

IRF3 signaling pathway serves an important role in poly(I:C)-induced procollagen reduction in human skin fibroblasts

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Abstract. Pattern recognition receptors (PRRs) are part of the immune system. They can recognize pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) are 2 types of PRR in the innate immune system. Double-stranded RNA (dsRNA) can exist as a PAMP, including dsRNA viruses. dsRNA is known as a ligand not only for TLR3 but also for RLRs, including melanoma differentiation-associated gene 5 and RIG-I. Collagen is the main structural protein in the extracellular space in the skin. Recently, it was reported that treatment of a synthetic dsRNA, poly(I:C), decreases procollagen expression in skin fibroblasts. However, signaling pathways involved in this process have not yet been fully elucidated. The present study further explored the underlying signaling pathways involved in the processes. It was demonstrated by western blotting that treatment of poly(I:C), but not another PAMP, Pam3CSK4, inhibited procollagen expression in cultured human skin fibroblasts. Treatment of poly(I:C) and Pam3CSK4 induced activation of the mitogen-activated protein kinases and the nuclear factor- κ B pathways. However, only poly(I:C), but not Pam3CSK4, induced the activation of the interferon regulatory factor 3 (IRF3) pathway. By using specific inhibitors, it was demonstrated that inhibition of IRF3 pathway relieved poly(I:C)-induced procollagen reduction. In conclusion, IRF3 signaling pathway serves an important role

in poly(I:C)-induced procollagen reduction in skin fibroblasts. This suggests that the IRF3 signaling pathway may be a key target for collagen regulation in the skin.

Introduction

Pathogen-associated molecular patterns (PAMPs) are molecules associated with groups of pathogens. PAMPs include molecules from Gram-positive and -negative bacteria, DNA and RNA viruses (1). PAMPs can be recognized by Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs); two types of pattern recognition receptors (PRRs) in the innate immune system (2,3). PAMPs recognized by TLRs and RLRs include lipids, lipoproteins, proteins and nucleic acids derived from bacteria, viruses, parasites and fungi (4). For example, triacyl lipopeptide and diacyl lipopeptide are PAMPs that can be recognized by TLR1/2 and TLR2/6, respectively (5-7). Double-stranded RNA (dsRNA) is a ligand not only for TLR3, but also for RLRs including melanoma differentiation-associated gene 5 (MDA5) and retinoic acid-inducible gene 1 (RIG-I) (8). MDA5 and RIG-I are cytosolic RNA helicases capable of unwinding dsRNA molecules (9). Once recognized by receptors, PAMPs can activate nuclear factor (NF) κ B, activator protein 1 (AP-1), interferon regulatory factor 3 (IRF3), and IRF7 signaling pathways which induce the expression of inflammatory cytokines (3,10,11).

Collagen is the main structural protein in the extracellular space of tissues. Collagen-related diseases can arise from genetic defects or environmental stresses that affect the biosynthesis, assembly, secretion or other processes involved in normal collagen production. Scleroderma results from an overproduction and accumulation of collagen in tissues (12). Skin aging may result from decreased synthesis of collagen and/or induced collagen degradation (13).

Poly(I:C) is a synthetic dsRNA that has frequently been used as a representative dsRNA ligand in numerous studies (14,15). Upon binding to receptors, poly(I:C) is able to selectively activate NF- κ B, AP-1 and IRF3 signaling pathways depending on different experiment conditions (8,16,17). It has been suggested that poly(I:C) treatment inhibited procollagen expression by autocrine interferon signaling in skin

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fibroblasts (18). However, the signaling pathways involved in poly(I:C)-induced procollagen reduction have yet to be fully elucidated.

The present study identified that treatment of poly(I:C), but not another PAMP, Pam3CSK4, inhibited procollagen expression in cultured human skin fibroblasts. Although treatment of poly(I:C) and Pam3CSK4 induced activations of the mitogen-activated protein kinases (MAPK) and the NF- κ B pathways, only poly(I:C), not Pam3CSK4, induced the activation of IRF3 pathway. By using two different specific inhibitors, it was identified that inhibition of IRF3 signaling pathway relieved poly(I:C)-induced procollagen reduction in skin fibroblasts.

Materials and methods

Reagents. Pam3CSK4 and poly(I:C) were purchased from InvivoGen (San Diego, CA, USA) and Tank binding kinase 1 (TBK1) inhibitor BX795 from Calbiochem (EMD Millipore, Billerica, MA, USA). Another TBK1 inhibitor, SU6668, was purchased from Tocris Bioscience (Bristol, UK). For detecting procollagen protein, monoclonal anti-type I procollagen aminoterminal extension peptide antibody (clone SP1.D8; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) was diluted 1:10 in TBST to be used. Antibody for matrix metalloproteinase-1 (MMP-1) was made by Lab Frontier Co., Ltd. (Seoul, Korea). Antibody for β -actin (cat. no. sc-1616) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies for phosphorylated-extracellular signal-regulated kinase (p-ERK)1/2 (cat. no. 9101), p-c-Jun N-terminal kinases (p-JNK) (cat. no. 9251), p-p38 (cat. no. 9211), p-nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (p-I κ B- α) (cat. no. 9246), and p-IRF3 (cat. no. 4947) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated anti-mouse (cat. no. sc-2031), anti-rabbit (cat. no. sc-2030) or anti-goat (cat. no. sc-2020) IgG (Santa Cruz Biotechnology, Inc.) were used as secondary antibodies. Primary antibodies other than type I procollagen antibody were diluted 1:1,000 and secondary antibodies were diluted 1:10,000 in TBST for western blotting.

Cell culture. From December 2013 to January 2014, three young healthy volunteers provided foreskin samples at the Department of Dermatology, Seoul National University Hospital (Seoul, Korea). Human foreskin fibroblasts from young healthy volunteers were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Geyongsan, Korea) supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 mg/ml), and 10% fetal bovine serum (FBS; Welgene) in a humidified 5% CO₂ atmosphere at 37°C. Skin fibroblasts were used for the experiments at passages 6-8. For chemical treatment, skin fibroblasts were serum-starved for 24 h in DMEM containing 0.1% FBS. This study was approved by the Institutional Review Board at Seoul National University Hospital and conducted according to the Declaration of Helsinki (IRB no. 1101-116-353).

Western blotting. The amounts of procollagen and MMP-1 proteins secreted into culture media were analyzed. β -actin

was detected from equal volume of cell lysate as a loading control for procollagen and MMP-1. For the detection of p-ERK1/2, JNK, p38, I κ B α and IRF3 in cell lysates, cells were washed twice with ice-cold phosphate buffered saline, and then lysed in radioimmunoprecipitation assay (RIPA) buffer (EMD Millipore). Cell lysates were incubated in RIPA buffer for 30 min at 4°C, centrifuged for 15 min at 12,000 x g, 4°C, and then supernatants were collected. Protein concentrations were measured by Bradford protein assay using the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada). Protein (30 μ g) was separated by 10% SDS-PAGE. Separated proteins were transferred onto PVDF membranes, which were then incubated in blocking buffer consisting of 5% skim milk in TBST at room temperature for 30 min. Then, membranes were incubated with the appropriate primary antibodies at 4°C for 16 h and secondary antibodies at room temperature for 1 h. The signals were developed by enhanced chemiluminescence (GE Healthcare Life Sciences, Chalfont, UK). After being probed for procollagen and MMP-1, the same membrane was washed and stained with Coomassie Blue as a loading control. For certain experiments, the relative level of protein bands was quantified by densitometric analysis (ImageJ v1.47; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Experiments were carried out in triplicate. Data are expressed as mean values \pm standard error of the mean. Statistical analysis was performed using the Student's t-test (Microsoft Office Excel 2013; Microsoft Corporation, Redmond, WA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Treatment of poly(I:C), but not Pam3CSK4, reduces procollagen expression in skin fibroblasts. Poly(I:C) is commonly known as a viral PAMP mimic (19). It has been demonstrated that poly(I:C) inhibits procollagen expression in skin fibroblasts (18). To confirm this result in the present study, various doses of poly(I:C) were administered to cultured skin fibroblasts. Following 48 h of treatment, the protein expression of procollagen was analyzed. It was identified that poly(I:C) reduced procollagen expression dose-dependently in skin fibroblasts (Fig. 1A). Next, in order to investigate whether other PAMPs or PAMP mimics can also inhibit procollagen expression levels in skin fibroblasts, skin fibroblasts were treated with a bacterial PAMP mimic, Pam3CSK4, which is commonly used as a ligand for TLR1/2 (16,20). It was observed that treatment with 5 μ g/ml Pam3CSK4 and 20 μ g/ml poly(I:C) induced MMP-1 expression to a similar level. However, only poly(I:C), but not Pam3CSK4, reduced procollagen expression in skin fibroblasts (Fig. 1B). Thus, the data indicated that Pam3CSK4 has no effect on procollagen expression in skin fibroblasts.

Treatment of poly(I:C), but not Pam3CSK4, induces activation of IRF3 in skin fibroblasts. Since only poly(I:C), but not Pam3CSK4, reduced procollagen expression, it was hypothesized that specific signaling pathway(s) could be involved in poly(I:C)-induced procollagen reduction. Thus,

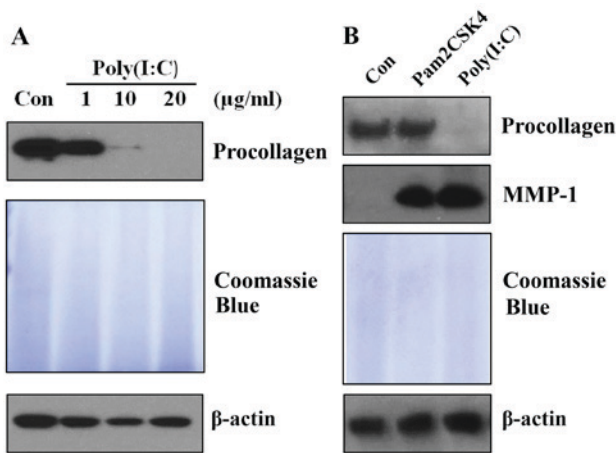


Figure 1. Treatment with poly(I:C), but not Pam3CSK4, reduces procollagen expression in skin fibroblasts. (A) Following serum-starvation, skin fibroblasts were incubated with various concentrations of poly(I:C) for 48 h. (B) Skin fibroblasts were incubated with 5 µg/ml Pam3CSK4 and 20 µg/ml poly(I:C) for 48 h. Protein level of procollagen and MMP-1 in the cell culture medium and β-actin in the cell lysates were analyzed by western blotting, and total protein in the cell culture medium was analyzed by Coomassie Blue staining of the same membrane used to detect procollagen and MMP-1. The data presented are representative of three experiments. Con, control; MMP-1, matrix metalloproteinase-1.

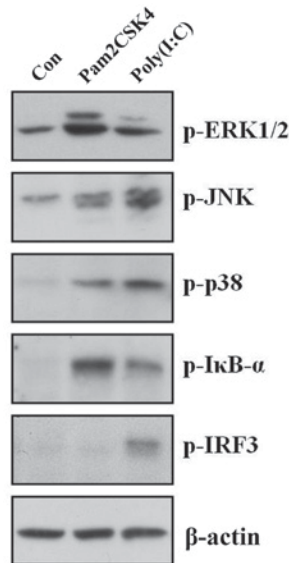


Figure 2. Treatment of poly(I:C), but not Pam3CSK4, induces activation of IRF3 in skin fibroblasts. Cultured skin fibroblasts were stimulated with 5 µg/ml Pam3CSK4 and 20 µg/ml poly(I:C) for 2 h. Cell lysates were fractionated by SDS-PAGE and then protein levels of MAPKs (p-ERK1/2, p-JNK, and p-p38), p-IκB-α and p-IRF3 were analyzed by western blotting. A single representative experiment is presented from three different experiments. Con, control; p, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IκB-α, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α; IRF3, interferon regulatory factor 3.

the activation of several signaling pathways was checked and compared following treatment of poly(I:C) and Pam3CSK4. At 2 h following treatment, it was observed that Pam3CSK4 and poly(I:C) induced phosphorylation of ERK1/2, JNK, p38 and IκB-α. However, only poly(I:C), not Pam3CSK4, induced the phosphorylation of IRF3 (Fig. 2).

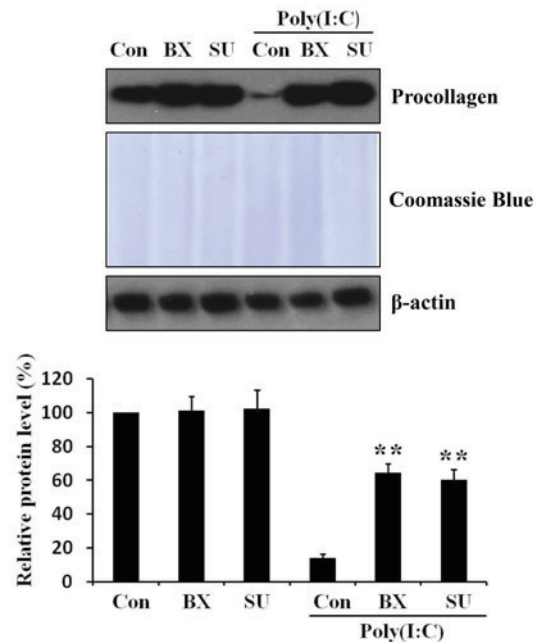


Figure 3. Inhibition of IRF3 signaling pathway mitigates poly(I:C)-induced procollagen reduction in skin fibroblasts. Following serum-starvation, skin fibroblasts were pretreated 1 h with two TBK1 inhibitors: 0.1 µM BX795 and 10 µM SU6668. The cells were then further incubated with 20 µg/ml poly(I:C) for 48 h. The protein level of procollagen in the cell culture medium and β-actin in the cell lysates were analyzed by western blotting, and total protein in the cell culture medium was analyzed by Coomassie Blue staining of the same membrane used to detect procollagen. The data presented are representative of three independent experiments. Values are mean ± standard error of the mean. **P<0.01 vs. poly(I:C)-treated control. IRF3, interferon regulatory factor 3; TBK1, Tank binding kinase 1; Con, control; BX, BX795; SU, SU6668.

Inhibition of IRF3 signaling pathway relieves poly(I:C)-induced procollagen reduction in skin fibroblasts. To investigate whether IRF3 signaling pathway is involved in the reduction of procollagen by poly(I:C) in skin fibroblasts, cells were pretreated for 1 h with two different TBK1 (an upstream molecule of IRF3) inhibitors: BX795 (21) and SU6668 (8). Then poly(I:C) was further added to the cell cultured medium. After 48 h, it was observed that pretreatment of IRF3 pathway inhibitors mitigated poly(I:C)-induced procollagen reduction (Fig. 3). Thus, the results indicated that poly(I:C)-induced procollagen reduction is regulated by IRF3 signaling pathways in skin fibroblasts.

Discussion

dsRNA can act as a form of genetic information carried by certain viruses including retroviruses (22). dsRNA can also exist as an endogenous TLR3 ligand. For example, it was identified that UV-damaged self-noncoding RNA can be detected by TLR3 (23). Poly(I:C) is a synthetic dsRNA which has frequently been used as a representative dsRNA ligand in a number of studies (14,15). The effects of poly(I:C) on collagen expression have been extensively investigated in a number of studies (15,18) and it has been demonstrated that subcutaneous poly(I:C) delivery by osmotic pumps induces epidermal hyperplasia and increased matrix deposition in mice. TGF-β-related genes were elevated in lesional mouse

skin and Farina *et al* (14) concluded that chronic TLR3 stimulation can induce cutaneous fibrosis in mice. However, other studies have provided evidence of the anti-fibrosis effects of poly(I:C) in mice. For example, studies have identified that injection of poly(I:C) ameliorated lung and liver fibrosis in mice (24,25). Conflicting results of poly(I:C) on collagen expression were also identified in several *in vitro* studies (15,18). Sugiura *et al* (15) identified that activation of TLR3 by poly(I:C) augments collagen production in cultured human fetal lung fibroblasts. A NF- κ B-TGF- β 1-dependent pathway was identified to be involved in the processes. It has been suggested (15) that IRF3 signaling pathway is not associated with collagen production by poly(I:C) in fetal lung fibroblasts. Another study (18) demonstrated that poly(I:C) reduces TGF- β -induced collagen expression in cultured skin fibroblasts. It was suggested that poly(I:C) upregulates the expression of Smad7 which inhibits TGF- β -induced collagen production.

The present study identified that treatment with poly(I:C), but not another PAMP, Pam3CSK4, inhibited procollagen expression in skin fibroblasts (Fig. 1). It was hypothesized that poly(I:C) could activate specific factor(s) that mediate(s) procollagen reduction in skin fibroblasts. Indeed, poly(I:C), but not Pam3CSK4, induced activation of IRF3 (Fig. 2). Apart from the IRF3 pathway, the other signaling pathways, including MAPKs and NF- κ B pathways, were activated by treatment with the two PAMPs (Fig. 2). To understand the role of IRF3 in poly(I:C)-induced collagen reduction, two inhibitors were used for the IRF3 pathway (Fig. 3). The data indicated that poly(I:C)-induced procollagen reduction is regulated by IRF3 pathway in skin fibroblasts (Fig. 3). At present, only a small number of studies have investigated the relationship between IRF3 and collagen. It has been suggested that the IRF3 pathway is not associated with increased collagen production by poly(I:C) in cultured fetal lung fibroblasts (15). Recently (26), it was demonstrated that inhibition of IRF3 significantly decreases the expression of type I collagen in human hepatic stellate cells: Indeed, overexpression of IRF3 increases collagen expression. The data appears to conflict with the data of the present study, which demonstrated that activation of IRF3 decreased collagen expression. However, another study by Xu *et al* (27) demonstrated that poly(I:C) suppresses TGF- β -induced Smad3 signaling through activation of IRF3 in human HepG2 hepatoma cells. This result may imply that poly(I:C) can suppress TGF- β -induced collagen expression through IRF3 in human HepG2 hepatoma cells, although the direct evidence was not revealed (27). Thus, the data from the present study and the published results may indicate that IRF3 pathway can be either a suppressor or inducer of collagen production depending on experiment conditions.

In conclusion, it has been demonstrated that the IRF3 signaling pathway is involved in poly(I:C)-induced procollagen reduction in skin fibroblasts. In particular, it is for the first time, to the best of the authors' knowledge, demonstrated that activation of the IRF3 signaling pathway is involved in procollagen reduction in skin fibroblasts. Understanding the relation between IRF3 and collagen may aid the treatment of fibrotic diseases, including scleroderma and liver fibrosis.

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