The expression profile of the toll-like receptor family in scleroderma dermal fibroblasts

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ABSTRACT

Objectives. *The toll-like receptor (TLR) family is thought to be expressed in many cell types in the skin and play a role in various diseases. The expression pattern and role of TLRs in systemic sclerosis (SSc) is to be clarified. We investigated the expression profiles of TLR-related genes in SSc fibroblasts, and tried to clarify their roles in the pathogenesis of this disease.*

Methods. *The expression profile of TLR-related genes was assessed by gene array. Real-time PCR was used to confirm the array result. The protein expression of TLRs and type I collagen was determined by immunoblotting and immunohistochemistry.*

Results. *PCR array revealed that several genes were up- or down-regulated in SSc fibroblasts compared to normal cells. Among them, both mRNA and protein levels of TLR5 and TLR10 were up-regulated in SSc fibroblasts. The transfection of Smad3 siRNA into SSc fibroblasts resulted in the down-regulation of TLR proteins. There was no significant difference in mRNA half-lives of TLR5 and TLR10 between normal and SSc fibroblasts. Immunohistochemical staining revealed that TLRs expression was strongly detected in SSc fibroblasts in vivo. The stimulation of TLR5 signal with flagellin reduced the expression of type I collagen in SSc fibroblasts, but not in normal fibroblasts.*

Conclusion. *TLR5 and TLR10 expression is increased in SSc fibroblasts in vitro and in vivo, probably at transcript level via the TGF-β/Smad3 activation. Furthermore, TLR5 itself may have suppressive effects on collagen expression, and its overexpression in SSc fibroblasts may be the negative feedback against tissue fibrosis.*

Introduction

Systemic sclerosis (SSc) is an autoimmune disorder characterised by tissue fibrosis of the skin and internal organs. Although the pathogenesis of this disease is still unclear, recent techniques such as genome-wide associated study suggested the involvement of various genes in the pathogenesis of SSc (1). Inflammation, autoimmune attack, and vascular damage may lead to the activation of fibroblasts and abnormal accumulation of extracellular matrix (ECM), mainly collagen (2, 3). Thus, abnormal SSc fibroblasts responsible for the fibrosis may develop from cells that have escaped from normal control mechanisms (4, 5).

Although the mechanism of fibroblast activation in SSc is unknown, many of the characteristics found in SSc fibroblasts resemble those in healthy fibroblasts stimulated by transforming growth factor (TGF)-β1 (6, 7). The principal effect of TGF-β1 on fibroblasts is induction of ECM deposition. Fibroblasts from affected SSc skin cultured *in vitro* can produce excessive amounts of collagens (8, 9), indicating that the activation of dermal fibroblasts in SSc may be a result of stimulation by TGF-β1 signalling. This notion is supported by the findings that the phosphorylated levels and DNA-binding activity of Smad3, an intracellular signal molecule of TGF-β1, are constitutively up-regulated in SSc fibroblasts (10). TGF-β1 may also contribute to the activation of Abelson kinase (c-Abl) in SSc fibroblasts: the inhibition of c-Abl by imatinib mesylate treatment is reported to improve the skin sclerosis of SSc (11).

There may be other factors that mediate fibroblast activation. The toll-like receptor (TLR) family is thought to play

a key role in the innate immune system. Mammalians are known to have more than 10 types of TLRs on dendritic cells, monocyte/macrophages and B cells which control immune responses by detecting common molecular motifs, including RNA ligands by TLR3, TLR7 and TLR8, DNA ligands by TLR9 and bacterial cell surface proteins by TLR4 (12). In the skin, TLRs also express on many of cell types including keratinocytes and Langerhans cells in the epidermis, or lymphocytes, macrophages, and fibroblasts in the dermis (13).

The expression levels of TLRs are reported to be up- or down-regulated in various diseases, probably contributing to the pathogenesis. Recently, the up-regulation of TLR2, TLR3, and TLR4 in SSc dermal fibroblasts is reported (14-17), suggesting the possibility that immune dysregulation may contribute to fibroblast activation. In this study, we performed PCR array for TLR-related genes using RNA from SSc dermal fibroblasts, and found that the expression of TLR5 and TLR10 is also up-regulated. Therefore, we tried to clarify the mechanism of the up-regulation of the TLRs in these cell types and their roles in the pathogenesis of this disease.

Patients and methods

Cell culture

Human dermal fibroblasts were obtained by skin biopsy of the affected areas (dorsal forearm) from patients with SSc who had <2 years of skin thickening. Control fibroblasts were obtained by skin biopsies from healthy donors. Primary explant cultures were established in 75-cm^2 culture flasks in modified Eagle's medium (MEM; Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal calf serum (FCS) and Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA) as described previously (18,19). Fibroblast cultures independently isolated from different individuals were maintained as monolayers at 37°C in 95% air, 5% CO_2, and studied between the third and sixth subpassages. Before experiments, cells were serum-starved for 24 hours.

Ethics

Institutional review board approval and written informed consent were obtained before patients and healthy volunteers were recruited into this study according to the Declaration of Helsinki.

RNA isolation and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted with ISOGEN (Nippon Gene, Tokyo, Japan), following the protocol provided by the manufacturer. Then, RNAs were reverse-transcribed into the first strand cDNA using an RT^2 First Strand Kit (SABioscience, Frederick, MD). For RT^2 Profiler PCR Array, the cDNA was mixed with RT^2 Real-Time SYBR GREEN/ROX PCR Master Mix (SABioscience) and the mixture was added into a 96-well RT^2 Profiler PCR Array (Human Toll-Like Receptor Signalling Pathway; SABioscience) that included primer pairs for 84 TLR-related genes. PCR was performed on Takara Thermal Cycler Dice (TP800)® following the manufacturer's protocol. The threshold cycle (Ct) for each gene was extracted using Thermal Cycler Dice Real Time System ver2.10B (Takara Bio Inc., Shiga, Japan). The raw Ct was normalised using the values of housekeeping genes. For quantitative real-time PCR, primers and templates were mixed with the SYBR Premix Ex TaqII (Takara Bio Inc.). Primer sets for 13 TLR-related genes including TLR5, TLR10 as well as GAPDH were purchased from Takara. DNA was amplified for 50 cycles of denaturation for 15s at 95°C, annealing for 35s at 60°C and extension for 30s at 72°C. Transcript levels of each gene of interest were normalised to GAPDH.

Cell lysis and Immunoblotting

Fibroblasts were washed with cold phosphate-buffered saline twice and lysed in Denaturing Cell Extraction Buffer (BIOSOURCE, Camarillo, CA). Aliquots of cell lysates (normalised for protein concentrations) were subjected to electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to PVDF membranes, which were blocked in blocking One P buffer (Nacalai Tesque, Kyoto, Japan) for 1 hour and incubated overnight at 4°C with primary antibody for TLR5 (Imgenex corporation, San Diego, CA), TLR10 (Novus Biologicals, Littleton, CO), type I collagen (Southern Biotech, Birminghan, AL) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were washed with Tris-buffered saline and 0.1%Tween 20 (TBS-T), probed with HRP-conjugated secondary antibody for 1 hour, and then washed with TBS-T again. The detection was performed using ECL system (Amersham Biosciences, Arlington Heights, IL) according to the manufacturer's recommendations.

Transient transfection

SiRNA against Smad3 was purchased from Santa Cruz Biotechnology. Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) was used as a transfection reagent. For reverse transfection, control siRNA or Smad3 siRNA (30nM) mixed with transfection reagent were added when fibroblasts were plated, followed by the incubation for 96 hours at 37°C in 5% CO_2.

Immunohistochemical staining

Skin specimens were obtained from 5 SSc patients and 5 control subjects. Control and patient samples were collected and processed immediately after resection in parallel.

Deparaffinised sections were retrieved by incubation with citrate buffer pH6 for 5 minutes with autoclave treatment. Endogenous peroxidase activity was inhibited, after which sections were blocked with 5% normal goat serum for 60 min and then reacted with the antibodies for TLR5 or TLR10 (1:100) overnight at 4°C. After excess antibody was washed out with PBS, samples were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (Nichirei, Tokyo, Japan) for 30 minutes. The reaction was visualised by the diaminobenzidine substrate system (Dojin, Kumamoto, Japan). Slides were lightly counterstained with Mayer's haematoxylin, and examined under a light microscope (BX50, OLYMPUS, Tokyo, Japan).

Statistical analysis

Statistical analysis was carried out with the Mann-Whitney test for the comparison of medians, and Fisher's exact

probability test for the analysis of frequency. P-values less than 0.05 were considered significant.

Results

Expression profile of TLR-related genes in normal and SSc fibroblasts

As an initial experiment, to determine which genes involved in human TLR signalling pathway were up- or down-regulated in SSc, we performed PCR array analysis, consisting of 84 TLR-related genes (Table I). The gene expression profile in 5 dermal fibroblasts derived from the involved skin of SSc patients was compared with that of normal fibroblasts. When 2-cycle difference (=4-fold difference in ΔΔCT method) was considered meaningful, several genes were up- or down-regulated in SSc fibroblasts than normal cells. Among them, 13 genes (BTK, CLEC4E, CSF3, CD180, LY86, NFRKB, PELI1, SIGIRR, ECSIT, TNF, TLR5, TLR9 and TRL10) have not previously investigated in SSc. Real-time PCR using specific primer for each gene with increased number of samples (n=8) revealed that only the expression of TLR5 and TLR10 were still significantly higher in SSc fibroblasts than in normal fibroblasts (Fig. 1a and b). The difference in the expression of other genes became insignificant by increasing sample number; for example, although SIGGER expression was increased in SSc fibroblasts by the array, but it was down-regulated in SSc fibroblasts by real-time PCR albeit insignificant, probably due to the variation among samples.

We also found that the expression of TLR5 in the array were up-regulated in TGF-β-treated normal fibroblasts (7.01-fold difference compared with untreated normal fibroblasts), as well as in SSc fibroblasts (47.2-fold difference) (Table I). Also, TLR10 was increased in both TGF-β-treated normal fibroblasts and SSc fibroblasts. Because many characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by TGF-β as described above, we expected that genes up- or down-regulated both in normal fibroblasts treated with TGF-β and in SSc fibroblasts play a role in the pathogenesis of SSc. Thus, we focused on TLR5 and TLR10 in the following experiments.

Table I. Summary of TLR-related gene expression in normal and SSc fibroblasts by PCR analysis.

Gene name	NS	NS+TGFβ	SSc	Gene name	NS	NS+TGFβ	SSc
BTK	1.00	0.58	8.46	MAP3K1	1.00	0.55	1.48
CASP8	1.00	0.45	0.26	MAP3K7	1.00	0.66	0.66
CCL2	1.00	3.18	0.80	MAP3K7IP1	1.00	6.02	1.42
CD14	1.00	58.89	224.41	MAP4K4	1.00	0.88	0.87
CD80	1.00	0.00	10.70	MAPK8	1.00	1.34	0.98
CD86	0.00	1.00	0.00	MAPK8IP3	1.00	0.74	0.69
CHUK	1.00	0.99	0.66	MYD88	1.00	1.64	0.62
CLEC4E	0.00	1.00	5.78	NFKB1	1.00	1.45	1.23
CSF2	1.00	1.31	3.10	NFKB2	1.00	0.24	0.00
CSF3	1.00	7.26	162.02	NFKBIA	1.00	0.46	0.74
CXCL10	0.00	1.00	4.72	NFKBIL1	1.00	1.69	2.55
EIF2AK2	1.00	0.72	0.59	NFRKB	1.00	1.77	0.25
ELK1	1.00	0.80	1.30	NR2C2	1.00	0.55	2.00
FADD	1.00	1.29	0.65	PELI1	1.00	9.51	7.78
FOS	1.00	2.01	0.75	PPARA	1.00	0.57	0.75
HMGB1	1.00	0.57	0.24	PRKRA	1.00	1.25	0.97
HRAS	1.00	1.28	1.21	PTGS2	1.00	3.32	0.47
HSPA1A	1.00	1.42	1.72	REL	1.00	3.20	0.33
HSPD1	1.00	2.46	1.97	RELA	1.00	1.45	1.34
IFNA1	1.00	6.50	73.52	RIPK2	1.00	0.78	0.64
IFNB1	0.00	1.00	0.00	SARM1	1.00	0.88	0.97
IFNG	0.00	1.00	0.19	SIGIRR	0.00	0.00	1.00
IKBKB	1.00	1.42	0.63	ECSIT	1.00	31.12	3743.05
IL10	1.00	0.62	10.06	TBK1	1.00	0.50	0.33
IL12A	1.00	1.66	1.32	TICAM2	1.00	0.57	0.47
IL1A	1.00	0.59	0.67	TIRAP	1.00	1.52	1.67
IL1B	1.00	4.29	23.10	TLR1	1.00	1.34	3.05
IL2	0.00	1.00	1.17	TLR10	0.00	1.00	0.03
IL6	1.00	1.93	1.25	TLR2	1.00	0.00	1.11
IL8	1.00	2.27	0.36	TLR3	1.00	0.84	0.34
IRAK1	1.00	2.35	0.64	TLR4	1.00	2.13	47.18
IRAK2	1.00	0.75	0.52	TLR5	1.00	7.01	47.18
IRF1	1.00	0.20	0.40	TLR6	1.00	2.31	0.98
IRF3	1.00	1.23	0.87	TLR7	0.00	1.00	0.00
JUN	1.00	1.16	0.76	TLR8	0.00	1.00	0.00
LTA	0.00	1.00	7.21	TRL9	1.00	36.76	0.00
CD180	0.00	0.00	1.00	TNF	1.00	1.61	1.08
LY86	0.00	0.00	1.00	TNFRSF1A	1.00	0.87	0.67
LY96	1.00	0.55	0.74	TOLLIP	1.00	4.59	2.75
MAP2K3	1.00	0.67	1.64	TRAF6	1.00	1.40	1.57
MAP3K4	1.00	0.90	0.97	TICAM1	1.00	1.06	0.59

A mixture of equal amounts of total RNAs from 5 normal fibroblasts (NS), 5 normal fibroblasts stimulated with TGF-β for 12 hours (NS+TGFβ) or 5 SSc fibroblasts was prepared, and TLR-related gene expression profile in each cell group was evaluated using PCR Array. The fold-change was calculated as 1/2 (raw Ct of each miRNA – mean Ct of small RNA housekeeping genes). The mean fold-change of each miRNA is shown. The values in the normal skin samples were set at 1. When the value of normal skin was 0, the mean value of another sample was set at 1.

TLR5/10 expression is increased at the transcriptional level in SSc dermal fibroblasts

We then determined whether the protein expression of TLR5 and TLR10 was also up-regulated both in normal fibroblasts treated with TGF-β and in SSc fibroblasts. As expected, the protein expression of TLR5 and TLR10 in normal fibroblasts was induced by the ectopic stimulation of TGF-β (Fig. 2a). In addition, we found constitutive up-regulation of TLR5 and TLR10 in SSc fibroblasts compared to normal fibroblasts (Fig. 2b). Therefore, the increase of TLR5 and TLR10 mRNA expression in normal fibroblasts treated with TGF-β and in SSc fibroblasts, which was shown by the PCR array, was thought to result in the overexpression of the TLR5 and TLR10 proteins.

Thus, to examine the possibility that

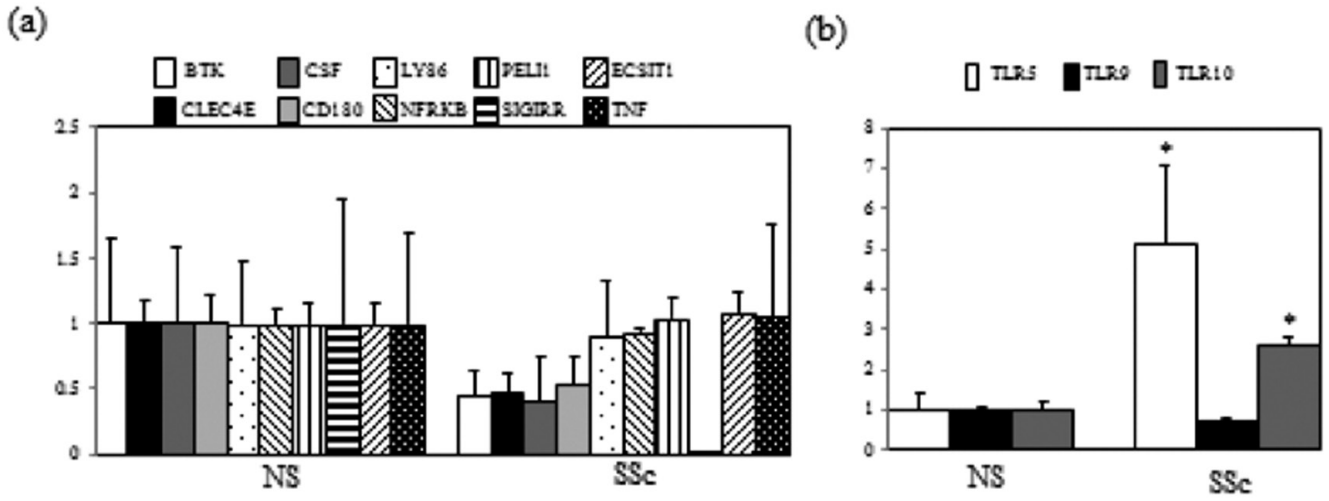

Fig. 1. Expression profiles of TLR-related genes. (**a**, **b**) The relative gene expression levels in 8 normal fibroblasts (NS) and 8 scleroderma fibroblasts (SSc) determined by real-time PCR. Error bars represent standard error. *p<0.05 compared with the values in NS (1.0).

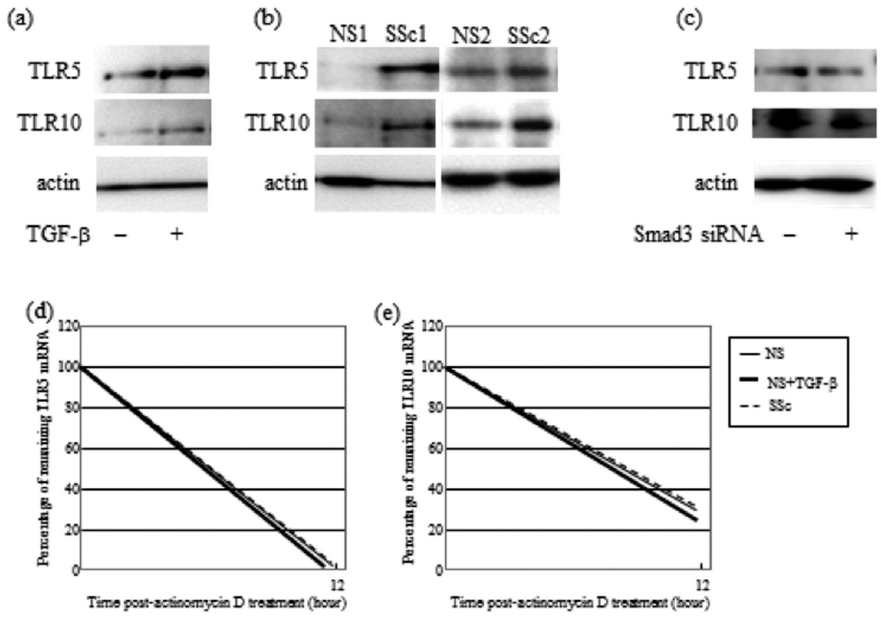

Fig. 2. TLR5/10 overexpression *in vitro*. (**a**) Normal fibroblasts were incubated in the presence or absence of TGF-β (2ng/ml) for 24 hours. Cell lysates were subjected to immunoblotting with antibody for TLR5 or TLR10. The same membrane was reprobed with anti-β-actin antibody as a loading control.
(**b**) Normal (NS) and scleroderma (SSc) fibroblasts were cultured independently under the same conditions until they were confluent, serum-starved for 24 hours, and harvested. Cell lysates were subjected to immunoblotting as described in Figure 1a.
(**c**) Scleroderma fibroblast at a density of 2×10^4 cells/well in twenty four-well culture plates were transfected with control siRNA or Smad3 siRNA for 96 hours. Cell lysates were subjected to immunoblotting as described in Figure 1a.
(**d**, **e**) Normal fibroblasts (NS, black lines), normal fibroblasts stimulated with TGF-β for 12 hours (NS+ TGF-β, bold lines) and scleroderma fibroblasts (SSc, dotted lines) were incubated with 5μg/ml of actinomycin D for 12 hours. mRNA expression of TLR5 (**d**) and TLR10 (**e**) was analysed by real-time PCR. The mRNA levels in cells before actinomycin D treatment were set at 100.

the up-regulation of TLR5 and TLR10 in SSc fibroblasts is due to the stimulation of endogenous TGF-β signalling, we determined the effect of the TGF-β blockade by Smad3 siRNA on TLR expression. The transfection of Smad3 siRNA into SSc fibroblasts resulted in the down-regulation of TLR proteins (Fig. 2c). Therefore, the up-regulated mRNA and protein expression of TLR5 and TLR10 in SSc fibroblasts is likely to be caused by the TGF-β activation in these cells.

The steady-state level of mRNA can be affected by the level of gene transcription and/or the stability of mRNA. To determine whether the increase of TLR5 and TLR10 mRNA in SSc fibroblasts takes place at the transcriptional or post-transcriptional level, de novo mRNA synthesis was blocked by actinomycin D, a RNA synthesis inhibitor, in normal and SSc fibroblasts in the presence or absence of TGF-β (Fig. 2d and e). There were no significant differences in mRNA decrease rate of TLR5 and TLR10 between normal and SSc fibroblasts in the presence or absence of TGF-β, indicating that the mRNA stability of TLR5 and TLR10 is not changed in SSc fibroblasts as well as TGF-β–treated normal fibroblasts. Taken together, the expression of TLR5 and TLR10 was likely to be increased at a transcriptional level in TGF-β-treated normal fibroblasts and SSc dermal fibroblasts.

Immunohistochemical staining using paraffin-embedded sections revealed that TLR5 and TLR10 expression was hardly detected in normal dermal fibroblasts, but was strongly detected in SSc fibroblasts between the thickened collagen bundles (Fig. 3). The result was consistent in 5 normal skin and 5 SSc skin. Thus, expression of TLR5 and TLR10 is increased in SSc both *in vivo* and *in vitro*.

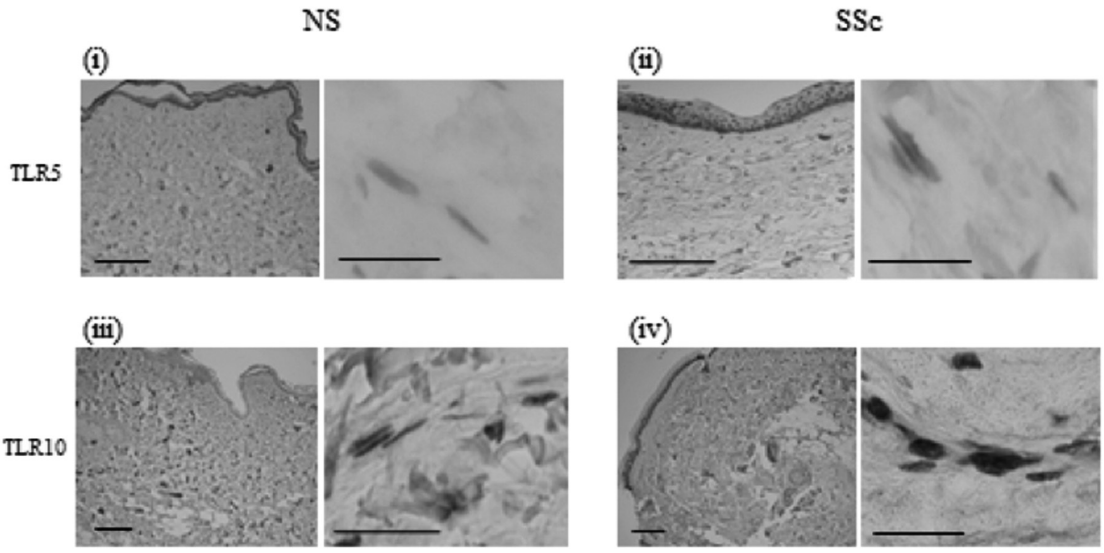

Fig. 3. *In vivo* expression of TLR5/10 in scleroderma.
Immunostaining of TLR5 and TLR10 in the normal skin (NS: i, iii) and scleroderma skin (SSc; ii, iv). Tissue sections were stained with antibodies against TLR5 or TLR10 as described in Materials and Methods. One experiment representative of five independent experiments is shown. Scale bar = 250μm (left), 20μm (right).

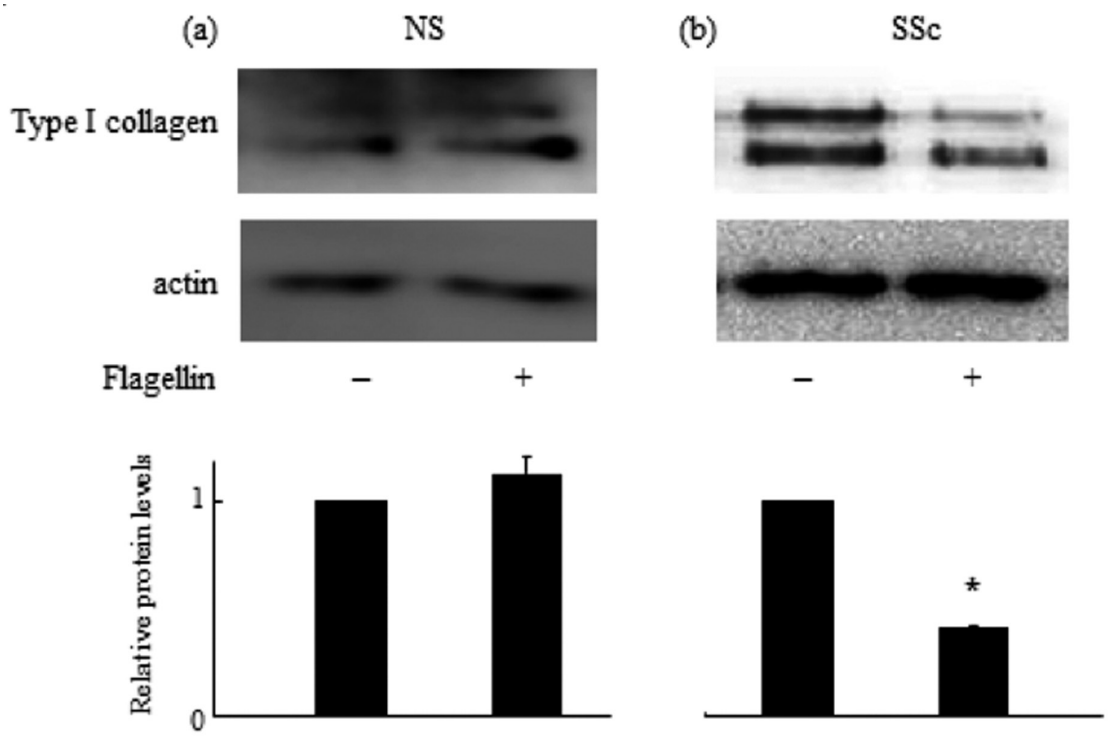

Fig. 4. TLR5 signalling regulates type I collagen expression *in vitro*.
Normal (NS, a) and scleroderma (SSc, b) fibroblasts at a density of 2×10^4 cells/well in twenty four-well culture plates were stimulated with control agonist or flagellin for 48 hours. Cell lysates were subjected to immunoblotting with antibody for type I collagen. The same membrane was reprobed with anti-β-actin antibody as a loading control. Levels of TLR5 protein quantitated by scanning densitometry and corrected for the levels of β-actin in the same samples are shown relative to the levels in control cells (1.0). *$p<0.05$.

The function of TLR5 in SSc fibroblasts

Next, we tried to examine the role of TLR5 in SSc fibroblasts using flagellin, the specific agonist of the TLR5. Treatment of normal fibroblasts with flagellin (0.1μg/ml, Enzo Life Science) for 48 hours did not affect the expression of type I collagen (Fig. 4a). On the other hand, the type I collagen expression was significantly reduced by the addition of flagellin in SSc fibroblasts (Fig. 4b). Accordingly, TLR5 activation may have negative effect on the collagen expression only in SSc fibroblasts.

Discussion

This study demonstrated the expression and role of TLR5 and TLR10 in SSc by three major findings. First, we showed the expression profile of genes involved in human TLR signalling pathway in SSc fibroblasts, and identified that TLR5 and TLR10 is overexpressed specifically in SSc fibroblasts compared with normal fibroblasts. TLR5 recognises bacterial flagellin (20, 21). On the other hand, the exact function of TLR10 remains largely unexplored due to the fact that it is not present in mice. Although the expression of TLR5 and TLR10 has been found in breast and prostate cancer (22-24), our study is the first to demonstrate the expression of TLR5 and TLR10 in autoimmune diseases including SSc.

Next, we found that up-regulation of TLR5 and TLR10 in SSc fibroblasts was at a transcriptional level. In SSc

fibroblasts, various molecules including collagen, tenascin and MMP-1 are controlled by TGF-β/Smad3 signalling (25-27). Our study suggests the possibility that up-regulation of TLR5 or TLR10 in SSc fibroblasts is also induced by TGF-β/Smad3 activation.

Lastly, we investigated the effects of TLR signalling on the collagen expression in SSc fibroblasts. Since TLR5 activation inhibited the collagen expression only in SSc fibroblasts, TLR5 itself may have suppressive effects on collagen expression. Thus, TLR5 overexpression in SSc fibroblasts may be one of the negative feedback mechanisms for the collagen overexpression in SSc fibroblasts. The effect of flagellin was not seen in normal fibroblasts, probably because the expression level of TLR5 was low. On the other hand, at present, ligand of TLR10 is not identified and not commercially available, but the function of TLR10 in SSc needs to be clarified in the future.

In summary, TLR5 and TLR10 may play some roles in the pathogenesis of SSc. Investigation of the regulatory mechanisms of fibrosis by TLRs may lead to new therapeutic approaches of this disease.

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