Suppression of TNF-alpha-induced MMP-9 expression by a cell-permeable superoxide dismutase in keratinocytes

Ha Yong Song, Sung Mi Ju, Ah Ra Goh, Dong-Joo Kwon, Soo Young Choi & Jinseu Park*
Department of Biomedical Science, Research Institute for Bioscience & Biotechnology, Hallym University, Chunchon 200-702, Korea

Up-regulation of selected matrix metalloproteinases (MMPs) such as MMP-9 contributes to inflammatory processes during the development of various skin diseases, such as atopic dermatitis. In this study, we examined the effect of a cell-permeable superoxide dismutase (Tat-SOD) on TNF-α-induced MMP-9 expression in human keratinocyte cells (HaCaT). When Tat-SOD was added to the culture medium of HaCaT cells, it rapidly entered the cells in dose- and time-dependent manners. Tat-SOD decreased TNF-α-induced reactive oxygen species (ROS) generation. Tat-SOD also inhibited TNF-α-induced NF-κB DNA binding activity. Treatment of HaCaT cells with Tat-SOD significantly inhibited TNF-α-induced mRNA and protein expression of MMP-9, as measured by RT-PCR and Western blot analysis. In addition, Tat-SOD suppressed TNF-α-induced gelatinolytic activity of MMP-9. Taken together, our results indicate that Tat-SOD can suppress TNF-α-induced MMP-9 expression via ROS-NF-κB-dependent mechanisms in keratinocytes, and therefore can be used as an immunomodulatory agent against inflammatory skin diseases related to oxidative stress. [BMB reports 2011; 44(7): 462-467]

INTRODUCTION

Characteristic features of inflammatory skin diseases include dysregulation of pro-inflammatory mediators such as cytokines/chemokines, adhesion molecules, and enzymes, including matrix metalloproteinases (MMPs), which may accelerate migration of immune cells into the inflamed area of the skin (1-3). MMPs belong to the family of neutral endopeptidases, which participate in many physiological processes, including tissue remodeling and inflammation (Reviewed in Ref. 4). MMP-9 can mediate the degradation of extracellular matrix proteins such as type IV collagen during the inflammatory response. It has been implied that dysregulation of MMP activity is associated with inflammatory skin diseases such as atopic dermatitis (AD) (5, 6). Previous studies reported that elevated levels of MMP-9 are present in the plasma of patients with AD (5). Recently, it was demonstrated that various MMPs such as MMP-8 and 9 exhibit increased activity in skin wash samples from AD lesions (6), suggesting that MMP activity contributes to the inflammatory process in AD.

Stimulation of keratinocytes with tumor necrosis factor-α (TNF-α) results in activation of NF-κB, which is responsible for up-regulation of MMP-9 (7, 8). Previous studies have implied the involvement of TNF-α-induced oxidative stress in up-regulation of MMP-9. TNF-α can increase the levels of reactive oxygen species (ROS) both directly and indirectly, which in turn activates NF-κB, a redox-sensitive transcriptional factor (9-11). ROS such as superoxide anion (O2-·) and hydrogen peroxide (H2O2) can act as second messengers in signaling cascades leading to expression of many pro-inflammatory genes (12, 13).

Since antioxidants exert broad effects on cellular physiology, antioxidant enzymes such as superoxide dismutase (SOD) and catalase are considered to be candidates to control ROS generation. For this aim, we generated a cell-permeable SOD using HIV-1 Tat protein transduction domain (PTD), which facilitates the uptake of target proteins into cells (reviewed in Ref. 14, 15). Previous studies demonstrated that Tat-SOD is capable of inhibiting ROS generation in various types of cells under oxidative stress (16-18). Recently, it was demonstrated that a cell-permeable catalase attenuates TNF-α-induced production of cytokines such as IL-8, IL-6, and TNF-α itself in primary human keratinocytes (11). Taken together, these studies suggest a potential role of antioxidant enzymes in regulating the inflammation processes induced by ROS.

Although we previously reported that Tat-SOD exerts a protective effect against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation in mice (18), the molecular mechanism by which Tat-SOD exerts its anti-inflammatory activity is not yet completely understood. Therefore, in this study, we examined the regulatory effect of a cell-permeable Tat-SOD on TNF-α-induced MMP-9 expression in HaCaT cells. We found that a cell-permeable Tat-SOD inhibited TNF-α-induced MMP-9 expression via ROS-NF-κB-dependent mechanisms in keratinocytes.
RESULTS

Delivery efficiency of SOD fusion proteins into HaCaT cells

A cell-permeable Tat-SOD was previously shown to efficiently enter cells and tissues, exerting its protective effect against oxidative stress (16-18). To assess the amount of SOD fusion proteins taken up into the cells, SOD fusion proteins at concentrations ranging from 0.25 to 2 μM were added to the culture medium of HaCaT cells for 1 h, after which cell lysates were prepared and analyzed by Western blotting. As shown in Fig. 1B, Tat-SOD efficiently entered the cells, whereas control SOD did not. Next, we analyzed the kinetics of Tat-SOD delivery into HaCaT cells. As shown in Fig. 1A, transduced Tat-SOD was initially detected after 15 min, and the level was maintained until 1 h. To investigate the stability of Tat-SOD, HaCaT cells were incubated with Tat-SOD for 1 h, washed, and incubated further for 3-26 h. The intracellular levels of SOD were measured by Western blotting. The amount of transduced Tat-SOD was constant for 6 h and then declined gradually (Fig. 1C).

Effect of introduced Tat-SOD on TNF-α-induced ROS generation

Several studies reported that TNF-α can induce ROS generation, which is involved in intracellular signaling cascades leading to pro-inflammatory genes, and this is suppressed by various antioxidants (10, 11). These results suggest that prevention of ROS generation is one strategy to control inflammatory skin diseases. To evaluate the kinetics of ROS generation, HaCaT cells were exposed to TNF-α for the indicated times, and the levels of ROS generation were evaluated using DCF-DA as a probe. Consistent with previous results (11), the intracellular levels of ROS were increased in a time-dependent manner, reaching a peak after 30 min, after which they decreased back to control level after 24 h (Fig. 2A). A general NADPH oxidase inhibitor, diphenylene iodonium (DPI), along with the antioxidants NAC and vitamin C efficiently suppressed TNF-α-induced ROS generation (Fig. 2B). Next, we ex-
Effects of SOD on TNF-α-induced ROS generation
In the previous study, it was demonstrated that TNF-α-induced NF-κB activation was involved in up-regulation of MMP-9 in keratinocytes (7, 8). Next, we examined the effect of Tat-SOD on the TNF-α-induced DNA binding ability of NF-κB in HaCaT cells. HaCaT cells were treated with SOD fusion proteins for 1 h, followed by exposure to TNF-α for 30 min. DNA binding activity of NF-κB was strongly induced in TNF-α-stimulated HaCaT cells. As shown in Fig. 3, treatment with Tat-SOD significantly suppressed TNF-α-induced DNA binding activity of NF-κB in a dose-dependent manner.

Effects of SOD on TNF-α-induced MMP-9 expression
We next analyzed the effects of SOD fusion proteins on TNF-α-induced MMP-9 expression in HaCaT cells. Cells were pretreated with SOD fusion proteins for 1 h, stimulated with TNF-α, and then mRNA expression of MMP-9 was analyzed by RT-PCR. As shown in Fig. 4, Tat-SOD efficiently inhibited TNF-α-induced MMP-9 mRNA expression. To examine the effects of Tat-SOD on TNF-α-induced protein expression of MMP-9, the culture supernatants were analyzed for expression and gelatinolytic activity of MMP-9 by Western blot assay and zymography, respectively. Tat-SOD significantly suppressed TNF-α-induced protein expression and gelatinolytic activity of MMP-9, whereas control SOD had a minimal effect.

DISCUSSION
In this study, we showed that Tat-SOD exerts its inhibitory effect on MMP-9 gene expression in TNF-α-stimulated HaCaT cells. Tat-SOD inhibited TNF-α-induced mRNA and protein expression of MMP-9 as well as its enzymatic activity. These inhibitory effects were based on the ability of Tat-SOD to suppress TNF-α-induced ROS generation and NF-κB activation. Since ROS plays a major role in a variety of inflammatory skin diseases such as atopic dermatitis (19), the use of antioxidant enzymes such as SOD and catalase is one of the strategies for protection against ROS-mediated diseases (20). Antioxidant enzymes are often used for controlling ROS generation, with limited success due to the lack of their efficient transduction ability into cells. In this study, we generated a cell-permeable SOD using HIV-1 Tat protein transduction domain (PTD), which is capable of delivering protein into cells. As shown in Fig. 2, Tat-SOD efficiently inhibited TNF-α-induced ROS generation in HaCaT cells under inflammatory conditions. These results suggest a potential role for Tat-SOD in regulating inflammation processes induced by ROS. Consistent with our results, a cell-permeable catalase with nine arginine residues was previously shown to suppress TNF-α-induced production of cytokines, such as IL-8 and IL-6, in primary human keratinocytes (11). We previously reported that Tat-SOD exerts anti-inflammatory responses by inhibiting expression of pro-inflammatory mediators such as iNOS and COX-2 in LPS-stimulated macrophages (16, 17). Taken together, these studies demonstrated that availability of cell-permeable antioxidant enzymes, such as SOD and catalase, sig-
nificantly contribute to the development of therapeutic tools for controlling ROS-mediated inflammation.

Up-regulation of MMP-9 expression in the inflamed area of the skin may lead to the degradation of extracellular matrix proteins, resulting in increased infiltration of immune cells into the skin. Previous studies have shown that the activity of MMPs such as MMP-9 is increased in skin wash samples from AD lesions (6). ROS is considered to play a major role in inflammation processes during the development of inflammatory skin diseases (21). One approach to controlling these ROS-mediated skin diseases is to supplement various antioxidants or antioxidant enzymes in an effort to modulate oxidative stress induced by a variety of stimuli, including cytokines. Young et al. reported that a cell-permeable catalase efficiently inhibits ROS generation as well as cytokine production in TNF-α-stimulated keratinocytes (11), suggesting that antioxidant enzymes inhibit production of pro-inflammatory mediators by removing intracellular ROS. As shown in Fig. 3C, Tat-SOD inhibited TNF-α-induced ROS generation in HaCaT cells. The exact sources of ROS generation induced by TNF-α in keratinocytes have not been elucidated. However, as shown in Fig. 3B, the general NADPH oxidase inhibitor DPI inhibited TNF-α-induced ROS generation, suggesting the involvement of a NADPH oxidase. Further studies are required to define the exact molecular source of ROS generation upon TNF-α stimulation. Although the signaling pathways for expression of MMP-9 are complex (22), previous studies demonstrated that TNF-α induces up-regulation of MMP-9 expression and release via an NF-xB-dependent pathway in keratinocytes (7, 8). We performed experiments to analyze the effect of Tat-SOD on activation of NF-xB in TNF-α-stimulated HaCaT cells. We found that Tat-SOD inhibited TNF-α-induced NF-xB activation as well as MMP-9 expression in keratinocytes (Fig. 3 and 4). These results suggest that Tat-SOD modulates NF-xB activity, leading to production of pro-inflammatory mediators such as MMP-9.

In conclusion, our results imply that SOD has a regulatory function in the TNF-α-induced ROS and NF-xB-dependent signaling pathways, leading to MMP-9 expression in keratinocytes. Therefore, cell-permeable SOD can be used as a therapeutic agent against ROS-associated inflammatory skin diseases.

MATERIALS AND METHODS

Cell culture
The immortalized human keratinocyte cell line, HaCaT, was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin G, and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air, as previously described (18).

Reagents
Recombinant human TNF-α was purchased from R&D systems (Minneapolis, MN, USA). Primary antibodies against MMP-9 (Cell Signaling Technology, Beverly, MA, USA) were obtained commercially. Gelatin, 2',7'-dichlorofluorescin diacetate (DCF-DA), Diphenyl iodonium (DPI), N-Acetylcysteine (NAC), vitamin C, and HRP-conjugated anti-rabbit or goat antibodies were supplied by Sigma (St. Louis, MO, USA).

Expression and purification of SOD fusion proteins
Recombinant SOD fusion proteins were prepared under denaturing conditions as described previously (18). E. coli BL21 cells transformed with plasmids encoding control SOD or Tat-SOD fusion proteins were induced with IPTG to express SOD fusion proteins. To prepare denatured SOD fusion proteins, the induced cells were pelleted and lysed in binding buffer containing 6 M urea. SOD fusion proteins were subjected to affinity chromatography on a Ni⁺⁺-IDA column, followed by desalting with a PD10 column (Amersham). The SOD preparation eluted from the column was applied to a Detoxi-Gel™ Endotoxin Removing Gel (Pierce, Rockford, IL, USA) to remove endotoxin. Endotoxin levels for SOD preparation were below the detection limit (<0.1 EU/ml) as tested by Limulus Amoeобocyte Lysate assay (BioWhitaker, Walkersville, MD, USA). The purified SOD fusion proteins dissolved in PBS containing 20% glycerol were then aliquoted and stored at −80°C.

Western blot analysis
Cell lysates were prepared by incubating cells in lysis buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 10% v/v glycerol) at 4°C for 30 min. Cell lysates (50 μg of protein) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane, which was blocked with 10% dry milk in PBS. The membrane was then probed with the indicated antibodies, followed by HRP-conjugated anti-rabbit or goat IgG secondary antibody. The immunoreactive bands were detected by chemiluminescence using an ECL system (Amersham).

Measurement of intracellular ROS
The levels of ROS in TNF-α-stimulated HaCaT cells were determined using the ROS-sensitive dye 2',7'-dichlorofluorescein diacetate (DCF-DA), which is converted by ROS into highly fluorescent 2',7'-dichlorofluorescein (DCF) as described previously (23, 24). Briefly, HaCaT cells were exposed to TNF-α (10 ng/ml) and incubated for various times (5 min-24 h). Cells were washed twice with PBS and incubated with DCF-DA (10 μM) for 30 min. The cellular fluorescent images were obtained using a Zeiss Axiosvert S100 microscope equipped with a confocal laser-scanning system (Bio-Rad MRC-1024ES) at a laser excitation wavelength of 494 nm.
RT-PCR analysis
Total RNA was prepared from HaCaT cells using a Trizol RNA isolation kit (Invitrogen, Gaithersburg, MD, USA). Total RNA (2 μg) was then reverse transcribed to 10,000 U of reverse transcriptase and 0.5 μg/μl of oligo(dT)$_{15}$ primer (Promega, Madison, WI, USA). Transcribed cDNA aliquots were then amplified in 50 μl of 10 mmol/l Tris-HCL (pH 8.3), 25 mmol/l of MgCl$_2$, 10 mmol/l of dNTP, 100 U of Taq DNA polymerase, and 0.1 μmol/l of each primer and then terminated by heating at 70°C for 15 min. For PCR amplification, we used the following sense and antisense primers (5’→3’): MMP-9 sense, GTG CTG GGC TGC TGC TTG GGT GT; MMP-9 antisense, GCC CTC AAA GGT TTG GAA T; beta-actin sense, GCG GGA ATG GGT GGG TGA CAT T; and beta-actin antisense, GAT GGA GTT GAA GGT AGT TTC GTG. PCR products were resolved on 1% agarose gel and visualized with UV light after ethidium bromide staining.

Assay of MMP-9 activity by gelatin zymography
MMP-9 activity was assayed by gelatin zymography as described previously (25). Briefly, cells were pretreated with control SOD (2 μM) or Tat-SOD (0.5, 1, and 2 μM) for 1 h, followed by exposure to TNF-α (10 ng/ml) for the indicated periods. Culture supernatants (20 μl) were mixed with SDS sample buffer without reducing agent, and proteins were subjected to SDS-PAGE in 8% polyacrylamide gels containing 0.2% gelatin (w/v). After electrophoresis, the gels were washed twice in 10 mM Tris-Cl (pH 7.5) and 2% Triton X-100 for 1 h at room temperature to remove SDS, followed by incubation for 24 h at 37°C in buffer containing 10 mM Tris-Cl (pH 7.5), 10 mM CaCl$_2$, and 150 mM NaCl. The gels were then stained with Coomassie Brilliant Blue R250 (Bio-Rad, CA) (0.25%) for 30 min and destained for 1 h in a solution of acetic acid and methanol. Clear bands (zone of gelatin degradation) against the blue background of stained gelatin indicate proteolytic activity.

Electrophoretic mobility shift assay (EMSA)
Nuclear extracts of HaCaT cells were prepared and analyzed for NF-κB binding activity by EMSA as described previously (17). An NF-κB consensus oligonucleotide (Promega) was used in the EMSA. The complementary oligonucleotide was annealed and end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. EMSA was performed in a total volume of 20 μl at 4°C. Five micrograms of nuclear extracts were equilibrated for 15 min in binding buffer (10 mM Tris-HCl, pH 8.0, 75 mM KCl, 2.5 mM MgCl$_2$, 0.1 mM EDTA, 10% glycerol, 0.25 mM dithiothreitol) containing 1 μg of poly d(dC). [γ-32P]-labeled oligonucleotide probe (20,000 cpm) was then added, and the reaction was incubated on ice for an additional 20 min. Bound and free DNA were then resolved by electrophoresis on 6% native polyacrylamide gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA).

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Inhibition of MMP-9 by SOD in keratinocytes
Ha Yong Song, et al.


