Abstract

Ultraviolet (UV) irradiation on the skin triggers photoaging-related phenotypes such as formation of wrinkles. UV ray up-regulates matrix metalloproteinase-1 (MMP-1), which in turn degrades extracellular matrix proteins, mostly collagens. Also, excessive UV irradiation on the skin causes cutaneous inflammation known as sunburn. Serum amyloid A1 (SAA1) is an acute-phase protein of which plasma concentration increases in response to inflammation. Although the expression of SAA1 in the skin was reported, its function in the skin is yet to be studied. In this research, I found that the expression of SAA1 was increased in acute UV-irradiated human buttock skin and photoaged human forearm skin *in vivo* as well as in acute UV-irradiated mouse dorsal skin *in vivo.* UV irradiation also increased SAA1 in normal human epidermal keratinocytes (NHEK) and treatment of recombinant human SAA1 (rhSAA1) induced MMP-1 and proinflammatory cytokines such as IL-6 and IL-8 in normal human dermal fibroblasts (NHDF); however, the effect of rhSAA1 on NHEK was limited compared to

NHDF. Next, I demonstrated that NHDF treated with UV-irradiated keratinocyte conditioned media showed the increased MMP-1, IL-1β, IL-6, and IL-8 expression; however those increases in NHDF was inhibited by knockdown of SAA1 in NHEK. In addition, knockdown of Toll-like receptor 4 (TLR4) inhibited rhSAA1-induced MMP-1, IL-6, and IL-8 expression in NHDF. Taken together, my data showed that UV induced SAA1 production in NHEK, and this secreted SAA1 induced MMP-1, IL-6, and IL-8 expression in NHDF in a paracrine manner through TLR4 signaling pathway. Therefore, my results suggest that SAA1 can be a potential mediator for UV-induced MMP-1, IL-6, and IL-8 expression in human skin.

Key words: UV, serum amyloid A1, Toll-like receptor 4

Student number: 2012-21813

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Introduction

1. The effect of ultraviolet (UV) on the skin

UV light is a major environmental stimulus that leads to inflammation, carcinogenesis, hyperpigmentation, and photoaging [1]. In photoaged skin, the amount of collagen is down-regulated because of the decreased procollagen synthesis and the elevated collagen degradation by matrix metalloproteinases (MMPs) [2]. MMP-1, also known as collagenase-1, belongs to a family of structurally related zinc-dependent enzymes that are capable of degrading extracellular matrix (ECM) components [3]. The level of MMP-1 is increased by various stimuli including UV, cytokines, and growth factors [4]. MMP-1 is known to play roles in remodeling and destructive processes such as wound healing, angiogenesis, tumor invasion, and skin aging [2].

UV-induced cutaneous inflammation, known as sunburn, has been well documented clinically. Proinflammatory cytokines such as Interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor (TNF)-α are known to play important roles in skin inflammation as well as affect

ECM alterations. However, despite many previous efforts, it is not fully understood which molecules mediate UV-induced MMPs and proinflammatory cytokines in the skin.

2. Serum amyloid A1 (SAA1)

Serum amyloid A (SAA) proteins comprise a family of apolipoproteins that circulate in the plasma in association with high-density lipoprotein [5]. SAA is synthesized mostly in liver but many reports show the extrahepatic production of SAA [6]. SAA has been classified into two groups: acute-phase SAA (A-SAA) and constitutive SAA (C-SAA). A-SAA is one of the acute-phase proteins of which plasma concentration increases up to 1,000-fold as a result of inflammation, infection, and tissue injury [7]. Thus, A-SAA can be used as a marker for diagnosis of inflammatory diseases. On the other hand, C-SAA level in the plasma does not change during the inflammatory responses [8]. Two isoforms of A-SAA (SAA1 and SAA2) and one isoform of C-SAA (SAA4) have been reported to be expressed in human [9]. Studies have shown that SAA has cytokine-like properties in that it induces cytokines,

including IL-1β, IL-8, IL-10, IL-12, and IL-23, from granulocytes, lymphocytes, and monocytes [10]. It also increases expression of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, which in turn recruits immune cells to inflammatory sites [11]. Furthermore, SAA plays a role in certain diseases by stimulating the production of MMPs. For example, arthritis patients have elevated SAA level in blood and synovial fluids. SAA induces the expression of MMPs in cells from the joint including chondrocytes and fibroblast-like synoviocytes, thereby breaking down ECM, which eventually gives rise to the progress of the arthritis [11].

SAA causes various biological activities including induction of MMPs and cytokines by activating receptors as a ligand. Studies have indicated several candidate receptors for SAA [12]. Toll-like receptors (TLRs) mediate the innate immune system by detecting and responding to harmful pathogens. They recognize exogenous ligands from fragments of pathogens and endogenous ligands from damaged cells as well [13]. SAA has been suggested as an endogenous ligand for TLR4 [14].

Though the expression of SAA in the skin has been demonstrated, its function in the skin has been unknown.

Purposes

Chapter I. Serum amyloid A1 secreted from UV-irradiated keratinocytes induces matrix metalloproteinase-1 in fibroblasts through Toll-like receptor 4.

The main objectives of this chapter are: (1) to investigate whether SAA1 mediates UV-induced MMP-1 in human skin and (2) to elucidate its mechanism of action.

Chapter II. Serum amyloid A1 is induced by UV irradiation and detected by Toll-like receptor 4 to cause skin inflammation

The main objectives of this chapter are: (1) to investigate whether SAA1 mediates UV-induced proinflammatory cytokines in human skin and (2) to elucidate its mechanism of action.

Chapter I.

Serum amyloid A1 secreted from UV-irradiated keratinocytes induces matrix metalloproteinase-1 in fibroblasts through Toll-like receptor 4.

Materials and methods

Human skin samples and UV irradiation

To investigate the effect of acute UV irradiation on human skin, 2 minimal erythema dose (MED) UV-irradiated or non-irradiated buttock skin samples were provided from 8 healthy Korean male volunteers (mean age 47.2±3.0, age range 41-52 years). UV was irradiated on buttock skin as described previously. In brief, UV irradiation to the buttock skin was performed with a Waldmann UV-800 (Waldmann Co., Villingen-Schwenningen, Germany) phototherapy device. A F75/85W/UV21 fluorescent lamp with emission spectrum between 275 and 380 nm (peak at 310-315 nm) was fitted in the device and used as the UV light source. A Kodacel filter (TA401/407; Kodak, Rochester, NY, USA) was used to remove wavelengths below 290 nm (UVC). MED was individually determined at 24 hours after UV irradiation. Then, the buttock skin areas were irradiated with 2 MED of UV, or non-irradiated, and these tissues were obtained at 24 hours after UV irradiation by 6

mm punch biopsy.

To investigate the effect of chronic UV irradiation on human skin, photo-protected buttock and photoaged forearm skin samples were provided from 8 elderly Korean male volunteers (mean age 81.9±6.7, age range 75-93 years). Buttock and forearm skin samples were obtained from all subjects by 6 mm punch biopsy. The epidermis was peeled off from the biopsied skin samples by the heat separation method. Briefly, whole skin specimens were incubated in 55° C phosphate-buffered saline (PBS) for 2 minutes, and the epidermis was separated from the dermis using forceps.

This study was received prior approval from the Seoul National University Institutional Review Board and all human subjects were given written informed consent according to the Declaration of Helsinki Principles.

Cell culture and UV irradiation

Primary normal human epidermal keratinocytes (NHEK) and normal human dermal fibroblasts (NHDF) from foreskin were isolated as

described previously [15, 16]. NHEK were cultured in keratinocyte growth medium (Clonetics, San Diego, CA, USA). Cultured NHEK at passages of 3-4 were used for the experiments. NHDF were cultured in Dulbecco's Modified Essential Medium (DMEM, Gibco, Rockville, MD, USA) with 10% fetal bovine serum (Welgene, Daegu, Republic of Korea) and 1% Penicillin-Streptomycin (Welgene). Cultured NHDF at passage of 10-15 were used for the experiments.

For UV irradiation, Philips TL 20W/12RS fluorescent sun lamps (Philips, Eindhoven, Netherlands) with an emission spectrum between 275 and 380 nm (peak, 310-315 nm) were used as the UV source. The power output distribution of the UV emission spectrum was 10.2% UVC (275-290 nm), 53.3% UVB (290-320 nm), 25.3% UVA1 (320-340 nm), and 11.2% UVA2 (340-380 nm; Park et al., 2006). A Kodacel filter (TA401/407; Kodak) was used to block UVC (< 290 nm).

NHEK were incubated with keratinocyte starvation medium composed of keratinocyte basal medium (Clonetics) mixed with Hank's balanced salt solution (Gibco) (1:1) for 48 hours. NHEK were washed with PBS twice and irradiated with UV (50, 100, and 150 mJ/cm^2) in PBS. After UV irradiation, PBS was removed and replaced by keratinocyte

starvation medium. Cell viability was determined at 24, 48, and 72 hours after UV irradiation by WST-1 reagent (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Briefly, NHEK were treated with WST-1 at each time point after UV irradiation, and cultured media were collected at 2 hours after treatment. Then, the absorbance at 450 nm of the collected media was determined by microplate reader (Molecular Devices, Sunnyvale, CA, USA). For mRNA and protein analysis, NHEK were harvested at 24 and 48 hours, and 48 and 72 hours after UV irradiation, respectively.

Treatment with recombinant human SAA1 (rhSAA1)

To examine whether rhSAA1 (ProSpec, East Brunswick, NJ, USA) induces MMP-1, NHEK were starved in keratinocyte starvation medium and NHDF were starved in serum-free DMEM for 24 hours. Then, cells were treated with several concentrations (0, $5x10^{-12}$, $5x10^{-11}$, $5x10^{-10}$, $5x10^{-9}$ and $5x10^{-8}$ g/mL) of rhSAA1and harvested for mRNA and protein analysis at 24 hours and 48 hours after treatment, respectively.

To examine whether rhSAA1 activates mitogen-activated protein kinases (MAPK)/activator protein-1 (AP-1) signaling pathway, NHDF were starved with serum-free DMEM for 24 hours and treated with rhSAA1 ($5x10^{-8}$ g/mL). Then, NHDF were harvested at different time-points (0, 5, 15, 30, 60, 120, and 240 minutes) after treatment for protein analysis from cell lysates.

Transfection with siRNA

For siRNA transfection, NHDF were seeded and transfected with scrambled negative control or TLR4 siRNA (Bioneer, Daejeon, Republic of Korea) simultaneously using G-fectin (Genolution, Seoul, Republic of Korea) method as the manufacturer's instructions. At 48 hours after transfection, NHDF were starved for 24 hours, and were treated with SAA1 ($5x10^{-8}$ g/mL) for mRNA and protein analysis for 24 hours and 48 hours, respectively.

Treatment with keratinocyte conditioned media (KCM) to

NHDF

For KCM experiments, NHEK were cultured in keratinocyte growth medium until 80-90% confluence. Then the NHEK were incubated with keratinocyte starvation medium, and the cells were transfected with scrambled negative control or SAA1 siRNA (Bioneer) using G-fectin. At 48 hours later, UV (100 mJ/cm^2) was irradiated on NHEK, and KCM was collected at 72 hours after irradiation. Each KCM was mixed with serum-free DMEM (1:1) and treated to NHDF which had been starved with serum-free DMEM 24 hours before. Then NHDF were harvested for protein analysis at 72 hours after KCM treatment.

Reverse transcriptase (RT)-PCR

Total RNA was isolated from whole skin, from epidermis to upper dermis, of normal human buttock, and cells using RNA iso (Takara Bio), and the same amount of RNA was converted to cDNA using First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. To estimate the mRNA expression, semi-quantitative RT-PCR was performed using AccuPower PCR

PreMix (Bioneer), with the following primer pairs: SAA1 (forward, 5’-CTGCAGAAGTGATCAGCG-3’; reverse, 5’-ATTGTGTACCCTCTCCCC-3’) and GAPDH (forward, 5’-ATTGTTGCCATCAATGACCC-3’; reverse, 5’-AGTAGAGGCAGGGATGATGT-3’). The RT-PCR conditions were 95℃ for 5 minutes, followed by 30 cycles for SAA1 and 24 cycles for GAPDH at 95℃ for 30 seconds, at 60℃ for 30 seconds, and at 72℃ for 30 seconds, and 72℃ for 7 minutes for final extension. PCR products were loaded onto 1% agarose gel, separated electrophoretically, and visualized by UV exposure. GAPDH was used as endogenous control.

Quantitative real-time RT-PCR

Total RNA was isolated from skin samples and cells using RNA iso, and the same amount of RNA was converted to cDNA using First Strand cDNA Synthesis Kit according to the manufacturer’s instructions. To estimate the mRNA expression quantitatively, PCR was performed on a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (Takara Bio)

according to the manufacturer’s instructions, with primer pairs in Table 1. The PCR conditions were 50° C for 2 minutes, 95° C for 2 minutes, followed by 40 cycles at 95° C for 15 seconds and 60° C for 1 minute. The data were analyzed using the comparative ⊿⊿Ct method, and presented as mean ± standard error (SE) of relative mRNA expressions against corresponding controls, normalized to 36B4.

Western blot analysis

Skin samples and cells were homogenized, and proteins were extracted using RIPA buffer (Merck Millipore, Billerica, MA, USA) containing protease inhibitor mixture (Roche Applied Science, Rockford, IL) and phosphatase inhibitor mixture (Sigma Aldrich, St. Louis, MO, USA). Tissue extracts and cell lysates were centrifuged at 12,000 rpm, 4° C for 15 minutes, and supernatants were collected. Protein concentration of samples was determined by the Bradford protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). To analyze secreted protein including MMP-1 and SAA1, cultured media were collected. Especially for SAA1, cultured media were concentrated by

heating at 95° C. Proteins were loaded onto SDS polyacrylamide gels, separated by gel electrophoresis and transferred to polyvinylidene difluoride membranes (Roche Applied Science). Membranes were blocked with Tris-buffered saline containing 0.1% Tween 20 and 5% skim milk, and incubated with mouse monoclonal antibody against SAA1 (Abcam, Cambridge, United Kingdom), rabbit polyclonal antibody against MMP-1 (Lab Frontier, Seoul, Korea), phospho-extracellular signal-regulated kinaes-1/2 (ERK1/2), ERK1/2, phospho-c-Jun N-terminal kinase (JNK), JNK, phospho-p38, and p38 (Cell Signaling Technology, Beverly, MA, USA), and a goat polyclonal antibody against actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat IgG (Santa Cruz Biotechnology) were used as secondary antibodies. Blots were visualized by enhanced chemiluminescence detection system (Thermo Fisher Scientific, Waltham, MA, USA). Signal intensity was quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Gelatin zymography analysis

To analyze the expression of MMP-2 and MMP-9, cultured media were collected. Collected media mixed with zymography sample buffer consisting of 60mM Tris-HCl, 25% glycerol, 2% SDS, and 0.1% bromophenol blue were loaded onto SDS polyacrylamide gels with 0.1% gelatin (Sigma Aldrich), and separated by gel electrophoresis. Gels were incubated in renaturing buffer consisting of 2.5% Triton X-100 (Amresco LLC, Solon, OH, USA) at 37° C for 1 hour, and incubated in developing buffer consisting of 50 mM Tris-HCl (pH 8.0) and 5 mM $CaCl_2$ at 37° C for 40 hours for NHEK and 4 hours for NHDF. Then, the gels were stained with 0.25% Coomassie Brilliant Blue R (Sigma Aldrich) solution for 4 hours, and destained with 10% acetic acid till the bands appear.

Immunohistochemistry (IHC) staining

Skin biopsies were fixed with 10% formalin for 24 hours, and processed into paraffin wax. Paraffin-embedded samples were sectioned into 4 μm, and mounted onto silane-coated slides (Dako, Glostrup, Denmark). Slides of skin specimens were dewaxed in xylene

substitutes, rehydrated with graded ethanol and washed with distilled water. Catalyzed Signal Amplification System (Dako) was used to stain the specimens according to the manufacturer's instructions. A mouse monoclonal antibody against SAA1 (Abcam) diluted (1:100) in diluent buffer (Dako) was used to detect SAA1.

Statistical analysis

Data are presented as mean ± standard error. Significance was determined using the paired *t*-test. *P*-values of less than 0.05 were considered statistically significant.

Table 1. Primer sequences for human genes for quantitative real-time PCR

Human gene	Forward	Reverse
h36B4	TCGACAATGGCAGCATCTAC	TGATGCAACAGTTGGGTAGC
hSAA1	CTGCAGAAGTGATCAGCG	ATTGTGTACCCTCTCCCC
hMMP-1	ATTCTACTGATATCGGGGCTTTGA	ATGTCCTTGGGGTATCCGTGTAG

Results

SAA1 is mainly expressed in the epidermis

To determine the main source of SAA1 expression in the skin, I performed IHC staining with human buttock skin samples. SAA1 was expressed mainly in the epidermis compared to negative control staining (Fig. 1A). To confirm whether epidermal keratinocytes are major cells producing SAA1 in human skin, I tested cultured NHEK and NHDF for SAA1 expression. SAA1 mRNA was expressed in NHEK as well as whole skin, while rarely expressed in NHDF when analyzed by RT-PCR (Fig. 1B).

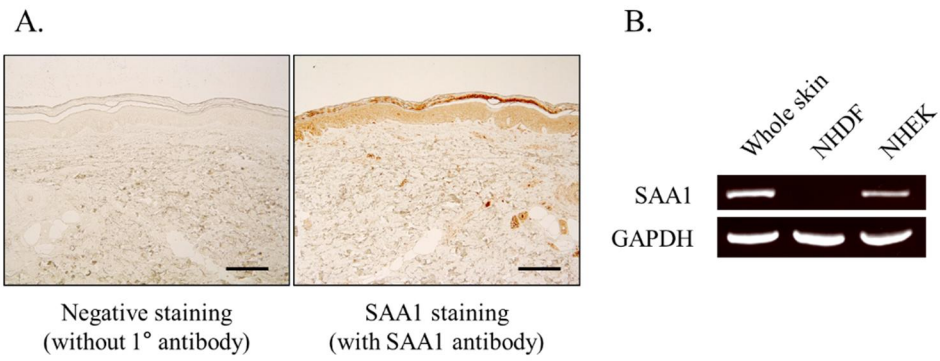

Figure 1. SAA1 is mainly expressed in the epidermis

(A) SAA1 expression in human buttock skin was analyzed by IHC staining. (B) SAA1 mRNA expressions from whole skin, NHDF, and NHEK were analyzed by RT-PCR. The PCR products were visualized on 1% agarose gels. Scale bar represents 200 μm.

Acute UV irradiation increases SAA1 in the human buttock skin

To investigate whether UV irradiation induces the expression level of SAA1, buttock skin of healthy volunteers were irradiated with 2 MED of UV, and biopsied at 24 hours after UV irradiation. The expressions of SAA1 mRNA and protein were analyzed by quantitative real-time RT-PCR and Western blot, respectively. The expressions of SAA1 mRNA and protein were significantly higher in UV-irradiated skin than non-irradiated skin (Fig. 2A and 2B). In addition, I performed IHC staining with the UV-irradiated or non-irradiated skin samples. The expression of SAA1 was elevated in the epidermis of UV-irradiated skin compared to non-irradiated skin (Fig. 1C).

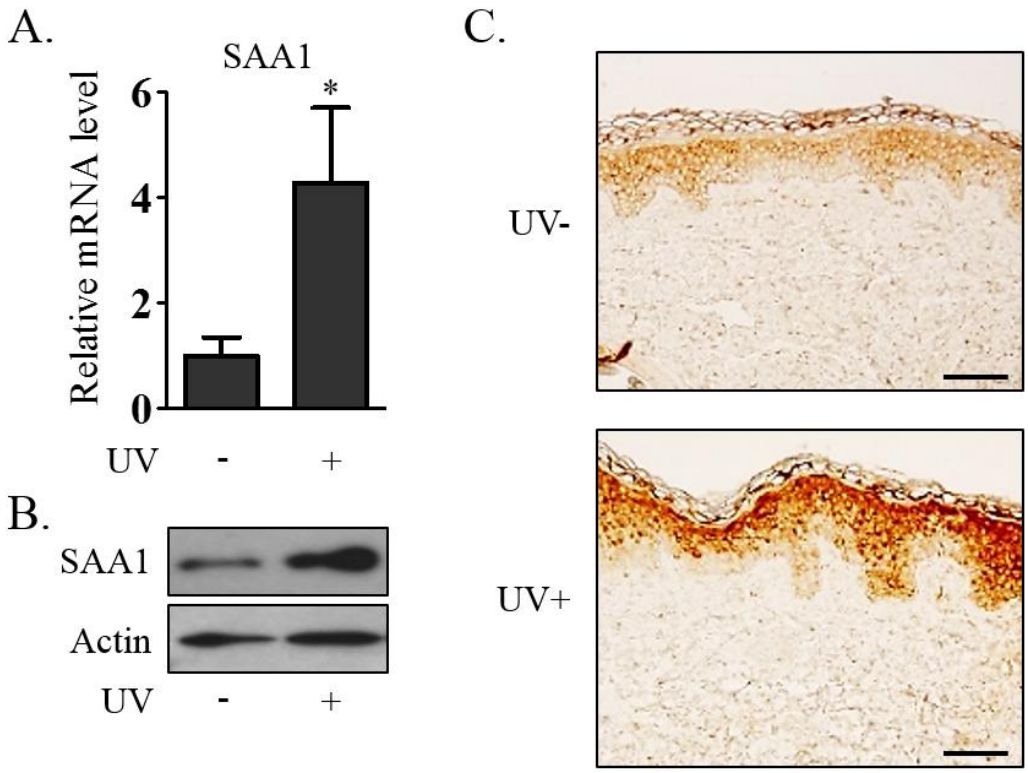

Figure 2. Acute UV irradiation increases SAA1 in the human buttock skin

Human buttock skin was irradiated with 2 MED of UV or non-irradiated and the skin specimens were obtained at 24 hours after UV irradiation. (A) SAA1 mRNA was analyzed by quantitative real-time RT-PCR and (B) SAA1 protein was analyzed by Western blot analysis. Data represent mean ± SE of relative mRNA expressions (n=8). $*P< 0.05$ versus non-irradiated control. (C) SAA1 expression level from UV-irradiated and non-irradiated skin of each subject was analyzed by IHC

staining (n=8). Scale bar represents 100 μm.

SAA1 is increased in the epidermis of photoaged forearm skin

Since photoaging is a chronic process in which wrinkles are formed by sustained collagen degradation by MMP-1, I wondered if chronic UV irradiation up-regulates SAA1 as does acute UV irradiation. Photo-protected buttock skin and photoaged forearm skin from the same individual were biopsied from healthy elderly volunteers (mean age 81.9 ±6.7, age range 75-93 years). I separated the epidermis from the dermis, and checked SAA1 mRNA and protein level in the epidermis because SAA1 is expressed in the epidermis. I found that the mRNA level of SAA1 was higher in photoaged forearm epidermis than photo-protected buttock epidermis (Fig. 3A and 3B). In addition, IHC staining revealed that the expression of SAA1 was much higher in photoaged forearm skin than in photo-protected buttock skin (Fig. 3C).

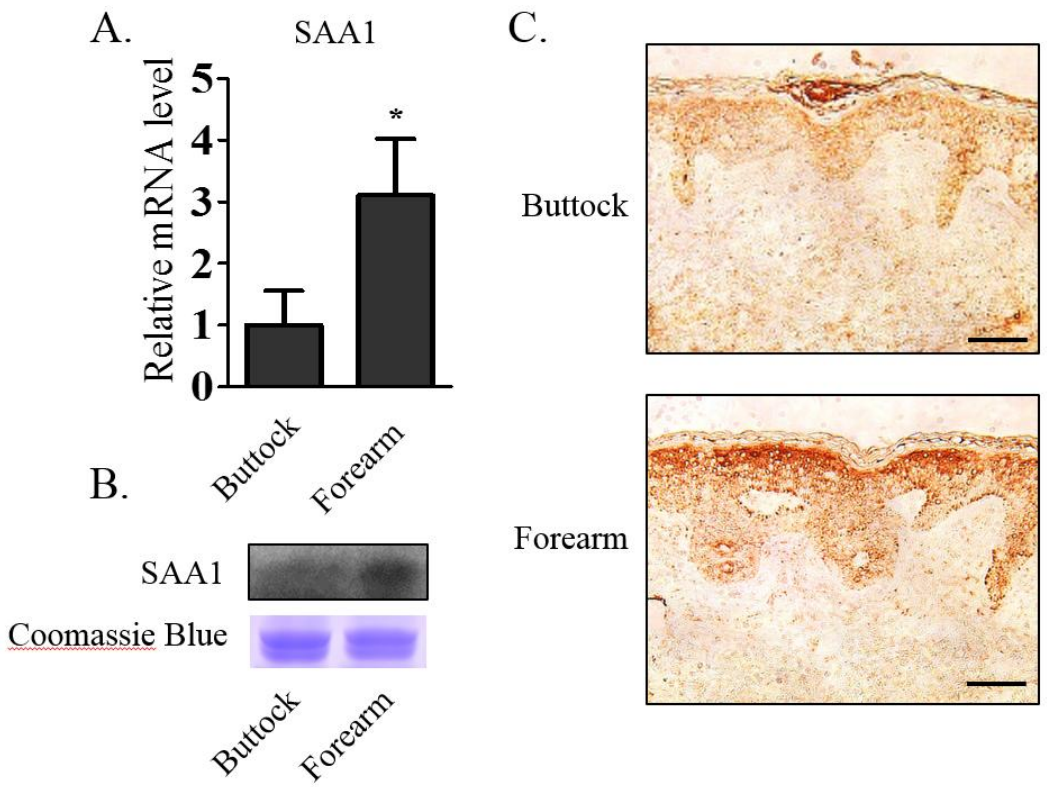

Figure 3. SAA1 is increased in the epidermis of photoaged forearm skin

(A) SAA1 mRNA and (B) protein in photo-protected buttock skin and photoaged forearm skin samples from elderly subjects (mean age 81.9±6.7, age range 75-93 years) were analyzed. Data represent mean ± SE of relative mRNA expressions (n=8). *P< 0.05, versus buttock skin. (C) SAA1 expression level in photo-protected buttock skin and photoaged forearm skin of each subject was analyzed by IHC staining (n=8). Scale bar represents 100 μm.

UV irradiation increases SAA1 in NHEK

To confirm the UV effect on SAA1 production in NHEK, I irradiated NHEK with 100 mJ/cm^2 of UV, which had no effect on NHEK viability at 72 hours after irradiation (Fig. 4A), and analyzed SAA1 mRNA and protein level. The mRNA level of SAA1 was significantly increased at 24 hours and 48 hours after UV irradiation (Fig. 4B), and protein level was also increased in cultured media at 48 hours and 72 hours after UV irradiation (Fig. 4C).

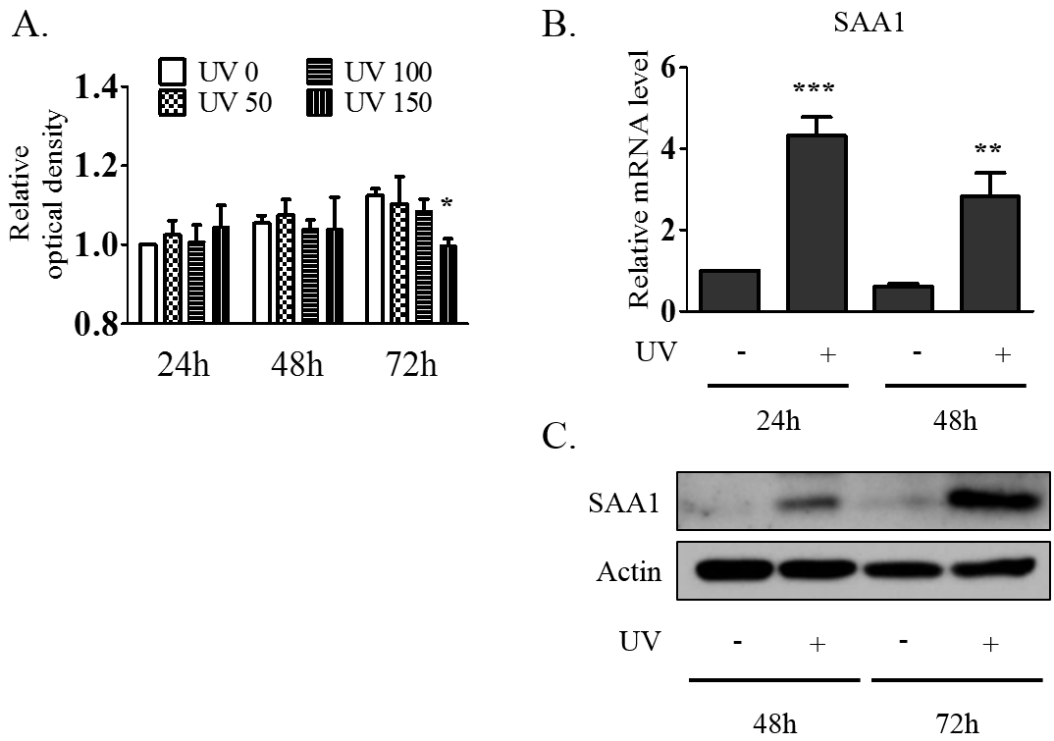

Figure 4. UV irradiation increases SAA1 in NHEK

(A) NHEK were irradiated with several doses of UV (50, 100, and 150 mJ/cm^2), and the cell viability was determined at 24, 48, and 72 hours after UV irradiation. NHEK were irradiated with UV (100 mJ/cm^2), harvested at 24 and 48 hours for mRNA and 48 and 72 hours for protein after irradiation, and (B) mRNA and (C) protein expressions of SAA1 were analyzed. Data represent mean ± SE of relative mRNA expressions (n=4). *P< 0.05, **P< 0.01, and ***P< 0.001 versus non-irradiated control.

Treatment with rhSAA1 induces MMPs in NHDF but not in NHEK

Since SAA1 is a secreted protein [17], I hypothesized that UV-induced SAA1 could affect either NHEK in an autocrine manner or NHDF in a paracrine manner. To elucidate the effect of SAA1 on NHEK and NHDF, I treated these cells with rhSAA1 with diverse concentrations (0, $5x10^{-12}$, $5x10^{-11}$, $5x10^{-10}$, $5x10^{-9}$, and $5x10^{-8}$ g/mL) and checked MMP-1 induction. Treatment with rhSAA1 did not induce the expressions of MMP-1, MMP-2, nor MMP-9 (Fig. 5A and 5B) in NHEK; however, it significantly increased the expressions of MMP-1 mRNA and protein in NHDF in a dose-dependent manner and also the expression of MMP-2 while the expression of MMP-9 was not detected (Fig. 5C and 5D).

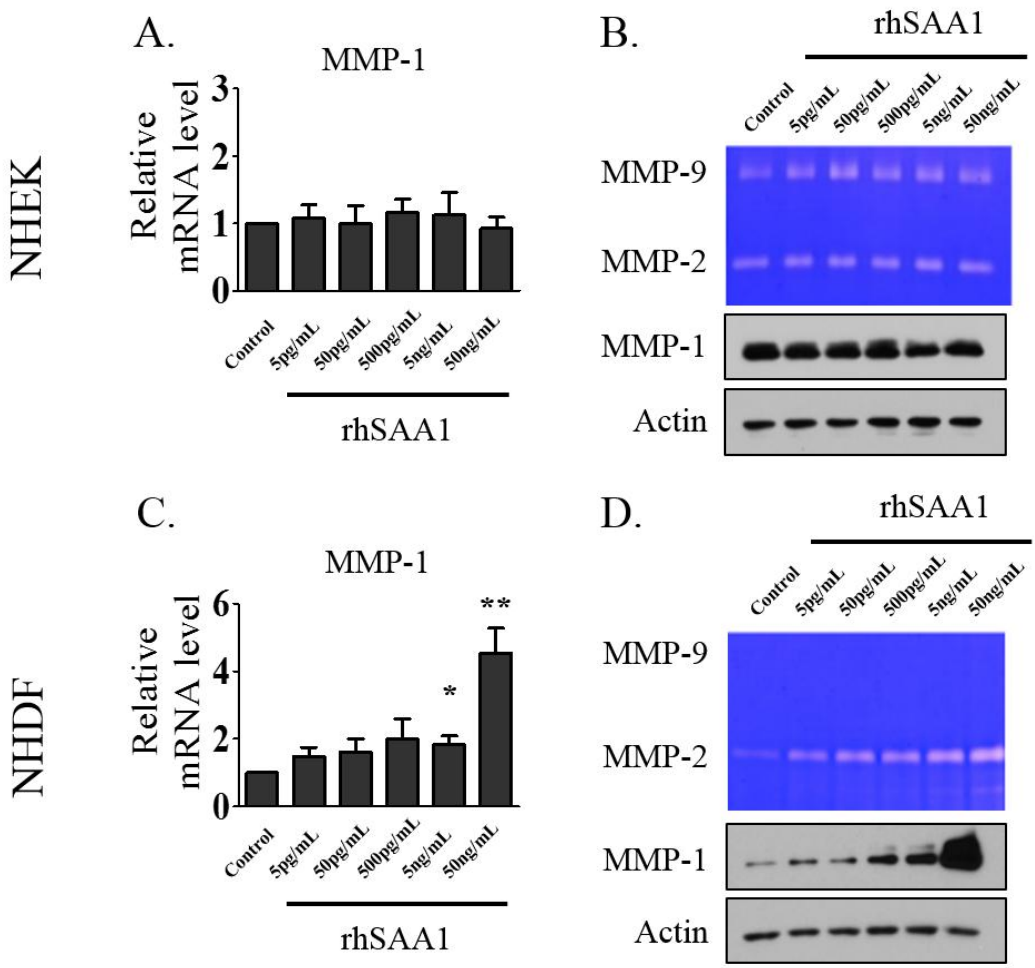

Figure 5. Treatment with rhSAA1 induces MMPs in NHDF but not in NHEK

Numerous doses (0, $5x10^{-12}$, $5x10^{-11}$, $5x10^{-10}$, $5x10^{-9}$, and $5x10^{-8}$ g/mL) of rhSAA1 were treated to NHDF and NHEK, and the expression of MMP-1 mRNA and protein were detected at 24 hours and 48 hours

after treatment, respectively. The expressions of MMP-1 mRNA from (A) NHEK and (C) NHDF were detected by quantitative real-time RT-PCR, and the expressions of MMP-1, MMP-2, and MMP-9 protein in cultured media from (B) NHEK and (D) NHDF were detected by Western blot analysis or zymography analysis. Data represent mean ± SE of relative mRNA expressions (n=4). *P< 0.05 and **P< 0.01 versus control.

SAA1 secreted from UV-irradiated NHEK induces MMP-1 in NHDF

To prove that elevated SAA1 from UV-irradiated NHEK could increase the expression of MMP-1 in NHDF, I treated NHDF with KCM. I found that the expression of MMP-1 in NHDF was increased by treatment with UV-irradiated KCM, compared to non-irradiated controls; however, this MMP-1 induction in NHDF treated with UV-irradiated KCM was attenuated significantly by knockdown of SAA1 in NHEK (Fig. 6A). On the other hand, UV-irradiated KCM did not induce MMP-2 or MMP-9 in NHDF (Fig. 6B).

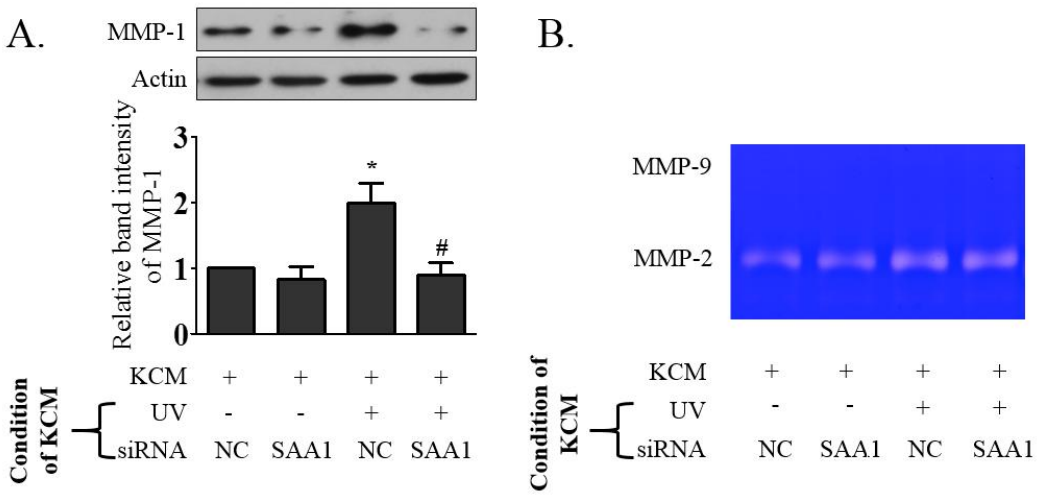

Figure 6. SAA1 secreted from UV-irradiated NHEK induces MMP-1 in NHDF

NHEK were transfected with siRNA of negative control (NC) or SAA1 and then irradiated with UV (100 mJ/cm^2) or non-irradiated. At 72 hours after UV irradiation, the conditioned media were collected from NHEK and mixed with serum-free DMEM (1:1). Thereafter, NHDF were treated with theses mixture of KCM and DMEM (1:1) and their cell lysates and cultured media were harvested at 72 hours after treatment. (A) The expression of MMP-1 in cultured media was determined by Western blot analysis and actin in the cell lysates was determined as a loading control. (B) The expression of MMP-2 and MMP-9 in cultured media were determined by zymography analysis. Data represent mean

± SE of relative protein expressions of MMP-1, normalized to actin (n=4). *P< 0.05 versus non-irradiated KCM with NC siRNA and $^{\#}P$ < 0.05 versus UV-irradiated KCM with NC siRNA.

Treatment with rhSAA1 induces MMP-1 through TLR4 signaling pathway in NHDF

Since SAA1 has been speculated as an endogenous ligand of TLR4, I examined whether downregulation of TLR4 could attenuate the increase of MMP-1 induced by rhSAA1 treatment in NHDF. NHDF were transfected with scrambled negative control or TLR4 siRNA, and treated with rhSAA1 ($5x10^{-8}$ g/mL) afterwards. To determine the expression level of MMP-1, cell lysates were harvested for mRNA analysis at 24 hours, and cultured media were collected for protein analysis at 48 hours after treatment with rhSAA1. I observed that treatment with rhSAA1 increased the expression of MMP-1, and this increase was inhibited by knockdown of TLR4 in NHDF in both mRNA and protein level (Fig. 7A and 7B).

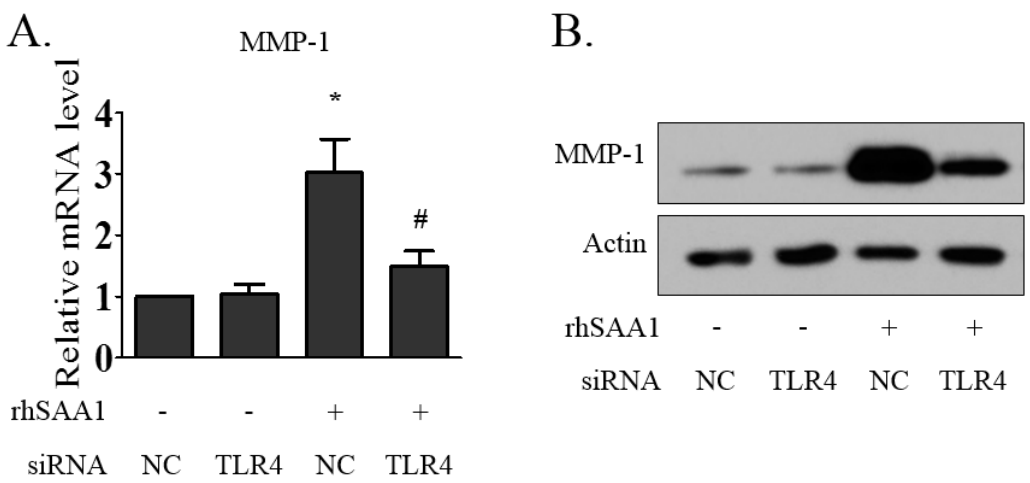

Figure 7. Treatment with rhSAA1 induces MMP-1 through TLR4 signaling pathway in NHDF

NHDF were transfected with negative control (NC) or TLR4 siRNA, and then treated with rhSAA1 ($5x10^{-8}$ g/mL). (A) The expression of MMP-1 mRNA was determined at 24 hours after treatment by quantitative real-time RT-PCR and (B) the expression of MMP-1 protein in cultured media were determined at 48 hours after treatment by Western blot analysis (n=4). Data represent mean ± SE of relative mRNA of expressions. $*P < 0.05$ versus control group with NC siRNA and $^{\#}P < 0.05$ versus rhSAA1-treated group with NC siRNA.

Treatment with rhSAA1 induces MAPK/AP-1 signaling pathway in NHDF

As AP-1 is an essential transcription factor of MMP-1 gene and the expression and activity of AP-1 is induced by MAPK [18], I investigated if SAA1 activates MAPK/AP-1 signaling pathway. NHDF were treated with rhSAA1 ($5x10^{-8}$ g/mL) for different periods of time (0, 5, 15, 30, 60, 120, and 240 minutes), and expressions and phosphorylations of MAPKs, and c-Jun and c-Fos, two main components of AP-1, were determined by Western blot analysis. The phosphorylations of ERK1/2, JNK and p38 MAPK were induced within 5 minutes and sustained for 120 minutes after rhSAA1 treatment (Fig. 8A). Also, c-Jun was phosphorylated by rhSAA1 within 15 minutes and kept phosphorylated until at least 240 minutes and the expression of c-Fos was remarkably increased at 60 minutes after rhSAA1 treatment (Fig. 8B).

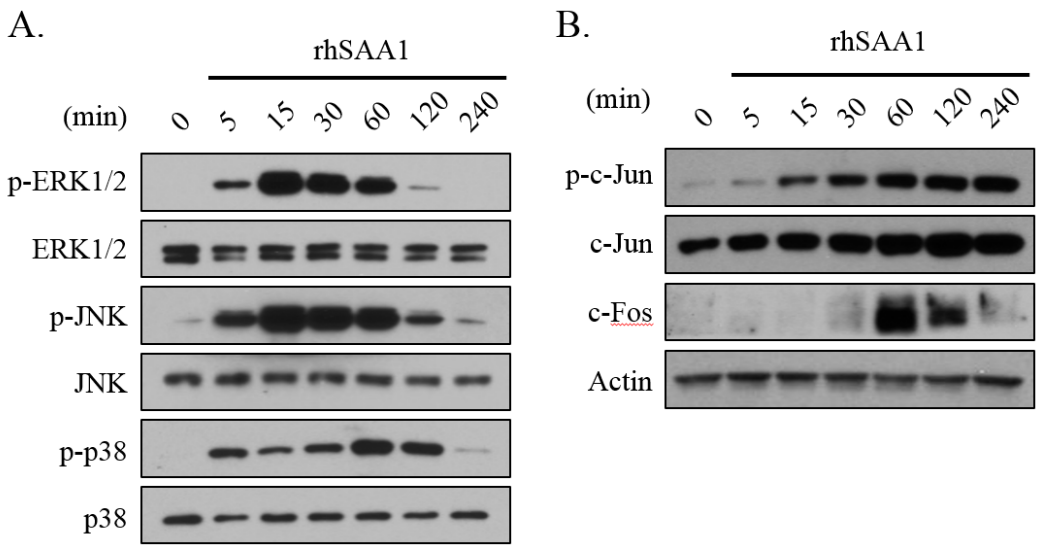

Figure 8. Treatment with rhSAA1 induces MAPK/AP-1 signaling pathway in NHDF

NHDF were treated with rhSAA1 ($5x10^{-8}$ g/mL) and cell lysates were harvested at the indicated times. (A) Phosphorylated and total form of ERK1/2, JNK, and p38 in the cell lysates were analyzed by Western blot analysis. (B) The expression of phospho-c-Jun, c-Jun and c-Fos in the cell lysates were analyzed and actin was determined as a loading control (n=4).

Chapter II.

Serum amyloid A1 is induced by UV irradiation and detected by Toll-like receptor 4 to cause skin inflammation

Materials and methods

Mouse experiments

C57BL/6 mice were purchased (Orientbio, Seongnam, South Korea) and housed under specific pathogen-free conditions. Seven-week-old female mice were used and hair from dorsal skin of the mice was removed using Veet cream (Reckitt Benckiser, Slough, UK) at 48 hours before the following experiments.

For UV irradiation, Philips TL 20W/12RS fluorescent sun lamps (Philips, Eindhoven, Netherlands) with an emission spectrum between 275 and 380 nm (peak, 310-315 nm) were used as the UV source. The power output distribution of the UV emission spectrum was 10.2% UVC (275-290 nm), 53.3% UVB (290-320 nm), 25.3% UVA1 (320-340 nm), and 11.2% UVA2 (340-380 nm). A Kodacel filter (TA401/407; Kodak, Rochester, NY) was used to block UVC (< 290 nm). Dorsal skin of mice was irradiated with 200 mJ/cm of UV and the skin was obtained at 48 hours post-UV irradiation.

To investigate the effect of SAA1 in mouse skin *in vivo*, PBS or 2.5 mg/kg body weight of recombinant mouse SAA1 (rmSAA1, MyBioSource, San Diego, CA) was injected intradermally into the dorsal skin of the mice. Photographs were taken and skin samples were obtained at 24 hours post-injection. The protocol for this study was approved by the Seoul National University Hospital Institutional Animal Care and Use Committee (IACUC 15-0019-S1A0).

Cell culture

Primary normal human dermal fibroblasts (NHDF) and normal human epidermal keratinocytes (NHEK) were isolated. NHDF were cultured in Dulbecco's Modified Essential Medium (DMEM, Gibco, Rockville, MD) with 10% fetal bovine serum (FBS, Welgene, Daegu, South Korea) and 1% Penicillin-Streptomycin (Welgene) and normal human epidermal keratinocytes (NHEK) were cultured in keratinocyte growth medium (Clonics, San Diego, CA). Cultured NHDF at passage of 10-15 and NHEK at passage of 3-5 were used for the experiments.

Treatments, UV irradiation, and transfections on cells

To examine if SAA1 induces proinflammatory cytokines, NHDF and NHEK, starved in serum-free DMEM or keratinocyte starvation medium, which is a mixture (1:1) of keratinocyte basal medium (Clonics) and Hank's balanced salt solution (Gibco), for 24 hours, were treated with various doses (0, 5, 50, and 500 ng/ml) of recombinant human SAA1 (rhSAA1, ProSpec, East Brunswick, NJ) and harvested for mRNA and protein analysis at 6 or 24 hours post-rhSAA1 treatment, respectively.

For siRNA transfection and UV irradiation to NHEK, 80% confluent NHEK were washed with PBS for 2 times and starved and transfected simultaneously with 300 pmol/ml of negative control or SAA1 siRNA (Bioneer, Daejeon, South Korea) using G-fectin (Genolution, Seoul, South Korea) in keratinocyte starvation medium for 48 hours. Then, NHEK were irradiated with UV (100 mJ/cm), and keratinocyte conditioned media (KCM) were collected at 72 hours post-UV irradiation. Afterwards, mixtures (1:1) of KCM and serum-free DMEM were treated to NHDF, which had been starved in serum-free DMEM for 24 hours, and mRNA and protein were harvested at 6 hours

or 48 hours post-treatment, respectively.

For siRNA transfection to NHDF, NHDF were seeded and transfected with 300 pmol/ml of negative control or TLR4 siRNA (Bioneer) using G-fectin according to the manufacturer's instructions. After 48 hours, NHDF were starved in serum-free DMEM for 24 hours and treated with PBS or rhSAA1 (50 ng/ml) and harvested for mRNA and protein analysis at 6 or 24 hours post-treatment, respectively.

To investigate whether SAA1 activates NF-κB signaling pathway, NHDF were starved in serum-free DMEM for 24 hours and treated with PBS or rhSAA1 (50 ng/ml). Then, NHDF were harvested at given time points (0, 5, 15, 30, 60, 120, and 240 minutes) after rhSAA1 treatment for protein analysis.

To analyze if SAA1 induces proinflammatory cytokines through NF-κB signaling pathway, NHDF were starved in serum-free DMEM for 24 hours and treated with either DMSO (Merck, Millipore, Billerica, MA) or 5 μM BAY 11-7082 (Sigma-Aldrich, St. Louis, MO), and subsequently treated with PBS or rhSAA1 (50 ng/ml). Then, NHDF were harvested for mRNA and protein analysis at 6 or 24 hours post-treatment, respectively.

Quantitative real-time reverse transcriptase (RT)-PCR

Total RNA was isolated from mouse skin samples and cells using RNAiso (Takara Bio, Shiga, Japan), and 4 μg of RNA used to synthesize cDNA using First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. PCR was performed on a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq (Takara Bio) with primer pairs in Table 2. The PCR conditions were 50° C for 2 minutes, 95° C for 2 minutes, followed by 40 cycles at 95° C for 15 seconds and 60° C for 1 minute. The data were analyzed using the comparative ΔΔCt method, and normalized to 36B4 and presented as fold changes± standard error (SE).

Western blot analysis

Mouse skin samples and cells were homogenized and lysed in RIPA buffer (Merck) containing protease inhibitor mixture (Roche

Applied Science, Rockford, IL) and phosphatase inhibitor mixture (Sigma-Aldrich). Tissue extracts and cell lysates were centrifuged at 12,000 rpm, 4° C for 15 minutes, and supernatants were collected for Western blot analysis. Protein concentration of the samples was determined by Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Proteins with the same amount were loaded onto SDS polyacrylamide gels, separated electrophoretically, and transferred to PVDF membranes (Roche Applied Science). Membranes were blocked with 5% skim milk, and incubated with goat polyclonal antibody against SAA1 (R&D Systems, Minneapolis, MN) and actin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody against phopho-p65, p65, and IκBα, and mouse polyclonal antibody against phospho-IκBα (Cell Signaling Technology, Beverly, MA). Horseradish peroxidase-conjugated anti-goat, anti-rabbit, and anti-mouse IgG (Santa Cruz Biotechnology) was used as secondary antibodies. Bands were visualized by enhanced chemiluminescence detection system (Thermo Scientific, Waltham, MA).

Bead-based multiplex immunoassays

Mouse skin samples were homogenized and proteins were extracted using lysis buffer (Bio-Rad) containing protease inhibitor mixture. Tissue extracts and cultured media from NHDF were centrifuged at 13,000 rpm, 4 ° C for 15 minutes, and supernatants were collected. Protein concentration of the tissue samples was determined by Bradford reagent and the samples with the same concentration were prepared. Protein levels of mouse IL-1 β and IL-6 and human IL-1 β, IL-6 and IL-8 were measured by Bio-Plex multiplex system (Bio-Rad).

Haematoxylin and Eosin (H&E) staining

Mouse skin samples were fixed in 10% formalin for 24 hours and processed into paraffin wax. Paraffin-embedded samples were sectioned into 4 μm and mounted onto silane-coated slides (Dako, Glostrup, Denmark). Slides of skin specimens were dewaxed in xylene substitutes, rehydrated with graded ethanol and washed with distilled water. Then, H&E staining was performed and the specimens were examined using an optical microscope (Leica, Solms, Germany).

Statistical analysis

Data are presented as mean ± standard error. Significance was determined using Student's *t*-test for mouse *in vivo* experiments and paired *t*-test for *in vitro* experiments. *P*-values of less than 0.05 were considered statistically significant.

Table 2. Primer sequences for human and mouse genes for quantitative real-time PCR

Human gene	Forward	Reverse
h36B4	TCGACAATGGCAGCATCTAC	TGATGCAACAGTTGGGTAGC
hIL-1β	CTGTCCTGCGTGTTGAAAGA	TTCTGCTTGAGAGGTGCTGA
hIL-6	GCAGATGAGTACAAAAGTCC	GCAGAATGAGATGAGTTGTC
hIL-8	CAGGAATTGAATGGGTTTGC	AAACCAAGGCACAGTGGAAC
Mouse gene	**Forward**	**Reverse**
m36B4	TGGGCTCCAAGCAGATGC	GGCTTCGCTGGCTCCCAC
mSAA1	GGACATGAGGACACCATTGC	GTAGGAAGAAGCCCAGACCC
mIL-1β	GACTCATGGGATGATGATGATAAC	CCATACTTTAGGAAGACACGGATT
mIL-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA

Results

Acute UV irradiation increases SAA1 in the mouse dorsal skin

To validate whether UV can induce SAA1 expression in the skin, C57BL/6 mice (n=4) were irradiated with 200 mJ/cm of UV and the dorsal skin was harvested at 48 hours post-UV irradiation. SAA1 mRNA and protein levels were evaluated by quantitative RT-PCR and Western blot analysis, respectively (Fig. 9A and 9B).

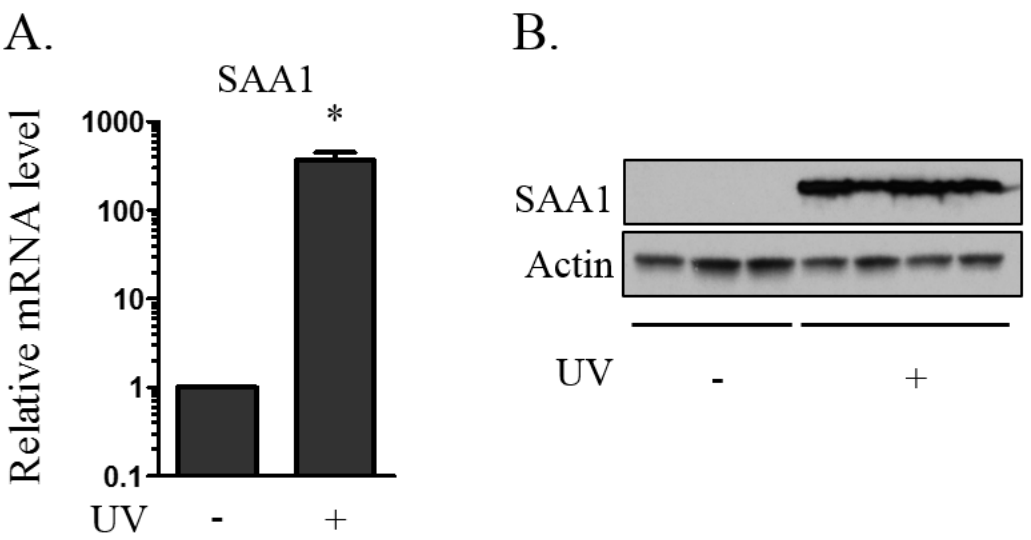

Figure 9. Acute UV irradiation increases SAA1 in the mouse dorsal skin

Depilated dorsal skin of 7-week-old C57BL/6 mice was irradiated with 200 mJ/cm or non-irradiated. Biopsy was performed at 48 hours post-UV irradiation. SAA1 (A) mRNA and (B) protein levels were determined by quantitative RT-PCR and Western blot analysis, respectively (n=4). Data are expressed as means ± SE. *P<0.05 versus control group.

Intradermal injection of rmSAA1 induces inflammation in the mouse dorsal skin

To further examine the effect of SAA1 administration, PBS or recombinant mouse SAA1 (2.5 mg/kg body weight) was injected intradermally into the dorsal skin of C57BL/6 mice (n=4) and the skin was biopsied at 24 hours post-injection. Intradermal injection of rmSAA1 caused local swelling and erythema on the dorsal skin of mice (Fig. 10A). I performed H&E staining to assess histological changes in the skin caused by rmSAA1 injection. In the rmSAA1-injected group, heavy inflammatory cell infiltration into the dermis was observed (Fig. 10B), which is consistent with findings in UV-irradiated skin. The mRNA and protein levels of proinflammatory cytokines were evaluated by quantitative RT-PCR and bead-based multiplex immunoassays, respectively. Injection of rmSAA1 significantly increased the mRNA and protein levels of IL-1β and IL-6 (Fig. 10C and 10D).

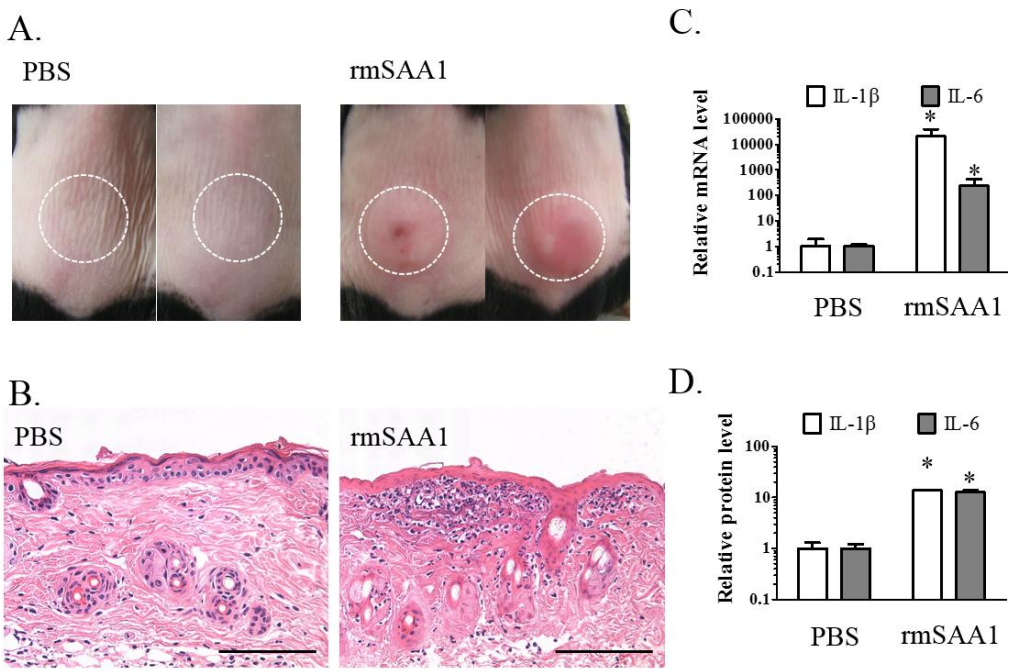

Figure 10. Intradermal injection of rmSAA1 induces inflammation in the mouse dorsal skin

Seven-week-old C57BL/6 mice were injected intradermally with PBS or rmSAA1 (2.5 mg/kg body weight) into the depilated dorsal skin. (A) Photographs were taken and the injected area of the skin was biopsied 24 hours post-injection. (B) Histology was analyzed by H&E staining (n=4). Scale bar represents 50 μm. IL-1β and IL-6 (C) mRNA and (D) protein levels were determined by quantitative RT-PCR and multiplex bead assay, respectively. Data are expressed as means ± SE. *P<0.05 versus control group.

Treatment with rhSAA1 induces proinflammatory cytokines in NHDF

I subsequently examined SAA1 ability to induce proinflammatory cytokines in skin cells and determined its mechanism of action. NHDF and NHEK were treated with different concentrations of rhSAA1 (0, 5, 50, and 500 ng/ml). I found that rhSAA1 effect on NHEK was limited although a significant increase in IL-1β and IL-8 expression was observed (Fig. 11A and 11B). On the other hand, rhSAA1 treatment of NHDF significantly increased the IL-1β, IL-6, and IL-8 mRNA levels at 6 hours post-treatment, and the IL-6 and IL-8 protein levels at 24 hours post-treatment (Fig. 11C and 11D).

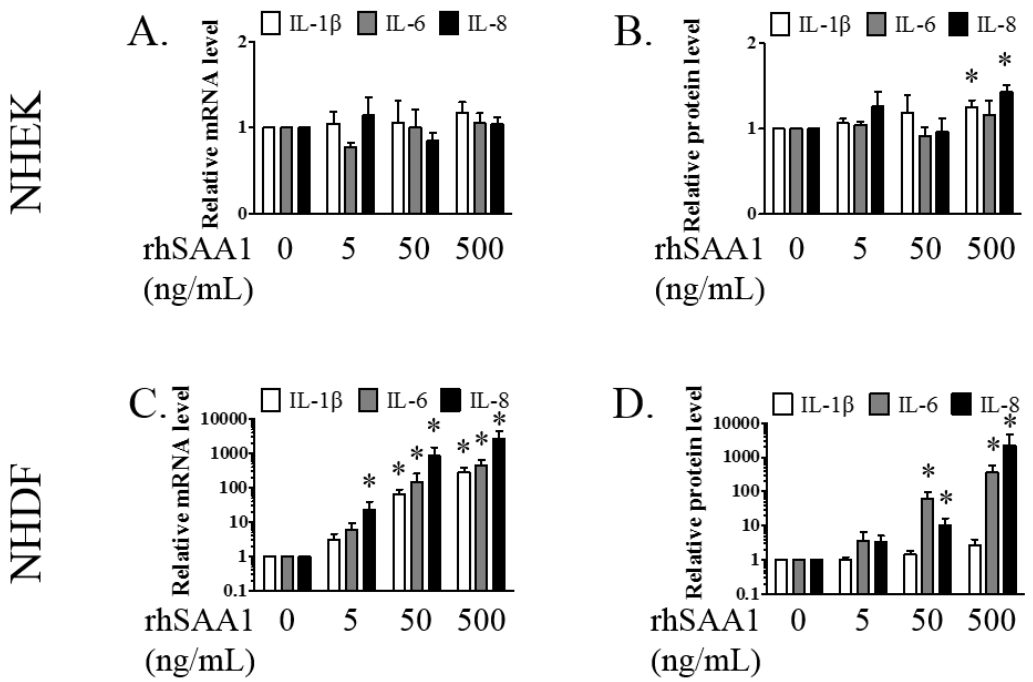

Figure 11. Treatment with rhSAA1 induces proinflammatory cytokines in NHDF

(A, B) NHEK and (C, D) NHDF cells were treated with rhSAA1 (0, 5, 50, and 500 ng/ml). The cells were harvested 6 hours post-treatment to assess IL-1β, IL-6, and IL-8 mRNA levels. Culture media were collected 24 hours post-treatment for protein analysis. Data are expressed as means ± SE. *P<0.05 versus control group.

SAA1 secreted from UV-irradiated NHEK induces proinflammatory cytokines in NHDF

Since UV-induced SAA1 in NHEK was found to exert its action on NHDF in a paracrine manner, I treated KCM of following conditions; KCM from NHEK with or without UV irradiation (100 mJ/cm) and with transfection of negative control or SAA1 siRNA. I confirmed SAA1 mRNA expression level after siRNA transfection and UV irradiation in NHEK. UV irradiation increased and SAA1 siRNA transfection decreased SAA1 expression in NHEK (Fig. 12A). The expression of proinflammatory cytokine mRNAs and proteins were analyzed at 6 or 24 hours post-KCM treatments. UV-irradiated KCM induced proinflammatory cytokines; however, these were attenuated by SAA1 knockdown on NHEK (Fig. 12B and 12C).

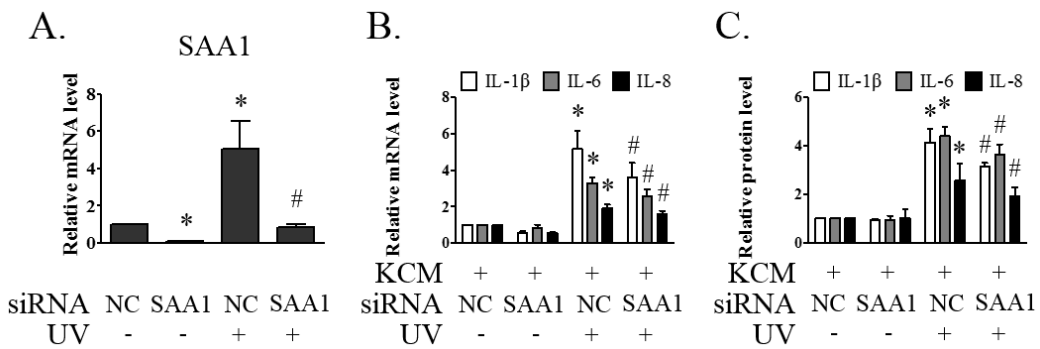

Figure 12. SAA1 secreted from UV-irradiated NHEK induces proinflammatory cytokines in NHDF

NHEK were transfected with negative control (NC) or SAA1 siRNA for 48 hours. Then NHEK were non-irradiated or irradiated with UV (100 mJ/cm), and (A) the expression of SAA1 mRNA were analyzed at 72 hours post-irradiation. NHDF were treated with various conditions of KCM. (B) The cells were harvested 6 hours post-treatment to assess IL-1β, IL-6, and IL-8 mRNA levels. (C) Culture media were collected 24 hours post-treatment for protein analysis. Data are expressed as means ± SE. $*P<0.05$ versus control group and $^{\#}P<0.05$ versus rhSAA1 treated group.

Treatment with rhSAA1 induces proinflammatory cytokines through TLR4 signaling pathway in NHDF

TLR4 was previously identified as a functional SAA1 receptor. Thus, to explore the mechanism of proinflammatory cytokine induction by SAA1, NHDF were transfected with negative control or TLR4 siRNA. TLR4 was knocked-down significantly at 24 hours post-transfection (Fig. 13A). Subsequently, the cells were treated with PBS or rhSAA1 (50 ng/ml). TLR4 knockdown was able to attenuate the increase of proinflammatory cytokine mRNAs and proteins at 6 or 24 hours post-rhSAA1 treatment, respectively (Fig. 13B and 13C).

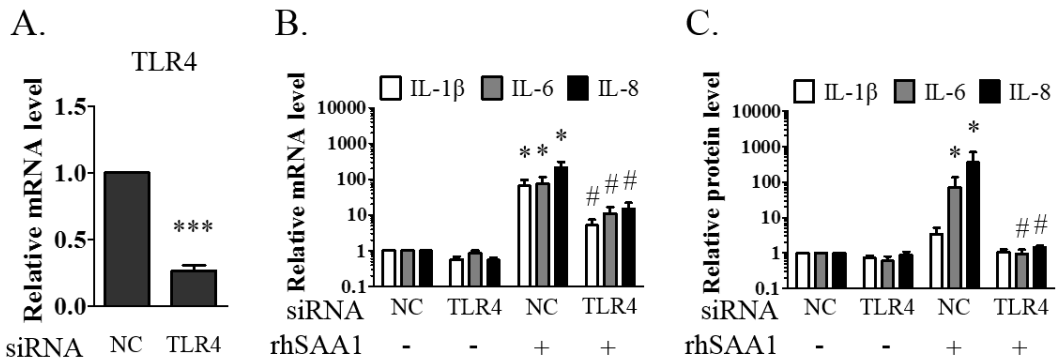

Figure 13. Treatment with rhSAA1 induces proinflammatory cytokines through TLR4 signaling pathway in NHDF

(A) NHDF were transfected with negative control (NC) or TLR4 siRNA and harvested 24 hours post-transfection. (B, C) NHDF were treated with PBS or rhSAA1 (50 ng/ml) 72 hours post-transfection. The cells were harvested 6 hours post-treatment to assess IL-1β, IL-6, and IL-8 mRNA levels. Culture media were collected 24 hours post-treatment for protein analysis. Data are expressed as means ± SE. $^{*}P<0.05$ versus control group and $^{\#}P<0.05$ versus rhSAA1 treated group.

Treatment with rhSAA1 induces proinflammatory cytokines through NF-κB signaling pathway in NHDF

As NF-κB is the key regulator of cytokine expressions during UV-induced inflammatory responses, I investigated the role of NF-κB signaling pathway in the SAA1-induced proinflammatory cytokine expressions. IκBα and p65 phosphorylations were assessed by Western blot analysis to evaluate NF-κB activation by SAA1. I found that IκBα phosphorylation was increased while total IκBα level was decreased at 30 minutes to 4 hours post-rhSAA1 treatment (50 ng/ml). Additionally, the levels of phosphorylated and total p65 were increased at 30 minutes to 4 hours post-rhSAA1 treatment (Fig. 14A). To further assess the role of NF-κB signaling pathway, NHDF were treated with either DMSO or 5 μM BAY 11-7082 (an irreversible inhibitor of IκBα phosphorylation), and subsequently treated with PBS or rhSAA1 (50 ng/ml). Inhibition of the NF-κB signaling pathway was able to block the rhSAA1-induced upregulation of proinflammatory cytokines in the mRNA and protein levels at 6 or 24 hours post-rhSAA1 treatment, respectively (Fig. 14B).

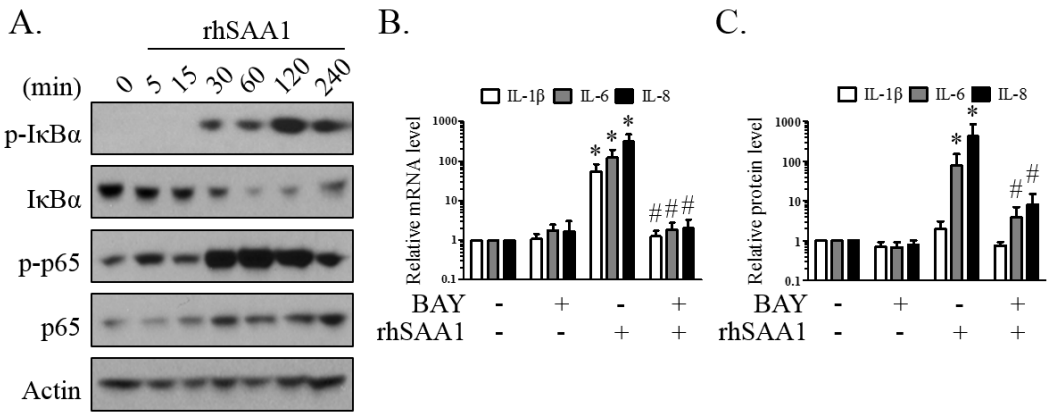

Figure 14. Treatment with rhSAA1 induces proinflammatory cytokines through NF-κB signaling pathway in NHDF

(A) rhSAA1-treated NHDF were harvested at indicated time points to evaluate levels of phosphorylated and total IκBα and p65 by Western blot analysis with actin as a loading control. A representative Western blot analysis was shown (n=4). (B and C) NHDF were incubated with DMSO or 5 μM BAY 11-7082 (BAY) for 30 minutes and treated with rhSAA1. Data are expressed as means ± SE. *P<0.05 versus control group and $^{\#}P$<0.05 versus rhSAA1 treated group.

Discussion

Photoaging alters the structure of the skin with the major detrimental effects in the connective tissue of the dermis. MMP-1 is one of the key proteases involved in chronic UV irradiation-induced photoaging process [19]. It is well known that UV irradiation on skin directly increases MMP-1 and MMP-1 from the epidermis can spread to and act in the dermis [20]. It is also known that UVA and UVB can reach the dermis though UVB can reach only upper dermis [21], and either UVA or UVB irradiation directly increases MMP-1 in dermal fibroblasts in vitro [22, 23]. It is recently reported that major source of increased MMP-1 expression in photoaged skin is dermal fibroblasts, rather than epidermal keratinocytes [24]. Moreover, since the basement membrane in the skin is known to play a role as a barrier in the cellular level not the protein level, epidermal keratinocyte-derived protein could diffuse through the basement membrane to the dermis to affect dermal fibroblasts. Therefore, it is possible that UV could indirectly induce

MMP-1 in the dermis through a certain molecule which could be induced in the epidermis and diffuse to the dermis.

Studies have shown that proinflammatory cytokines such as IL-1 and IL-6 are induced by UV irradiation in NHEK and these subsequently induce MMP-1 in NHDF [25, 26]. As cytokines reach their peak level and start to decline to their basal level before 24 hours after UV irradiation in NHEK [27], they may be involved in MMP-1 increase in NHDF in early time. However, since increased expression of SAA1 by UV was sustained until at least 72 hours which is relatively longer and later time than those cytokines, it could contribute to the increased expression of MMP-1 in NHDF in late time.

Importance of SAA1 as a paracrine factor mediating indirect UV-induced responses in NHDF was also suggested by a cell type-specific responding way. To clarify the function of SAA1 in the skin, I treated NHEK and NHDF with several doses of rhSAA1, and found that rhSAA1 induced MMP-1 and MMP-2 dose-dependently in NHDF but did not induce any changes of MMP-1, MMP-2, and MMP-9 in NHEK. I demonstrated that rhSAA1 induced MMP-1 in NHDF via TLR4 signaling pathway, which is revealed in this study. It is controversial whether

NHEK express TLR4 or not; some insist that NHEK express functional TLR4 [28], while others insist not [29]. In any case, previous study of my laboratory showed that relative expression level of TLR4 in NHEK was considerably lower than that in NHDF [30]. I also found that the expression levels of TLR4 in the epidermis and primary keratinocytes were considerably low. Therefore, lack of or at least low expression of TLR4 in NHEK could be attributable to the fact that rhSAA1 did not induce MMP-1 in NHEK in the given concentrations. Additionally, SAA1 can exert its action through various receptors. However, I found that the relative expression levels of those receptors other than TLR4 (i.e., TLR2, FPRL1, and RAGE) were much lower than that of TLR4 in NHDF. Thus, I assume that this is why knockdown of TLR4 alone could inhibit the effect of rhSAA1 treatment to NHDF almost completely.

TLRs are known to mediate the innate immune responses. In the skin, both dermal fibroblasts and epidermal keratinocytes express functional TLRs [30, 31]. Importantly, TLRs were found to participate in UV-induced inflammatory responses. TLR2 was reported to mediate the expressions of IL-1β, IL-6, and matrix metalloproteinase-13 induced by UV [32], while TLR3 was reported to recognize noncoding RNAs

produced by UV-damaged keratinocytes which caused increased TNF-α and IL-6 expressions [33]. Furthermore, UV-induced inflammation was found to promote angiotropism and metastasis through a TLR4/MyD88-dependent signaling pathway in melanoma [34]. Thus, I propose that SAA1 serves as an endogenous ligand for TLR4 in the skin. SAA1 recognition by TLR4 may result in the induction of proinflammatory cytokines during UV-induced acute responses.

Previous studies showed that UV irradiation on human skin induces several MMPs but not MMP-2 [35]. While rhSAA1 induced MMP-2 in NHDF, UV-irradiated KCM, despite including alarmin SAA1, did not increase MMP-2 in NHDF. Unlike MMP-1, MMP-3, and MMP-9, expression of MMP-2 is not regulated by AP-1 signaling pathway, and its regulatory mechanism is largely unknown [36]. It is possible that unknown factors in UV-irradiated KCM govern the MMP-2 regulation more powerfully than SAA1.

A recent study described that increased MMP-1 in the chronically photoaged skin occurs from the dermis not the epidermis [24]. I showed SAA1 expression was increased in chronically photodamaged forearm skin as well as in acute UV-irradiated skin. Thus,

I insist that repetitive UV irradiation maintain high level of SAA1, and this increase MMP-1, consequently leading to the formation of wrinkling.

Three A-SAA genes (SAA1, SAA2, and SAA3) have been identified in humans, though SAA3 is a pseudogene that generates neither mRNA nor protein [37]. Nucleotide and amino acid sequences of SAA1 and SAA2 are highly homologous and they are expressed concomitantly responding to inflammation [38]. Therefore, it is presumable that SAA2 could also be involved in UV-induced photoaging process similar to SAA1. When I checked the mRNA expression level of SAA2, it was substantially lower than that of SAA1. Hence, even if SAA2 might play a role in UV-induced responses in the skin, it would not be fairly important.

In this study, I focused on the local expression and function of SAA1 in the skin by UV irradiation. Besides the epidermis-originated SAA1, serum SAA1 induced by many inflammatory conditions is also available so that it may affect ECM regulation in the skin. In chronic inflammatory diseases such as rheumatoid arthritis, patients have sustained high serum SAA level [39]. I suggest this may contribute to the skin aging but this remains to be studied further.

Also, serum SAA level was found to positively correlate with the clinical severity in several inflammatory diseases, such as rheumatoid arthritis and osteoarthritis, indicating that SAA is a good predictor of disease progression. Moreover, SAA contributes to the disease pathogenesis by stimulating cytokines and enzymes that regulate the extracellular matrix [40]. Because SAA activates the NF-κB signaling pathway and induces the expression of various cytokines, SAA may also play an important role in inflammatory skin diseases such as psoriasis where elevated SAA level was reported [41].

To summarize, I demonstrated that SAA1 level was increased by acute UV irradiation and SAA1 level of photoaged skin was higher than that of photo-protected skin. I found that UV increased SAA1 in NHEK and this SAA1 in turn induced MMP-1 and proinflammatory cytokines in NHDF in a paracrine manner. Additionally, SAA1 induced MMP-1 and proinflammatory cytokine production through TLR4 signaling pathway and also activated MAPK/AP-1 signaling pathway in NHDF. In this study, I suggest that SAA1 be induced by UV irradiation in the epidermis and diffuse to the dermis to up-regulate MMP-1 and proinflammatory cytokines, resulting in photoaging and skin inflammation.

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국 문 초 록

반복된 자외선 조사는 피부에서 주름, 탄력 저하 등과 같은 광노화 현상을 유발한다. 자외선 조사는 피부에서 matrix metalloproteainse-1 (MMP-1)을 증가시키는데, 이는 세포외기질, 특히 콜라겐을 분해하여 피부에 주름 형성을 촉진시킨다. 또한, 피부에 과도한 자외선 조사는 화상과 같은 피부 염증을 일으킬 수 있다. Serum amyloid A1 (SAA1)은 급성기단백질로, 체내 염증이 일어나면 이에 반응하여 혈중 농도가 증가하는 것으로 잘 알려져 있다. 피부에서 SAA1이 발현하는 것은 알려졌지만, 피부에서 SAA1의 기능은 잘 알려져 있지 않다. 본 연구에서는 급성 자외선을 조사한 사람 엉덩이 피부에서, 광노화된 사람 전완 피부에서, 그리고 급성 자외선을 조사한 쥐 등 피부에서 SAA1의 발현이 증가하는 것을 발견하였다. 또한 자외선 조사는 피부 각질형성세포에서 SAA1을 증가시켰고, 재조합 SAA1 단백질을 피부 섬유아세포에 처리하였을 때 MMP-1과 IL-6과 IL-8 같은 염증성 싸이토카인이 유도되었다. 다음으로 자외선을 조사한 피부 각질형성세포로부터의 배양액을 피부 섬유아세포에 처리하였을 때 MMP-1, IL-1β, IL-6, 그리고 IL-8이 증가함을 보였다. 이러한 증가는 피부 각질형성세포에서 SAA1을 감소시켰을 때 완화되었다. 그리고 피부 섬유아세포에서 Toll-like receptor 4 (TLR4)를

감소시켰을 때 SAA1에 의한 MMP-1, IL-6와 IL-8의 증가 또한 완화되었다. 종합해보면, 자외선은 피부 각질형성세포에서 SAA1을 증가시키고, 분비된 SAA1은 TLR4를 통하여 피부 섬유아세포에 작용하여 MMP-1, IL-6, 그리고 IL-8의 발현을 유도한다. 결론적으로, 본 연구 결과는 SAA1이 자외선에 의해 피부에서 유도 되는 MMP-1, IL-6, 그리고 IL-8을 조절하는 잠재적 매개체임을 시사한다.

주요어: 자외선, serum amyloid A1, Toll-like receptor 4

학 번: 2012-21813