Introduction

Skin protects the body from external stress and controls homeostasis, maintaining moisture content and the temperature [19, 27]. Skin aging may be explained by both intrinsic and extrinsic factors [4, 9, 14]. Although intrinsic skin aging is related to the naturally occurring physiological process, extrinsic skin aging is associated with environmental factors, such as UV exposure, smoking, and environmental pollutants [2]. The most common extrinsic skin aging factor, or photoaging, is exposure to UV light, which has known harmful effects [1].

Chronic sun exposure causes problems such as skin aging, skin cancer, inflammation, roughness, laxity, and pigmentation. UV light is divided into three zones: UVA (320–400 nm), UVB (280–320 nm), and UVC (180–280 nm) [2, 4, 20, 24]. The ozone layer blocks UVC, but UVA and UVB penetrate this layer [9, 31, 33]. UVB causes eczema acutum, delayed pigmentation, and photo-hypersensitive reactions. UVB directly affects DNA, and UVB results in the production of reactive oxygen species (ROS), which play a role in photoaging [33]. As 96% of UV reaching the ground is UVA and it penetrates the epidermis and the dermis more deeply than UVB [15], UVA is more important in photoaging than UVB. It decreases type 1 collagen, the main factor of the extracellular matrix (ECM), and inhibits the synthesis of pro-collagen synthesis in dermal fibroblasts [9, 19]. UVA also induces cytokines-involved inflammation, such as tumor necrosis factor (TNF)-α, interleukin-1, and ROS [2, 28, 31]. In addition, UVA induces the phosphorylation of mitogen-activated protein kinase (MAPK) in dermal fibroblasts [17, 20]. The activation of the p38 and c-Jun N-terminal kinase (JNK) signals stimulates the level of the c-fos and c-jun transcription factor activator protein-1 (AP-1) [9]. After up-regulation, AP-1 binds to the matrix metalloproteinases (MMPs) gene, and this increases the mRNA level of the MMPs [24]. Although nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) does not binding
directly to the promoter site of the MMP-1 and the basic fibroblast growth factor (bFGF), it up-regulates both MMP-1 and bFGF [28]. MMPs have a highly conserved active center containing zinc, and play an important role in photoaging [9, 24]. Twenty types are recognized according to their structure and function. They are divided into collagenases, gelatinases, stromelysins, and membranes [22]. MMP-1 mainly exerts an effect on photoaging by initiating the breakage of the collagen cross-links [20]. The MMP-1 breaks the triple helix of collagen and the collagen cross-link in the ECM [21].

As 70–80% of the skin is composed of collagen, it sustains the structure of the skin [31]. Damage caused by MMP-1 leads to deeper wrinkles in the skin. Exposure to UVA causes secretion of TNF-α inducing MMP-1 in keratinocytes. The secreted TNF-α is transported to the fibroblasts and induces MMP-1 in normal human dermal fibroblasts (NHDFs) [6].

*Lactobacillus* species aid intestinal microbiota in the fermentation of various foods. Several studies have indicated that *Lactobacillus* species exert a favorable influence on the health of humans by decreasing the level of cytokines involved in inflammation [10, 28]. Lipoteichoic acid (LTA), a component of the cell wall of gram-positive bacteria, is one of the main active components of *Lactobacillus* species, and LTA of *Lactobacillus plantarum*-isolated *kimchi* is effective in suppressing UVA-induced MMP-1 [3].

Studies have not confirmed whether *L. sakei* LTA (sLTA) blocks various kinds of proinflammatory cytokines, including MMP-1. We investigated whether sLTA has strong effects on anti-photoaging and immunoregulation. The data collected, including the results of ELISA and western blot analysis, showed that sLTA inhibits the level of proinflammatory cytokines and the phosphorylation of the MAPK signal induced by lipopolysaccharide (LPS) in THP-1 cells. We confirmed remarkably protective effects of sLTA, which decreased the MMP-1 induced by not only proinflammatory cytokine and the phosphorylation of MAPK signal but also by the dose of TNF-α.

**Materials and Methods**

**Cell Viability**

The cells were grown to 70–80% confluency to confirm the cytotoxicity of sLTA. The NHDFs were cultured for 12–18 h to ensure their adhesion on a 12-well microplate and treated with 0.01–10 µg/ml sLTA for 18 h. The viability of the cells was measured with a WST-1 cell proliferation assay system (TAKARA, Japan). The test is based on the production of formazan dye, with the quantity of formazan associated with the number of metabolically active cells. The NHDFs were incubated with 50 µl of tetrazolium salt (WST-1 premixed reagent) per well for 30 min after sLTA treatment for 18 h. The absorbance of the medium was measured with an ELISA reader at 450 nm.

**Bacterial Strain and Preparation of the Components of the Cell Wall**

*L. sakei* (KCCM 11175P) was isolated from *kimchi*. It was confirmed by a BLAST search. Purified LTA of *L. sakei* was isolated by n-butanol extraction. The process of purifying LTA has been described previously. The purity of the purified LTA was determined by its endotoxin concentration and its protein level with the ToxinSensor Chromogenic Luminus Amboocyte Lysate Endotoxin Assay kit (Genscript, USA) and by silver staining after SDS-PAGE gel loading. DNA and RNA contaminations were determined by UV absorption at 260 and 280 nm, respectively.

**Cell Culture and UVA Irradiation**

The THP-1 cells, a human monocyte-like cell line, and U937 cells, were grown in a RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin sulfate (100 g/ml) (Welgene, USA). They were cultured at 37°C in a humidified 5% CO2 atmosphere. Before the cells were pretreated with sLTA, they were cultured for 8–12 h to ensure adhesion on a 6-well microplate and 96-well microplate. After THP-1 cells had treated by 0.1 to 10 µg/ml of sLTA for 18 h, it activated by LPS (0.5 µg/ml) for 1 h or 4 h.

The NHDFs isolated from human neonatal foreskin were grown in an FGM-2 fibroblast bullet kit (Lonza, USA) supplemented with 0.1% insulin, 0.1% rhFGF-B, 0.1% GA-1000, and 1% FBS. They were cultured at 37°C in a humidified 5% CO2 atmosphere. The NHDFs were used between the third and the seventh passages. The cells were transferred by trypsin-EDTA (Welgene, South Korea) when they had grown to 80% confluency. Before the cells were pretreated with sLTA, they were cultured for 12–18 h to ensure adhesion on a 6-well microplate. The cells were washed twice with 1× PBS Dulbeco’s solution without Mg and Ca (DPBS, pH 7.4) and exposed to UVA irradiation (VILBER Loumat, France; 360 nm) in 1 ml of DPBS. The dose of UVA irradiation was 5 J/cm², and the dose of TNF-α was 100 ng/ml. After the UVA irradiation and the TNF-α treatment, the NHDF medium was replaced with FGM without FBS, and the cells were incubated at 37°C in a humidified 5% CO2 atmosphere.

**Enzyme-Linked Immunosorbent Assay**

The THP-1 cells were stimulated with LPS and incubated at 37°C in a humidified 5% CO2 atmosphere for 4 h. The supernatant of the cells was collected, and the production of TNF-α was assayed by a standard sandwich enzyme-linked immunosorbent assay (ELISA). The ELISA detection of TNF-α was performed with human TNF-α antibody-monoclonal mouse IgG clone for capturing (R&D systems, USA). The human TNF-α-specific detection polyclonal (goat IgG) antibodies were biotinylated. The binding streptavidin HRP was confirmed using o-phenylenediamine as a substrate.
The OD was detected at a wavelength of 450 nm and using a reference wavelength of 550 nm.

The NHDFs were stimulated with UVA and incubated at 37°C in a humidified 5% CO₂ atmosphere for 18 h. The supernatant of the cells was collected, and the production of MMP-1 in the culture supernatant was analyzed by a standard sandwich enzyme-linked immunosorbent assay (ELISA). The ELISA detection of MMP-1 was conducted with human MMP-1 antibody-polyclonal goat IgG (R&D Systems, USA). The human MMP-1-specific detection polyclonal (goat IgG) antibodies were biotinylated. Streptavidin HRP was used for detection of bound antibody, and the binding streptavidin HRP was confirmed using o-phenylenediamine as a substrate. The OD was detected at a wavelength of 450 nm and using a reference wavelength of 550 nm.

Western Blot Analysis

At 30 min, 6 h, and 18 h, whole cell extractions of NHDFs were harvested in pro-prep buffer (iNTron Biotechnology) to detect the MAPK signal and the MMP-1 protein level. The THP-1 cells were harvested in pro-prep buffer at 1 h to detect the MAPK signal. The protein concentration was determined with the Bradford assay (Bio-Rad, USA). The cell lysate was boiled at 100°C for 5 min with 5x SDS gel-loading dye (250 mM Tris-Cl (pH 6.8), 10% SDS, 0.5% bromophenol blue, 50% glycerol, and 500 mM β-mercaptoethanol). The protein samples were resolved by 12% SDS-PAGE in a Tris-base/glycine/SDS buffer and transferred to polyvinylidene fluoride membranes (Millipore, USA) at 100 V for 1 h. The membranes were blocked in 5% skim milk (BD, France) and washed three times in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20). The membranes were blotted overnight at 4°C with p38 MAPK (T180/Y182) rabbit Ab, p-SAPK/JNK (T183/Y185) rabbit Ab, phosphor-NF-kappa B P65 (Ser 276) antibody (Cell Signaling, USA), and anti-MMP-1 (R&D Systems, USA), diluted with 5% BSA in TBST-T. The membranes were washed three times and incubated with secondary HRP-conjugated anti-rabbit IgG (R&D Systems, USA), anti-mouse IgG from sheep (GE Healthcare), and donkey anti-goat IgG (Santa Cruz, USA) for 2 h. The bands were detected by ECL reagent (Animal Genetics, Inc., USA) and developed with X-ray film processing.

Statistical Analysis

All the experiments were performed at least three times. The data shown are the representative results of the mean ± SD of triplicate experiments. Differences were judged to be statistically significant when the p value was <0.05.

Results

**sLTA Modulates TNF-α by Controlling MAPK Signal**

The role of LPS in systemic inflammation responses is well known. These are caused by phosphorylation of MAPK signal, which increases the mRNA of the pro-

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**Fig. 1.** Immune-regulating effects of sLTA in THP-1 cells. 
(A) THP-1 cells were treated by dose-dependent sLTA for 8 h. The induction test in sLTA-treated cells was measured using the TNF-α ELISA. sLTA induced TNF-α production dose-dependently. (B) THP-1 cells were pretreated by dose-dependent sLTA for 18 h and activated by 0.5 µg/ml of LPS for 4 h. TNF-α production was decreased in a dose-dependent manner from 0.1 to 10 µg/ml of sLTA. (C) THP-1 cells were pretreated by dose-dependent sLTA for 18 h and activated by 0.5 µg/ml of LPS for 1 h. sLTA reduced the MAPK signal, which was induced by LPS. The values for nitrite are the mean ± SD from three independent experiments.
inflammatory cytokine in THP-1 cells. We conducted TNF-α ELISA and MAPK western blot analysis to identify whether sLTA blocks the phosphorylation of MAPK signals and the TNF-α induced by LPS, in human monocyte THP-1 cells. The THP-1 cells were treated with sLTA for 8 h, which induced the level of TNF-α dose-dependently (Fig. 1A). The THP-1 cells were pretreated with sLTA for 18 h and activated by LPS for 4 h. Pretreatment of the sLTA reduced the LPS-induced production of TNF-α in the THP-1 cells (Fig. 1B). The production of TNF-α was decreased in a dose-dependent manner from 0.1 to 10 µg/ml of sLTA. A total of 10 µg/ml of sLTA suppressed TNF-α at concentrations of 7,000 to 2,000 pg/ml. The result shows that sLTA significantly decreased the production of TNF-α in the THP-1 cells.

Furthermore, phosphorylation of the MAPK signal was up-regulated by LPS and inhibited by 0.1 to 10 µg/ml of sLTA dose-dependently, as shown in Fig. 1C. The results of the western blot analysis suggest that sLTA may be effective in the prevention of inflammation. We confirmed the effects of sLTA on reducing the release of the TNF-α and the MAPK signal.

**sLTA has an Effect on the Viability of the Cells**

To confirm the cytotoxicity of sLTA, the viability of the cells was assessed with the WST-1 assay. Tetrazolium salts in the WST-1 reagent are cleaved to formazan dye by the mitochondrial respiratory chain that is active in viable cells. The level of the dye is associated with the viability of the cells. The viability of the cells was not significantly altered by the sLTA at up to 10 µg/ml (Fig. 2). In the subsequent experiments, NHDFs were treated with sLTA at a concentration range of 0.01–10 µg/ml.

**sLTA Inhibits UVA-Induced MMP-1 in NHDFs**

Previous studies have shown that UVA irradiation causes photoaging due to the induction of MMP-1. MMP-1 is a collagenase and matrix metalloprotease; it breaks cross-links in collagen, including those of triple helix structure. To investigate whether sLTA can inhibit UVA-induced MMP-1, we examined the level of the MMP-1 protein by western blot analysis and ELISA. The results confirmed that sLTA inhibited the production of MMP-1 in a dose-
dependent manner at 0.1 to 10 µg/ml of sLTA. Fig. 3A indicates that UVA induces MMP-1 and sLTA inhibits the level of the MMP-1 protein dose-dependently. The inhibition effect by sLTA of MMP-1 was similar to that of retinoic acid, which is a well-known anti-photoaging molecule, thereby confirming its potential to help prevent the effects of UVA exposure. Fig. 3B show the results of the western blot of MMP-1 in the cultured media and Fig. 3C is conducted in cell lysate. These data suggest that sLTA inhibits UVA-induced MMP-1.

sLTA Blocks the Phosphorylation of MAPK Induced by UVA

MAPKs play important roles not only in the regulation of cell growth and differentiation but also in the control of cellular responses to cytokine. Shown as several data, UVA up-regulated MMP-1 by inducing the phosphorylation of the MAPK signal in NHDFs. The MAPK signal, p38, JNK, and NF-κB are induced by UVA, and this increases the transcription of MMP-1. We expected that the suppression of the MAPK signal, which is induced by UVA, would inhibit the production of MMP-1, resulting in anti-photoaging. Fig. 4 indicates that sLTA reduces the MAPK signal, which was activated by UVA.

sLTA Down-Regulates TNF-α-Induced MMP-1 in NHDFs

Induced production of TNF-α in keratinocytes increases MMP-1 in NHDFs. UVA up-regulates TNF-α in keratinocytes, and it is transported to dermal fibroblasts, resulting in an increase in MMP-1. We examined whether recombinant TNF-α induced MMP-1 and whether sLTA inhibited the production of MMP-1 in NHDFs. The production of MMP-1 was measured by MMP-1 ELISA and western blot analysis. As shown in Fig. 5A, the secretion of MMP-1 was up-regulated by recombinant TNF-α and suppressed by sLTA in the NHDFs. Fig. 5B illustrates the effect of sLTA on the inhibition of TNFα damage-induced MMP-1 in the western blot assay. We postulate that sLTA blocks the production of MMP-1 induced by TNF-α via the inhibition of MMP-1 ELISA.

**Discussion**

*Lactobacillus* spp. are probiotics with many health benefits, such as helping to combat diarrhea and stomach ache [13, 18]. They have inhibitory effects on atherosclerosis, which is caused by inflammatory cytokines in blood vessels [10, 28, 30]. *Lactobacillus* spp. also act as immunomodulators, which control the induction and the inhibition of proinflammatory cytokines [5, 13, 28]. In addition, they have well-known...
antimicrobial properties and help to combat bacterial pathogens [7, 16]. Owing to the known function of *L. plantarum* as an immunomodulator, this bacterium is added to dairy products [10]. The components of the cell walls of *Lactobacillus* spp. play a key role in their effects [5]. Several papers have demonstrated that LTA, a component of the cell wall, binds the cytoplasmic membrane and acts as an important immunomodulator [5, 8, 10, 25]. LTA is a constituent of the cell wall of gram-positive bacteria. It is formed by linking a hydrophilic polyphosphate polymer to a glycolipid [8]. Different LTAs from various *Lactobacillus* spp. have different properties and immune activities. Although the effects of *L. plantarum* and *L. rhamnosus* GG are well known, those of *L. sakei* have not been examined to date. We investigated the possible role of *L. sakei* LTA (sLTA) as an immune-modulating and anti-photoaging agent. First, our data suggest that sLTA induces TNF-α, a proinflammation cytokine, dose-dependently in human monocyte THP-1 cells. We predict that it elicits physiologically relevant levels of immune activity in the human body. The result of the sLTA inhibition test also indicated the ability of sLTA effects to reduce the level of TNF-α, which is activated by LPS, a component of the cell wall, in gram-negative bacteria. LPS leads to up-regulation of proinflammatory cytokines through the MAPK signal cascade. Our data suggest that sLTA significantly blocks the MAPK signal, such as JNK, p38, and NF-κB. They indicate that the inhibition of TNF-α is the result of down-regulation of the MAPK signal. The results also revealed the effect of sLTA which represses excessive inflammation reactions produced by LPS.

UV light is divided into UVA, UVB, and UVC depending on the wavelength range. As UVC is absorbed by the ozone layer, it does not reach the ground. However, as UVA and UVB wavelengths are longer than UVC, they pass through the ozone layer and affect human skin [14, 26, 27]. UV exposure causes some skin diseases, such as skin cancer, as well as skin aging. In particular, UVA results in the reduction of collagen synthesis and breakage of links of the ECM. Type I collagen is the main component of the dermal ECM [6, 22]. Pro-collagen, a type I collagen precursor, is produced in dermal fibroblasts and transported to the ECM where it matures to type I collagen, which supports the strength and the elasticity of the ECM by forming cross-links with each other [22]. UVA-caused photoaging is well known. It damages dermal fibroblasts by penetrating deep into the dermis, as opposed to UVB radiation, which only penetrates the epidermis [28]. It breaks collagen cross-links, including the collagen triple helix structure of the ECM. Furthermore, it hinders pro-collagen synthesis in dermal fibroblasts. Alterations in the amount of collagen reduce the resilience of the skin and leads to wrinkling [11, 22]. Therefore, we used UVA induction for demonstrating the effect of sLTA on the NHDFs. UVA exposure elevates MMP-1, MMP-3, and MMP-9 *in vivo* [2, 15].

MMP-1 is the most important photoaging marker, due to its role in the initiation of the breakdown of collagen [22]. The stimulation of MMP-1 by UVA exposure damages the structure of collagen, including its cross-links. MMP-1 also inactivates COL1A1 and COL1A2, both of which are involved in the synthesis of collagen [12, 29, 31]. UVA induces MMP-1 through activation of the MAPK signal cascades, induced by ROS [17, 26]. ROS damages cell components, such as proteins, lipids, and DNA, because it breaks down the antioxidant defense system. In addition to altering the pattern of genetic translation, ROS causes skin cancer and skin aging. ROS makes O₂⁻ and it is the main cause of the synthesis of MMP-1 [5, 12, 23]. p38 and JNK lead to the c-fos and c-jun complex, which is AP-1. As AP-1 is a transcription factor binding the MMP-1 gene, activated AP-1 induces the transcription of MMP-1 [23, 26, 32]. Up-regulation of NF-κB induces bFGF, which increases excessive physical process, leading to photoaging. NF-κB plays an important role in the induction of MMP-1. Although NF-κB does not directly bind to the promoter site of MMP-1 or bFGF, it up-regulates MMP-1 and bFGF. UVA stimulates not only dermal fibroblasts in the dermis but also keratinocytes. Keratinocytes secrete TNF-α when exposed to UVA. The secreted TNF-α moves into the ECM, leading to the induction of MMP-1 [6].

After confirming the immune-modulating effect of sLTA in the THP-1 cells, we conducted experiments to determine the role of sLTA as an anti-photoaging molecule in NHDFs. First, at concentrations of 0.01 to 10 μg/ml, sLTA did not affect the viability of the NHDFs. It clears the stability of sLTA and so we can use sLTA in photoaging experiments. Our results show that UVA induced MMP-1 and that the MAPK signal cascades stimulated MMP-1. sLTA significantly blocked the expression level of MMP-1 by reducing the of MAPK signal, such as p38, JNK, and NF-κB signal. The data indicate that the reduction in production of MMP-1 is caused by blocking the MAPK signal. Blocking p38 and JNK lead to inhibition of AP-1, the MMP-1 transcription factor, and blocking NF-κB suppresses level of MMP-1 and bFGF. This results in anti-photoaging effects and increasing amounts of collagen. In addition, sLTA inhibits MMP-1, which was induced by TNF-α in the NHDFs.
We conclude that sLTA appears to have the potential to dramatically suppress UVA-induced damage and it has not only anti-photoaging properties but also acts as an immunomodulator.

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References