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Roles of TLR3 in poly(I:C)- and UV-induced gene expressions in human dermal fibroblasts and mouse skin

사람진피섬유아세포와 마우스피부에서 Poly(I:C)와 자외선에 의해 유도되는 유전자 발현에서 TLR3의 역할

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Roles of TLR3 in poly(I:C)- and UV-induced gene expressions in human dermal fibroblasts and mouse skin

by Yao Cheng

A Thesis Submitted to the Department of Biomedical Sciences in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medical Sciences at Seoul National University College of Medicine
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Abstract

Toll-like receptors (TLRs) are known to recognize not only pathogen-associated molecular patterns (PAMPs) but also danger-associated molecular patterns (DAMPs). Recent studies have characterized the expressions and functions of TLRs in human epidermal cells. However, the characteristics of TLR family members in human dermal fibroblasts have not been thoroughly studied.

Ultraviolet (UV) irradiation can result in premature skin aging (photoaging) which is characterized by decreased expression of collagen and increased expression of matrix metalloproteinases (MMPs). Double strand RNA (dsRNA) can be generated at various conditions including virally infected cells or UV-damaged skin cells. TLR3 is one of the receptors for dsRNA. However, little is known about the effect of dsRNA on the expression of procollagen and MMPs in skin fibroblasts.

Recently, it is known that UV-irradiated keratinocytes could release damaged self-noncoding RNAs that serve as TLR3 ligands. This finding is suggested to be involved in UV-induced increase of inflammatory cytokines, such as TNF-α and IL-6. However, whether TLR3 is involved in UV-induced reduction of procollagen and induction of MMP expressions *in vivo* in unknown.

In chapter Ⅰ, I systematically investigated the expression of TLRs and their functional responses to each ligand in skin fibroblasts. I found that all 10 TLRs are expressed in skin fibroblasts. Stimulation of skin fibroblasts with each TLR ligand resulted in increase of IL-6, IL-8, and MMP-1 proteins. I also found that the expression level of each TLR was much higher in fibroblasts than in keratinocytes. In particular, I found that the fold-increases in IL-6 and IL-8 mRNA levels upon exposure to a TLR1/2 ligand were much higher in fibroblasts than in keratinocytes, which appears to reflect the difference in expression levels of TLR1 and 2 in between fibroblasts and keratinocytes.

In chapter Ⅱ, I observed that treatment of TLR3 ligand, poly(I:C), but not other TLR ligands reduced procollagen and induces IFN-β expression in skin fibroblasts. I found poly(I:C) induced IFN-β expression through interferon regulatory factor 3 (IRF3)-dependent pathway in skin fibroblasts. IRF-3 pathway was not activated by other TLR ligands except poly(I:C). Further experiments revealed that poly(I:C) reduced procollagen expression through induction of IFN-β in skin fibroblasts. I also showed that knockdown or knockout of TLR3 relieved poly(I:C)-induced reduction of procollagen expression.

In chapter Ⅲ, I examined the effect of poly(I:C) on MMP-1, -2, and -3 expressions in skin fibroblasts. I found that poly(I:C) treatment induced expressions of MMP-1, -2, and -3, which were dependent on

TLR3. Poly(I:C) treatment also induced activations of the mitogen-activated protein kinase (MAPK), the nuclear factor-kappaB (NF-κB) and the IRF3 pathways. By using specific inhibitors, I found that poly(I:C)-induced expressions of MMP-1, -2, and -3 were differentially regulated by these signaling pathways. In particular, inhibition of IRF3 signaling pathways attenuated poly(I:C) induced expressions of MMP-1, -2, and -3.

In chapter Ⅳ, I checked whether TLR3 is involved in UV-induced reduction of procollagen and induction of MMP expressions *in vivo*. I found that the expression of procollagen was not significantly different between UV-irradiated WT and TLR3-/- mice skin. In contrast to procollagen expression, I found that UV radiation-induced expression of MMP-13 was significantly higher in WT than in TLR3-/- mice skin. I suggested that the effect of higher expression of MMP-13 in WT skin fibroblasts might be due to released RNA from necrotic keratinocytes.

In summary, I showed that all 10 TLRs were constitutively expressed and functional in skin fibroblasts. Expression levels of TLR family members were higher in skin fibroblasts than in skin keratinocytes. Activation of TLR3 reduced procollagen expression through induction of IFN-β in skin fibroblasts. I also found that MMP-1, -2, and -3 could be induced by dsRNA in skin fibroblasts. Poly(I:C) induced expressions of MMP-1, -2, and -3 through various signaling pathways

including TLR3 and IRF3. In the end, by using WT and TLR3-/- mice, I determined that UV-induced increase of MMP-13, but not reduction of procollagen expression, was dependent on TLR3.

My results in chapter Ⅰ may provide information for studies of certain skin diseases in which fibroblasts are involved in the pathogenesis, such as keloid, hypertrophic scar and scleroderma. My study in chapter Ⅱ provides the first evidence that dsRNA has potent anti-fibrotic activity in skin. My data in chapter Ⅲ suggests that TLR3 and/or IRF3 may be good targets for regulating the expressions of MMP-1, -2, and -3 induced by dsRNAs. My study in chapter Ⅳ indicates that TLR3 could be viewed as a therapeutic target for UV-induced MMP induction.

Keywords: Toll-like receptor, photoaging, collagen, MMP

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Introduction

1: Toll-like receptors (TLRs)

TLRs are a group of receptors that recognize conserved pathogen-associated molecular patterns (PAMPs) (Akira, Takeda et al. 2001; Medzhitov 2001), playing a key role in host defense against infection. To date, ten members of TLRs with distinct PAMP ligands have been identified in humans (Takeda and Akira 2005). Triacyl lipopeptide and diacyl lipopeptide are recognized by TLR1/2 and TLR2/6, respectively (Takeuchi, Kaufmann et al. 2000; Takeuchi, Kawai et al. 2001; Takeuchi, Sato et al. 2002). TLR3 recognizes double-stranded RNA (dsRNA) (Alexopoulou, Holt et al. 2001). The receptor for lipopolysaccharide (LPS) is TLR4 (Tapping, Akashi et al. 2000). TLR5 recognizes bacterial flagellin (Hayashi, Smith et al. 2001). Imidazoquinolines and ssRNA are recognized by TLR7 and TLR8 (Heil, Hemmi et al. 2004), respectively. TLR9 recognizes bacterial and viral CpG DNA motifs (Hemmi, Takeuchi et al. 2000). In addition to sensing exogenous ligands from microbial components, TLRs can also recognize endogenous ligands, called danger-associated molecular patterns (DAMPs) (Sloane, Blitz et al. 2010). For example, it is well-known that HSPs can be locally released upon injury and then activate TLR2 or 4 (Calderwood, Mambula et al. 2007). Some self non-coding RNAs damaged by UV and released from necrotic cells can be recognized by TLR3 to induce inflammatory cytokines (Bernard, Cowing-Zitron et al. 2012).

The TLR-mediated signaling pathways can be roughly divided into two major pathways: the myeloid differentiation primary response protein 88 (MyD88) - and the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways. MyD88 is an adapter protein used by almost all 10 TLRs (except TLR3) to initiate TLR signaling (Takeda and Akira 2005). However, the signal transduction pathway of TLR3 requires the other adaptor protein, named TRIF (Takeda and Akira 2005). TLR4 can utilize both MyD88 and TRIF as signaling adaptors (Takeda and Akira 2005; Sasai and Yamamoto 2013). After recognition of different ligands, TLRs trigger signaling pathways and activate NF-kappaB (NF-κB) and activator protein-1 (AP-1), which results in the induction of inflammatory cytokines and chemokines (Medzhitov 2001; Takeda and Akira 2005).

In skin, TLRs are expressed not only in professional immune cells (e.g., Langerhans cells) but also in non-immune cells (e.g., keratinocytes and melanocytes) (Flacher, Bouschbacher et al. 2006; Lebre, van der Aar et al. 2007; Yu, Zhang et al. 2009). Several studies have shown that skin keratinocytes and melanocytes display TLRs and respond to corresponding PAMPs by producing pro-inflammatory cytokines (Lebre, van der Aar et al. 2007; Yu, Zhang et al. 2009). Although recent studies have reported that TLRs are expressed in skin fibroblasts (Wang, Hori et al. 2011; Jang, Park et al. 2012), the expressions and functional responses of TLRs in skin fibroblasts have not been thoroughly studied.

2: Skin aging

Skin aging is recognizable by wrinkles. One of the hallmarks of wrinkle is disrupted expression of collagen. Decreased collagen expression may be due to decreased synthesis of collagen and/or induced collagen degradation (Fisher, Wang et al. 1997; Rittie and Fisher 2002). Matrix metalloproteinases (MMPs) are a large family of zinc-requiring endoproteases with a broad range of substrate specificities. MMPs have the capacity to degrade various extracellular matrix proteins, including collagen (Rittie and Fisher 2002). The functions of several MMPs have been extensively studied in skin. For instance, MMP-1 (interstitial collagenase or collagenase 1) is found to initiate degradation of types I and III fibrillar collagens, and then MMP-2 (gelatinase A) further degrade collagen fragments generated by collagenases, and MMP-3 (stromelysin 1) can degrade type IV collagen of the basement membrane of skin and activate proMMP-1 (Patterson, Atkinson et al. 2001; Rittie and Fisher 2002).

Reduction of procollagen and induction of MMPs expressions can be achieved by various stimuli *in vitro* and *in vivo*, for example, by ultraviolet (UV) irradiation, and by cytokines such as tumor necrosis factor alpha (TNF-α) (Lee, Kim et al. 2009; Park, Moon et al. 2010). However, there is little knowledge on the effect of dsRNA on procollagen and MMPs in human skin fibroblasts. Whether TLR3 is involved in UV-induced procollagen reduction and MMP induction is unknown.

Purposes

Chapter Ⅰ. Toll-like receptor family members in skin fibroblasts are functional and are expressed higher than those in skin keratinocytes.

This chapter has two main objectives: to investigate (1) which TLRs are expressed in skin fibroblasts and whether they are functional; (2) compared to skin keratinocytes, what the relative expression level of TLRs in skin fibroblasts are.

Chapter Ⅱ. Activation of TLR3 by poly(I:C) reduces procollagen expression through induction of IFN-β in human skin fibroblasts.

The main objectives of this chapter are: to investigate which TLR(s) affect(s) procollagen expression after being activated in skin fibroblasts; and find out the mechanism.

Chapter Ⅲ. Poly(I:C) induces expressions of MMP-1, -2, and -3 through various signaling pathways including IRF3 in human skin fibroblasts.

The main objectives of this chapter are: to investigate (1) whether poly(I:C) can induce several MMPs expression in skin fibroblasts; (2) what signaling

pathways are involved in the processes.

Chapter Ⅳ. TLR3 is involved in UV-induced increase of MMP-13 expression in mice.

The main objective of this chapter is to investigate whether TLR3 is involved in UV-induced reduction of procollagen and induction of MMP-13 expressions in mice skin.

Chapter Ⅰ

Toll-like receptor family members in skin fibroblasts are functional and are expressed higher than those in skin keratinocytes.

Materials and Methods

Reagents

All TLR ligands were purchased from Invivogen (San Diego, CA, USA). For Western blot analysis, antibodies to phospho-IκBα and phospho- and total- ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody to MMP-1 was made by Lab Frontier Ltd. (Seoul, Korea). Antibodies to TLR2, 3, 4 and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Human foreskin fibroblasts and keratinocytes were established from biopsies obtained from four different healthy donors. Skin fibroblasts were

cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 mg/ml), and 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37℃. Skin fibroblasts were used for the experiments at passages 3-6. For treatment, skin fibroblasts were serum-starved for 48 h in DMEM containing 0.1% FBS.

Skin keratinocytes were cultured in keratinocyte growth medium (Clonetics, San Diego, CA, USA) composed of MCDB 153 medium supplemented with epidermal growth factor (10 ng/ml), bovine pituitary extract (70 μg/ml), hydrocortisone (0.5 μg/ml), penicillin (100 μg/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml). Skin keratinocytes were used at passage 3–4 in the experiments. For treatment, skin keratinocytes were maintained in keratinocyte basal medium (MCDB 153 medium only) for 48 h.

Ligand treatment

To induce IL-6, IL-8, and MMP-1 production, skin fibroblasts were plated at the concentration of 0.5 $x10^5$ cells/500ul in 24-well plates for 48 h in the presence of different concentrations of Pam3CSK4 (TLR1/2 ligand, 0.01, 0.1, and 1 μg/ml), poly(I:C) (TLR3 ligand, 0.1, 1, and 10 μg/ml), LPS (TLR4 ligand, 0.001, 0.01, and 0.1 μg/ml), flagellin (TLR5 ligand, 0.05, 0.5, and 5 μg/ml), FSL-1 (TLR2/6 ligand, 0.001, 0.01, and 0.1 μg/ml), imiquimod (TLR7 ligand, 0.1, 1, and 10 μg/ml), ssRNA40 (TLR8 ligand,

0.1, 1, and 10 μg/ml) and ODN2006 (TLR9 ligand, 0.05, 0.5, and 5 μM). The concentrations of each TLR ligand were screened and determined using the information in previous published works (Lebre, van der Aar et al. 2007; Yu, Zhang et al. 2009).

RNA isolation and semi-quantitative and quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). One μg of total RNA was used to make cDNA using a First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania).

Semi-quantitative RT-PCRs were performed to detect TLRs 1–10 and 36B4 mRNAs using PCR premix (Promega, Wisconsin, USA). PCR conditions were as follows: 95℃ for 2 min, 35 cycles of 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 1 min. The PCR products were visualized on 2% agarose gels and ethidium bromide staining.

Quantitative RT-PCRs were performed to measure the relative levels of TLRs 1–10 mRNAs using an ABI Prism 7500 (Applied Biosystems, Darmstadt, Germany). Input cDNA was normalized according to 36B4 or actin as internal control genes. The primer sequences for quantitative RT-PCR are the same as we used in semi-quantitative RT-PCR.

Enzyme-linked immunosorbent assay (ELISA)

After stimulation for 48 h with TLR ligands, cell culture supernatants

were harvested and tested by ELISA for IL-8 and IL-6, following the manufacturer's instructions (Endogen, Woburn, MA, USA).

Western blotting

For MMP-1 measurement, equal volume of cell culture supernatants was prepared for western blot analysis. β-actin was detected from equal volume of cell lysate as a loading control for MMP-1. For detection of protein in cell lysates, cells were washed twice with ice-cold phosphate buffered saline to remove all serum proteins and then lysed in cell lysis buffer. Separated proteins were transferred to PVDF membranes, which were then blocked with Tris-buffered saline containing 0.1% Tween 20 and 5% non-fat dry milk. Membranes were incubated with the appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody. Antibody-bound proteins were visualized by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK).

Statistical analysis

Statistical analyses were performed using the Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant. Results are presented as mean values ± SEM.

Primer sequences

	Forward primer	Reverse primer
TLR1	CTATACACCAAGTTGTCAGC	GTCTCCAACTCAGTAAGGTG
TLR2	GCCAAAGTCTTGATTGATTG	TTGAAGTTCTCCAGCTCCTG
TLR3	GATCTGTCTCATAATGGCTTG	GACAGATTCCGAATGCTTGTG
TLR4	TGGATACGTTTCCTTATAAG	GAAATGGAGGCACCCCTTC
TLR5	ATTGCCAATATCCAGGATGC	CACCACCATGATGAGAGCAC
TLR6	TTGGGCTAACATTAGAGCCG	AGCTCAGTTCCCCAGATGAA
TLR7	AATGTCACAGCCGTCCCTAC	GCGCATCAAAAGCATTTACA
TLR8	TCAACAAATCCGCACTTGAA	CAGGACTGGCACAAATGACA
TLR9	TACGATGCCTTCGTGGTCTT	CTCAAAGAGGGTTTTGCCAG
TLR10	TCTCCCTGGATGCAGTCATT	AACTTCCTGGCAGCTCTGAA
36B4	TGGGCTCCAAGCAGATGC	GGCTTCGCTGGCTCCCAC
β-actin	AGAGATGGCCACGGCTGCTT	ATTTGCGGTGGACGATGGAG
IL-6	CTCCTTCTCCACAAGCGCC	GCCGAAGAGCCCTCAGGC
IL-8	CTCTTGGCAGCCTTCCTG	TTGGGGTCCAGACAGAGC

Results

All of 10 TLR family members are expressed in human skin fibroblasts

In order to gain insight into the functions of TLRs in skin fibroblasts, I first checked the expressions of TLR family members by RT-PCR. Consistently with the previously published works (Lebre, van der Aar et al. 2007; Wang, Hori et al. 2011; Jang, Park et al. 2012), I found that all of 10 TLR family members are constitutively expressed in skin fibroblasts (Fig. 1).

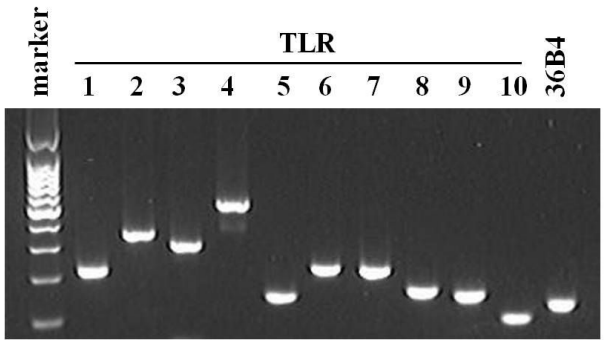

Figure 1. All of 10 TLR family members are expressed in human skin fibroblasts.

Total RNA was prepared from cultured skin fibroblasts and subjected to RT-PCR using TLRs 1–10 specific primers. The PCR products were visualized on 2% agarose gels and ethidium bromide staining. The data shown are representative of three independent experiments.

Activations of TLRs induce expression of IL-6, IL-8, and MMP-1 in human skin fibroblasts

Activations of TLRs in skin keratinocytes and melanocytes are known to produce various cytokines and chemokines (Lebre, van der Aar et al. 2007; Yu, Zhang et al. 2009; Lee, Kim et al. 2010). As all 10 TLRs are expressed in skin fibroblasts, I questioned whether these TLRs are functional or not. Therefore, I stimulated skin fibroblasts with each available TLR ligand (TLR10 ligand is unavailable to be purchased) for 48h and analyzed protein expression of IL-6 and IL-8 induced by each TLR ligand. The concentrations of each TLR ligand were screened and determined using the information in previous published works (Lebre, van der Aar et al. 2007; Yu, Zhang et al. 2009). I selected IL-6 and IL-8 as representatives of cytokines and chemokines, respectively. I found that all of the TLR ligands induced the production of IL-6 (Fig. 2A) and IL-8 (Fig. 2B) in a dose-dependent manner, except IL-8 and IL-6 in activation of TLR8 and 9, respectively. I observed that activation of TLR8 and 9 slightly induced IL-6 and IL-8 as compared with activation of the other TLRs.

The expression of members of MMP family is important during inflammation, cell migration and during the remodeling of damaged tissues (McMillan, Kearley et al. 2004; Lee, Li et al. 2008). Besides IL-6 and IL-8, I wondered whether activation of TLRs in skin fibroblasts can induce MMPs, for example, MMP-1. I found that all of the TLR ligands induced the production of MMP-1 in a dose-dependent manner (Fig. 3). Activation

of TLR8 and 9 slightly induced MMP-1 as compared with activation of the other TLRs.

Taken together, my results suggest that TLRs1-9 in human skin fibroblasts are functional and that activation of these TLRs can induce expression of IL-6, IL-8, and MMP-1.

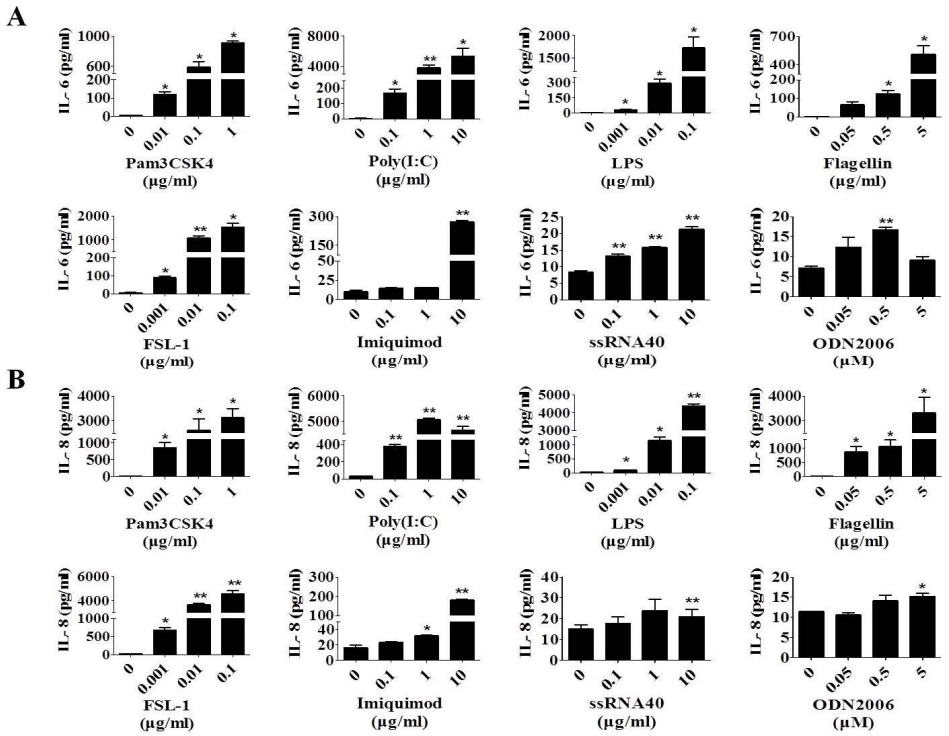

Figure 2. Activations of TLRs induce expression of IL-6 and IL-8 in human skin fibroblasts. After cultured in basal medium for 48 h, skin fibroblasts were incubated with different concentrations of Pam3CSK4 (TLR1/2 ligand), poly(I:C) (TLR3 ligand), LPS (TLR4 ligand), flagellin (TLR5 ligand), FSL-1 (TLR2/6 ligand), imiquimod (TLR7 ligand), ssRNA40 (TLR8 ligand), and ODN2006 (TLR9 ligand). At 48 h after treatment, the concentrations of IL-6 (A) and IL-8 (B) in the cell culture medium were measured with specific ELISA. The results expressed as mean ± SEM of triplicate cultures are from one experiment representative of three with different donors. *$P < 0.05$, **$P < 0.01$ versus corresponding control.

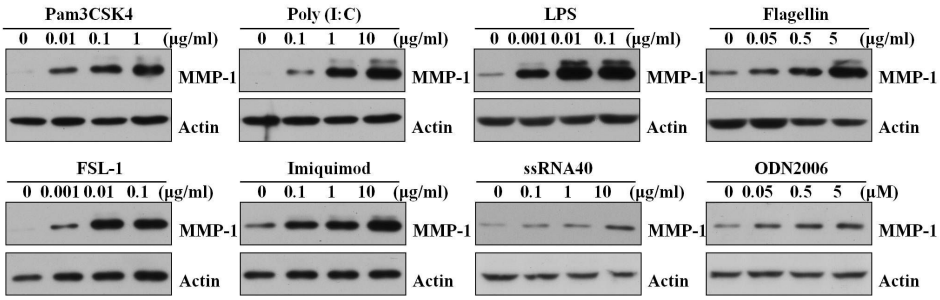

Figure 3. Activations of TLRs induce expression of MMP-1 protein in human skin fibroblasts. At 48 h after incubation with different TLR ligands, protein levels of MMP-1 in the cell culture medium and actin in the cell lysates were analyzed with Western blotting. The data shown are representative of three experiments.

Triggering TLR1/2, 3 and 4 by each ligand induces phosphorylation of IκBα and activation of ERK in human skin fibroblasts

Activation of NF-κB and MAP kinases has been shown to play a central role in TLR-mediated cellular activation and gene expression in a variety of cell types (Lebre, van der Aar et al. 2007; Yu, Zhang et al. 2009; Lee, Kim et al. 2010). Therefore, I also tested whether triggering TLRs with each ligand can induce activation of NF-κB and MAP kinase signaling pathways in skin fibroblasts. I stimulated skin fibroblasts with TLR1/2, 3, and 4 ligands, harvested at 3 h after treatment, and assessed, by Western blot analysis, phosphorylation of IκBα, which is frequently used as one of the markers for activation of the NF-κB pathway, and active phosphorylation of ERK, which is representative of MAP kinases. Treatment of TLR1/2, 3, and 4 ligands induced phosphorylation of IκBα and active phosphorylation of ERK (Fig. 4).

In summary, my data suggest that activation of TLR can induce activation of the NF-κB and the ERK pathways in skin fibroblasts.

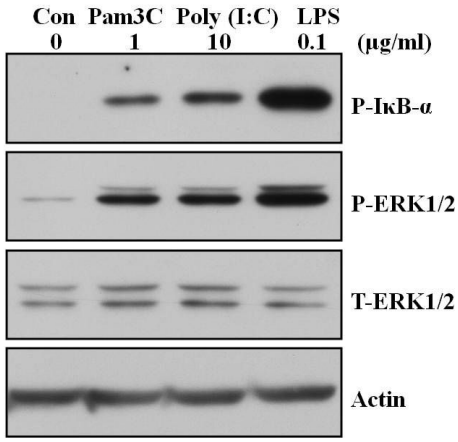

Figure 4. Triggering TLR1/2, 3 and 4 by each ligand induces phosphorylation of IκBα and activation of ERK in human skin fibroblasts. Cultured skin fibroblasts were stimulated with Pam3CSK4 (1 μg/ml), poly(I:C) (10 μg/ml), and LPS (0.1 μg/ml) for 3 h. Cell lysates were fractionated by SDS-PAGE and then protein levels of p-IκBα and p-ERK1/2 were analyzed by Western blotting. A single representative experiment is shown from three different experiments.

Expression levels of TLR family members are higher in skin fibroblasts than in skin keratinocytes

Skin keratinocytes are the major cells expressed in epidermis, which is the first defense line of skin against pathogens. Expressions of TLRs were well studied in skin keratinocytes in several research papers (Kollisch, Kalali et al. 2005; Lebre, van der Aar et al. 2007). To better understand the expression levels of TLRs in skin fibroblasts, I decided to compare expression levels of TLRs in skin keratinocytes with fibroblasts. To eliminate the possibility that expression level of TLRs could be affected by different cell culture conditions, I used both growth culture condition (Fig. 5A) and basal culture condition (Fig. 5B). Consistently with the previous published work (Lebre, van der Aar et al. 2007), I found that all TLRs are constitutively expressed in skin keratinocytes, except TLR7 and 8 (Fig.5 A and B). Regardless of culture conditions, each TLR was expressed much higher in skin fibroblasts than in skin keratinocytes (Fig. 5A and B). Next, I confirmed the protein expression levels of some TLRs between fibroblasts and keratinocytes. I found that the protein expression levels of TLR2, 3 and 4 are much higher in skin fibroblasts than in keratinocytes (Fig. 5C).

Taken together, my data indicate that the expression level of each TLR is higher in skin fibroblasts than in skin keratinocytes.

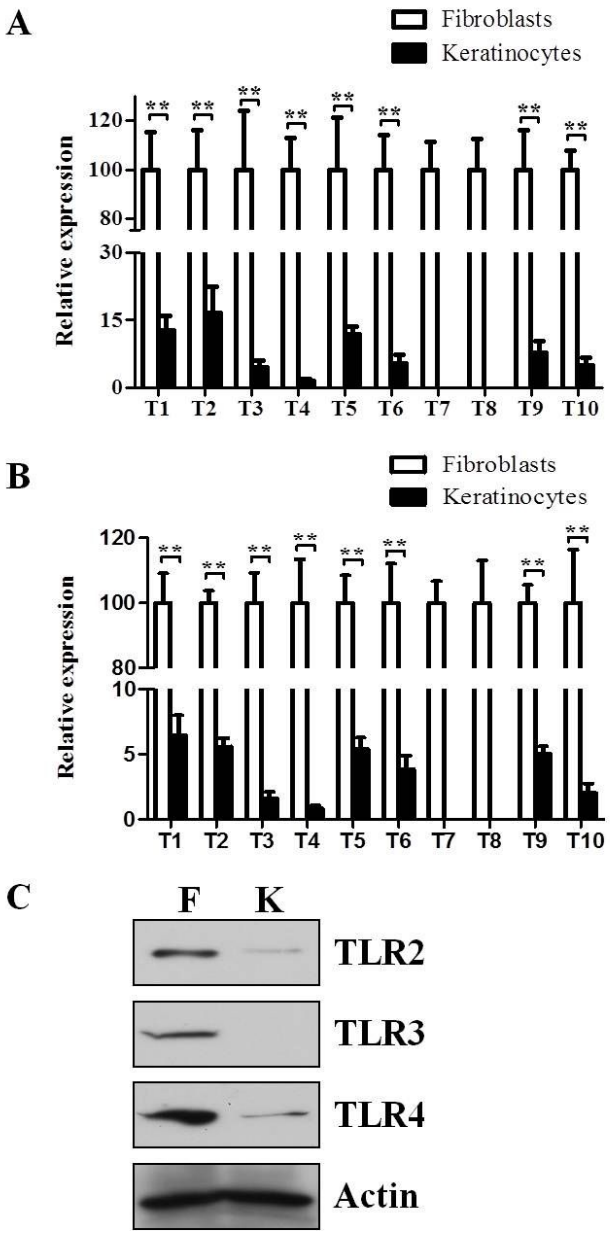

Figure 5. Expression levels of TLR family members are higher in skin fibroblasts than in skin keratinocytes. RNA was extracted from skin fibroblasts and keratinocytes obtained from four different donors. Equal amount of cDNA was used to assess mRNA expression of TLRs by real-

time RT-PCR. 36B4 was used as an endogenous control. (A) Cells were cultured in growth medium (DMEM containing 10% FBS for fibroblasts and keratinocyte growth medium for keratinocytes, respectively). (B) Cells were first cultured in growth medium for 24 h and then maintained in basal medium (DMEM containing 0.1% FBS for fibroblasts and keratinocyte basal medium for keratinocytes, respectively) for 48 h. Data are presented as mean ± SEM (N=4,**P < 0.01). (C) Cells were cultured in growth medium for 24 h and then maintained in basal medium, after that lysates were prepared and then protein levels of TLR2, TLR3 and TLR4 were analyzed by Western blotting. F, fibroblasts; K, keratinocytes. A single representative experiment is shown from three different experiments.

Fold-increases in IL-6 and IL-8 mRNA expression level upon exposure to a TLR1/2 ligand were much higher in skin fibroblasts than in skin keratinocytes.

To confirm the competence of TLR signaling in skin fibroblasts, I compared the activation of TLR1/2 signaling pathways in skin fibroblasts with that in skin keratinocytes. I treated fibroblasts and keratinocytes with Pam3CSK4 and harvested the cells at 10 h and 24 h after treatment. Then I analyzed mRNA levels of IL-6 and IL-8 by real-time RT-PCR, and calculated the fold-increases of mRNA levels of IL-6 and IL-8 in fibroblasts and keratinocytes. Pam3CSK4 treatment increased mRNA levels of IL-6 (Fig. 6A) and IL-8 (Fig. 6B) in both fibroblasts and keratinocytes. However, at both time points and both concentrations, Fold-increases in both IL-6 and IL-8 mRNA expression levels induced by Pam3CSK4 treatment were much higher in fibroblasts than in keratinocytes.

Therefore, these results indicate that TLR signaling pathways in skin fibroblasts are functional and very active, and suggest that TLRs in skin fibroblasts may provide strong responses to pathogens and/or danger signals.

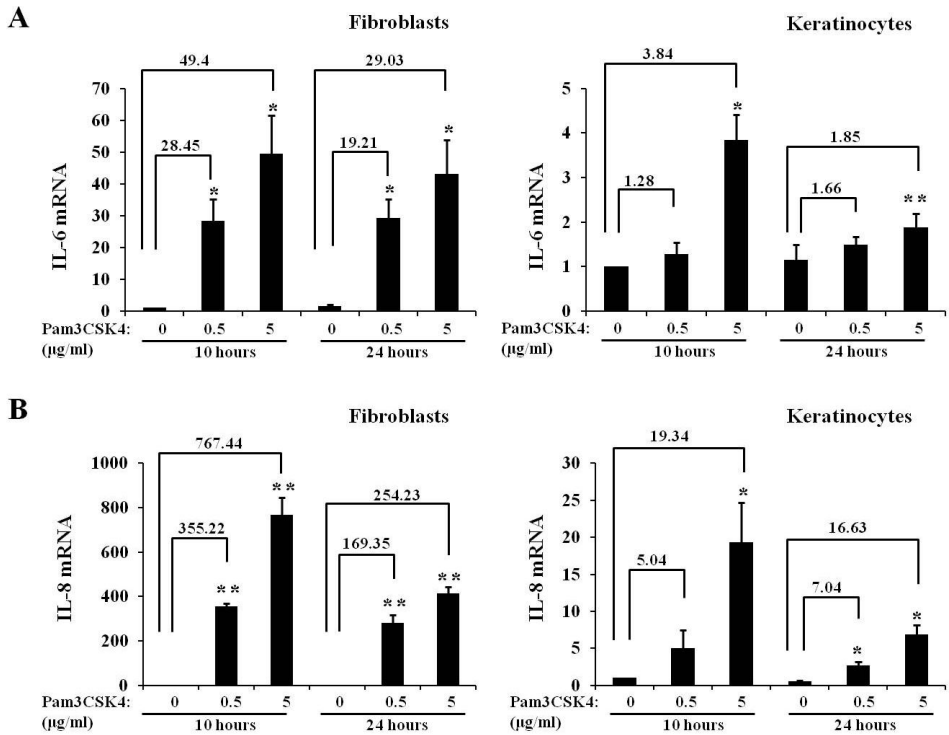

Figure 6. Fold-increases in IL-6 and IL-8 mRNA expression level upon exposure to a TLR1/2 ligand were much higher in skin fibroblasts than in skin keratinocytes. Fibroblasts and keratinocytes were starved in basal medium for 48 h. Cells were then stimulated with TLR1/2 ligand Pam3CSK4 (0.5 and 5 μg/ml) for 10 h and 24 h. IL-6 and IL-8 mRNA level were checked by RT- qPCR. Experiments were performed using fibroblasts and keratinocytes obtained from four different donors. (a) IL-6 mRNA level in fibroblasts and keratinocytes, (b) IL-8 mRNA level in fibroblasts and keratinocytes. Data are presented as mean± SEM. *P < 0.05, **P < 0.01 versus corresponding control. Values of fold-increases are also shown in the graph.

Chapter Ⅱ

Activation of TLR3 by poly(I:C) reduces procollagen expression through induction of IFN-β in human skin fibroblasts

Materials and Methods

Reagents

Jak inhibitor JAKI and TBK1 inhibitor BX795 were purchased from Calbiochem (San Diego, CA, USA). Recombinant Human interferon-β (IFN-β) was bought from PeproTech (New Jersey, NJ, USA). Antibodies for phospho- or total- IRF3 were purchased from Cell Signaling Technology (Beverly, MA, USA). The mouse monoclonal anti-type I procollagen antibody, SP1.D8, was used. β-Actin antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA).

Cell culture and treatment

Human foreskin fibroblasts were established from biopsies obtained from healthy donors. Wild-type (WT) and TLR3 knockout (TLR3-/-) mice (C57/BL6 background) were purchased from Jackson Laboratories (Sacramento, CA, USA). Skin fibroblasts from WT and TLR3-/- mice were

isolated. Cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 mg/ml), and 10% fetal bovine serum (FBS) in a humidified 5% CO2 atmosphere at 37℃. Human skin fibroblasts were used for the experiments at passages 5-10. Mice skin fibroblasts were used for the experiments at passages 5-6. For treatment, human and mice skin fibroblasts were first serum starved for 24 h in DMEM containing 0.1% FBS, and then treated with various chemicals.

RNA interference

Knockdown of TLR3, MDA5 and RIG-I expressions were performed by the RNA interference method using small interfering RNAs (siRNAs). The control scrambled siRNA and siRNAs specific for TLR3, MDA5 and RIG-I were obtained from Bioneer (Daejeon, Korea). For the knockdown of TLR3 expression, skin fibroblasts were transfected with TLR3 siRNA at 600 nM. For the knockdown of MDA5 and RIG-I expressions, 300 nM siRNAs were used. 600 nM control scrambled siRNA was used as negative control. G-fectin from Genolution (Seoul, Korea) was used as transfection reagent.

Western blotting

The amounts of procollagen proteins secreted into culture media were analyzed as described previously (Park, Moon et al. 2010). Briefly, for procollagen measurements equal volume of cell culture supernatants was

prepared for Western blotting analysis. β-Actin was detected from equal volume of cell lysate as a loading control. For detection of phospho- and total- IRF3 in cell lysates, same amount of total protein were used. The details for performing Western blotting were described in "Materials and Methods" in Chapter Ⅰ.

Total RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

The details for performing RT-qPCR were described in "Materials and Methods" in Chapter Ⅰ. The sequences of forward and reverse primers for procollagen and 36B4 are designed as the following: procollagen forward, 5'- CTC GAG GTG GAC ACC ACC CT -3'; reverse, 5'- CAG CTG GAT GGC CAC ATC GG -3', 36B4 forward, 5'- TGG GCT CCA AGC AGA TGC -3'; reverse, 5'- GGC TTC GCT GGC TCC CAC -3'.

ELISAs

Supernatants from cultured human or mice skin fibroblasts incubated for 48 h with various chemicals were collected. Levels of secreted human or mice IFN-β were determined in triplicate using ELISA kits (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using the Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant. Results are presented as mean values ± SEM.

Results

Treatment of TLR3 ligand poly(I:C), but not other TLR ligands, reduces procollagen expression in skin fibroblasts

As I observed that TLRs in skin fibroblasts are functional (Fig. 2 and 3), I next questioned whether activation of TLRs affect expressions of fibroblasts' specific genes, such as procollagen, in skin fibroblasts. Therefore, I stimulated skin fibroblasts with each available TLR ligand for 48h and analyzed protein expression of procollagen. As shown in Figure 7, I observed that poly(I:C) inhibited procollagen expression dose-dependently. However, treatment of the other TLR ligands had no effect on procollagen expression. Moreover, I further checked the mRNA level of procollagen. I found that 24 h after poly(I:C) treatment, procollagen mRNA level was dose-dependently decreased in skin fibroblasts (Fig. 8). Taken together, my results suggest that treatment of TLR3 ligand poly(I:C), but not other TLR ligands, reduces procollagen expression in skin fibroblasts.

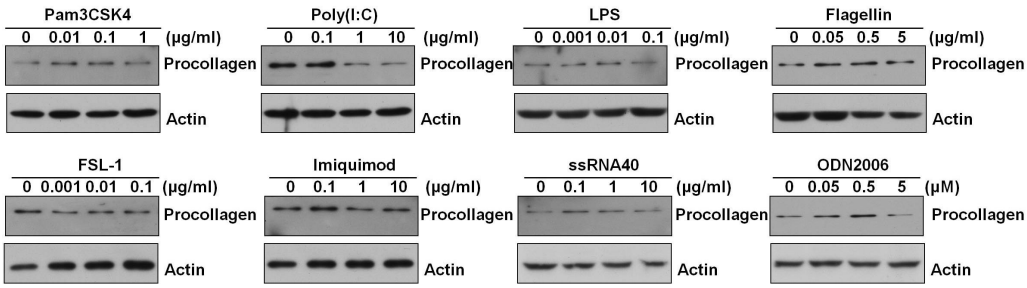

Figure 7. Treatment of TLR3 ligand poly(I:C), but not other TLR ligands, reduces procollagen expression in skin fibroblasts. At 48 h after incubation with different TLR ligands, protein levels of procollagen in the cell culture medium and actin in the cell lysates were analyzed with Western blotting. The data shown are representative of three experiments.

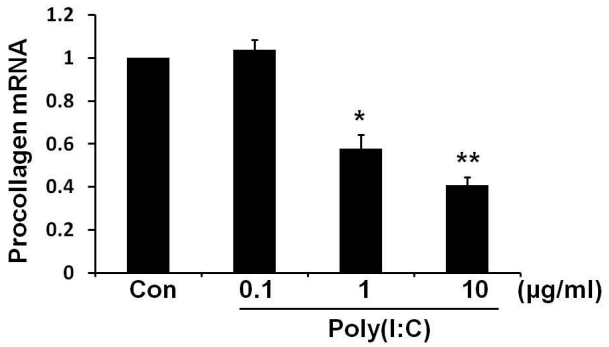

Figure 8. Poly(I:C) reduces procollagen mRNA level in skin fibroblasts. At 24 h after incubation with poly(I:C), relative procollagen mRNA levels were analyzed by real-time qPCR. Data are presented as mean ± SEM. (N = 3, *P < 0.05, **P < 0.01).

Treatment of TLR3 ligand poly(I:C), but not other TLR ligands, induces IFN-β expression in skin fibroblasts

Activation of TLR3 by poly(I:C) induces IL-6, -8 and MMP-1 which can also be induced by activation of other TLRs (Fig. 2 and 3). It indicates that activation of different TLRs may induce common pathways which lead to induction of same proteins. Given the fact that treatment of TLR3 ligand poly(I:C), but not other TLR ligands, reduces procollagen expression in skin fibroblasts (Fig. 7), I hypothesized that poly(I:C) treatment may specifically induce expressions of certain molecule which further reduces procollagen expressions.

It was suggested that ligands to TLR3 and TLR7/8, but not other TLRs, induced IFN-β in bronchial epithelial cells (Sykes, Edwards et al. 2013). Ligands to TLR3, but not TLR2 and 9, induced IFN-β in skin fibroblasts (Hamidi, Schafer-Korting et al. 2014). I further tested the expression of IFN-β after treatment of TLR ligands to skin fibroblasts in my experiment conditions. As shown in Figure 9A, poly(I:C) highly induced IFN-β expression. However, treatment of the other TLR ligands had no effect on IFN-β expression. The dose-dependent induction manner of IFN-β by poly(I:C) was also observed (Fig. 9B).

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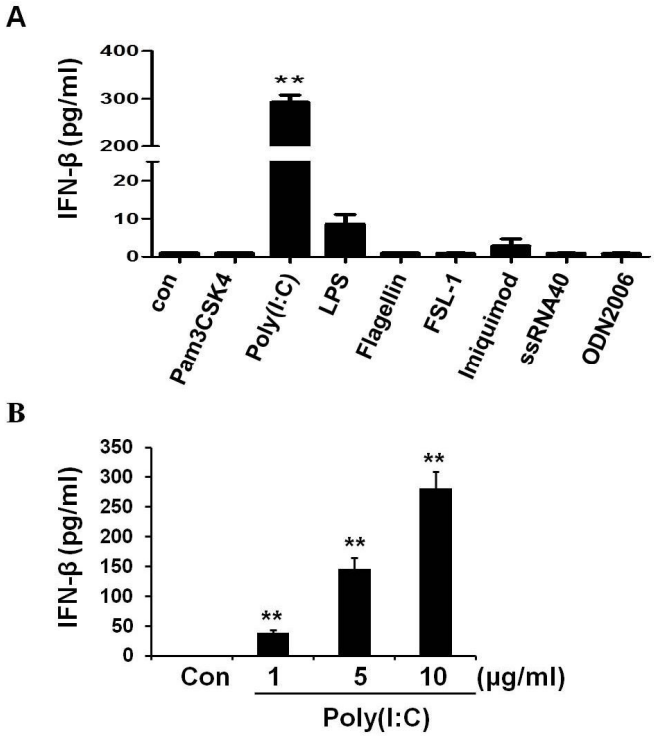

Figure 9. Treatment of TLR3 ligand poly(I:C), but not other TLR ligands, induces IFN-β expression in skin fibroblasts. (A) After cultured in basal medium for 48 h, skin fibroblasts were incubated with Pam3CSK4 (1 μg/ml), poly(I:C) (10 μg/ml), LPS (0.1 μg/ml), flagellin (5 μg/ml), FSL-1 (0.1 μg/ml), imiquimod (10 μg/ml), ssRNA40 (10 μg/ml), and ODN2006 (5 μM). At 48 h after incubation with each TLR ligand, protein expressions of IFN-β in cell culture medium were analyzed by ELISA. Data are presented as mean ± SEM. (N = 3, **P < 0.01). (B) Skin fibroblasts were treated with several doses of poly(I:C) for 48 h, protein expressions of IFN-β in cell culture medium were analyzed by ELISA. Data are presented as mean ± SEM. (N = 3, **P < 0.01).

Poly(I:C) reduces procollagen expression through induction of IFN-β in skin fibroblasts

Type I interferons, such as IFN-α and β, signal through a Janus kinase (Jak) to signal transduction and activator of transcription (STAT) pathway to stimulate gene expression. In response to ligand binding, the receptors dimerize, Jaks phosphorylate STAT1 and STAT2, which then stimulate gene expression (Horvath 2004). Since poly(I:C) highly induced IFN-β expression, I next investigated whether induction of IFN-β is involved in poly(I:C)-induced reduction of procollagen expression. Thus, I pretreated a Jak inhibitor (JAKI) to skin fibroblasts, then incubated cells with poly(I:C) for 48 h. As shown in Fig. 10A, pretreatment of JAKI prevented poly(I:C)-induced reduction of procollagen expression. The effect of IFN-β on procollagen expression was confirmed by recombinant IFN-β. Treatment of recombinant IFN-β decreased procollagen expression (Fig. 10B). Thus, these data indicated that poly(I:C) reduced procollagen expression through induction of IFN-β in skin fibroblasts.

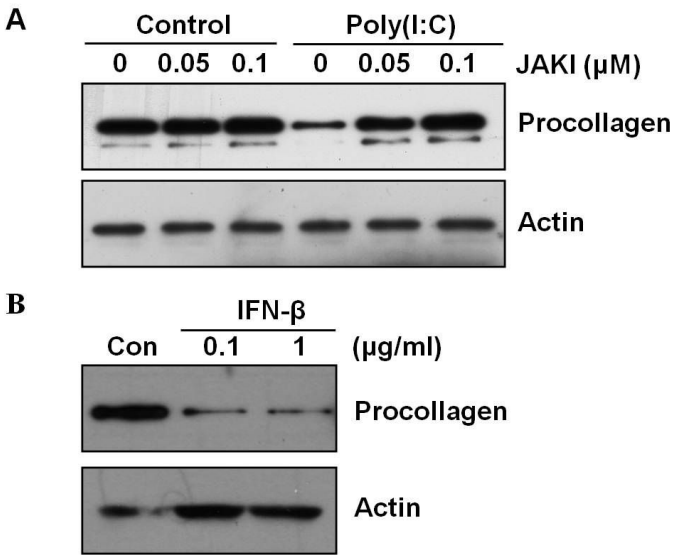

Figure 10. Poly(I:C) reduces procollagen expression through induction of IFN-β in skin fibroblasts. (A) After serum starvation, skin fibroblasts were incubated with JAKI (0.05 and 0.1 μM) for 1 h. Then, cells were stimulated with 10 μg/ml poly(I:C) for 48 h. (B) After serum starvation, human recombinant IFN-β cytokine (0.1 and 1 μg/ml) was added to cell culture medium for 48 h. Procollagen expression in cell culture medium was analyzed by Western blotting. The data shown are representative of three experiments.

Poly(I:C) induces IFN-β expression through IRF3-dependent pathway in skin fibroblasts.

I next investigated the signaling pathways involved in poly(I:C)-induced IFN-β expression. Activation of TLR3 can also lead to the activation of TANK-binding kinase-1 (TBK-1), which results in the phosphorylation and nuclear translocation of transcription factors such as interferon regulatory factor 3 (IRF3) (Sankar, Chan et al. 2006). Many studies have shown that IRF3 plays an important role in IFN-β regulation (Sin, Li et al. 2012; Khan, Do et al. 2015). Thus, I checked whether poly(I:C) can induce activation of IRF3 in my experiment condition. As shown in Fig. 11A, poly(I:C), but not Pam3CSK4 and LPS, induced phosphorylation of IRF3 in skin fibroblasts. To confirm whether activation of IRF3 is involved in poly(I:C)-induced IFN-β expression, I used a inhibitor of TBK-1 which is a upstream molecule of IRF3. I found that pretreatment of TBK-1 inhibitor BX795 inhibited poly(I:C)-induced IFN-β expression (Fig. 11B). Moreover, inhibition of IRF3 by BX795 relieved poly(I:C)-induced reduction of procollagen expression (Fig. 11C). Thus, the data indicated that poly(I:C) induced IFN-β expression through IRF3-dependent pathway in skin fibroblasts.

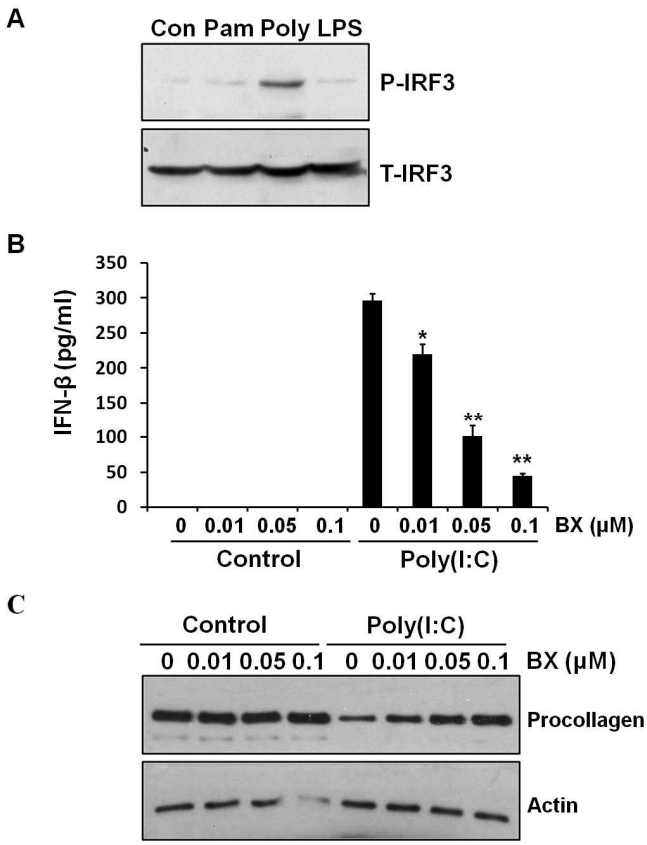

Figure 11. Poly(I:C) induces IFN-β expression through IRF3-dependent pathway in skin fibroblasts. (A) Skin fibroblasts were treated with Pam3CSK4 (1 μg/ml), poly(I:C) (10 μg/ml) or LPS (0.1 μg/ml) for 2 h. P-IRF3 expression in cell lysate was analyzed by Western blotting. The data shown are representative of three experiments. (B) Skin fibroblasts were pretreated with BX795 for 1 h, and then incubated with poly(I:C) for 48 h. IFN-β expression in cell culture medium was analyzed by ELISA. Values are mean ± SEM. (N=3, *P < 0.05, **P < 0.01 versus poly(I:C)-treated control). (C) Procollagen expression in medium was analyzed by Western blotting. The data shown are representative of three experiments.

Knockdown of TLR3, MDA5, and RIG-I attenuates poly(I:C)-induced reduction of procollagen expression.

In order to determine whether dsRNA receptors such as TLR3, MDA5 and RIG-I are involved in poly(I:C)-induced reduction of procollagen expressions in human skin fibroblasts, I transfected fibroblasts with each specific siRNA for 48 h and then treated with poly(I:C) for 48 h. I found that transfection of TLR3, MDA5, and RIG-I siRNAs relieved poly(I:C)-induced reduction of procollagen expression (Fig. 12). These data indicate that dsRNA receptors such as TLR3, MDA5, and RIG-I are involved in poly(I:C)-induced procollagen expression reduction in human skin fibroblasts.

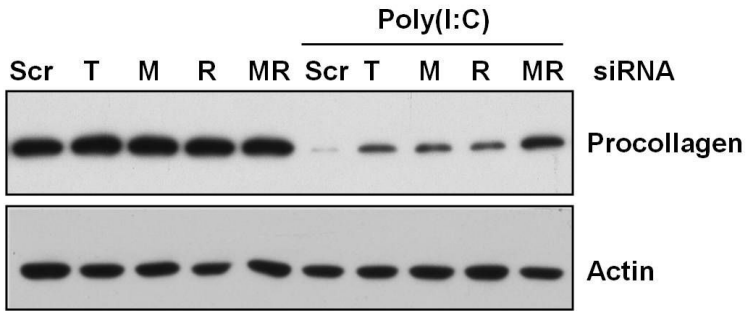

Figure 12. Knockdown of TLR3, MDA5, and RIG-I attenuates poly(I:C)-induced reduction of procollagen expression. Fibroblasts were transfected with scrambled control (Scr), TLR3 (T), MDA5 (M), RIG-I (R) and MDA5 plus RIG-I (MR) siRNAs for 48 h. The transfected cells were then serum starved for 24 h. Cells were then incubated with poly(I:C) (20 μg/ml) for a further 48 h. Protein levels of procollagen in the cell culture medium and actin in the cell lysates were analyzed with Western blotting. The data shown are representative of three independent experiments.

Absence of TLR3 relieves poly(I:C)-induced reduction of procollagen expression

Besides TLR3, poly(I:C) could also be recognized by other receptors, such as melanoma differentiation-associated gene 5 (MDA5) and retinoic acid inducible gene-1 (RIG-I) (Kato, Takeuchi et al. 2006). In order to confirm whether TLR3 is involved in poly(I:C)-induced reduction of procollagen expression, I checked the effect of poly(I:C) on procollagen expression in cultured wild-type (WT) and TLR3 knock-out (TLR3-/-) mice skin fibroblasts. I found that poly(I:C) induced much higher expression of IFN-β in cultured WT skin fibroblasts than in TLR3-/- skin fibroblasts (Fig. 13A). Furthermore, in contrast to WT skin fibroblasts, poly(I:C) failed to suppress procollagen expression in dose of 20 μg/ml in TLR3-/- skin fibroblasts. In dose of 100 μg/ml, poly(I:C) highly suppressed procollagen in WT skin fibroblasts compared to TLR3-/- skin fibroblasts (Fig. 13B). Thus, the data indicate that TLR3 is involved in poly(I:C)-induced reduction of procollagen expression in skin fibroblasts.

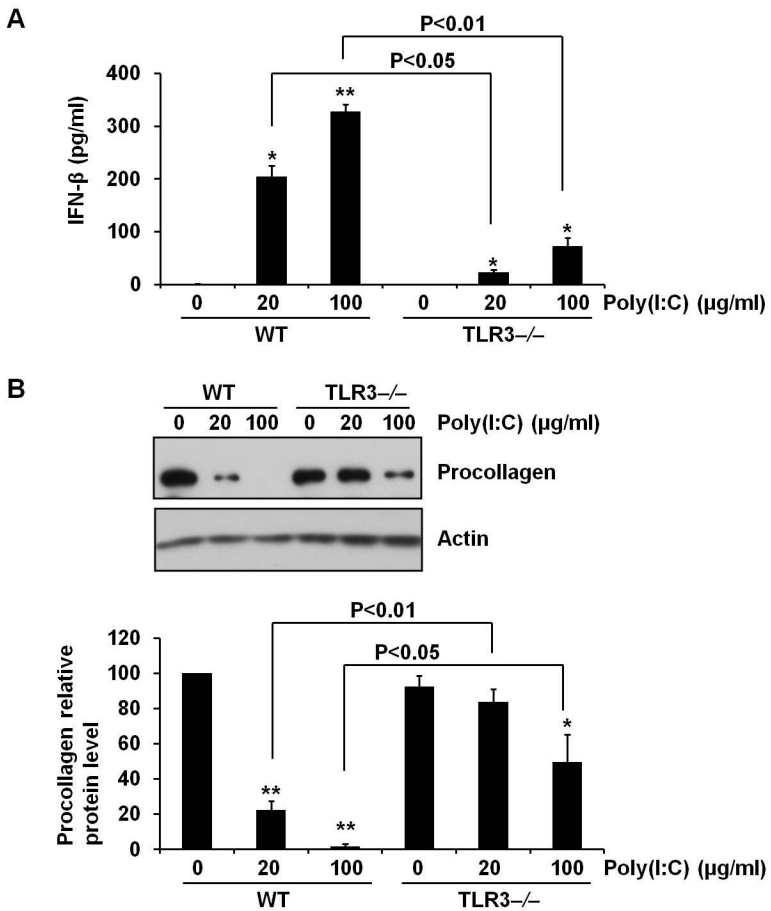

Figure 13. Absence of TLR3 relieves poly(I:C)-induced reduction of procollagen expression. Cultured WT and TLR3-/- skin fibroblasts were incubated with poly(I:C) (20 or 100 μg/ml) for 48 h. (A) Expression of IFN-β in cell culture medium was analyzed by ELISA. Values are mean ± SEM of triplicate experiments. *P < 0.05, **P < 0.01 versus control. (B) Procollagen expression in cell culture medium was analyzed by Western blotting. Signal intensity was quantified using a densitometric program (ImageJ, W. Rasband, NIH, USA).The data shown are representative of four experiments.

Chapter Ⅲ

Poly(I:C) induces expressions of MMP-1, -2, and -3 through various signaling pathways including IRF3 in human skin fibroblasts

Materials and Methods

Reagents

MEK1/2 inhibitor U0126, JNK inhibitor SP600125, p38 MAPK inhibitor SB203580, NF-κB inhibitor BAY11-7082, and TBK1 inhibitor BX795 were purchased from Calbiochem (San Diego, CA, USA). Antibodies for phospho- or total- ERK1/2, JNK, p38, and IRF3, and phospho- IκBα were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for MMP-1 and MMP-2 were made by Lab Frontier Ltd. (Seoul, Korea). Antibody for MMP-3 was purchased from NeoMarkers (Fremont, CA, USA).

Cell culture and treatment

Human foreskin fibroblasts were established from biopsies obtained from healthy donors. Cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 mg/ml), and 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37℃. Human skin fibroblasts were used for the experiments at passages 5-10. For treatment, skin fibroblasts were first serum starved for 24 h in DMEM containing 0.1% FBS, and then treated with various chemicals.

RNA interference

Knockdown of TLR3 or IRF3 expression was performed by the RNA interference method using small interfering RNAs (siRNAs). Negative control siRNA (scrambled siRNA) and the siRNA specific for TLR3 (siRNA No.1151756) or IRF3 (siRNA No. 1075322) was obtained from Bioneer (Daejeon, Korea). For the knockdown of TLR3 expression, skin fibroblasts were transfected with control scrambled siRNA or TLR3 siRNA at 300 nM using G-fectin transfection reagent from Genolution (Seoul, Korea). For the knockdown of IRF3 expression, 600 nM control scrambled siRNA or IRF3 siRNA was used.

Western blotting

The amounts of MMP-1, -2, and -3 proteins secreted into culture media were analyzed as described previously (Park, Moon et al. 2010). Briefly, for

MMP-1, -2, and -3 measurements, equal volume of cell culture supernatants was prepared for Western blotting analysis. β-Actin was detected from equal volume of cell lysate as a loading control for MMP-1, -2, and -3. For detection of phospho- and total- ERK1/2, JNK, p38, and IRF3 and phospho-IκBα in cell lysates, equal amount of total protein were used. The details for performing Western blotting were described in "Materials and Methods" in Chapter Ⅰ.

Total RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

The details for performing RT-qPCR were described in "Materials and Methods" in Chapter Ⅰ. The sequences of forward and reverse primers for TLR3 and 36B4 are designed as the following: TLR3 forward, 5'- GAT CTG TCT CAT AAT GGC TTG -3'; reverse, 5'- GAC AGA TTC CGA ATG CTT GTG -3', 36B4 forward, 5'- TGG GCT CCA AGC AGA TGC -3'; reverse, 5'- GGC TTC GCT GGC TCC CAC -3'.

Statistical analysis

Statistical analyses were performed using the Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant. Results are presented as mean values ± SEM.

Results

Poly(I:C) induces the expressions of MMP-1, -2, and -3 dose-dependently in cultured human skin fibroblasts

MMPs have the capacity to degrade various extracellular matrix proteins, including collagen (Rittie and Fisher 2002). Poly(I:C) was shown to induce the expressions of various MMPs in diverse types of cells including skin keratinocytes. However, whether poly(I:C) can regulate MMPs in skin fibroblasts has not been studied yet. To investigate the effect of poly(I:C) on MMP-1, -2, and -3 expressions in human skin fibroblasts, I treated cultured fibroblasts with 1, 10, and 20 μg/ml of poly(I:C) for 48 h. By Western blotting, I observed that the protein levels of MMP-1, -2, and -3 in cell culture medium were increased by poly(I:C) in a dose-dependent manner (Fig. 14).

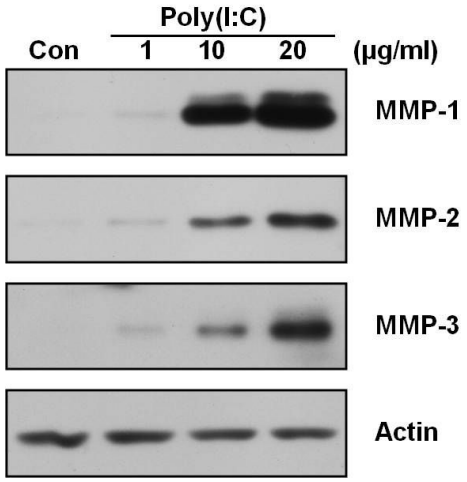

Figure 14. Poly(I:C) induces MMP-1, -2, and -3 expressions dose-dependently in cultured human skin fibroblasts. After serum-starvation for 24 h, skin fibroblast were treated with various indicated doses of poly(I:C) for 48 h. Protein levels of MMP-1, -2, and -3 in the cell culture medium and actin in the cell lysates were analyzed with Western blotting. The data shown are representative of three independent experiments.

Poly(I:C) induces MMP-1, -2, and -3 expressions via TLR3-dependent pathways in human skin fibroblasts

In order to determine whether TLR3 is involved in poly(I:C)-induced MMP-1, -2, and -3 expressions in human skin fibroblasts, I transfected fibroblasts with scrambled control siRNA or TLR3 siRNA for 48 h and then treated the transfected cells with poly(I:C) for 48 h . I found that the mRNA level of TLR3 was significantly reduced by TLR3 siRNA (Fig. 15A) and that the transfection of TLR3 siRNA relieved poly(I:C)-induced MMP-1, -2, and -3 expressions (Fig. 15B). These data indicate that poly(I:C) induces MMP-1, -2, and -3 expressions via TLR3-dependent pathways in human skin fibroblasts.

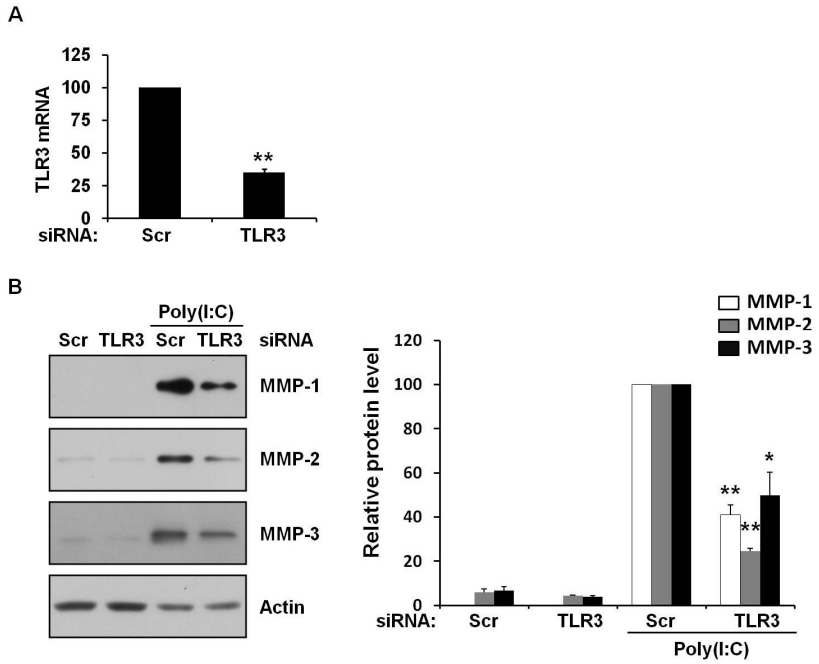

Figure 15. Poly(I:C) induces MMP-1, -2, and -3 expressions via TLR3-dependent pathways in human skin fibroblasts. (A) Fibroblasts were transfected with scrambled control (Scr) siRNA or TLR3 siRNA for 48 h. The transfected cells were then serum starved for 24 h. Then, relative TLR3 mRNA levels were analyzed by real-time qPCR. Data are presented as mean ± SEM (N=3, **P < 0.01). (B) After transfection for 48 h and further starvation for 24 h, cells were incubated in fresh starvation media with or without poly(I:C) (20 μg/ml) for a further 48 h. Protein levels of MMP-1, -2, and -3 in the cell culture medium and actin in the cell lysates were analyzed with Western blotting. The data shown are representative of three independent experiments. Values are mean ± SEM. *P < 0.05, **P < 0.01 versus poly(I:C)-treated control.

Poly(I:C) induces activation of MAPKs, degradation of IκBα and activation of IRF3 in human skin fibroblasts

Activations of MAPKs and NF-κB have been shown to play essential roles in TLR -mediated cellular activation and gene expression in many different cell types (Lebre, van der Aar et al. 2007; Yu, Zhang et al. 2009; Lee, Kim et al. 2010). Besides MAPKs and NF-κB, IRF3 pathways have been reported to be activated by TLR3 ligands (Doyle, Vaidya et al. 2002). Interestingly, depending on different cell types and cell culture conditions, poly(I:C) treatment was shown to selectively activate MAPKs, NF-κB and IRF3 pathways. For example, poly(I:C) treatment activates JNK and p38 MAPK, but not ERK1/2 pathways in skin keratinocytes (Lee, Kim et al. 2010). Therefore, I tested whether poly(I:C) can induce activation of these signaling pathways in human skin fibroblasts. After serum-starvation for 24 h, cells were stimulated with poly(I:C) (20 μg/ml) for 2 h or 4 h. Poly(I:C) induced phosphorylation of ERK, JNK, and p38 MAPK which are representative of MAPKs (Fig. 16A). Poly(I:C) also induced phosphorylation and further degradation of IκBα which is frequently used as one of the markers for activation of the NF-κB pathway (Fig. 16B). I also observed that poly(I:C) induced activation of IRF3 pathways (Fig. 16C). Taken together, my data indicate that poly(I:C) can induce activation of MAPKs, degradation of IκBα and activation of IRF3 in human skin fibroblasts.

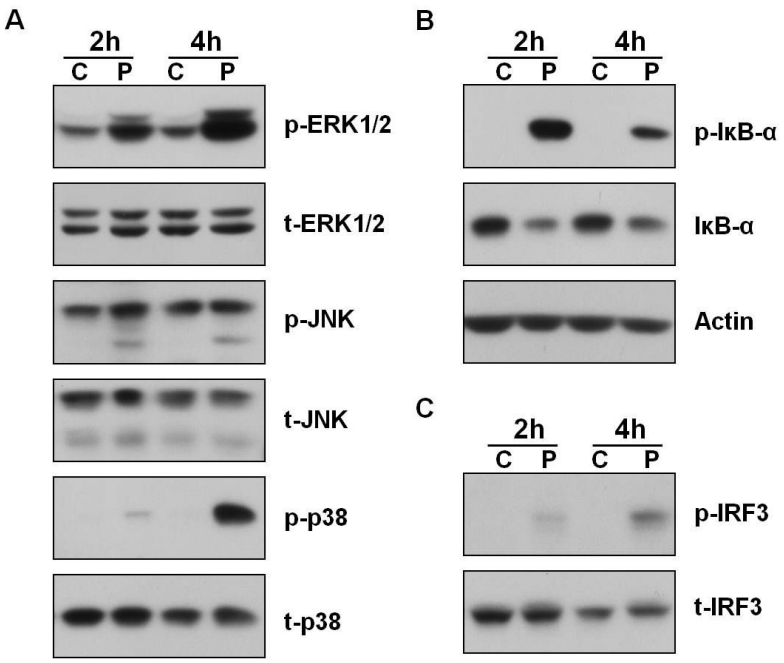

Figure 16. Poly(I:C) treatment induces activation of MAPKs, degradation of IκBα and activation of IRF3 in human skin fibroblasts. Cultured skin fibroblasts were first serum-starved and then stimulated with poly(I:C) (20 μg/ml) for 2 h or 4 h. Cell lysates were fractionated by SDS-PAGE and then protein levels of MAPKs (p-ERK1/2, p-JNK, and p-p38 MAPK) (A), p-IκBα (B), and p-IRF3 (C) were analyzed by Western blotting. C, control; P, poly(I:C). A single representative experiment is shown from three different experiments.

Poly(I:C)-induced MMP-1, -2, and -3 expressions are differentially regulated through various signaling pathways in human skin fibroblasts

To study the signaling pathways involved in the induction of MMP-1, -2, and -3 by poly(I:C) stimulation in human skin fibroblasts, I pretreated cells for 1 h with the MEK1/2 (upstream molecule of ERK1/2) inhibitor U0126, the JNK inhibitor SP600125, the p38 MAPK inhibitor SB203580, the NF-κB inhibitor BAY11-7082, and the TBK1 (upstream molecule of IRF3) inhibitor BX795. Then, the cells were stimulated in the presences of these inhibitors with 20 μg/ml poly(I:C) for 48 h. The MMP-1 expression induced by poly(I:C) was inhibited by pretreatment with all the inhibitors. The MMP-2 expression induced by poly(I:C) was inhibited by pretreatment with NF-κB and IRF3 pathway inhibitors, but not by MAPK pathway inhibitors. The MMP-3 expression induced by poly(I:C) was inhibited by pretreatment with ERK1/2, p38 MAPK, and IRF3 pathway inhibitors, but not by JNK and NF-κB pathway inhibitors (Fig. 17). Thus, my data indicate that poly(I:C)-induced expressions of MMP-1, -2, and -3 are differentially regulated by these signaling pathways and that IRF3 pathway seems to be particularly involved in the induction of all the three MMPs in human skin fibroblasts.

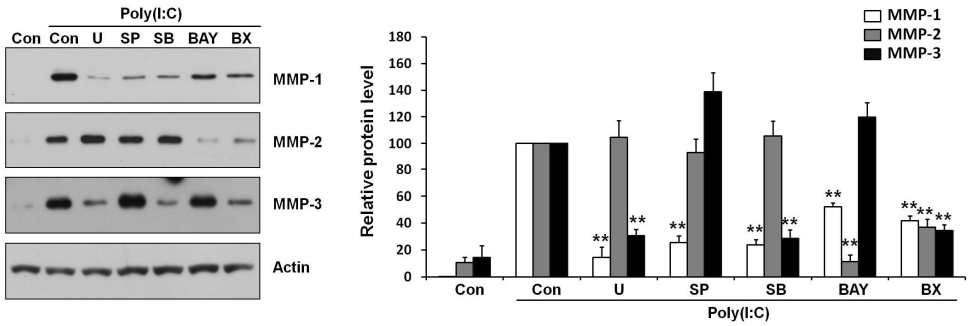

Figure 17. Poly(I:C) induces MMP-1, -2, and -3 through different signaling pathways in human skin fibroblasts. After serum-starvation for 24 h, skin fibroblast were pretreated 1 h with 10 μM of the MEK1/2 inhibitor U0126 (U), 10 μM of the JNK inhibitor SP600125 (SP), 10 μM of the p38 MAPK inhibitor SB203580 (SB), 5 μM of the NF-κB inhibitor BAY11-7082 (BAY), and 0.1 μM of the TBK1 inhibitor BX795 (BX). Then, cells were stimulated in the presence of these inhibitors with 20 μg/ml poly(I:C) for 48 h. Protein levels of MMP-1, -2, and -3 in the cell culture medium and actin in the cell lysates were analyzed with Western blotting. Con, control. The data shown are representative of three independent experiments. Values are mean ± SEM. **P < 0.01 versus poly(I:C)-treated control.

Knockdown of IRF3 attenuates poly(I:C)-induced increase in MMP-1, -2, and -3 expressions

To confirm the role of IRF3 in poly(I:C)-induced MMP-1, -2, and -3 expressions, I transfected fibroblasts with scrambled control siRNA or IRF3 siRNA. As shown in Fig. 18A, the level of IRF3 protein was significantly reduced by IRF3 siRNA. After poly(I:C) treatment for 48 h, I found that knockdown of IRF3 attenuated poly(I:C)-induced MMP-1, -2, and -3 expressions (Fig. 18B). These data indicate that IRF3-dependent pathways play an important role in poly(I:C)-induced increase in MMP-1, -2, and -3 expressions in human skin fibroblasts.

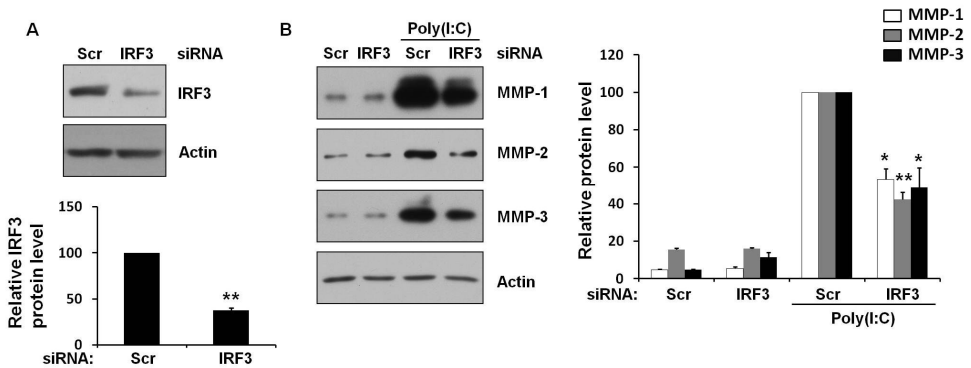

Figure 18. Knockdown of IRF3 attenuates poly(I:C)-induced increase in MMP-1, -2, and -3 expressions. (A) Fibroblasts were transfected with scrambled control (Scr) siRNA or IRF3 siRNA for 48 h. The transfected cells were then serum starved for 24 h. Then, IRF3 protein level was analyzed by Western blotting. The data shown are representative of three independent experiments. Signal intensity was quantified using a densitometric program (ImageJ, W. Rasband, NIH, USA). Values are mean ± SEM. **P < 0.01. (B) After transfection for 48 h and further starvation for 24 h, cells were incubated in fresh starvation media with or without poly(I:C) (20 μg/ml) for a further 48 h. Protein levels of MMP-1, -2, and -3 in the cell culture medium and actin in the cell lysates were analyzed with Western blotting. The data shown are representative of three independent experiments. Values are mean ± SEM. *P < 0.05, **P < 0.01 versus poly(I:C)-treated control.

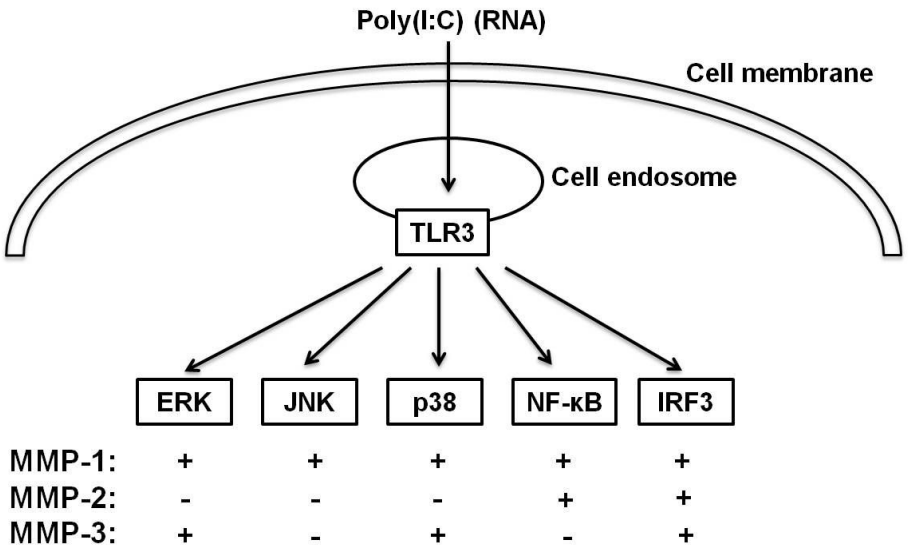

Figure 19. A schematic diagram showing the regulatory pathways of poly(I:C)-induced expressions of MMP-1, -2, and -3 in human skin fibroblasts. My data indicate that the expressions of MMP-1, -2, and -3 induced by poly(I:C) are TLR3-dependent and differentially regulated by various downstream signaling pathways. The ERK, the JNK, the p38, the NF-κB, and the IRF3 pathways are involved in poly(I:C)-induced MMP-1 expression. The NF-κB and the IRF3 pathways are involved in poly(I:C)-induced MMP-2 expression. The ERK, the p38 and the IRF3 pathways are involved in poly(I:C)-induced MMP-3 expression.

Chapter Ⅳ

TLR3 is involved in UV-induced increase of MMP-13 expression in mice

Materials and Methods

Mice

Wild-type (WT) and TLR3 knockout (TLR3-/-) mice (C57/BL6 background) were purchased from Jackson Laboratories (Sacramento, CA, USA). All mice were bred under specific pathogen-free conditions in the facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Eight-week-old female mice were used in my experiments. Animal experiments were approved by the institutional animal care and use committee (IACUC) of Seoul National University Hospital (IACUC No. 14-0241-S1A0 (1)). Four mice per group were assigned to the non-UV-irradiated WT and TLR3-/- groups. Five mice per group were assigned to the UV-irradiated WT and TLR3-/- groups.

UV irradiation on mice skin

Mice were irradiated with a UV device equipped with TL 20W/12 RS

fluorescent sunlamp (Philips, Amsterdam, The Netherlands), with an emission spectrum between 275 and 380 nm (peak, 310–315 nm). A TA401/407 Kodacel filter (Kodak, NY, USA) was used to remove UVC (wavelengths <290 nm). Irradiance was measured using a Waldmann UV meter (Model 585100) (Kim and Chung 2008). Dorsal skin was shaved with an electric clipper and chemically depilated using a depilatory cream (Veet, Oxy-Reckitt Benckiser, Seoul, Korea) 48 hours before UV irradiation. The hairless skin was then exposed to a single dose of UV (600mj/cm^2). After 48 h, photographs of dorsal skin were acquired. Dorsal skin thickness was measured using a digital caliper (Mitutuyo, Kawasaki, Japan). After skin thickness measurement, dorsal skin samples were obtained. For protein analysis, biopsied skin samples were homogenized in 1X Radioimmunoprecipitation assay buffer (RIPA buffer). Lysates were centrifuged and supernatants were collected for protein quantification using Bradford protein assay. Equal amount of protein extracts were prepared for ELISA or Western blotting analysis. RT-qPCR was used for mRNA analysis.

Treatment of poly(I:C) to cultured WT and TLR3-/- mice skin fibroblasts

Skin fibroblasts from WT and TLR3-/- mice were isolated. Cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 mg/ml),

and 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37℃. For treatment, skin fibroblasts were first serum starved for 24 h in DMEM containing 0.1% FBS, and then treated with poly(I:C). In some experiments, collected cells were treated with RNase for 1 h in room temperature. RT-qPCR was used for mRNA analysis. The sequences of forward and reverse primers for IL-1β and MMP-13 are designed as the following: IL-1β forward, 5'- GAC TCA TGG GAT GAT GAT GAT AAC -3'; reverse, 5'- CCA TAC TTT AGG AAG ACA CGG ATT -3', MMP-13 forward, 5'- CAT CCA TCC CGT GAC CTT AT -3'; reverse, 5'- GCA TGA CTC TCA CAA TGC GA -3'.

UV irradiation-induced cell necrosis

Necrotic cells were generated by UV irradiation as described previously, with slight modifications (Lai, Di Nardo et al. 2009; Bernard, Cowing-Zitron et al. 2012; Borkowski, Kuo et al. 2015). I irradiated cultured human keratinocytes by UV at 400mj/cm^2. After 24 h, I collected the floating and attached cells. I checked that more than 90% of the collected cells were propidium iodide – positive cells (data not shown). I then added these UV-irradiated cells to cultured WT and TLR3-/- mice skin fibroblasts. I used non-UV irradiated human keratinocytes as controls. In some experiments, collected cells were treated with 100 μg/ml of RNase for 2 h in room temperature. 24 h after incubation with the added cells, mRNA

level of IL-1β and MMP-13 in WT and TLR3-/- fibroblasts were analyzed by RT-qPCR.

Statistical analysis

Statistical analyses were performed using the Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant. Results are presented as mean values ± SEM.

Results

UV radiation induces higher skin inflammation in WT than in TLR3-/- mice skin

Recently, researchers found that UV damage can be detected by TLR3. UV damaged RNA in skin cells contain stem-loop structures that could form dsRNAs and thereby activate TLR3 (Bernard, Cowing-Zitron et al. 2012). Since it was suggested that UV radiation induces more skin inflammation in WT than in TLR3-/- mice skin (Bernard, Cowing-Zitron et al. 2012), I first confirmed this data. It was suggested that high dose of UV can induce necrosis of cells which may release damaged dsRNA (Bernard, Cowing-Zitron et al. 2012; Borkowski, Kuo et al. 2015). Thus, I irradiated mice skin using high dose of UV (600mj/cm2). At 48 h after UV irradiation, I found that UV induces much more redness (Fig. 20A), swelling (Fig. 20B) and inflammatory cytokines (Fig. 20C) in the skin of WT than the skin of TLR3-/- mice. This result is correspondent with the published results (Bernard, Cowing-Zitron et al. 2012).

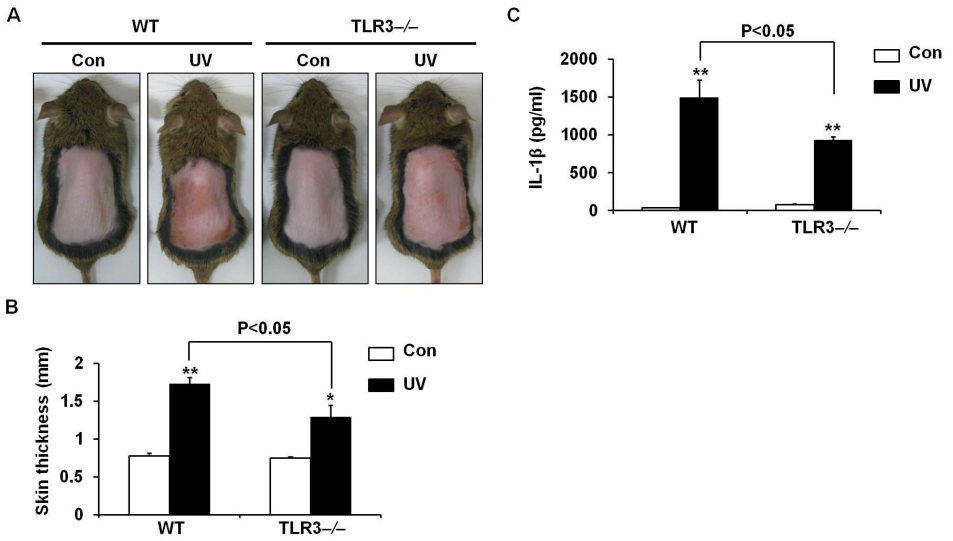

Figure 20. UV radiation induces higher skin inflammation in WT than in TLR3-/- mice skin. (A) Representative image of WT and TLR3-/- mice 48 h after exposure to 600mj/cm^2 UV on hair-removed back skin. (B) Skin thickness was measured using a digital caliper. (C) IL-1β protein expression was measured by ELISA. n=4, control groups; n=5, UV irradiated groups. Values are mean ± SEM. *P < 0.05, **P < 0.01 versus control groups.

TLR3 is involved in UV radiation-induced induction of MMP-13, but not reduction of procollagen in mice

Since poly(I:C) is one kind of dsRNA that may reduce procollagen and induce MMP expressions through TLR3 (Fig. 7 and 14), I further investigated whether UV reduces procollagen and induces MMP expressions through TLR3. I checked expressions of procollagen and MMP-13 which is a main collagenase gene in mice (Schorpp, Mattei et al. 1995). I found that UV radiation reduced expression of procollagen in WT as well as TLR3-/- mice skin. And expression of procollagen was not significantly different between UV-irradiated WT and TLR3-/- mice skin. However, I found that UV-induced expression of MMP-13 is significantly higher in WT than in TLR3-/- mice skin (Fig. 21A). I have shown that IFN-β is involved in dsRNA-induced reduction of procollagen *in vitro* (Fig. 10). And it is known that UV irradiation can induce endogenous RNA which activates TLR3 (Bernard, Cowing-Zitron et al. 2012). Thus, I checked IFN-β expression after UV irradiation on mice. I found that UV radiation had no effect on IFN-β expression both in WT and TLR3-/- mice skin (Fig. 21B). I also checked mRNA level of MMP-13. UV-induced mRNA level of MMP-13 is significantly higher in WT than in TLR3-/- mice skin (Fig. 21C).

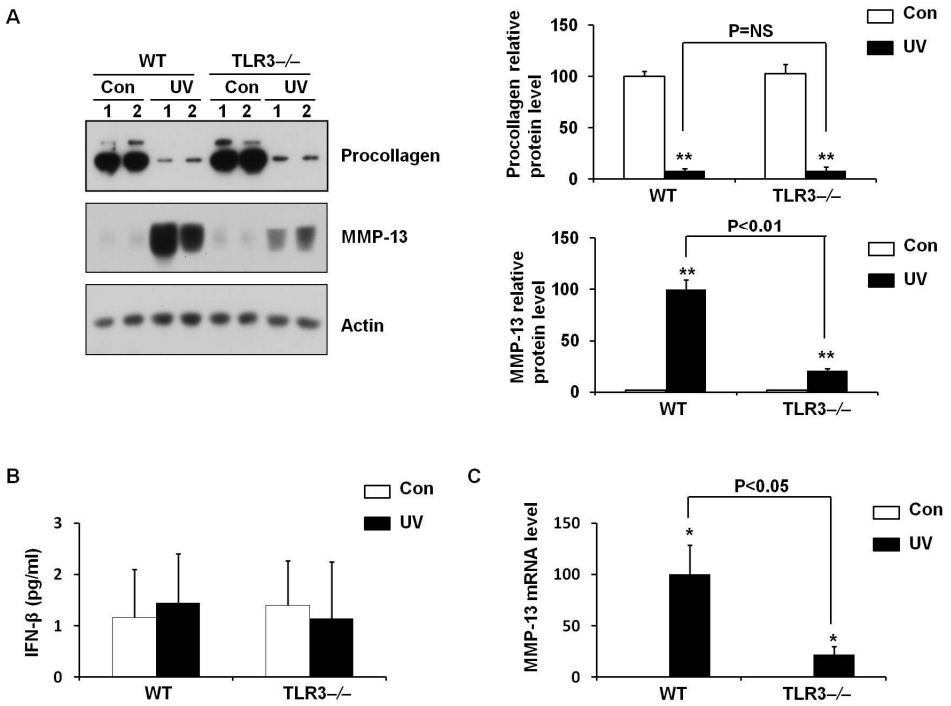

Figure 21. TLR3 is involved in UV radiation-induced induction of MMP-13, but not reduction of procollagen in mice. (A) Procollagen and MMP-13 protein expressions were measured by using Western blot analysis. Signal intensity was quantified using a densitometric program (ImageJ, W. Rasband, NIH, USA). (B) IFN-β protein expression was measured by ELISA. (C) MMP-13 mRNA level was measured by RT-qPCR. n=4, control groups; n=5, UV irradiated groups. Values are mean ± SEM. *P < 0.05, **P < 0.01 versus control groups.

Poly(I:C) increases mRNA levels of IL-1β and MMP-13 in WT but not TLR3-/- mice skin fibroblasts

Since UV radiation induced IL-1β and MMP-13 expressions in mice, I assumed that UV-induced necrotic cells might release RNAs which induced IL-1β and MMP-13 expressions through TLR3 (Bernard, Cowing-Zitron et al. 2012). To understand whether the necrotic cells induced by UV can increase MMP-13 expression through TLR3, I first checked the effect of poly(I:C), a mimic of RNA, on MMP-13 expression *in vitro*. My data indicated that poly(I:C) can induce IL-1β (Fig. 22A) and MMP-13 (Fig. 22B) expressions in cultured WT but not TLR3-/- mice skin fibroblasts. And incubation with RNase abrogated the effect of poly(I:C) on IL-1β (Fig. 22A) and MMP-13 (Fig. 22B). Thus, my data indicated that poly(I:C), a mimic of RNA, increases mRNA levels of IL-1β and MMP-13 in WT but not TLR3-/- mice skin fibroblasts.

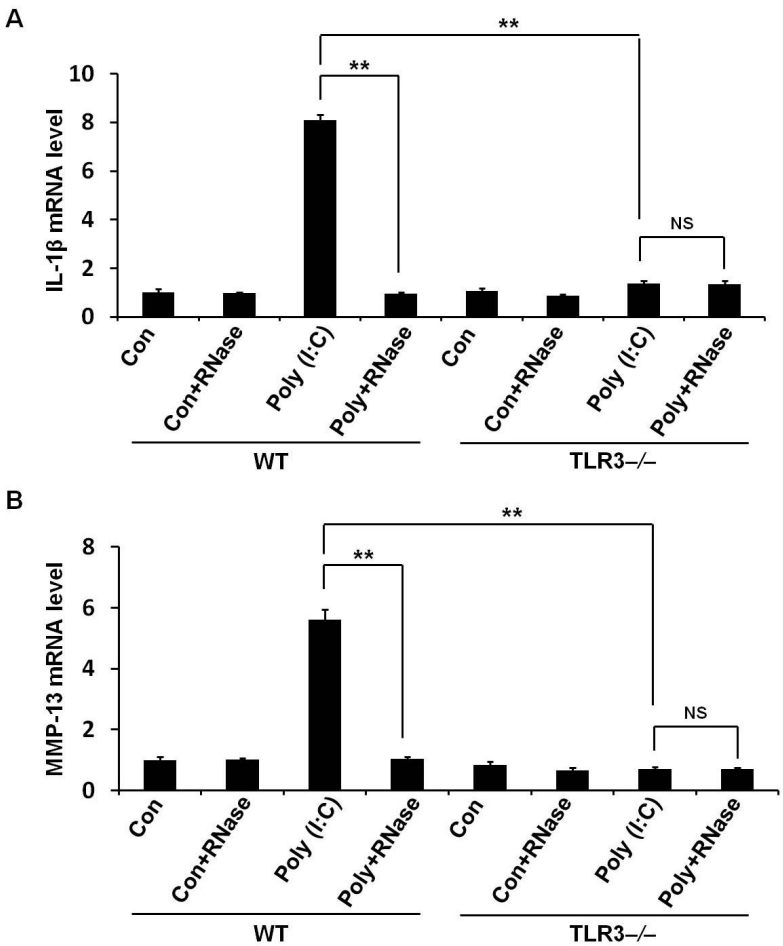

Figure 22. Poly(I:C) increases mRNA levels of IL-1β and MMP-13 in WT but not TLR3-/- mice skin fibroblasts. Fibroblasts isolated from WT and TLR3-/- mice skin were cultured and starved for 24 h. After treatment of poly(I:C) or poly(I:C) plus RNase for 24 h, mRNA levels of IL-1β (A) and MMP-13 (B) were measured by RT-qPCR. n=3. Values are mean ± SEM. **P < 0.01.

UV-irradiated keratinocytes induced higher mRNA levels of IL-1β and MMP-13 in WT than in TLR3-/- mice skin fibroblasts

Since it was suggested that UV radiation-induced necrotic cells could release RNAs capable of increasing the expression of cytokines from non-irradiated cells through TLR3 ((Bernard, Cowing-Zitron et al. 2012)), I first confirmed this result. To get necrotic cells, I exposed normal human epidermal keratinocytes to UVB radiation (400 mJ/cm^2). I used non-irradiated normal human epidermal keratinocytes as controls. Treatment of UV radiation-induced necrotic keratinocytes induced higher mRNA level of IL-1β (Fig. 23A) in WT than in TLR3-/- mice skin fibroblasts. The data was correspondent with the previously published study in which IL-6 was checked instead of IL-1β (Bernard, Cowing-Zitron et al. 2012). I also found that treatment of UV radiation-induced necrotic keratinocytes induced higher mRNA level of MMP-13 (Fig. 23B) in WT than in TLR3-/- mice skin fibroblasts. Incubation with RNase relieved the effect of UV radiation-induced necrotic keratinocytes on IL-1β (Fig. 23A) and MMP-13 (Fig. 23B) in WT skin fibroblasts. My data indicated that UV-irradiated keratinocytes induced higher mRNA levels of IL-1β and MMP-13 in WT than in TLR3-/- mice skin fibroblasts.

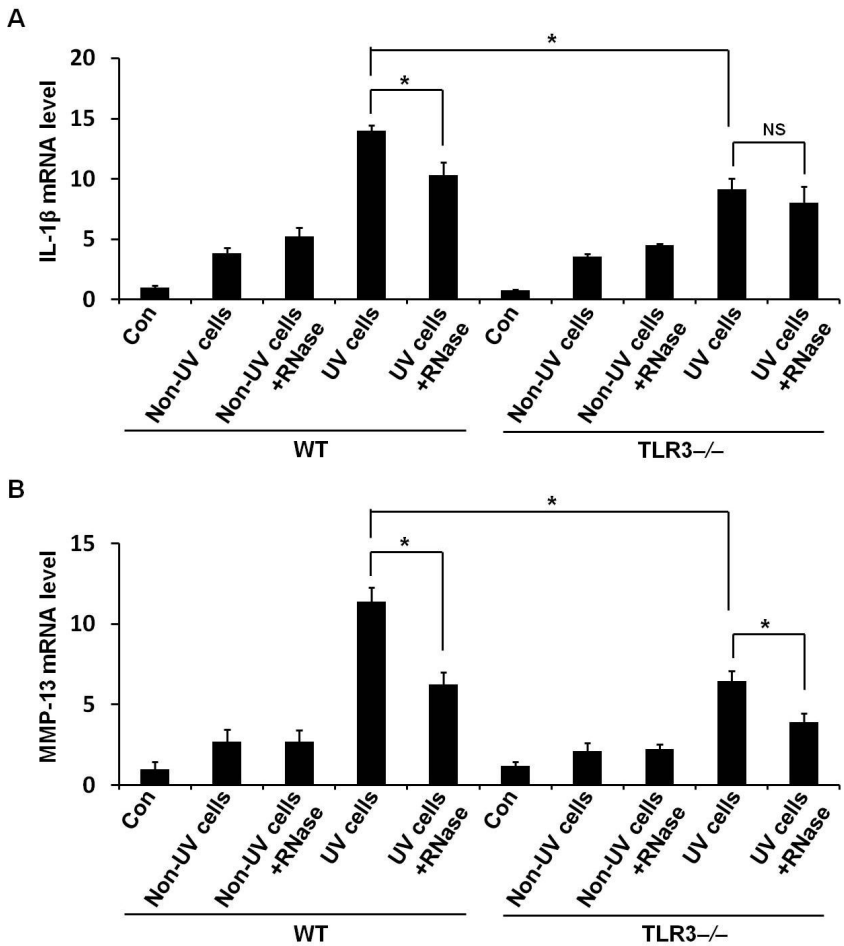

Figure 23. UV-irradiated keratinocytes induced higher mRNA levels of IL-1β and MMP-13 in WT than in TLR3-/- mice skin fibroblasts.

Fibroblasts isolated from WT and TLR3-/- mice skin were cultured and starved for 24 h. UV radiation-induced necrotic keratinocytes or normal keratinocytes were treated with RNase, and then treated to cultured WT and TLR3-/- mice skin fibroblasts. After incubation for 24 h, mRNA levels of IL-1β (A) and MMP-13 (B) were measured by RT-qPCR. n=3. Values are mean ± SEM. *P < 0.05.

Discussion

Many studies have characterized the expressions and functions of TLRs in human epidermal cells. It is well known that keratinocytes constitutively express TLR1-6, 9, and 10, but not TLR7 and 8 (Lebre, van der Aar et al. 2007). Melanocytes constitutively express TLR1-4, 6, 7, and 9, but not TLR5, 8 and 10 (Yu, Zhang et al. 2009). However, the characteristics of TLR family members in skin fibroblasts have not been thoroughly studied.

In chapter Ⅰ, I showed that cultured human skin fibroblasts express functional TLR1-10 (Fig. 1-3). Activation of NF-κB and MAP kinases plays a central role in TLR-mediated cellular activation and gene expression in a variety of cell types (Lebre, van der Aar et al. 2007; Yu, Zhang et al. 2009; Lee, Kim et al. 2010). I found that treatment of several TLR ligands can induce phosphorylation of IκBα and ERK1/2 (Fig. 4). It suggests that NF-κB and MAP kinases pathways can be activated by TLR ligands, and activation of NF-κB and MAP kinases may contribute to up-regulation of IL-6, -8, and MMP-1 expressions by TLR ligands in skin fibroblasts.

It is generally considered that epidermis are the first defense line of skin against pathogens and that skin keratinocytes are major cells in the epidermis. To this point, I assumed that the expression level of some (if not all) TLR family members might be higher in skin keratinocytes than in skin fibroblasts. Thus, I decided to compare expression levels of TLRs in skin keratinocytes with fibroblasts. Surprisingly, I found that relative expression

levels of each TLR family member were much higher in fibroblasts than in keratinocytes, regardless of cell culture conditions. These results were somewhat surprising, considering that epidermis is the first defense line of skin against pathogens and that skin keratinocytes are major cells in the epidermis. However, many studies also show that in addition to exogenous ligands derived from pathogens, various endogenous ligands can activate TLRs and initiate responses to danger signals (Liu-Bryan, Scott et al. 2005; Calderwood, Mambula et al. 2007; Bernard, Cowing-Zitron et al. 2012). In addition, a recent study has shown that some TLR4 endogenous ligands such as tenascin C and hyaluronic acid are elevated in scleroderma skin lesion, and has suggested that TLR4 signaling activated by endogenous ligands in skin fibroblasts may be involved in pathogenesis of scleroderma (Bhattacharyya, Kelley et al. 2013). Thus, high expression levels of TLRs found in skin fibroblasts may play important roles when they are activated by endogenous ligands.

I also compared the activities of TLR1/2 signaling pathways in fibroblasts with that in keratinocytes. As shown in Fig.6, my data have shown that Pam3CSK4 treatment increased mRNA level of IL-6 and IL-8 in both fibroblasts and keratinocytes and that Pam3CSK4-induced expression folds of IL-6 and IL-8 were much higher in fibroblasts than in keratinocytes, at every time point and concentration. These data may result, at least partially, from the fact that expression levels of TLR1 and 2 are higher in fibroblasts than in keratinocytes (Fig. 5). Thus, my data show that activation

of TLR signaling pathway seems to be much stronger in fibroblasts than in keratinocytes. TLR signaling pathways in fibroblasts may play important roles in host defense against infection and/or in response to danger signals.

In chapter Ⅱ, I focused on studying the effect of activation of TLRs on procollagen expression in skin fibroblasts. Collagen is the main structural protein in the extracellular space in the various connective tissues in human (Di Lullo, Sweeney et al. 2002). In photoaged skin, collagen production is found to be decreased (Chung, Hanft et al. 2003). In this chapter, I found that only poly(I:C), but not other TLR ligands, inhibited procollagen synthesis in skin fibroblasts. It is very well known that TLR3 is a receptor for dsRNA (Lester and Li 2014). However, in many different cell types, dsRNA can also be recognized by MDA5 and RIG-I (Kato, Takeuchi et al. 2006). In certain cases, dsRNA-induced effects can be TLR3-independent (Kalali, Kollisch et al. 2008). Thus, I investigated whether poly(I:C)-induced reduction of procollagen expression is TLR3-dependent in human skin fibroblasts. My data indicated that knockdown of TLR3, MDA5, and RIG-I by siRNAs relieved poly(I:C)-induced reduction of procollagen expression (Fig. 12). Although siRNAs are widely used for studying gene functions, siRNAs have disadvantages, such as the variability and incompleteness of knockdowns (Boutros and Ahringer 2008). Thus, to further confirm the role of TLR3 on procollagen expression, I used cultured skin fibroblasts from WT and TLR3-/- mice in which TLR3 was inoperative. My data showed that knockout of TLR3 relieved poly(I:C)-induced

production of IFN-β and reduction of procollagen expression (Fig. 13). Interestingly, in TLR3-/- skin fibroblasts poly(I:C) still induced IFN-β expression. It may be due to the fact that poly(I:C) can also be recognized by MDA5 and RIG-I. Activation of MDA5 and RIG-I can induce IFN-β expression (Kalali, Kollisch et al. 2008).

The effect of activation of TLR3 on procollagen production in lung was reported previously. A study showed that activation of TLR3 by poly(I:C) induced collagen production in lung fibroblasts (Sugiura, Ichikawa et al. 2009). However, an *in vivo* study showed that poly(I:C) ameliorated bleomycin-induced lung fibrosis in mice (Hyde and Giri 1990). This data suggested an anti-collagen production effect of poly(I:C) in lung fibrosis. A potential explanation for these conflicting observations regarding the pro- or anti- fibrotic effects of poly(I:C) might lie in the dual effect of poly(I:C): inducing fibrotic inflammatory cytokines, such as IL-6, and inducing anti-fibrotic cytokines, such as IFN-β. The net result of these fibrotic and anti-fibrotic responses then might be dependent on different experiment conditions. Thus, the published results and my data indicated that poly(I:C) may differentially regulate procollagen expression in different cell types or experiment conditions.

MMPs are a group of proteins that are capable of degrading many kinds of extracellular matrix proteins, including collagen. Whether poly(I:C) can regulate MMP expressions in skin fibroblasts has not been thoroughly studied. In chapter Ⅲ, I investigated the effect of poly(I:C) on the

expressions of MMPs in human skin fibroblasts. I found that poly(I:C) can induce MMP-1, -2, and -3 expressions in a dose-dependent manner in human skin fibroblasts and that the inductions of MMP-1, -2, and -3 by poly(I:C) are TLR3-dependent. Interestingly, by using specific inhibitors, I found that poly(I:C)-induced expressions of MMP-1, -2, and -3 are differentially regulated by these signaling pathways. In particular, IRF3 pathway was shown to be involved in the induction of all the three MMPs. It is the first time to report that IRF3-dependent pathways are involved in MMP inductions in human skin cells. A recent study has shown that poly(I:C) can induce MMP-1, -2, and -9 expressions via IRF3-dependent pathways in human lung fibroblasts (Ichikawa, Sugiura et al. 2014). Taken together, these results suggest that TLR3- and IRF3-dependent pathways may play an important role in the induction of MMPs by poly(I:C).

It is well known that UV irradiation can result in premature skin aging (photoaging) which is characterized by decreased expression of collagen and increased expression of MMPs (Gilchrest and Yaar 1992). Through *in vitro* and *in vivo* experiments, it has been suggested that UV irradiation may directly impair transforming growth factor (TGF)- β signaling to decrease collagen synthesis (Quan, He et al. 2001) and that UV irradiation may activate cell surface cytokine and growth factor receptors, resulting in the increased transcription factor AP-1 activity, and thus, the increased expression of MMPs (Rosette and Karin 1996; Fisher and Voorhees 1998).

Recently, it has been shown that UV radiation damages self noncoding

RNA which is detected by TLR3 (Lai, Di Nardo et al. 2009; Bernard, Cowing-Zitron et al. 2012; Borkowski, Kuo et al. 2015). Treatment with UV-induced necrotic keratinocytes to healthy keratinocytes increased expressions of inflammatory cytokines, such as IL-6 and TNF-α. Further studies showed that TLR3 siRNA and RNase inhibited the capacity of UV-induced necrotic keratinocytes to increase the expressions of IL-6 and TNF-α. It indicated that UV-induced necrotic keratinocytes increased the production of IL-6 and TNF-α through TLR3 dependent pathway via released RNAs (Lai, Di Nardo et al. 2009; Bernard, Cowing-Zitron et al. 2012). Whole-transcriptome RNA sequencing (RNA-Seq) revealed that UV-damaged cells release certain noncoding RNAs contain stem-loop structures that could form dsRNAs and thereby activate TLR3 (Bernard, Cowing-Zitron et al. 2012). Thus, UV-damaged RNA could act as a DAMP that serves as an endogenous signal of solar injury.

In the last chapter, chapter Ⅳ, I studied the role of TLR3 in UV-induced decrease of procollagen and increase of MMP expressions in mice. Poly(I:C) is a synthetic dsRNA analog and has been regarded as a representative RNA ligand for TLR3 and extensively used instead of both exogenous and endogenous RNA ligands in many studies (Kalali, Kollisch et al. 2008; Fang, Ooka et al. 2013). Since activation of TLR3 by poly(I:C) decreased procollagen (Fig. 10) and increased MMP expressions in vitro (Fig. 14), I speculated that RNAs released from UV-induced necrotic cells might inhibit procollagen and increase MMP expressions through TLR3-

dependent pathways. Thus, I did UV-irradiation on WT and TLR3-/- mice skin. Since IFN-β is the main factor involved in procollagen reduction by poly(I:C), I then checked procollagen, IFN-β and MMP expressions in skin tissue.

I found that the expression of procollagen was not significantly different between UV-irradiated WT and TLR3-/- mice skin. Expression level of IFN-β in non-irradiated mice skin was very low. Moreover, UV irradiation did not affect IFN-β expression either in WT or TLR3-/- mice skin (Fig. 21B). Several studies suggested that UV radiation can inhibit IFN-β expression (Aragane, Schwarz et al. 1997; Bielenberg, Bucana et al. 1998). It was suggested that UV radiation could down-regulate the expression of IFN response factors (IRF-1 and IRF-2) that might inhibit de novo transcription of IFN-β mRNA (Aragane, Schwarz et al. 1997). Also, UV radiation might induce cytokines such as IL-10 that act in paracrine fashion to inhibit the production of IFN-β (Bielenberg, Bucana et al. 1998). Thus, the result indicated that TLR3 is not involved in UV-induced reduction of procollagen expression *in vivo*. It may be partially because UV inhibits IFN-β expression *in vivo* although RNAs could be released from necrotic cells.

In contrast to procollagen expression, I found that UV-induced expression of MMP-13 was significantly higher in WT than in TLR3-/- mice skin (Fig. 21A). I assumed that UV-induced necrotic cells might be involved in the mechanism. Although keratinocytes are in the first layer of skin, skin fibroblasts also play important roles in UV-induced skin aging (Rittie and

Fisher 2002; Kim and Chung 2008; Kim, Kim et al. 2008). Moreover, in my study, I used high dose of UV (600mj/cm2). Mice skin dermis was also found to be damaged by UV irradiation. Then, I isolated and cultured skin fibroblasts from WT and TLR3-/- mice. Treatment of UV radiation-induced necrotic keratinocytes induced higher mRNA level of MMP-13 (Fig. 23B) in WT than in TLR3-/- mice skin fibroblasts. Incubation with RNase relieved the effect of UV radiation-induced necrotic keratinocytes on MMP-13 in WT skin fibroblasts (Fig. 23B). To a less extent, RNase also relieved the effect of UV radiation-induced necrotic keratinocytes on MMP-13 in TLR3-/- mice skin fibroblasts (Fig. 23B). This data indicated that RNA could induce MMP-13 through TLR3 dependent or independent pathways. In fact, I previously showed that ssRNA induced MMP expressions through TLR8 (Fig. 3). TLR8 might be still expressed in TLR3-/- mice skin fibroblasts. Thus, my data indicated that UV-irradiated keratinocytes induced higher mRNA levels of MMP-13 in WT than in TLR3-/- mice skin fibroblasts. The effect of higher expression of MMP-13 in WT skin fibroblasts may be due to released RNA from necrotic keratinocytes. Furthermore, in my study, IRF3 was found to be involved in dsRNA-induced decrease of procollagen and increase of MMP expressions (chapter Ⅱ and Ⅲ). Further studies need to be done using wild-type and IRF3 knockout mice, in order to address whether IRF3 pathways play an important role in regulating UV-induced skin damages or expressions of procollagen and MMPs in vivo.

In summary, my results in chapter Ⅰ extend the knowledge to understand expression and activation of TLRs in skin fibroblasts. My data indicates that TLRs in skin fibroblasts may play an important role in detection of and response to different classes of pathogens and/or danger signals. My studies in chapter Ⅱ, Ⅲ, and Ⅳ provide the possible mechanisms involved in dsRNA-induced decrease of procollagen and increase of MMP expressions. It indicates that TLR3 could be viewed as a therapeutic target for UV-induced MMP induction, but not procollagen reduction *in vivo*.

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국 문 초 록

Toll-like receptors (TLRs)는 pathogen associated molecular pattern (PAMP)뿐 아니라 damage associated molecular pattern (DAMP)를 인식한다고 알려져 있다. 최근 들어 사람의 상피세포에서의 TLRs의

발현과 역할에 대한 보고가 이루어지고 있지만,
섬유아세포에서만의 TLR family members 에 대한 특성은 아직 충분히 보고 되지 않고 있다.

Ultraviolet (UV) 조사는 피부에서 collagen의 감소와 matix metalloproteinase (MMPs)의 증가와 같은 피부노화를 일으키는데 이를 photoaging (광노화)라 한다. 일반적으로 바이러스에 의한 감염이나 자외선 같은 여러 자극에 의해 손상을 입은 피부세포는 Double strand RNA (dsRNA)가 발생된다. TLR3는 dsRNA를 인식하는 수용체로 잘 알려져 있다. 하지만 섬유하세포에서 여러 자극과 손상으로부터 발생된 dsRNA가 TLR3를 통하여 procollagen 및 MMPs를 발생시키는지 여부에 대한 보고는 아직 이루어지지 않았다.

최근, 각질 형성 세포에 자외선을 조사하였을 때 TLR3 리간드로 작용하는 손상 받은 self-noncoding RNA를 분비한다는 것이 알려졌다. 이러한 발견은, 자외선에 의한 염증성 사이토카인들인 TNF-a와 IL-6 증가와 관련 있을 것으로 예상되지만, TLR3가 자외선에 의한 procollagen의 발현 감소와 MMP-1의 발현증가에 대한 상관성에 대해서는 알려져 있지 않다.

제 1장에서는, 우리는 TLR의 발현과 그것들 각각의 리간드에 의한 반응을 피부 섬유아세포에서 체계적으로 관찰하였다. 우리는

10가지 TLR 모두가 피부 섬유아세포에서 발현하는 것을 발견하였으며, TLR 리간드로 자극함에 따라 염증성 사이토카인인 IL-6, IL-8 뿐만 아니라, MMP-1 단백질 발현도 증가되는 결과를 확인하였다. 또한, 우리는 TLR 발현 정도가 각질형성세포에 비해 섬유아세포에서 더 많이 발현된다는 것을 관찰하였다. 특히, TLR1/2의 리간드를 처리하였을 때, IL-6와 IL-8의 mRNA 발현 양이 각질형성세포에 비해, 섬유아세포에서 높게 발현된다는 것을 확인되었다. 이를 종합적으로 볼 때, TLR1과 2 발현과 그 반응 정도가 섬유아세포와 각질형성세포가 다르다는 것을 시사한다.

제 2장에서는 피부 섬유아세포에 TLR3 리간드인 poly(I:C)를 처리하면 procollagen을 줄이고 IFN-β를 늘이는 것을 관찰했다. 다른 TLR 리간드들은 이런 반응을 보이지 않았다. Poly(I:C)는 IRF-3의존 경로를 통해 IFN-β를 유도했다. IRF-3 경로는 poly(I:C)이외 다른 TLR 리간드들에 의해서는 활성화되지 않았다. 추가 실험에서, poly(I:C)는 IFN-β를 올림으로써 procollagen을 줄였다. 또한 TLR3 발현을 감소시키거나 없애면 poly(I:C)에 의한 procollagen이 감소 현상이 완화되는 것을 보였다.

제 3장에서는 피부 섬유아세포에서 poly(I:C)에 대한 MMP-1, -2, -3 발현에 주는 영향을 관찰하였다. 그리고 poly(I:C)에 의해서 MMP-1,2,3이 증가하는 것을 관찰했으며, 이 현상은 TLR3

의존적으로 일어났다. 그 밖에도 poly(I:C)를 처리하면 MAPK, NF-kB, IRF3 pathway가 활성화 되는 것도 관찰할 수 있었다. 여러가지 특이적 Inhibitor를 이용하여 MMP-1, -2, -3의 발현 패턴을 연구해본 결과 위의 Signaling pathway를 통해서 각각 다르게 발현양 조절 되는 것을 관찰하였다. 특히, IRF3 signaling pathway를 억제하게되면 poly(I:C)에 의한 MMP-1, -2, -3의 발현이 모두 감소하는 것을 관찰하였다.

제 4장에서, 우리는 TLR3가 *in vivo*상에서 자외선에 의해 유도되는 procollagen발현 감소와 MMP 발현의 증가에 대해 관련이 있는지 확인하였다. 자외선을 조사한 WT와 TLR3-/- mice 피부에서 procollagen의 발현이 크게 변하지 않은 것을 발견했다. Procollagen 발현과는 반대로, UV에 의해 유도된 MMP-13발현은 TLR3-/- mice보다 WT mice에서 크게 증가하였다. 따라서 WT 피부 섬유모세포에서 MMP-13의 발현 증가는 괴사형 각질형성세포로부터 RNA 방출에 의한 결과라고 생각된다.

요약하면, 우리는 10종류의 TLR 모두 피부 섬유아세에서 지속적으로 발현하고 기능하는 것을 알아냈으며 이러한 TLR family member의 발현양은 피부 각질형성세포에서 보다 섬유아세포에서 훨씬 증가되어 있었다. 섬유아세포에서 TLR3의 활성화는 IFN-β의 유도를 통해 procollagen의 감소를 불러일으켰다.

또한 dsRNA에 의해 MMP-1, -2, -3가 증가되어 있는 것을 관찰할 수 있었고 Poly(I:C)는 TLR3, IRF3를 포함한 여러 신호전달과정을 통해 MMP-1, -2, -3의 발현을 증가시켰다. 마지막으로 우리는 WT과 TLR3-/- mice를 사용하여 UV에 의한 MMP-13의 증가가 procollagen 발현의 감소는 없이 TLR3에 의존하는 것을 확인할 수 있었다.

1장에서의 결과는 섬유모세포가 발병기전에 연관되어 있는 켈로이드와 비후흉터, 피부경화증 등 피부 질환에 대한 정보를 주었다. 우리는 2장에서 dsRNA가 피부에서 잠재적인 항섬유화 활성을 가진다는 단초를 제공한다. 그 다음으로 3장에서는 dsRNA에 의해 유도된 MMP-1, -2, -3의 발현을 조절하는 데 있어 TLR3과 IRF3 각각 또는 두 가지 모두가 좋은 타깃이 될 수 있다는 것을 보여주었다. 마지막으로 4장을 통해 TLR3가 UV에 의한 MMP 유도의 치료표적으로 이용될 수 있다는 것을 시사했다.