samples 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.

**TPA-Induced Skin Inflammation Mouse Model**

To induce skin inflammation, 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 [24]. SF peptide (3.0 µg/ear) was applied to the same surfaces and thereafter PEP-1-FK506BP or FK-506BP (3.0 µg/ear) was topically applied 1 h later. The treatments were repeated for three consecutive days. On the fourth day, ear punches 5 mm in diameter were taken from each mouse and ear thickness and the weights of each group were measured. For histological analysis, 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 Western blot analysis, ear biopsy samples were homogenized vigorously in tissue protein extraction reagent buffer with a protease inhibitor cocktail (Sigma-Aldrich) and incubated on ice for 15 min in the presence of 0.1% Triton X-100. Each homogenate was prepared by centrifuging at 10,000 × g for 10 min.

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'-TGGACGAGGTTTTTCCACCA-3'; COX-2 sense, 5'-CAAAGGCCCTCAATGGACAGA-3'; IL-6 antisense, 5'-TGGATGGTCTTGGTCCTTAGCC-3'; IL-6 sense, 5'-CAAGAAA GACAAAGCCCAAGGCTTT-3'; TNF-α antisense, 5'-TGCACTACCCTATTGGTACTACGATTGCACAGA-3'; TNF-α sense, 5'-CAAAGGCCTCCATTGACCAGA-3'; IL-1β antisense, 5'-AGCACTACGTGATCCATC-3'; IL-1β sense, 5'-TGGCACCACTAGTTGGTGTTTT-3'; IL-1α antisense, 5'-AGGTGCTCCATTGACAT-3'; IL-1α sense, 5'-CTGGCTGTCTTGGTCCTTAGCC-3'; β-actin antisense, 5'-GGACAGTGAGGCCAGGATGG-3'; β-actin sense, 5'-AGTGTGACGTTGACATCCGTAAAGA-3'. The PCR products were resolved on a 1% agarose gel and visualized with UV light after ethidium bromide staining.

Statistical Analysis
Data are expressed as the means ± SD. Comparison between groups was performed by Student’s t test. Values of P<0.01 and P<0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION
Effect of SF Peptide on In Vitro and In Vivo Transduction Efficiency of PEP-1-FK506BP
Increasing delivery of therapeutic molecules to target cells or tissues could be proportional to improvement of their therapeutic efficiency. Therefore, various ways of improving the delivery of therapeutic molecules have been extensively studied [3, 22, 26, 27]. We have also kept searching for molecules to improve the transduction efficiency and therapeutic utilization of many protein transduction domain (PTD) fusion proteins, and recently reported that some molecules such as bog blueberry anthocyanins (BBA) and pergolide mesylate enhance the transduction efficiency of PTD fusion proteins into cells or tissues, leading to improvement of the therapeutic delivery of PTD fusion proteins [15, 23].

In the present study, to investigate whether SF peptides have the potential to enhance to transduction efficiency of PEP-1-FK506BP, we tried to measure the transduction efficiencies of PEP-1-FK506BP and FK506BP in the absence or presence of SF peptide. Schematic structures of PEP-1-FK506BP and FK506BP are shown in Fig. 1A. We prepared the SF peptide as mentioned in the Materials and Methods section. HaCaT cells were pretreated with SF peptide (0.3 µg/ml) 1 h before treatment of PEP-1-FK506BP. FK506BP failed to be delivered to cells (Fig. 1B). However, PEP-1-FK506BP was delivered to cells in a dose-dependent manner (Fig. 1C). The addition of SF peptide had no effect on the transduction efficiency of PEP-1-FK506BP as well as FK506BP, compared with untreated samples. We also examined the extent of PEP-1-FK506BP to be delivered to cells as treatment concentrations of SF peptide gradually increased. The in vitro transduction data confirmed that PEP-1-FK506BP (3.0 µM) was equally delivered to cells regardless of the treatment concentration of SF peptide (Fig. 1D).

Next, we examined the influence of SF peptide on in vivo transduction of PEP-1-FK506BP into mouse skin. SF peptide (3.0 µg/ear) was topically applied to mouse skin 1 h prior to treatment with various concentrations of PEP-1-FK506BP or FK506BP (0.3 µg/ml) 1 h prior to treatment with various concentrations of PEP-1-FK506BP or FK506BP. In vivo transduction data from histological and Western blot
analysis indicated that FK506BP was not transduced into mice skin as in the untreated control sample (Fig. 2A and 2B), consistent with in vitro transduction results. On the other hand, PEP-1-FK506BP exhibited the similar penetration into dermis of the subcutaneous layer in the absence and presence of SF peptide. From these transduction data, we also surmised that SF peptide has no ability to enhance the transduction of PTD fusion proteins into HaCaT cells or animal tissues, and is likely neither to affect the membrane permeability nor to perforate the cellular membrane at concentrations used in our test. Collectively, our data indicate that SF peptide might not affect the transduction potential of PEP-1-FK506BP in vitro and in vivo.

SF Peptide Exerts Anti-Inflammatory Activity on Mice Ear Model

TPA induces the generation of ROS, resulting in inflammatory responses such as the expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and enzymes (COX-2, iNOS) [25, 30]. Moreover, the resultant increased ear thickness by inflammatory response is considered as another solid evidence of inflammation. Therefore, TPA is used as an established inflammatory stimulator in cells or animal models.

In our study, we examined the anti-inflammatory activity of FK506BP, PEP-1-FK506BP, and SF peptide using histological, Western blot, and RT-PCR analyses. As shown in Fig. 3A–3C, ear thickness and weight of the TPA-treated group increased three times compared with the control group. The FK506BP-treated group exhibited similar ear thickness to the TPA-treated group. However, not only the PEP-1-FK506BP but also SF peptide considerably inhibited the increase of ear thickness and weight induced by TPA. Co-treatment with PEP-1-FK506BP and SF peptide remarkably suppressed inflammation. Thus, it is assumable that PEP-1-FK506BP and SF peptide have a positive influence on each other’s anti-inflammatory effects, compared with the sample treated with PEP-1-FK506BP or SF peptide alone. Furthermore, we reconfirmed the anti-inflammatory effects of FK506BP, PEP-1-FK506BP, and SF peptide by measuring altered levels of pro-inflammatory mediators and cytokines in ear tissues. Fig. 4A–4D shows that the application of TPA completely induced the up-regulations of COX-2, IL-6, IL-1β, and TNF-α (lanes 1 and 2). As evidenced by our previous histological analysis, FK506BP did not suppress the expressions of pro-inflammatory mediators and cytokines (lane 3), but considerable inhibitions of pro-inflammatory mediators and cytokines expression were observed in both SF peptide and PEP-1-FK506BP treated samples (lanes 4 and 5). Most of all, among our data, the sample treated with both PEP-1-FK506BP and SF peptide dramatically suppressed the expression of COX-2, IL-6, IL-1β, and TNF-α (lane 6), suggesting that, although their action mechanism is unknown, SF peptide clearly enhanced the inhibitory activity of PEP-1-FK506BP against TPA-induced inflammation.

FK506BP has been suggested as a therapeutic target for several diseases by virtue of involvement in various biological responses [11, 21]. Thus, FK506BP, a member of the enzyme family catalyzing cis/trans peptidylpropyl isomerization, is involved in the protein folding process [11]. Moreover, without FK506, FK506BP stabilizes the ryanodine receptors (RyRs) by binding with RyRs, one of the Ca²⁺-releasing channels. Displacement of FK506BP from RyRs induces a Ca²⁺ leakage, thereby leading to several pathogenetic processes [16]. FK506BP is known to interact with various intracellular molecules such as transforming growth factor-beta (TGF-β), Activin type I receptor, Smad7, and epidermal growth factor (EGF) [11, 17, 28, 29]. In addition, we showed that PEP-1-FK506BP could be efficiently transduced into cells and animals and also inhibits inflammatory responses by blocking activation of NF-κB and p38 MAPK, suggesting that PEP-1-FK506BP could be a potential drug for inflammation and atopic dermatitis [13, 14]. Many previous studies have suggested the evidence that FK506BP plays crucial roles in protein–protein interaction and consequently modulates the biological activities of its binding partners. Although the mechanism related with the anti-inflammatory
effects of PEP-1-FK506BP, which were improved by SF peptide, is not clear from our present data, it is conceivable that SF peptide and PEP-1-FK506BP could individually exert their own anti-inflammatory effects and also that they could interact with each other or affect some regulators of each molecule, thereby being able to reciprocally enhance their biological activities. However, it is necessary that the action mechanism of SF peptide in its anti-inflammatory effects will be clarified through supplementary future studies.

Various biological functions of SF peptide have been extensively studied. Diverse results relating to the biological
activity of SF peptide may have arisen as a result of the type of cells, experiment schedule, and techniques used in each study. Depending on the preparation process of the SF peptide, SF peptides having different molecular sizes and characteristics might be produced, consequently leading to various biological responses. Inflammation is a particularly complex process, in which various factors and cells are involved, in response to traumatic, infectious, or toxic damage [20]. For usage of silk as matrix or suture, a potential to induce inflammation will be an obstacle to various clinical applications of silk. Therefore, SF peptide may be considered as a promising material acceptable for clinical application.

In conclusion, we demonstrated that SF peptide as well as PEP-1-FK506BP could significantly inhibit the inflammation induced by TPA through down-regulating the expression of several pro-inflammatory mediators or cytokines such as COX-2, IL-6, IL-1β, and TNF-α. First of all, co-administration of SF peptide and PEP-1-FK506BP could result in improved anti-inflammatory effects, which were not caused by way of enhanced transduction of PEP-1-FK506BP by SF peptide or other factors. These results demonstrated that co-treatment with PEP-1-FK506BP and SF peptide may be useful as a potential therapy against several inflammatory diseases.

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