

ORIGINAL ARTICLE

Involvement of interleukin-17A-induced expression of heat shock protein 47 in intestinal fibrosis in Crohn's disease

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ABSTRACT

Objective Intestinal fibrosis is a clinically important issue in Crohn's disease (CD). Heat shock protein (HSP) 47 is a collagen-specific molecular chaperone involved in fibrotic diseases. The molecular mechanisms of HSP47 induction in intestinal fibrosis related to CD, however, remain unclear. Here we investigated the role of interleukin (IL)-17A-induced HSP47 expression in intestinal fibrosis in CD.

Design Expressions of HSP47 and IL-17A in the intestinal tissues of patients with IBD were determined. HSP47 and collagen I expressions were assessed in intestinal subepithelial myofibroblasts (ISEMFs) isolated from patients with IBD and CCD-18Co cells treated with IL-17A. We examined the role of HSP47 in IL-17A-induced collagen I expression by administration of short hairpin RNA (shRNA) to HSP47 and investigated signalling pathways of IL-17A-induced HSP47 expression using specific inhibitors in CCD-18Co cells.

Results Gene expressions of *HSP47* and *IL-17A* were significantly elevated in the intestinal tissues of patients with active CD. Immunohistochemistry revealed HSP47 was expressed in α -smooth muscle actin (α -SMA)-positive cells and the number of HSP47-positive cells was significantly increased in the intestinal tissues of patients with active CD. IL-17A enhanced HSP47 and collagen I expressions in ISEMFs and CCD-18Co cells. Knockdown of HSP47 in these cells resulted in the inhibition of IL-17A-induced collagen I expression, and analysis of IL-17A signalling pathways revealed the involvement of c-Jun N-terminal kinase in IL-17A-induced HSP47 expression.

Conclusions IL-17A-induced HSP47 expression is involved in collagen I expression in ISEMFs, which might contribute to intestinal fibrosis in CD.

INTRODUCTION

IBD, including Crohn's disease (CD) and UC, is characterised by chronic relapsing intestinal inflammation of unknown aetiology.¹ Chronic intestinal inflammation results in intestinal fibrosis, which manifests as strictures in CD.² More than one-third of patients with CD develop a distinct fibrostenosing phenotype that results in recurrent intestinal stricture formation.³ Strictures are the endproduct of chronic transmural inflammation associated with an excessive and abnormal deposition of extracellular matrix consisting mainly of

Significance of this study

What is already known on this subject?

- The mechanisms of intestinal fibrosis related to Crohn's disease (CD) remain unclear.
- Heat shock protein (HSP) 47 is a glycoprotein that binds collagen in fibroblasts and plays a pivotal role as a collagen-specific chaperone.
- Expression of HSP47 in the fibroblasts was reported to be associated with several fibrotic diseases.
- Interleukin (IL)-17A is profoundly involved in the pathogenesis of IBD.

What are the new findings?

- Expressions of HSP47 and IL-17A were upregulated in inflamed intestinal tissues of patients with CD.
- IL-17A alone induced HSP47 and collagen I expressions in human intestinal subepithelial myofibroblasts (ISEMFs) and CCD-18Co cells.
- Knockdown of HSP47 in CCD-18Co cells and ISEMFs suppressed IL-17A-induced collagen I expression.

How might it impact on clinical practice in the foreseeable future?

- Based on the present study, IL-17A-induced HSP47 is involved in collagen I expression in ISEMFs, which could contribute to intestinal fibrosis in CD.

collagen I, which eventually disturbs wound healing.^{4 5} Abnormal contraction of collagen I leads to scar formation, tissue distortion and ultimately intestinal obstruction.⁶ Development of fibrostenotic strictures leading to intestinal obstruction is a frequent complication of CD, but it is rarely seen in UC.^{7 8} The pathophysiological mechanisms underlying fibrotic strictures in CD are poorly understood.

Heat shock protein (HSP) 47 is a basic glycoprotein that binds collagen in chick embryo fibroblasts and has a pivotal role as a collagen-specific molecular chaperone.^{9 10} HSP47 is mostly present in the endoplasmic reticulum of collagen-producing cells

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Inflammatory bowel disease

and is involved in collagen maturation reflected by the processing and secretion of procollagen.¹¹ HSP47 is predominantly expressed in the fibroblasts of various organs, and its expression is increased in several fibrotic diseases, such as connective tissue diseases, liver cirrhosis and pulmonary fibrosis.^{12–14} We previously demonstrated that HSP47 was critically involved in collagen production in the colon of murine experimental colitis, and serum levels of HSP47 were significantly higher in patients with CD than in those with UC and normal subjects.^{15–16} The molecular mechanisms of HSP47 induction in intestinal fibrosis related to human IBD, however, remain unclear.

An emerging hypothesis for the pathogenesis of fibrotic disorders is that there is an imbalance of several cytokines in response to tissue injury.¹⁷ In general, T helper (Th) 2 cytokines such as interleukin (IL)-4, IL-6 and IL-13 stimulate the synthesis of collagen by human fibroblasts, whereas Th1 cytokines such as interferon (IFN)- γ suppress collagen productions.^{18–20} Th17 cells, which were discovered in 2005, are a third helper T cell subset that can produce IL-17A, IL-17F, IL-21 and IL-22.^{21–22} IL-17 signalling pathways are implicated in the pathogenesis of autoimmune and inflammatory diseases, including rheumatoid arthritis, allergic asthma, multiple sclerosis and IBD.^{22–23} Of particular interest is that Th17 cells are thought to be involved in not only tissue inflammation but also fibrosis. Indeed, recent studies demonstrated that IL-17A signalling played an important role in the fibrogenesis of liver, skin and lung.^{24–26} The association between IL-17A and HSP47 expression, however, has not been investigated.

The aims of the present study were (1) to elucidate the role of HSP47 in intestinal fibrosis of IBD and (2) to characterise the molecular mechanisms underlying IL-17-mediated induction of HSP47 expression in not only the human intestinal myofibroblast cell line, CCD-18Co cells but also intestinal subepithelial myofibroblasts (ISEMFs) isolated from patients with IBD.

MATERIALS AND METHODS

Human tissue samples

Intestinal tissue samples obtained from patients with CD, UC and normal subjects undergoing surgical resection of the intestine were studied. IBD tissue samples defined as inflamed/active were histologically characterised by unequivocally increased mononuclear infiltrates with active lesions, such as crypt abscess/cryptitis, erosion or ulceration, and tissue samples defined as non-inflamed/inactive histologically had no such lesions and no or only a marginal increase in the content of mononuclear cells as compared with normal subjects. Normal tissue samples were obtained from the healthy tissue (at least 7 cm away from neoplastic tissue) of patients undergoing surgery for colon cancer. A total of 149 human tissue samples were obtained from 78 patients with IBD (35 with CD, 43 with UC) and 11 normal subjects undergoing surgical resection of the intestine in this study. In patients with CD, 35 samples were obtained from inflamed areas and 28 samples from non-inflamed areas. In patients with UC, 43 samples were obtained from inflamed areas and 32 samples were obtained from non-inflamed areas. The characteristics of patients and normal subjects are summarised in table 1. Informed consent was obtained from all patients and normal subjects, and the experimental design using these samples was approved by the Kyoto University Hospital Ethics Committee.

Cell cultures

The human intestinal myofibroblast cell line CCD-18Co was obtained from ATCC (Rockville, Maryland, USA) and cultured

Table 1 Characteristics of patients and normal subjects

	CD (n=35)	UC (n=43)	Normal subjects (n=11)
Sex (male/female)	28/7	25/18	4/7
Age (median, years)	34	41	66
Disease duration (median, months)	144	60	
Disease location (n)	Ileal only (13) Ileocolonic (15) Colonic only (7)	Paccolitis (12) Left side colitis (31) Proctitis (0)	
Lesions (n)			
Inflamed/active	35	43	
Non-inflamed/inactive	28	32	
Medications (%)			
None	43	23	
Steroids	37	65	
Thiopurines	20	30	
Anti-TNF	37	0	

CD, Crohn's disease; TNF, tumour necrosis factor.

in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin (50 U/mL)/streptomycin (50 μ g/mL). Before stimulation with each cytokine, 1.0×10^6 cells were plated in a 10 cm dish and starved for 24 h in 0% FBS/DMEM.

Isolation and culture of human ISEMFs

Primary human ISEMFs were prepared according to the method reported by Mahida *et al.*²⁷ In 78 patients with IBD and 11 normal subjects, ISEMFs were isolated from the intestines of patients with CD (inflamed/active, n=7; non-inflamed/inactive, n=3), UC (inflamed/active, n=6; non-inflamed/inactive, n=3) and normal subjects (n=3). Cells were cultured in DMEM containing 10% FBS and penicillin (50 U/mL)/streptomycin (50 μ g/mL). Studies were performed on passage-3 to passage-6 ISEMFs. Before stimulation with each cytokine, 3.0×10^5 ISEMFs were plated in a 6 cm dish and starved for 24 h in 0% FBS/DMEM.

Collagen assay

In 78 patients with IBD and 11 normal subjects, tissue samples were obtained from the intestines of patients with CD (inflamed/active, n=10; non-inflamed/inactive, n=10), UC (inflamed/active, n=10; non-inflamed/inactive, n=10) and normal subjects (n=5) for collagen assay. Human intestinal tissues were minced and homogenised in 0.5 M acetic acid containing 1 mg pepsin (at a concentration of 10 mg of tissue/5 mL acetic acid solution). The resulting mixture was incubated at 4°C for 24 h with stirring. Total soluble collagen I content of the mixture was determined with a Sircol Collagen Assay Kit (Biocolor, Carrickfergus, Northern Ireland, UK) according to the manufacturer's instructions. Acid-soluble collagen I supplied with the kit was used to generate a standard curve. The collagen content was normalised by the wet weight of the intestines.^{28–29}

Quantitative analysis of gene expression

Messenger RNA (mRNA) expression was assessed using human tissue samples, human ISEMFs and CCD-18Co cells. Extraction of total RNA, generation of complementary DNA (cDNA) and real-time reverse transcription-polymerase chain reaction

(RT-PCR) were performed as described previously.³⁰ Intestinal tissue samples for gene analysis were obtained from 78 IBD patients with CD (inflamed/active, n=35; non-inflamed/inactive, n=28), UC (inflamed/active, n=43; non-inflamed/inactive, n=32) and normal subjects (n=11). The primer sets of *human HSP47*, *collagen I $\alpha 1$ chain*, *IL-17A*, *IL-17F*, *tissue growth factor (TGF)- $\beta 1$* , *IL-22*, *IL-17 receptor A (RA)*, *IL-17 receptor C (RC)*, and *glyceraldehyde phosphate dehydrogenase (GAPDH)* are described in online supplementary table S1.

Immunohistochemistry

In 78 patients with IBD and 11 normal subjects, tissue samples were obtained from the intestines of patients with CD (inflamed/active, n=10; non-inflamed/inactive, n=10), UC (inflamed/active, n=10; non-inflamed/inactive, n=10) and normal subjects (n=5) for immunohistochemistry. Samples were fixed in 10% buffered

formalin, dehydrated in ethanol and embedded in paraffin. For double immunofluorescent staining, sections (4 μ m) were treated by boiling in citrate buffer (pH 6.0) at 120°C for 15 min in an autoclave for antigen retrieval. After blocking with 10% bovine serum albumin (BSA) in phosphate-buffered (PBS), sections were incubated with each diluted primary antibody at 4°C overnight. Dilutions in PBS with 1% BSA were prepared with mouse anti-human HSP47 monoclonal antibody (1:100 dilution; Stressgen Biotechnologies, Victoria, British Columbia, Canada) and rabbit anti-human α -smooth muscle actin (SMA) monoclonal antibody (1:100 dilution; Abcam, Tokyo, Japan). After washing with PBS, the sections were incubated for 30 min with Alexa Fluor 488 goat antimouse IgG and Alexa Fluor 594 goat antirabbit IgG (1:500 dilution; Invitrogen, Carlsbad, California, USA) as a secondary antibody. Immunofluorescent-stained slices were photographed with a fluorescent scope (Biozero BZ-8000, Keyence,

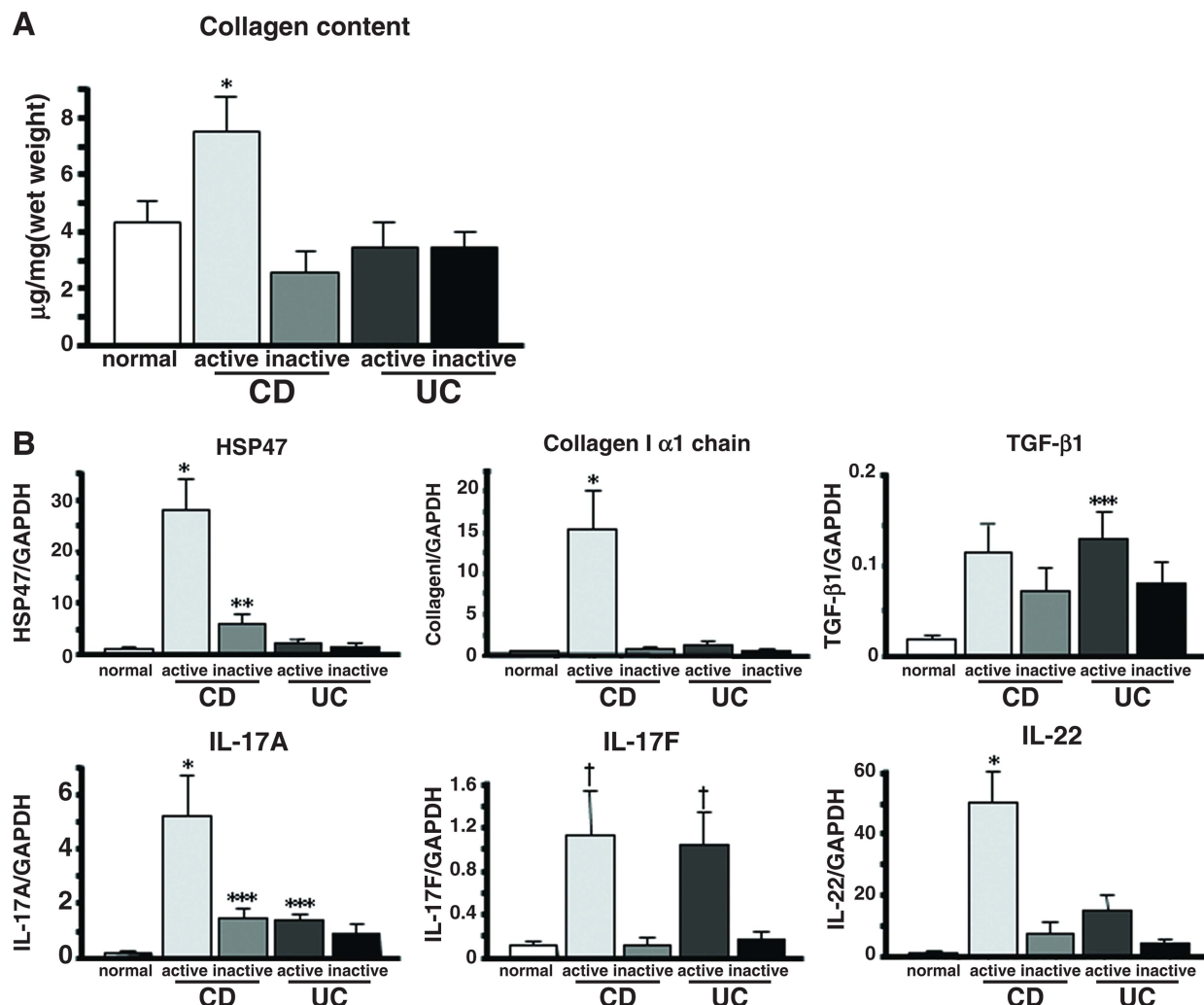


Figure 1 Analysis of collagen content and gene expression in the human intestinal tissues. (A) Collagen content was quantified by measuring pepsin-soluble collagen (collagen assay) in intestinal tissues. Tissue samples were obtained from the intestines of patients with CD (inflamed/active, n=10; non-inflamed/inactive, n=10), UC (inflamed/active, n=10; non-inflamed/inactive, n=10) and normal subjects (n=5). (B) Expression of genes encoding several proteins, including cytokines, in the human intestinal tissues. Gene expressions of *HSP47*, *collagen I $\alpha 1$ chain*, *TGF- $\beta 1$* , *IL-17A*, *IL-17F* and *IL-22* were determined by quantitative real-time RT-PCR. Gene expression of each target molecule was normalised by *GAPDH*. Intestinal tissue samples for gene analysis were obtained from 78 IBD patients with CD (inflamed/active, n=35; non-inflamed/inactive, n=28), UC (inflamed/active, n=43; non-inflamed/inactive, n=32) and normal subjects (n=11). (A, B) Data are expressed as mean $\pm$ SD of each group. * p <0.05 compared with inactive CD, active UC, inactive UC and normal subjects; ** p <0.05 compared with inactive CD, inactive UC and normal subjects; *** p <0.05 compared with normal subjects; † p <0.05 compared with inactive CD, inactive UC and normal subjects by one-way ANOVA with a Bonferroni correction. ANOVA, analysis of variance; CD, Crohn's disease; HSP, heat shock protein.

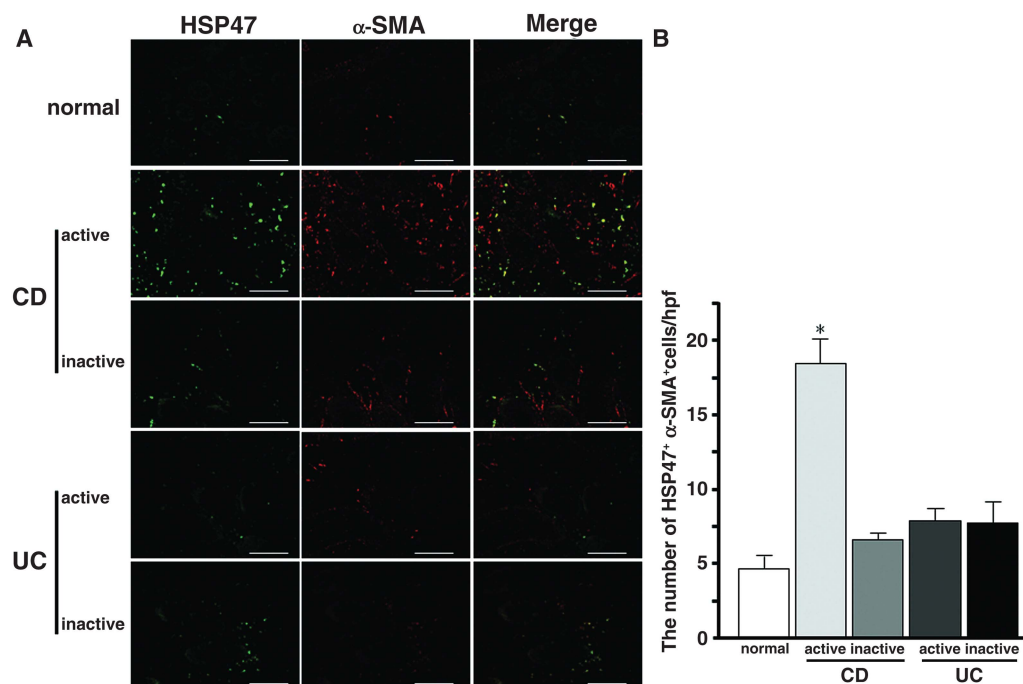


Figure 2 Immunohistochemical detection of HSP47-positive cells in the human intestinal tissues from IBD patients and normal subjects. (A) Double-immunofluorescent staining with anti-HSP47 plus anti- α -SMA was performed in the human intestinal tissues. Tissue samples were obtained from the intestines of patients with CD (inflamed/active, n=10; non-inflamed/inactive, n=10), UC (inflamed/active, n=10; non-inflamed/inactive, n=10) and normal subjects (n=5). (B) The number of merged cells for HSP47 and α -SMA per HPFs was counted at 400 $\times$ magnification. The mean count of five HPFs from one slide was used. (A) Data are representative images of each group. Bars indicate 100 μ m. (B) Data are expressed as mean $\pm$ SD of each group. *p<0.05 compared with inactive CD, active UC, inactive UC and normal subjects by one-way ANOVA with a Bonferroni correction. ANOVA, analysis of variance; CD, Crohn's disease; HPF, high-power field; HSP, heat shock protein; SMA, smooth muscle actin.

Osaka, Japan). Corresponding areas of sections for HSP47 were marked and high-power fields (HPFs) were counted at 400 $\times$ magnification. The mean count of five HPFs from one slide was used. In addition, to identify IL-17A-producing cells in the intestinal tissues of patients with active CD, double-immunofluorescent staining with rabbit antihuman IL-17A polyclonal antibody (1:100 dilution; Abcam) plus mouse antihuman CD3 (1:25 dilution; Abcam), CD68 (1:400 dilution; Abcam) or α -SMA (1:100 dilution; Abcam) was performed as described above. For the secondary antibody, Alexa Fluor 488 goat antirabbit IgG and Alexa Fluor 594 goat antimouse IgG (1:500 dilution; Invitrogen) were used.

Effect of cytokines on HSP47 induction in CCD-18Co cells and human ISEMFs

To examine the effect of several cytokines on the induction of HSP47 expression in human intestinal myofibroblasts cell line, CCD-18Co cells and isolated human ISEMFs were harvested after stimulation with recombinant human IL-17A, IL-17C, IL-17E, IL-22 and TGF- β 1 (R&D Systems, Minneapolis, Minnesota, USA).

HSP47 mRNA interference (RNAi) experiments

To knockdown HSP47 expression in CCD-18Co cells and ISEMFs, we used the Mission short hairpin RNA (shRNA) bacterial glycerol stocks against the human HSP47 (Sigma Aldrich, St. Louis, Missouri, USA). This stock consisted of individual shRNA lentiviral vectors in pLKO. 1-puro plasmids against different target sites of HSP47 (with clone IDs NM_001235. x-952s1c1, -1295s1c1). A 'control' vector, SHC002 (a non-targeting shRNA that activates the RNAi pathway without

targeting any known mouse gene), was also purchased from Sigma Aldrich. Cells were transfected using Lipofectamine LTX and Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions, and stimulation of IL-17A for these cells was started at 24 h after transfection.

Inhibition of IL-17A signalling in CCD-18Co cells

To inhibit IL-17A signalling pathways (nuclear factor- κ B (NF- κ B), signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK), c-Jun N-terminal kinase (JNK)) in CCD-18Co cells, we used specific inhibitors of these pathways. CCD-18Co cells were treated with an inhibitor of NF- κ B (BAY 11-7082, 2.5 μ M; InvivoGen, San Diego, California, USA), STAT3 (STAT3 inhibitor V, 5 μ M; Calbiochem, San Diego, California, USA), p38 MAPK (SB203580, 10 μ M; Calbiochem), ERK (PD98059, 100 μ M; Calbiochem) or JNK (JNK inhibitor II, 20 μ M; Calbiochem) for 1 h before IL-17A stimulation.

Western blot analysis

CCD-18Co cells and ISEMFs were then washed with PBS, and protein was extracted from isolated cells. Protein content was measured by the Bradford assay (Bio-Rad Laboratories, Hercules, California, USA). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membrane was blocked with Tris-buffered saline with 0.1% Tween-20 (TBS-T) and 5% skim milk at room temperature for 1 h. Blots were incubated with each diluted primary antibody at 4 $^{\circ}$ C overnight. Dilutions in TBS-T with 5% BSA were prepared with mouse antihuman HSP47 monoclonal antibody (1:1000 dilution;

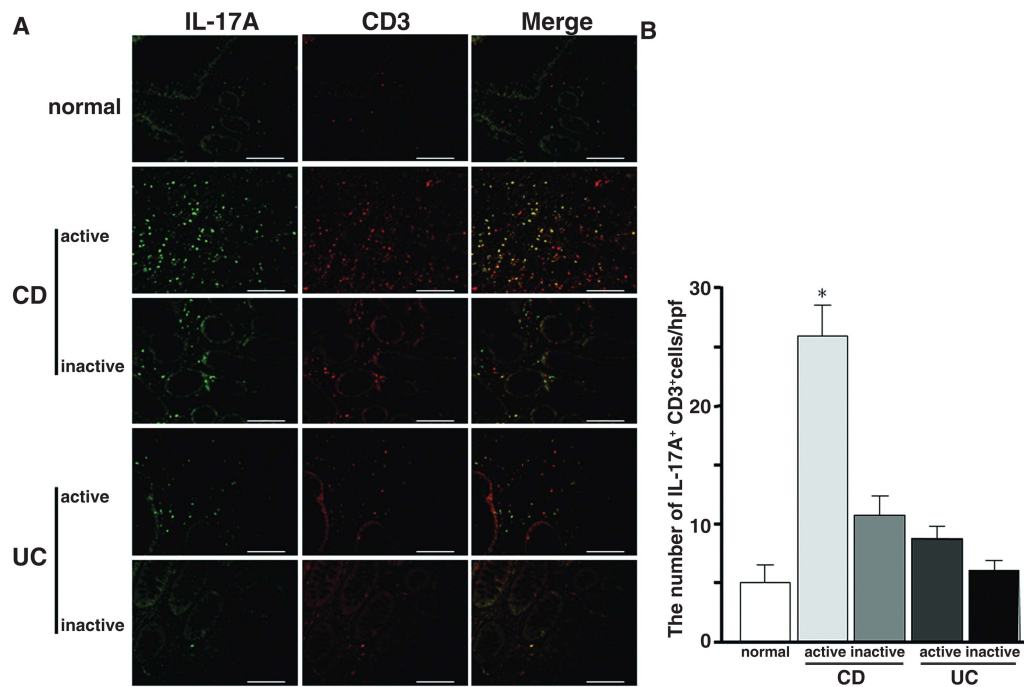


Figure 3 Cellular origin of IL-17A in the intestines of IBD patients and normal subjects. (A) Double-immunofluorescent staining with anti-IL-17A plus anti-CD3 was performed in the human intestinal tissues. Tissue samples were obtained from the intestines of patients with CD (inflamed/active, n=10; non-inflamed/inactive, n=10), UC (inflamed/active, n=10; non-inflamed/inactive, n=10) and normal subjects (n=5). (B) The number of merged cells for IL-17A and CD3 per HPFs was counted at 400× magnification. The mean count of five HPFs from one slide was used. (A) Data are representative images of each group. Bars indicate 100 μ m. (B) Data are expressed as mean $\pm$ SD of each group. *p<0.05 compared with inactive CD, active UC, inactive UC and normal subjects by one-way ANOVA with a Bonferroni correction. ANOVA, analysis of variance; CD, Crohn's disease; HPF, high-power field.

Stressgen Biotechnologies, Victoria, British Columbia, Canada), rabbit antihuman collagen I (1:250 dilution; Santa Cruz Biotechnology, Santa Cruz, California, USA), rabbit antihuman p38 MAPK, phospho-p38 MAPK (p-p38 MAPK), ERK, phospho-ERK (p-ERK), JNK, phospho-JNK (p-JNK), inhibitor of κ B α (I κ B α), p65, phospho-p65 (p-p65), STAT3, phospho-STAT3 (p-STAT3), and β -actin (1:1000 dilution; Cell Signaling Technology, Beverly, Massachusetts, USA). We used collagen I antibodies that could detect procollagen I and mature collagen I. After incubation, the membrane was washed three times for 5 min with TBS-T and incubated in TBS-T with 5% BSA containing antirabbit or mouse IgG antibody conjugated with horseradish peroxidase (1:1000 dilution; GE Healthcare, Little Chalfont, UK) at room temperature for 1 h. Immunoreactive bands were visualised using the Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore), and the image was recorded using a chemiluminescent image reader (ChemiDoc XRS plus, Bio-Rad Laboratories).

Statistical analysis

All numerical data are expressed as mean $\pm$ SD. The differences between groups were analysed by non-parametric Mann-Whitney U test or one-way analysis of variance with a Bonferroni correction for multiple comparisons. A p value <0.05 was considered statistically significant.

RESULTS

Collagen content was increased in intestinal tissues of patients with active CD

Because intestinal fibrosis comprises mainly collagen I, we first quantified the collagen content in the intestinal tissues of patients with IBD and normal subjects as an indicator of fibrosis by

measuring pepsin-soluble collagen (collagen assay). The pepsin-soluble collagen content was significantly increased in patients with active CD compared with that in patients with inactive CD, active UC, inactive UC and normal subjects (figure 1A).

Gene expressions of collagen-related molecules were upregulated in the intestinal tissues of patients with active CD

Gene expressions of *HSP47*, *collagen I α 1 chain*, *TGF- β 1*, *IL-17A*, *IL-17F*, and *IL-22* were examined in the intestinal tissues of patients with IBD and normal subjects. mRNA levels of *HSP47*, *collagen I α 1 chain*, *IL-17A* and *IL-22* in the intestinal tissues of patients with active CD were significantly upregulated in comparison with those of patients with inactive CD, active UC, inactive UC and normal subjects (figure 1B). As for expression of *TGF- β 1*, however, there was no significant difference among patients with IBD (figure 1B, upper-right panel). Expression of *IL-17F* in the intestinal tissues of patients with active CD and active UC was significantly higher than in that of patients with inactive CD, inactive UC and normal subjects (figure 1B, lower-middle panel). There was no significant difference in the expression of *IL-17C* among all patients (data not shown).

We also assessed IL-17 receptor (IL-17RA and IL-17RC) expression in isolated human ISEMFs (see online supplementary figure S1). Expression of *IL-17RA* in ISEMFs isolated from patients with active CD was significantly higher than those isolated from patients with inactive CD and normal subjects. No significant difference was observed, however, in the expression of *IL-17RC* in ISEMFs isolated from patients with IBD and normal subjects. These findings suggest that IL-17A and HSP47 may be involved in intestinal fibrosis of active CD.

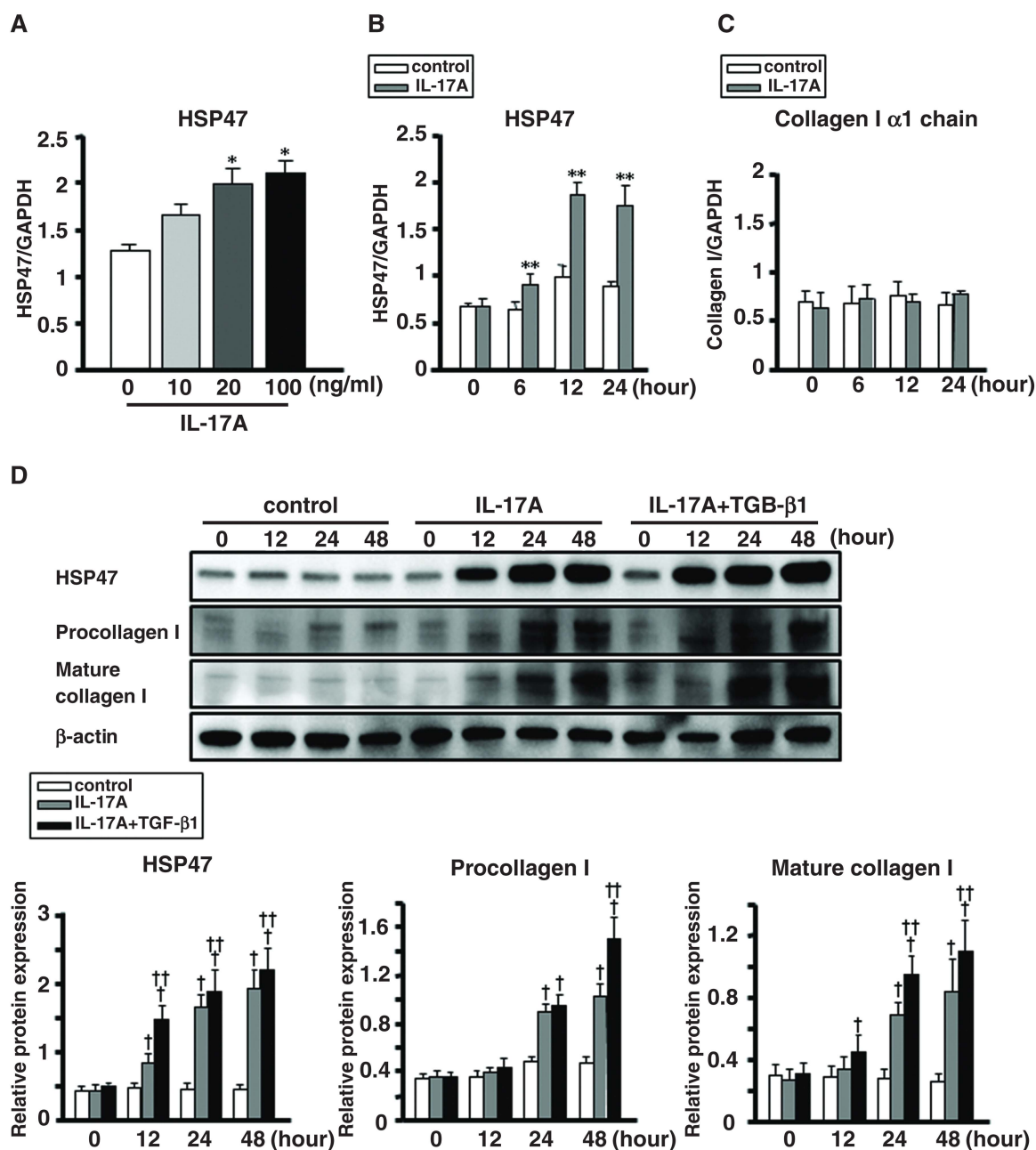


Figure 4 Effect of IL-17A on HSP47 and collagen I expressions in CCD-18Co cells ($n=12$ in each group). (A) Gene expression of HSP47 in CCD-18Co cells at 12 h after stimulation with IL-17A (0, 10, 20 and 100 ng/mL). (B, C) Gene expressions of *HSP47* and *collagen $\alpha 1$ chain* in CCD-18Co cells at 0, 6, 12 and 24 h after stimulation with IL-17A (20 ng/mL). *HSP47* and *collagen $\alpha 1$ chain* gene expressions were determined by quantitative real-time RT-PCR and normalised by *GAPDH*. (D) Protein expressions of HSP47, procollagen I and mature collagen I in CCD-18Co cells at 0, 12, 24 and 48 h after stimulation with IL-17A (20 ng/mL) and TGF- $\beta 1$ (1 ng/mL) were investigated by western blot analysis. Protein expressions of HSP47, procollagen I and mature collagen I were normalised by β -actin. Protein expression of collagen I is represented by distinct bands of procollagen I and mature collagen I. β -Actin is shown as control. (A) Data are expressed as mean $\pm$ SD of each group. * $p<0.05$ compared with 0 ng/mL group by one-way ANOVA with a Bonferroni correction. (B, C) Data are expressed as mean $\pm$ SD of each group. ** $p<0.05$ compared with control group of each time by non-parametric Mann–Whitney U test. (D) Data are representative images and expressed as mean $\pm$ SD of each group. † $p<0.05$ compared with control group; †† $p<0.05$ compared with IL-17A stimulation group of each time by one-way ANOVA with a Bonferroni correction. ANOVA, analysis of variance; HSP, heat shock protein.

HSP47-expressing myofibroblasts were increased in the intestinal tissues of patients with active CD

To examine the source and amount of HSP47 in the intestinal mucosa of patients with IBD, double-immunofluorescent staining was performed. Double-immunofluorescent staining revealed that α -SMA-positive cells mainly expressed HSP47 (figure 2A). In

addition, the number of HSP47-positive cells was significantly increased in the intestinal tissues of patients with active CD in comparison with those of patients with inactive CD, active UC, inactive UC and normal subjects (figure 2B). These results clearly demonstrated that HSP47-expressing myofibroblasts were increased in the intestinal mucosa of patients with active CD.

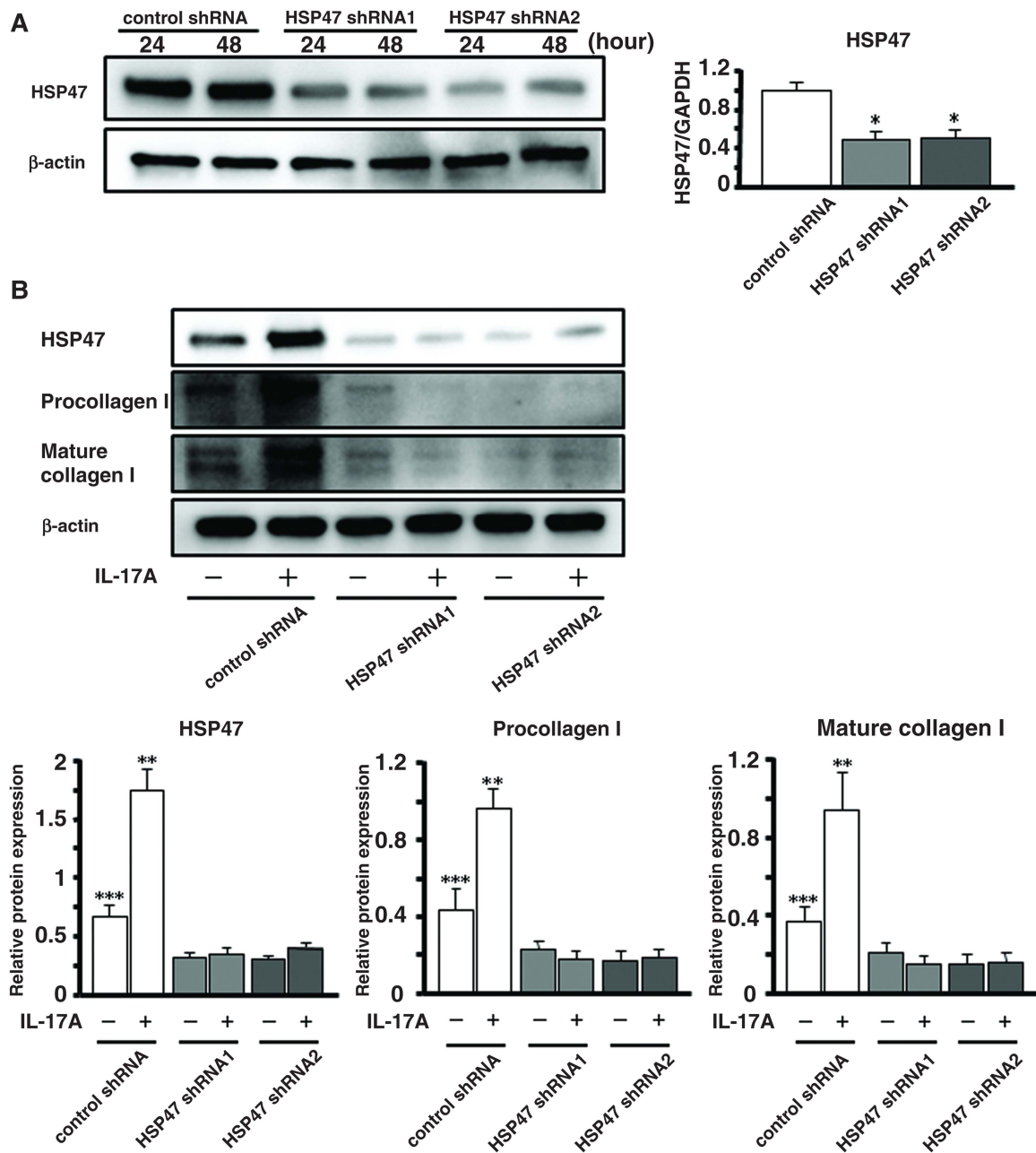


Figure 5 Knockdown analysis of HSP47 with shRNA targeting HSP47 in CCD-18Co cells (n=9 in each group). (A) Effect of shRNA on HSP47 expression by western blotting (24 and 48 h) and quantitative real-time RT-PCR (24 h). Two shRNA constructs decreased gene (left panel) and protein (right panel) expression of HSP47 in comparison with control shRNA (50–70% knockdown effect at mRNA level). Gene expression of each target molecule was normalised by *GAPDH*. (B) Protein expressions of HSP47, procollagen I and mature collagen I in HSP47 knockdown CCD-18Co cells at 48 h after stimulation with or without IL-17A (20 ng/mL). Protein expressions of HSP47, procollagen I and mature collagen I were normalised by β -actin. Protein expression of collagen I is represented by a distinct band of procollagen I and mature collagen I. β -Actin is shown as control. (A, B) Data are representative images and expressed as mean $\pm$ SD of each group. (A) * p <0.05 compared with control group by one-way ANOVA with a Bonferroni correction. (B) ** p <0.05 compared with control shRNA group (without IL-17A), HSP47 shRNA1 group (with or without IL-17A), HSP47 shRNA2 group (with or without IL-17A); *** p <0.05 compared with shRNA1 group (with or without IL-17A), HSP47 shRNA2 group (with or without IL-17A) by one-way ANOVA with a Bonferroni correction. ANOVA, analysis of variance; HSP, heat shock protein.

CD3-positive T cells expressing IL-17A were increased in the intestinal tissues of patients with active CD

Next, double-immunofluorescent staining demonstrated that CD3-positive cells expressing IL-17A and the total number of IL-17A positive cells were significantly increased in the intestinal tissues of patients with active CD in comparison with those of patients with inactive CD, active UC, inactive UC and normal subjects (figure 3A,B). α -SMA-positive cells and CD68-positive

cells, however, did not express IL-17A (see online supplementary figure S2).

IL-17A upregulated procollagen I and mature collagen I productions in CCD-18Co cells via HSP47 induction

To elucidate the role of IL-17A in HSP47-expressing fibroblasts, we examined the effect of IL-17A on HSP47 expression in CCD-18Co cells. IL-17A increased the gene expression of *HSP47*

Inflammatory bowel disease

in CCD-18Co cells in a dose-dependent manner (figure 4A). The significant induction of *HSP47* gene expression was observed at 6, 12 and 24 h with IL-17A (figure 4B), while it did not affect *collagen I $\alpha 1$ chain* gene expression (figure 4C). In contrast, *HSP47* expression did not change in CCD-18Co cells treated with IL-17C, IL-17F or IL-22 (see online supplementary figure S3).

Next, we assessed the protein levels of HSP47, procollagen I and mature collagen I. Interestingly, IL-17A increased the protein expressions of procollagen I and mature collagen I as well as HSP47 expression, and IL-17A and TGF- β 1 synergistically induced expressions of these proteins (figure 4D).

To further validate the relationship between IL-17A-induced HSP47 and collagen I expressions, we established CCD-18Co cells transfected with HSP47-specific shRNA or a scramble control shRNA. The HSP47-specific shRNA strongly suppressed HSP47 expression, whereas the control shRNA had no effect (figure 5A). Knockdown of HSP47 in CCD-18Co cells resulted in the inhibition of IL-17A-induced procollagen I and mature collagen I expressions (figure 5B). These findings suggest that IL-17A upregulates the productions of procollagen I and mature collagen I in CCD-18Co cells via HSP47 induction.

JNK pathway was involved in IL-17A-induced HSP47 expression

To gain a better understanding of the mechanism for HSP47 induction by IL-17A in CCD-18Co cells, we characterised the pathways that were involved in IL-17A-induced HSP47 expression using specific inhibitors of these pathways (see online supplementary figure S4). Although stimulation of CCD-18Co cells with IL-17A resulted in the phosphorylation of NF- κ B, STAT3 and MAPK, none of the inhibition of NF- κ B, STAT3, p38 MAPK or ERK suppressed the expression of HSP47, procollagen I and mature collagen I proteins (figure 6A–D). The JNK inhibitor, however, suppressed these protein expressions induced by IL-17A (figure 6E).

IL-17A increased procollagen I and mature collagen I in ISEMFs isolated from patients with IBD via HSP47 induction

Finally, we investigated the effect of IL-17A on HSP47 and collagen productions in ISEMFs isolated from patients with IBD and normal subjects. IL-17A stimulation significantly increased the protein levels of HSP47, procollagen I and mature collagen I in ISEMFs isolated from patients with active CD and active UC, whereas IL-17A induced only a slight increase of these

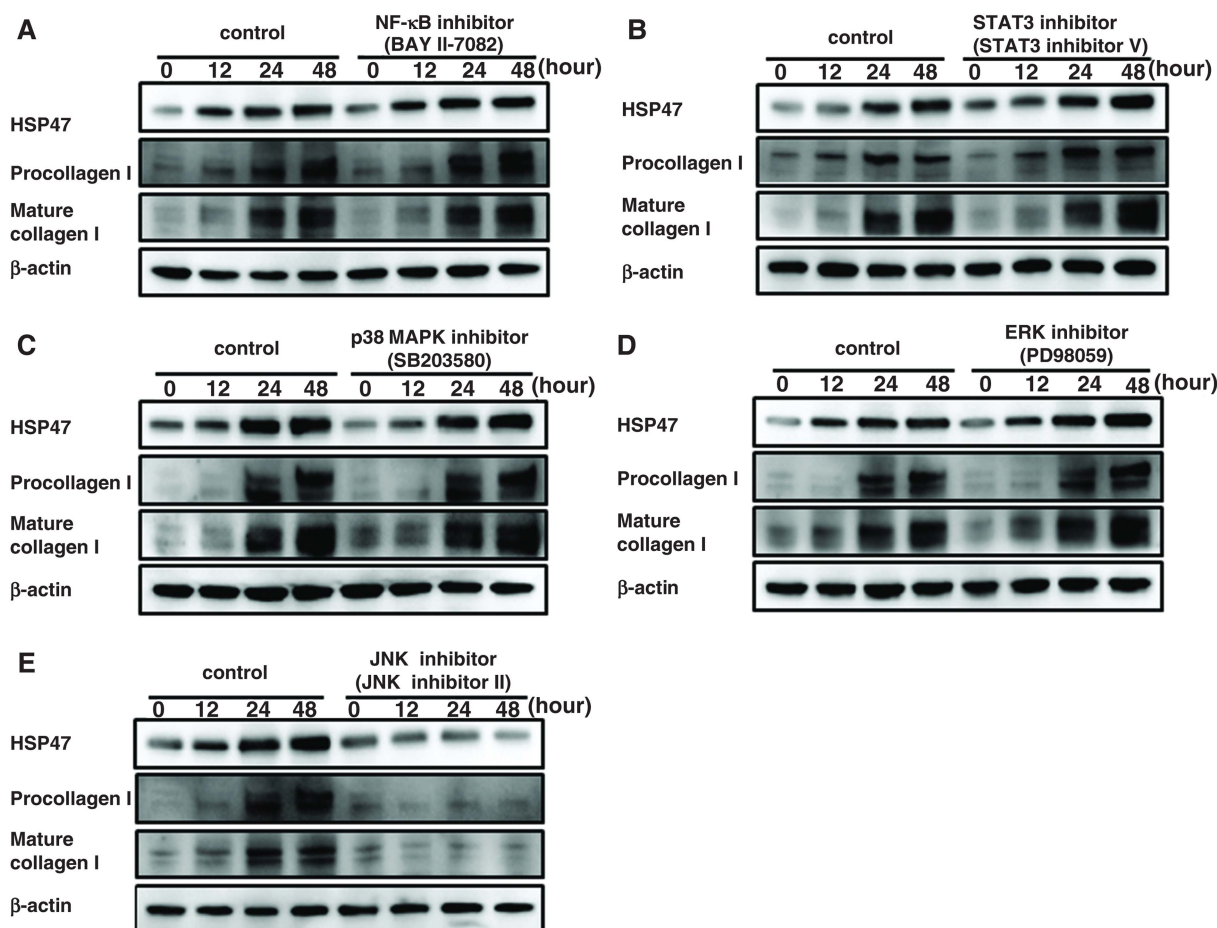


Figure 6 Signalling pathway on HSP47, procollagen I and mature collagen I expressions in CCD-18Co cells stimulated with IL-17A (n=9 in each group). Protein expressions of HSP47, procollagen I and mature collagen I in CCD-18Co cells at 0, 12, 24 and 48 h after stimulation with IL-17A (20 ng/mL) were evaluated by western blotting analysis. (A–E) CCD-18Co cells were treated with each specific inhibitor of NF- κ B (BAY 11-7082, 2.5 μ M), STAT3 (STAT3 inhibitor V, 5 μ M), p38 MAPK (SB203580, 10 μ M), ERK (PD98059, 100 μ M) or JNK (JNK inhibitor II, 20 μ M) for 1 h before stimulation with IL-17A (20 ng/mL). Protein expression of collagen I is represented by a distinct band of procollagen I and mature collagen I. β -Actin is shown as control. Data are representative images of each group. ERK, extracellular signal-regulated kinase; HSP, heat shock protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

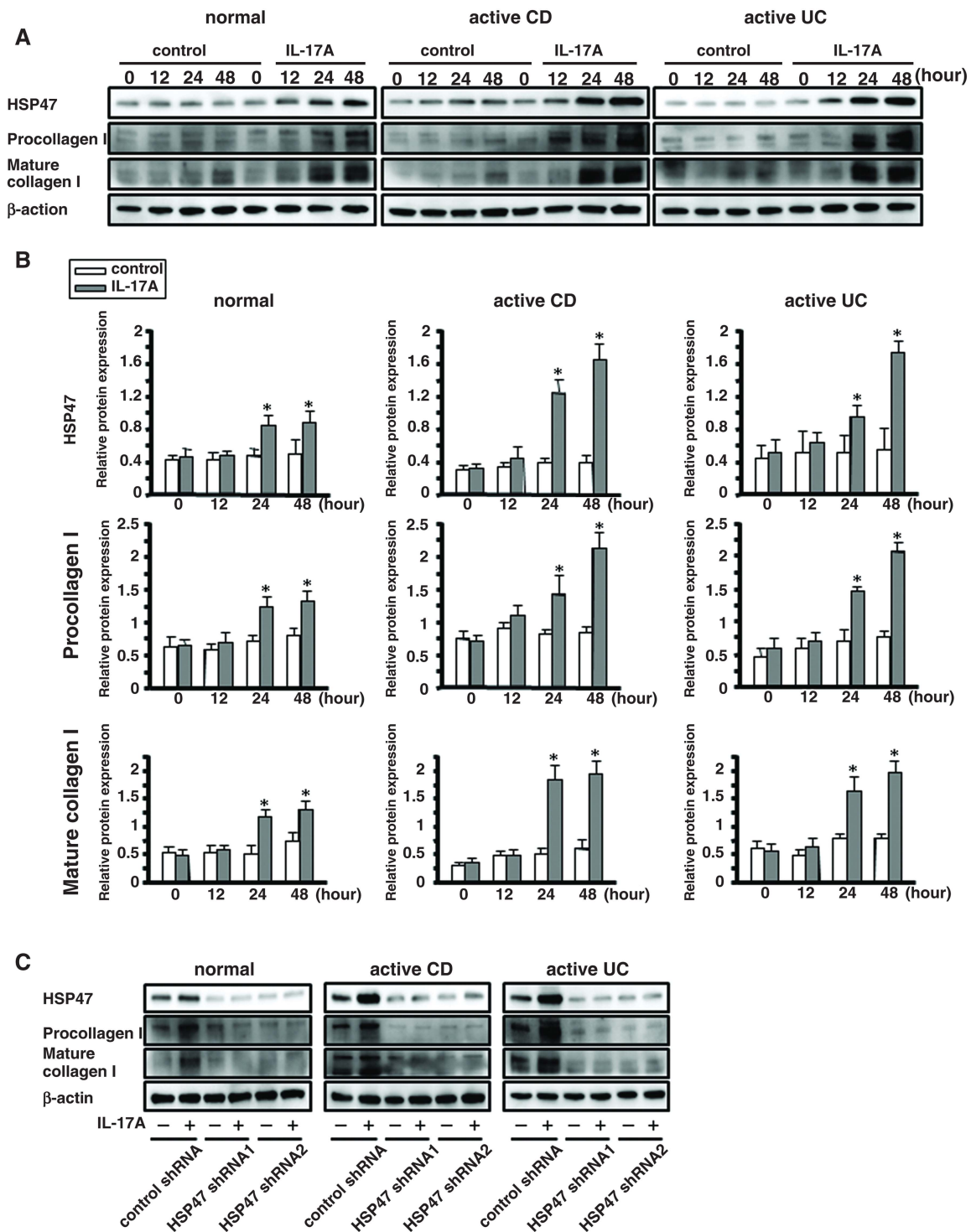


Figure 7 Analysis of IL-17A on HSP47, procollagen I and mature collagen I expressions in ISEMFs. Tissue samples were isolated from the intestines of patients with CD (inflamed/active, n=7), UC (inflamed/active, n=6) and normal subjects (n=3). (A, B) Protein expressions of HSP47, procollagen I and mature collagen I in ISEMFs isolated from patients with IBD and normal subjects at 0, 12, 24 and 48 h after stimulation with IL-17A (20 ng/mL) were determined by western blotting analysis. (C) Protein expressions of HSP47, procollagen I and mature collagen I in HSP47 knockdown ISEMFs at 48 h after stimulation with or without IL-17A (20 ng/mL) were evaluated by western blotting analysis. Protein expressions of HSP47, procollagen I and mature collagen I were normalised by β-actin. Protein expression of collagen I is represented by a distinct band of procollagen I and mature collagen I. β-Actin is shown as control. (A, C) Data are representative images of each group. (B) Data are expressed as mean±SD of each group. *p<0.05 compared with control group of each time by non-parametric Mann–Whitney U test. CD, Crohn's disease; HSP, heat shock protein; ISEMFs, intestinal subepithelial myofibroblasts.

protein levels in ISEMFs isolated from patients with inactive CD, inactive UC and normal subjects (figure 7A,B and online supplementary figure S5). In addition, knockdown of HSP47

with HSP47-specific shRNA in ISEMFs resulted in the inhibition of IL-17A-induced procollagen I and mature collagen I expressions (figure 7C). As we expected, IL-17A strongly

induced p-JNK in ISEMFs isolated from patients with active CD and active UC in comparison with those isolated from patients with inactive CD, inactive UC and normal subjects (see online supplementary figure S6).

DISCUSSION

The present study demonstrated significant increases in collagen content in the intestinal tissues of patients with active CD. In addition, we also showed significant increases in the number of HSP47-positive myofibroblasts and IL-17A-expressing CD3-positive cells in the intestinal mucosa in association with elevated expressions of collagen I, IL-17A and HSP47 mRNAs in the intestinal tissues of patients with active CD. Moreover, in vitro and ex vivo studies revealed that IL-17A upregulated not only procollagen I and mature collagen I but also HSP47 expressions in CCD-18Co cells and ISEMFs isolated from the intestinal mucosa of patients with active CD, and knockdown of HSP47 by HSP47-specific shRNA significantly inhibited IL-17A-induced procollagen I and mature collagen I expressions in these cells. Moreover, we first demonstrated that JNK was a major signalling molecule in IL-17A-induced collagen production via HSP47 expression. These findings strongly suggest that IL-17A-induced HSP47 in myofibroblasts contributes to intestinal fibrosis in patients with CD.

HSP47, a 47 kDa collagen-binding glycoprotein, acts as a molecular chaperone under stress conditions in the endoplasmic reticulum, and this molecule is known to be involved in the processing and transport of procollagen.^{9–11} Excessive HSP47 expression under pathological conditions such as chronic inflammatory disorders contributes to tissue fibrosis.^{12–15} Thus, the present finding of the increased expression of not only collagen I but also HSP47 in the intestinal mucosa of patients with active CD may support an idea that the increased levels of HSP47 play an important role in intestinal fibrosis of CD, similar to other fibrotic diseases.

In this study, we focused on local cytokine profiles in the intestinal mucosa of patients with IBD. TGF- β 1 is considered to be the most potent fibrogenic cytokine in CD.³ Indeed, we confirmed that TGF- β 1 stimulation increased collagen I and HSP47 expression in CCD-18Co cells and human ISEMFs (data not shown), consistent with previous reports.^{31–32} Our data, however, demonstrated no significant difference in TGF- β 1 expression in the intestinal mucosa among patients with IBD irrespective of disease activity. On the other hand, among IL-17 family members, expression of IL-17A in the intestinal mucosa was strongly increased specifically in patients with active CD. Our in vitro data revealed that only IL-17A but not IL-17F induced HSP47 expression in CCD-18Co cells. Thus, it is reasonable to speculate that this remarkably high expression of IL-17A has a key role in fibrogenic feature of CD. Similar to IL-17A, IL-22 expression was also significantly upregulated only in patients with active CD in this study, confirming a previous study.³³ A recent report demonstrated that IL-22 blocked the progression of tissue fibrosis in liver disease.²⁵ In this study, however, IL-22 did not affect HSP47 expression in CCD-18Co cells, suggesting that IL-22 might inhibit tissue fibrosis by another mechanism except for regulating HSP47 expression.

We found in this study that IL-17A increased HSP47 gene expression, whereas it did not affect collagen α 1 chain gene expression in CCD-18Co cells. In contrast, western blot data clearly demonstrated increases of all HSP47, procollagen I, and mature collagen I protein levels by IL-17A stimulation. Also, IL-17A strongly induced these proteins under the presence of TGF- β 1. Knockdown of HSP47 in CCD-18Co cells and ISEMFs

lead to the inhibition of IL-17A-induced increases of procollagen I and mature collagen I proteins. Taken together, IL-17A increases procollagen I and mature collagen I proteins by enhancing HSP47 expression. Of note, our study showed that not only collagen content but also collagen α 1 chain gene expression was increased in the intestinal tissues of patients with active CD, suggesting that several molecules independent of IL-17A could be involved in the enhancement of collagen chain gene expression.

To further clarify how IL-17A signalling stimulates HSP47 expression in collagen production, we next examined downstream signalling molecules of IL-17A (MAPK, NF- κ B and STAT3), the phosphorylation of which is regarded as the crucial intracellular step in IL-17A signal transduction. We found in this study that stimulatory effects of IL-17A on HSP47 and collagen I expressions were suppressed only by inhibition of the JNK pathway. Previous studies demonstrated that all of ERK, JNK and STAT3 were involved in collagen production in response to several cytokines.^{34–37} JNK is well known to have an important role in mediating many mechanisms that contribute to the pathogenesis of liver fibrosis.³⁶ However, it has remained unclear how JNK, as a downstream molecule of IL-17A, is involved in collagen synthesis in myofibroblasts. The present data are the first to demonstrate that JNK is a major signalling molecule in IL-17A-induced collagen production via HSP47 expression.

Finally, turning to the main focus of the present study, we investigated the effect of IL-17A on ISEMFs. Western blot analysis demonstrated that protein levels of HSP47, procollagen I and mature collagen I were strongly increased by IL-17A administration in ISEMFs isolated from patients with active CD and active UC in comparison with those isolated from patients with inactive CD, inactive UC and normal subjects. In addition, strong JNK phosphorylation in ISEMFs isolated from patients with active CD and active UC after IL-17A stimulation was observed in comparison with those isolated from patients with inactive CD, inactive UC and normal subjects. In agreement with our data, Lawrance *et al*³⁸ reported that collagen production of fibroblasts isolated from patients with CD and UC included an activated subset that was functionally distinct from normal intestinal fibroblasts. They also reported that collagen production did not differ between fibroblasts isolated from patients with active CD and active UC, similar to our results.³⁸ Thus, the present and previous data suggest that sustained mucosal inflammation could induce human fibroblast to develop fibrogenic phenotype, but diverse cytokine profiles in local intestinal tissues may result in the difference of collagen production between CD and UC.

A previous clinical trial demonstrated that anti-IL-17A antibody (secukinumab) was not effective for patients with moderate to severe CD.³⁹ This trial, however, assessed the effect of secukinumab on disease activity of enrolled patients with CD but not on intestinal fibrosis by several modalities such as ultrasound and MRI. In this regard, further investigation will be required to evaluate the clinical benefit of secukinumab for patients with CD.

In conclusion, the present study strongly suggests that IL-17A-induced HSP47 expression contributes to the pathophysiology of intestinal fibrosis in CD. Currently, inhibition of TGF- β 1 seems to be the most promising candidate treatment for fibrotic diseases.⁴⁰ Whether blockade of TGF- β 1 is an optimal treatment for intestinal fibrosis related to IBD, however, is under debate because TGF- β 1 has a potent immunosuppressive effect on intestinal inflammation.²⁹ Therefore, the present findings may provide novel potential antifibrotic therapies to treat intestinal fibrosis in CD.

Contributors HN was responsible for study concept and design. YH was responsible for acquisition of data. HN and YH were responsible for data analysis. HN and TC were responsible for critical revision of the manuscript and obtained funding. MS, HI, TY, MM, YK, HI, AA, YS and KN were responsible for technical support.

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Ethics approval Intestinal Tissues.

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