

Fibrocytes Are Involved in Inflammation as well as Fibrosis in the Pathogenesis of Crohn's Disease

Sayuri Sazuka · Tatsuro Katsuno · Tomoo Nakagawa · Masaya Saito · Keiko Saito · Daisuke Maruoka · Tomoaki Matsumura · Makoto Arai · Hideaki Miyauchi · Hisahiro Matsubara · Osamu Yokosuka

Received: 24 May 2013 / Accepted: 15 July 2013 / Published online: 22 August 2013
© Springer Science+Business Media New York 2013

Abstract

Background We previously showed that fibrocytes, a hematopoietic stem cell source of fibroblasts/myofibroblasts, infiltrated the colonic mucosa of a murine colitis model.

Aim We investigated whether fibrocytes were involved in the pathogenesis of Crohn's disease.

Methods Human surgical intestinal specimens were stained with anti-leukocyte-specific protein 1 and anti-collagen type-I (ColI) antibodies. Circulating fibrocytes in the human peripheral blood were quantified by fluorescence-activated cell sorting with anti-CD45 and anti-ColI antibodies. Cultured human fibrocytes were prepared by culturing peripheral CD14⁺ monocytes.

Results In the specimens of patients with Crohn's disease, the fibrocyte/total leukocyte percentage was significantly increased in inflammatory lesions (22.2 %, $p < 0.01$) compared with that in non-affected areas of the intestine (2.5 %). Interestingly, the percentage in fibrotic lesions was similar (2.2 %, $p = 0.87$) to that in non-affected areas. The percentages of circulating fibrocytes/total leukocytes were significantly higher in patients with Crohn's disease than in healthy controls. Both CXC-chemokine receptor 4⁺ and intercellular adhesion molecule 1⁺ fibrocyte numbers were significantly increased in Crohn's

disease, suggesting that circulating fibrocytes have a higher ability to infiltrate injured sites and traffic leukocytes. In cultured fibrocytes, lipopolysaccharide treatment remarkably upregulated tumor necrosis factor (TNF)- α mRNA (17.0 ± 5.7 -fold) and ColI mRNA expression (12.8 ± 5.7 -fold), indicating that fibrocytes stimulated by bacterial components directly augmented inflammation as well as fibrosis.

Conclusions Fibrocytes are recruited early in the inflammatory phase and likely differentiate into fibroblasts/myofibroblasts until the fibrosis phase. They may enhance inflammation by producing TNF- α and can directly augment fibrosis by producing ColI.

Keywords Collagen type-I (ColI) · CXC-chemokine receptor 4 (CXCR4) · Intercellular adhesion molecule 1 (ICAM1) · Leukocyte-specific protein 1 (LSP-1) · Lipopolysaccharide (LPS)

Introduction

The pathophysiology of Crohn's disease is characterized by persistent active inflammation followed by severe fibrosis within the intestinal walls. Intestinal fibrosis results in serious complications such as bowel obstructions and fistulas that potentially require surgery. Fibroblasts/myofibroblasts have a pivotal role in the development of intestinal fibrosis in Crohn's disease [1, 2]. The exposure of mucosal fibroblasts/myofibroblasts to inflammatory mediators and subsequent collagen production and tissue remodeling are considered key events in the development of Crohn's disease-associated fibrosis. Growth factors, such as transforming growth factor (TGF- β) and connective tissue growth factor, cytokines, such as interleukin

S. Sazuka · T. Katsuno (✉) · T. Nakagawa · M. Saito · K. Saito · D. Maruoka · T. Matsumura · M. Arai · O. Yokosuka
Department of Gastroenterology and Nephrology (K1), Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba-shi 260-8670, Japan
e-mail: katsuno@faculty.chiba-u.jp

H. Miyauchi · H. Matsubara
Department of Frontier Surgery (M9), Graduate School of Medicine, Chiba University, Chiba, Japan

(IL)-13, chemokines, such as monocyte chemoattractant protein-1, and toll-like receptor (TLR) ligands are mediators that modulate fibroblast/myofibroblast function [3, 4].

Bucala et al. [5] identified bone marrow-derived fibrocytes that comprise 0.1–0.5 % of the nonerythrocytic cells in peripheral blood, rapidly enter tissue injury sites, synthesize connective tissue matrices, and express fibrogenic cytokines. These fibrocytes are uniquely defined by the coexpression of both hematopoietic cell markers (panmyeloid antigen: CD13; leukocyte common antigen: CD34, CD45; and leukocyte-specific protein 1) and mesenchymal cell markers [collagen I (ColI), collagen III, fibronectin, and vimentin] [6, 7]. It was postulated that circulating progenitor fibrocytes undergo phenotypic changes to differentiate into immature fibrocytes that are recruited to wound sites. Within the wound sites, these early differentiated immature fibrocytes may further interact with recruited *T* cells and then fully differentiate into mature fibrocytes after exposure to TGF- β [8]. Mature fibrocytes, which produce collagen and other critical extracellular matrix molecules, are considered to further differentiate into fibroblasts/myofibroblasts that promote wound healing and fibrosis [7, 9].

Fibrocytes participate in aberrant wound repair and pathological fibroses, including hypertrophic scars and keloids, airway remodeling in asthma, interstitial pulmonary fibroses, systemic disorders, including scleroderma and nephrogenic systemic fibrosis, atherosclerosis, the stromal response to tumor invasion, and Graves' ophthalmopathy [8, 10]. However, the relationship between Crohn's disease-associated fibrosis and fibrocytes has not been elucidated. We have reported for the first time that significant numbers of fibrocytes emerged in the colonic mucosa of a murine model of Crohn's disease [11]. We hypothesized that fibrocytes are involved in the pathophysiology of human Crohn's disease accompanying severe fibrosis. We have addressed the following three questions in this study. First, are fibrocytes trafficked into the inflamed tissue of patient's with Crohn's disease as observed with the murine colitis model? Second, are there any differences between the number and expression levels of surface markers of circulating fibrocytes in patients with Crohn's disease and healthy controls? Finally, what is the pathogenesis of mature tissue fibrocytes in the intestinal mucosa exposed to bacterial components?

Materials and Methods

Patients and Clinical Samples

All studies were conducted with the approval of the ethics committee of Chiba University School of Medicine, Chiba,

Japan. Patients diagnosed with Crohn's disease and treated at Chiba University Hospital were included in the study. Healthy volunteers were recruited from the same hospital.

Immunohistochemistry of Surgical Specimens

Surgical specimens from patients with Crohn's disease were examined in this study. The patients had undergone partial resection of the intestine because of intestinal wall perforations or fistula formation. Surgical specimens were impregnated in O.C.T. compound (Sakura Finetek Tokyo, Tokyo, Japan), stored at -80°C , sectioned at a 5- μm thickness with a cryostat (Microedge Instruments, White Rock, BC, Canada).

Samples were fixed with 4 % paraformaldehyde in phosphate-buffered saline (PBS) for 8 min at room temperature. Bovine serum albumin (5 %; Sigma-Aldrich, St. Louis, MO, USA) was used as a blocking agent, and immunohistological staining was performed with Can Get Signal B (Toyobo, Tokyo, Japan). Sections were incubated with purified mouse anti-human leukocyte specific protein 1 (LSP-1) (1:200) (BD Biosciences, San Jose, CA, USA) and anti-collagen type I (ColI; rabbit) antibody (1:200) (Rockland Immunochemicals, Gilbertsville, PA, USA) at 4°C overnight, followed by incubation with Alexa Fluor 488 anti-mouse secondary antibody (1:200) (Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 610-R-PE anti-rabbit secondary antibody (1:800) (Invitrogen) for 1 h at room temperature. All sections were mounted with Vectashield mounting medium containing 1.5 $\mu\text{g}/\text{ml}$ of 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and covered with a cover slip. These samples were analyzed by confocal laser scanning microscopy (Carl Zeiss, Oberkochen, Germany). For quantitative measurement of tissue fibrocytes, stained cells were acquired in 3 nonoverlapping random fields ($\times 200$ magnification) and counted as the percentages of fibrocytes (LSP-1⁺ColI⁺ cells/LSP-1⁺ cells) in non-affected areas of the intestine and active inflammatory and fibrotic lesions.

Fluorescence-Activated Cell Sorting (FACS) Analysis

Human peripheral blood mononuclear cells (PBMCs) were isolated from 20 ml of whole blood using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions and the cells were stored frozen in Bambanker (Lymphotec, Tokyo, Japan). After thawing the frozen PBMCs, they were washed with PBS, and 1.0×10^6 cells were suspended in 50 μl of PBS. Then, they were stained with 1 μl of anti-CD45-FITC (Dako Denmark, Glostrup, Denmark), washed, and treated with a Cytotfix/Cytoperm kit (BD Biosciences) to permeabilize the cells according to the manufacturer's instructions. Cells

were incubated with 2 μ L of anti-ColII (rabbit) antibody (1:200) (Rockland Immunochemicals, Gilbertsville, PA, USA), washed, and finally counterstained with goat anti-rabbit Alexa647 (Invitrogen). Flow cytometry was performed with a BD FACS CantoII (BD Biosciences). For each sample, 20,000 events were collected by flow cytometry. The data were analyzed using FlowJo software (TreeStar, San Carlos, CA, USA).

Gene Expression of Cultured Fibrocytes Treated with TLR Ligands

Fibrocytes were harvested and cultured as previously described [5, 12]. In brief, after isolating PBMCs, a positive selection for CD14⁺ monocytes was performed with anti-CD14 Dynabeads (Invitrogen) according to the manufacturer's protocol. CD14⁺ monocytes were cultured for 48 h in fibronectin-coated 6-well culture dishes (BD Biosciences) at 1×10^5 CD14⁺ cells/ml in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 20 % fetal bovine serum (FBS; Invitrogen). Nonadherent cells were then removed by a single, gentle aspiration, and they were cultured for another 13–15 days. This protocol yielded a purity of approximately 95 % for cultured fibrocytes [12].

Isolated cultured fibrocytes were treated with 10 μ g/ml of lipopolysaccharide (LPS; a TLR4 agonist; InvivoGen, San Diego, CA, USA), 10 μ g/ml of flagellin (a TLR5 agonist; InvivoGen), 10 μ g/ml (5 μ M) of ODN2006 (a TLR9 agonist; InvivoGen), and 10 ng/ml of TGF- β -1 (Wako Pure Chemical Industries, Osaka, Japan) for 12 h [12]. Total cellular RNA was prepared using RNeasy mini kit (QIAGEN, Hilden, Germany). Reverse transcription-polymerase chain reaction (RT-PCR) measurements were performed with the ABI PRISM 7700 sequence Detector system (Applied Biosystems, Foster City, CA, USA). Taqman probes (Applied Biosystems, Wellesley, CA, USA) that were labeled with FAM [Hs01113624-g1 for tumor necrosis factor (TNF)- α , Hs01076780-g1 for ColII, and Hs00957562-m1 for matrix metalloproteinase-9 (MMP-9)] were used. The relative expression of each mRNA was calculated by the Δ Ct method, and the amount of target mRNA relative to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was expressed as 2^{-(Δ Ct)}. The data are presented as the ratio of target mRNA to GAPDH mRNA.

Statistical Analysis

A nonparametric Kruskal–Wallis analysis was used with a subsequent Mann–Whitney *U* test (two-tailed) to assess the significance of the quantitative measurements of tissue fibrocytes. The flow cytometric analyses and RT-PCR values

for TNF- α , ColII, and MMP-9 mRNA were analyzed with Mann–Whitney *U* tests. The analyses were performed using JMP statistical software, v.9.0.2 (SAS Institute, Cary, NC, USA). *p* values of <0.05 were considered statistically significant.

Ethical Considerations

All studies were conducted with the approval of the ethics committee of Chiba University School of Medicine, Chiba, Japan.

Results

LSP-1⁺ColII⁺ Fibrocytes Were Significantly Increased in the Inflammatory Lesions of Patients with Crohn's Disease

To examine the distribution of fibrocytes in the intestinal mucosa of patients with Crohn's disease, surgical specimens were immunostained with a primary antibody to LSP-1 and ColII. We chose a primary antibody to LSP-1 to detect leukocyte common antigens instead of CD34 and CD45 because the stable expression of LSP-1 has been reported in tissue fibrocytes compared with CD34 and CD45 [13, 14]. LSP-1 is localized to the internal surface of the plasma membrane, the cytoplasm, and insoluble actin filaments in leukocytes. In the non-affected areas of the intestine without inflammation or fibrosis, we found a constant number of LSP-1⁺ cells that were regarded as resident lymphocytes and macrophages, whereas LSP-1⁺ColII⁺ cells regarded as fibrocytes were scarcely found here (Fig. 1a, d). The percentage of LSP-1⁺ColII⁺ fibrocytes/total LSP-1⁺ cells was 2.48 %, which was equivalent to the percentage of fibrocytes in the peripheral blood. LSP-1⁺ cells in the non-affected tissue were distributed mainly in the proper mucosal layers. This result indicated that bone marrow-derived leukocytes were recruited into the mucosal layers without enhancing the recruitment of fibrocytes in the non-affected areas of the intestine.

In contrast, the percentage of LSP-1⁺ColII⁺ fibrocytes relative to total LSP-1⁺ cells was remarkably increased in the inflammatory lesions of the intestine that were close to active ulcers (22.15 %) compared with that in the non-affected areas (*p* < 0.01) (Fig. 1b, d). LSP-1⁺ColII⁺ fibrocytes were mainly distributed in the mucosal layers and the submucosal layers. This result demonstrated that LSP-1⁺ColII⁺ fibrocytes preferentially infiltrated into the inflammatory mucosa of patients with Crohn's disease, indicating that fibrocytes may play a role in the exacerbation of the inflammation in Crohn's disease. LSP-

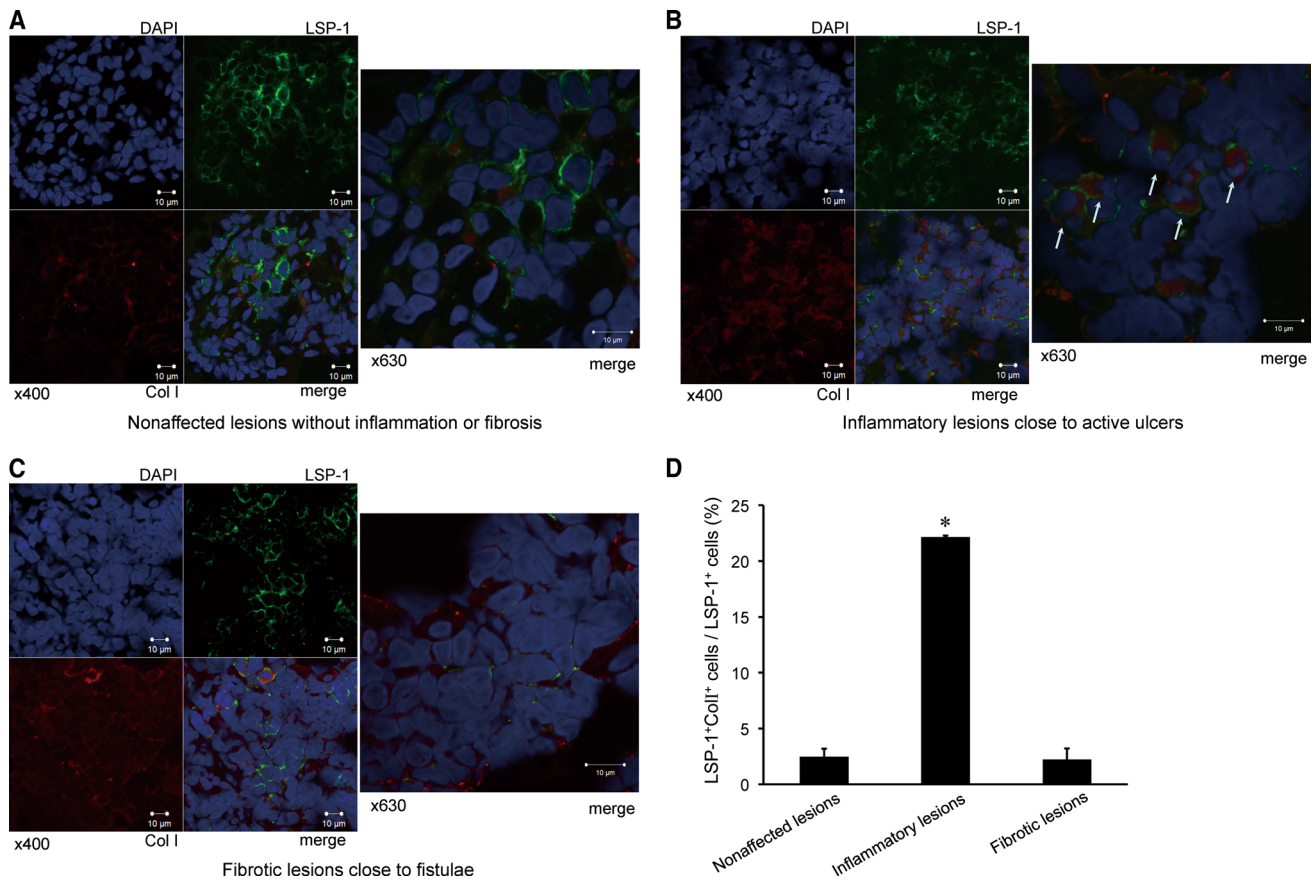


Fig. 1 Histological findings in the surgical specimens of patients with Crohn’s disease (immunohistochemical staining). **a–c** Specimens from non-affected areas of the intestine without inflammation or fibrosis (**a**), from inflammatory lesions close to active ulcers (**b**), and from fibrotic lesions close to fistulae (**c**) were immunostained with 4’,6-diamidino-2-phenylindole (DAPI) to label the nucleus, anti-leukocyte-specific protein-1 (LSP-1) antibody to stain the leukocytes, and anti-collagen type-I (ColI) antibody. *White arrows* indicate LSP-

1+ColI+ fibrocytes. The original magnifications were ×400 (*left*) and ×630 (*right*); *scale bar* 10 μm. **d** The number of LSP-1+ColI+ cells in the non-affected areas of the intestine and inflammatory and fibrotic lesions in surgical specimens from 3 patients with Crohn’s disease. The data are expressed as mean ± standard error of the mean (SEM). **p* < 0.01, as compared with the non-affected areas of the intestine

1+ColI+ fibrocytes showed an oval-type morphology instead of a spindle-type morphology, and this was regarded as immature tissue fibrocytes that had not yet differentiated into myofibroblasts [11]. In fibrotic lesions close to fistulae (Fig. 1c), the number of LSP-1-Coll+ cells, which were considered fibroblasts/myofibroblasts, was increased compared with that in inflammatory lesions. However, in contrast to our expectation, the number of LSP-1+Coll+ fibrocytes was significantly decreased compared with that in inflammatory lesions. The percentage of LSP-1+Coll+ fibrocytes relative to total LSP-1+ cells in the fibrotic lesions of the intestine close to fistulae was similar (2.23 %, *p* = 0.87) to that in the non-affected areas (Fig. 1c, d). These results indicated that fibrocytes were recruited early in the inflammatory phase and that most of them differentiated into fibroblasts/myofibroblasts until the fibrosis phase.

The Number of CD45+Coll+ Fibrocytes in the Peripheral Blood Was Increased in Patients with Crohn’s Disease

We sought to determine the number of circulating fibrocytes in patients with Crohn’s disease and compared it with that in healthy controls. In addition, we estimated the expression levels of CXC-chemokine receptor 4 (CXCR4), which is a receptor for CXC-chemokine ligand 12 (CXCL12), and intercellular adhesion molecule 1 (ICAM1) in the circulating fibrocytes because the increased expression of CXCR4 in the fibrocytes promotes their recruitment to sites of injury and ICAM1 facilitates leukocyte trafficking [15]. For each blood sample, monocytes were gated from PBMCs using a flow cytometric assessment of forward-scatter versus side-scatter characteristics (Fig. 2a-a). Unstained cells were used to determine negative gates

(Fig. 2a–b). As shown in Fig. 2a–c, CD45⁺ColI⁺ cells were defined as fibrocytes. The expression levels of CXCR4 or ICAM1 were determined in CD45⁺ColI⁺ fibrocytes (Fig. 2a–d, e, respectively).

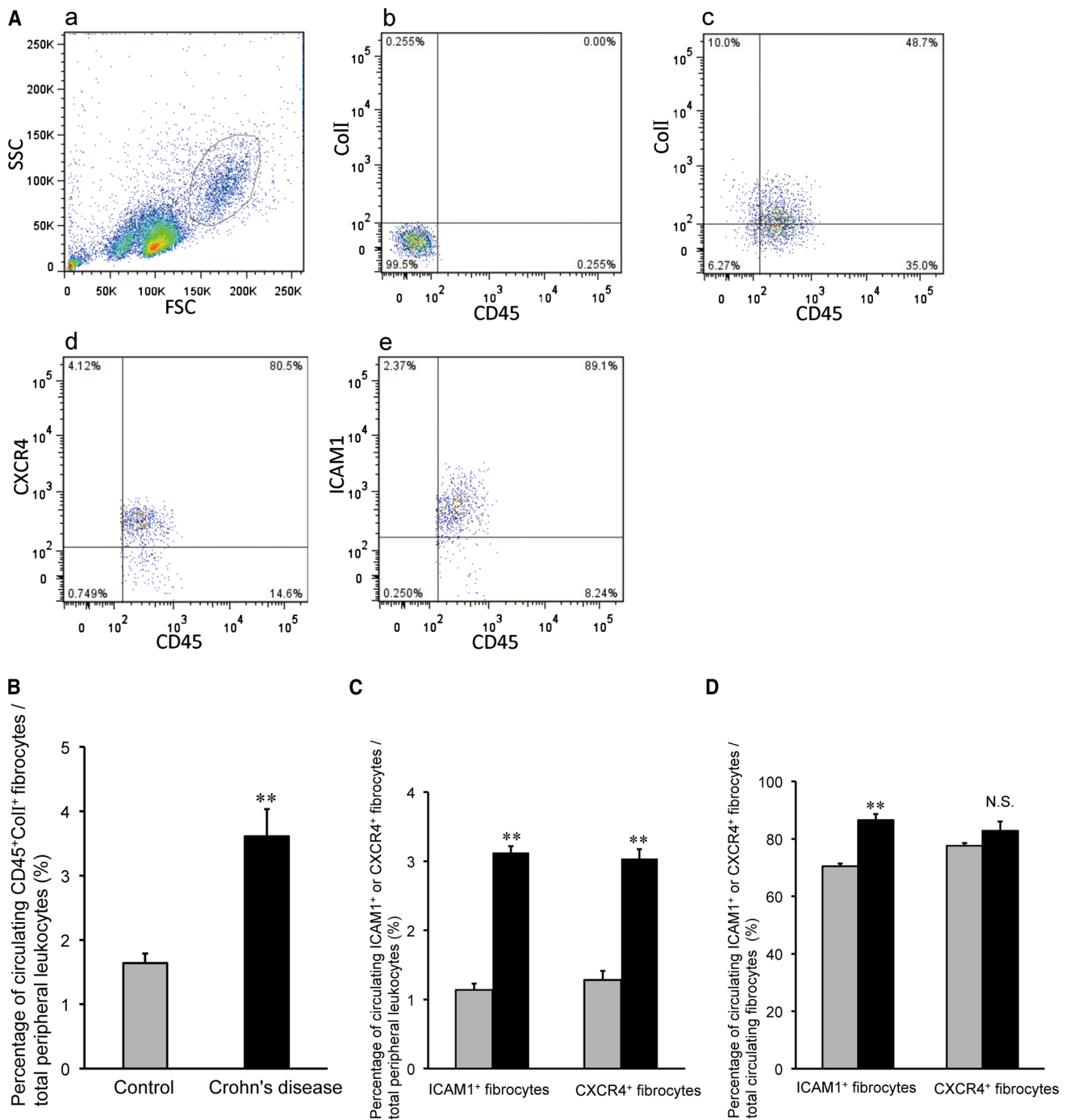
As shown in Fig. 2b, there was a significant increase in the percentage of CD45⁺ColI⁺ circulating fibrocytes in the peripheral blood of patients with Crohn's disease ($n = 13$) compared with that in healthy controls ($n = 10$) ($p = 0.002$). Furthermore, both circulating CD45⁺ColI⁺CXCR4⁺ fibrocytes and circulating CD45⁺ColI⁺ICAM1⁺ fibrocytes were significantly increased in the peripheral blood of patients with Crohn's disease compared with those in healthy controls ($p = 0.0001$ and $p = 0.004$, respectively; Fig. 2c). These results demonstrated that the percentage of CD45⁺ColI⁺ circulating fibrocytes that maintained their ability for their recruitment to wound sites and for lymphocyte trafficking was significantly increased in the peripheral blood of patients with Crohn's disease compared with that in healthy controls. Next, we compared the percentages of circulating CD45⁺ColI⁺CXCR4⁺ fibrocytes and circulating CD45⁺ColI⁺ICAM1⁺ fibrocytes per total circulating CD45⁺ColI⁺ fibrocytes between patients with Crohn's disease and healthy controls. As shown in Fig. 2d, the percentage of circulating CD45⁺ColI⁺ICAM1⁺ fibrocytes relative to the total circulating CD45⁺ColI⁺ fibrocytes was significantly increased compared to that in healthy controls (86.6 and 70.5 %, respectively, $p = 0.002$). The percentage of circulating CD45⁺ColI⁺CXCR4⁺ fibrocytes relative to the total circulating CD45⁺ColI⁺ fibrocytes in patients with Crohn's disease tended to be higher than that in healthy controls (82.8 and 77.6 %, respectively, $p = 0.110$). These results showed that the percentage of circulating CD45⁺ColI⁺ICAM1⁺ fibrocytes that were capable of leukocyte trafficking relative to the total circulating CD45⁺ColI⁺ fibrocytes was significantly increased in patients with Crohn's disease compared with that in healthy controls and there was a trend that the percentage of circulating CD45⁺ColI⁺CXCR4⁺ fibrocytes with a high ability for their recruitment to the wound sites relative to the total circulating CD45⁺ColI⁺ fibrocytes was higher in patients with Crohn's disease compared with that in healthy controls. Taken together, the FACS analysis showed that the percentage of circulating fibrocytes per total peripheral leukocytes was significantly increased in patients with Crohn's disease compared with that in healthy controls, and these fibrocytes maintained their ability for their recruitment to wound sites and for leukocyte trafficking. In particular, the percentage of circulating fibrocytes with a higher ability for leukocyte trafficking relative to the total circulating fibrocytes was significantly increased in patients with Crohn's disease compared with that in healthy controls.

TLR Ligands Enhance TNF- α and Collagen Gene Expression in Fibrocytes

We obtained spindle-shaped cultured fibrocytes by culturing CD14⁺ monocytes from patients with Crohn's disease ($n = 6$) and healthy controls ($n = 8$) for 14 days, as described in the “Materials and Methods” (Fig. 3a). In order to investigate whether TLR signaling affected fibrocyte function, the cultured fibrocytes were treated with 10 μ g/ml of LPS, a TLR4 agonist, 10 μ g/ml of flagellin, a TLR5 agonist, 10 μ g/ml of CpG-oligodeoxynucleotide (CpG-ODN), a TLR9 agonist, and 10 ng/ml of TGF- β . As shown in Fig. 3b, TNF- α mRNA was remarkably upregulated by treatment with LPS (17.0 \pm 5.7-fold in patients with Crohn's disease and 27.0 \pm 4.9-fold in healthy controls), whereas the other ligands had minimal effects on TNF- α mRNA expression levels. In addition, LPS treatment significantly upregulated ColI mRNA expression (12.8 \pm 5.7-fold in patients with Crohn's disease and 3.2 \pm 1.3-fold in healthy controls) (Fig. 3c). Interestingly, the levels of ColI mRNA expression were much higher after treatment with LPS than after treatment with TGF- β 1 (2.5 \pm 0.8-fold in patients with Crohn's disease and 2.7 \pm 0.7-fold in healthy controls). In contrast, MMP-9 mRNA levels were minimally changed by the ligands investigated in this study (1.4 \pm 0.3-fold in patients with Crohn's disease and 1.8 \pm 0.2-fold in healthy controls when treated with LPS) (Fig. 3d). Taken together, LPS treatment specifically and remarkably upregulated both TNF- α mRNA and ColI mRNA expression. No difference was observed between the function of fibrocytes obtained from patients with Crohn's disease and healthy controls. These data indicated that the responsiveness of matured fibrocytes to TLR ligands was not altered between patients with Crohn's disease and healthy controls when they were cultured in the same conditions, whereas cultured mature fibrocytes markedly produced TNF- α and ColI after treatment with LPS.

Discussion

This study demonstrated for the first time that a significant number of fibrocytes infiltrated into the intestinal mucosa of patients with Crohn's disease, suggesting their involvement in the pathogenesis of this disease. In addition, they may enhance inflammation by producing TNF- α and recruiting leukocytes, and they may directly augment fibrosis by producing ColI. Immunostaining the intestinal tissue of patients with Crohn's disease revealed that fibrocytes infiltrated early in the inflammatory phase, and they were likely to differentiate into fibroblasts/myofibroblasts up until the fibrosis phase. A FACS analysis of the peripheral blood showed that the percentage of circulating



fibrocytes per peripheral leukocytes was significantly increased in patients with Crohn’s disease compared with that in healthy controls, and these fibrocytes maintained their ability for their recruitment to the wound sites and for leukocyte trafficking. The cultured spindle-shaped mature fibrocytes from the peripheral blood of patients with Crohn’s disease responded to bacterial components, such as LPS, and the levels of gene expression were significantly enhanced, accelerating both inflammation and fibrosis.

Our previous study that used a murine model of colitis has demonstrated that oval-shaped fibrocytes are recruited into the colonic mucosa in the inflammatory phase of colitis, and these differentiated into spindle-shaped fibrocytes in the healing phase [11]. Another study showed that allergen exposure induces the accumulation of fibrocytes in the inflammatory bronchial mucosa of patients with asthma [16]. The results of the present study were in line with these studies in that fibrocytes emerged in the tissue early

◀ **Fig. 2** Flow cytometric analysis of human peripheral fibrocytes. **a** Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) from patients with Crohn's disease and healthy controls. (a) Forward-scatter (FSC) and side-scatter (SSC) analyses of PBMCs. Monocytes were selected. (b) Unstained cells were used to determine the negative gates for the monocyte population. (c) Extracting CD45⁺ColI⁺ cells (circulating fibrocytes) from the stained monocyte population. (d) Extracting CD45⁺, ColI⁺, and CXC-chemokine receptor 4 (CXCR4)⁺ cells from stained CD45⁺ColI⁺ cells. Negative gates were determined using unstained cells. (e) Extracting CD45⁺, ColI⁺, and intercellular adhesion molecule 1 (ICAM1)⁺ cells from stained CD45⁺ColI⁺ cells. Negative gates were determined using unstained cells. **b** Percentage of circulating CD45⁺ColI⁺ fibrocytes per total leukocytes in the peripheral blood from patients with Crohn's disease ($n = 13$; black bar) and healthy controls ($n = 10$; gray bar). Number of circulating CD45⁺ColI⁺ fibrocytes in each subject was determined by multiplying a peripheral blood monocyte count and a percentage of circulating CD45⁺ColI⁺ fibrocytes per monocytes obtained from flow cytometric analysis. The data are expressed as mean \pm SEM. $**p < 0.001$, as compared with healthy controls. **c** Percentage of CD45⁺ColI⁺CXCR4⁺ fibrocytes per total leukocytes and CD45⁺ColI⁺ICAM1⁺ fibrocytes per total leukocytes in the peripheral blood from patients with Crohn's disease ($n = 13$; black bar) and healthy controls ($n = 10$; gray bar). The data are expressed as mean \pm SEM. $**p < 0.001$, as compared with healthy controls. **d** Percentage of CD45⁺ColI⁺CXCR4⁺ fibrocytes per total CD45⁺ColI⁺ fibrocytes and CD45⁺ColI⁺ICAM1⁺ fibrocytes per total CD45⁺ColI⁺ fibrocytes in the peripheral blood from patients with Crohn's disease ($n = 13$; black bar) and healthy controls ($n = 10$; gray bar). The data are expressed as mean \pm SEM. $**p < 0.001$, as compared with healthy controls

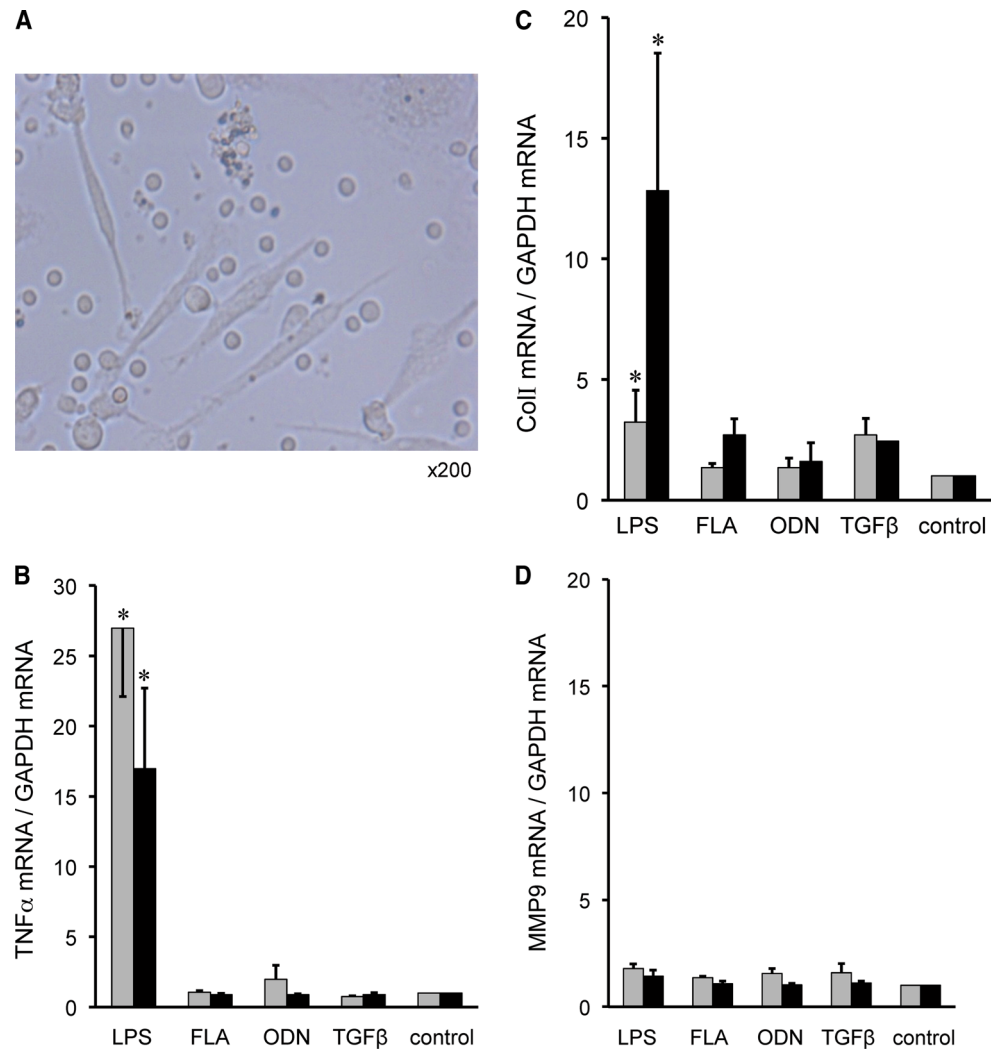
in the inflammatory phase. Recently, fibrocytes were revealed as effector cells in chronic inflammation and are implicated in the pathogenesis of the chronic inflammatory states in autoimmune diseases (scleroderma, Graves' disease, and rheumatoid arthritis), cardiovascular disease (atheroma), and asthma [6, 15]. Based on these findings, fibrocytes may influence chronic inflammation in the pathogenesis of Crohn's disease. Another possible explanation is that they were recruited to repair the damage—not to simply amplify inflammation. This study showed that the number of fibrocytes was decreased in the fibrotic tissue of patients with Crohn's disease, and this seemed to be paradoxical according to the hypothesis that fibrocytes are directly involved in the fibrogenesis of Crohn's disease. We speculated that, instead of fibrocytes by themselves, fibrocyte-derived fibroblasts/myofibroblasts participate the formation of fibrosis because fibrocytes are known to differentiate into fibroblasts/myofibroblasts in other human diseases [6]. Our previous study, which used a murine model of colitis, has also demonstrated that the appearance of spindle-shaped mature fibrocytes and α -SMA⁺ myofibroblasts occurred in the healing phase of colitis [11]. These observations indicate that fibrocytes are involved in the pathogenesis of Crohn's disease early in the inflammatory phase of the disease rather than late in the fibrotic phase.

The present study demonstrated a significant increase in circulating fibrocytes in patients with Crohn's disease compared with healthy controls. This result was consistent with previous reports that demonstrated that the number of circulating fibrocytes was significantly increased in idiopathic pulmonary fibrosis and scleroderma [17–19]. The recruitment of fibrocytes to the injured sites is promoted by chemokine receptors, such as CXCR4, which bind to CXCL12 [20]. In this study, we showed that the number of circulating CXCR4⁺ fibrocytes was significantly increased in patients with Crohn's disease, and this was similar to what is observed in patients with fibrotic interstitial lung disease, suggesting that the fibrocytes of patients with Crohn's disease have a high ability to infiltrate to the injured sites [17]. In addition, we showed that a significantly higher percentage of circulating fibrocytes expressed ICAM1 in patients with Crohn's disease compared with that in healthy controls, suggesting that circulating fibrocytes in Crohn's disease have a higher ability for leukocyte trafficking [17].

Finally, we showed that TLR signaling affected the function of the fibrocytes that were derived from the patients with Crohn's disease. A previous study showed that fibrocytes stimulated by TLR ligands produce a higher amount of IL-6 in a porcine model compared with plasmacytoid dendritic cells [21]. In this study, we demonstrated for the first time that cultured human fibrocytes derived from the peripheral blood of both patients with Crohn's disease and healthy controls express mRNA of inflammatory cytokines, such as TNF- α , and tissue remodeling factors such as ColI. Among the TLR ligands investigated in this study, LPS treatment specifically and remarkably upregulated the levels of TNF- α mRNA and ColI mRNA in fibrocytes derived from patients with Crohn's disease and healthy controls. Enterobacterium has emerged as an important player in the pathogenesis of Crohn's disease, and TLR ligands, such as LPS, play a critical role in exacerbating inflammation in the intestinal mucosa [22, 23]. Our data supported the notion that fibrocytes that infiltrate into the inflammatory intestinal tissue of patients with Crohn's disease can respond to TLR ligands in the bacterial component-rich environment. It is notable that the expression levels of both TNF- α mRNA and ColI mRNA were similar between fibrocytes derived from patients with Crohn's disease and healthy controls, suggesting that the fibrocytes themselves were not a primary pathogenetic factor in Crohn's disease but that they exacerbated inflammation and fibrosis when they were recruited to the bacterial component-rich environment.

There were some limitations in this study. The design of the present study was purely cross-sectional in that we did not show that the circulating fibrocytes infiltrated the inflammatory lesions of patients with Crohn's disease and

Fig. 3 Gene expression in cultured human fibrocytes. Cultured fibrocytes were obtained by incubating CD14⁺ cells from patients with Crohn's disease ($n = 6$; black bar) and healthy controls ($n = 8$; gray bar) for 14 days, as described in "Materials and Methods". They were incubated with lipopolysaccharide [LPS, a toll-like receptor (TLR)-4 agonist], flagellin (FLA, a TLR5 agonist), CpG-oligodeoxynucleotide (CpG-ODN, a TLR9 agonist), and transforming growth factor- β (TGF- β). **a** Isolated and cultured fibrocytes in a bright-field image. The cultured fibrocytes are spindle-shaped cells that attached surface of the culture dish. **b** TNF- α mRNA/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The data are expressed as mean \pm SEM. $*p < 0.01$, as compared with controls. **c** ColII mRNA/GAPDH mRNA. The data are expressed as mean \pm SEM. $*p < 0.01$, as compared with controls. **d** Matrix metalloproteinase-9 (MMP-9) mRNA/GAPDH mRNA. The data are expressed as mean \pm SEM. $*p < 0.01$, as compared with controls



that the immature fibrocytes in the inflammatory mucosa differentiated into fibroblasts/myofibroblasts. In order to address this question, fibrocyte tagging is required to assess their contribution.

The present study and our previous study that used a murine model of colitis [11] provide a new paradigm that fibrocytes infiltrate into the intestinal mucosa in the inflammatory phase and that they behave biphasically, similar to macrophages and myofibroblasts, in the pathogenesis of Crohn's disease. They have a proinflammatory phenotype that is induced by innate immune signals, such as LPS, and they have a profibrotic phenotype that is induced by TGF- β and innate immune signals such as LPS. Later in the fibrotic phase of Crohn's disease, after the inflammation regresses and repair and remodeling begins, the fibrocytes themselves disappear, and the fibroblasts/myofibroblasts transformed from fibrocytes continue to produce extracellular matrices in the intestinal walls of patients with Crohn's disease. It is plausible that a considerable number of fibrocytes that infiltrate into the

intestinal wall in the inflammatory phase may result in Crohn's disease becoming a profibrotic disease by directly producing extracellular matrices, activating resident fibroblasts/myofibroblasts through TGF- β production, and transforming to fibroblasts/myofibroblasts by themselves.

This study advocates fibrocytes as a novel target for controlling the disease activity of Crohn's disease, which is characterized by chronic inflammation that is accompanied by severe fibrosis in the intestinal wall. Advances in biologics, such as use of anti-TNF- α and anti-IL-6 agents, have made it possible to dramatically alleviate inflammation in the intestinal wall in patients with Crohn's disease. However, once inflammation is sustained in the intestinal wall, it is accompanied by fibrostenotic changes and fistula formation for which the patients with Crohn's disease need to undergo surgical treatment. The emergence of fibrocytes in tissue early in the inflammatory phase suggests that the best way to avoid fibrostenotic changes is to avoid inflammation in the intestinal walls of patients with Crohn's disease. Johnson et al. [24] recently reported that intestinal fibrosis

is reduced by the early elimination of inflammation in a murine model of colitis. The results of the present study support their notion and indicate that an early top-down approach for attenuating inflammation is beneficial for minimizing late fibrotic changes in the intestinal mucosa by reducing the infiltration of fibrocytes. We speculate that fibrocytes will be one of the targets of biologics and their apoptosis might be induced by treatment with anti-TNF- α agents because they produce a considerable amount of TNF- α in response to LPS stimulation as shown in this study. Collectively, our data suggest that fibrocytes are one of the determinants of the natural history of Crohn's disease and that regulating their function would be beneficial for treating patients with Crohn's disease.

In conclusion, this study demonstrated for the first time that fibrocytes were possibly involved in the pathogenesis of Crohn's disease. They may not only enhance inflammation by producing TNF- α and leukocyte recruitment but also directly augment fibrosis by producing ColI. Fibrocytes may be a novel target in the treatment of patients with Crohn's disease with the aim of changing its natural history.

Acknowledgments We are grateful to Yoshiko Noguchi and Fumie Saegusa for excellent technical assistance.

Conflict of interest None.

References

- Burke JP, Mulrow JJ, O'Keane C, Docherty NG, Watson RW, O'Connell PR. Fibrogenesis in Crohn's disease. *Am J Gastroenterol*. 2007;102:439–448.
- Rieder F, Brenmoehl J, Leeb S, Scholmerich J, Rogler G. Wound healing and fibrosis in intestinal disease. *Gut*. 2007;56:130–139.
- Rieder F, Fiocchi C. Intestinal fibrosis in inflammatory bowel disease: progress in basic and clinical science. *Curr Opin Gastroenterol*. 2008;24:462–468.
- Rieder F, Fiocchi C. Intestinal fibrosis in IBD—a dynamic, multifactorial process. *Nat Rev Gastroenterol Hepatol*. 2009;6:228–235.
- Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med*. 1994;1:71–81.
- Reilkoff RA, Bucala R, Herzog EL. Fibrocytes: emerging effector cells in chronic inflammation. *Nat Rev Immunol*. 2011;11:427–435.
- Quan TE, Cowper S, Wu SP, Bockenstedt LK, Bucala R. Circulating fibrocytes: collagen-secreting cells of the peripheral blood. *Int J Biochem Cell Biol*. 2004;36:598–606.
- Bellini A, Mattoli S. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. *Lab Invest*. 2007;87:858–870.
- Metz CN. Fibrocytes: a unique cell population implicated in wound healing. *Cell Mol Life Sci*. 2003;60:1342–1350.
- Herzog EL, Bucala R. Fibrocytes in health and disease. *Exp Hematol*. 2010;38:548–556.
- Uehara H, Nakagawa T, Katsuno T, et al. Emergence of fibrocytes showing morphological changes in the inflamed colonic mucosa. *Dig Dis Sci*. 2010;55:253–260.
- Garcia-de-Alba C, Becerril C, Ruiz V, et al. Expression of matrix metalloproteases by fibrocytes: possible role in migration and homing. *Am J Respir Crit Care Med*. 2010;182:1144–1152.
- Yang L, Scott PG, Dodd C, et al. Identification of fibrocytes in postburn hypertrophic scar. *Wound Repair Regen*. 2005;13:398–404.
- Wu Y, Wang J, Scott PG, Tredget EE. Bone marrow-derived stem cells in wound healing: a review. *Wound Repair Regen*. 2007;15:S18–S26.
- Peng H, Herzog EL. Fibrocytes: emerging effector cells in chronic inflammation. *Curr Opin Pharmacol*. 2012;12:491–496.
- Schmidt M, Sun G, Stacey MA, Mori L, Mattoli S. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *J Immunol*. 2003;171:380–389.
- Mehrad B, Burdick MD, Zisman DA, Keane MP, Belperio JA, Strieter RM. Circulating peripheral blood fibrocytes in human fibrotic interstitial lung disease. *Biochem Biophys Res Commun*. 2007;353:104–108.
- Moeller A, Gilpin SE, Ask K, et al. Circulating fibrocytes are an indicator of poor prognosis in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2009;179:588–594.
- Mathai SK, Gulati M, Peng X, et al. Circulating monocytes from systemic sclerosis patients with interstitial lung disease show an enhanced profibrotic phenotype. *Lab Invest*. 2010;90:812–823.
- Phillips RJ, Burdick MD, Hong K, et al. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest*. 2004;114:438–446.
- Balmelli C, Alves MP, Steiner E, et al. Responsiveness of fibrocytes to toll-like receptor danger signals. *Immunobiology*. 2007;212:693–699.
- Pasternak BA, D'Mello S, Jurickova II, et al. Lipopolysaccharide exposure is linked to activation of the acute phase response and growth failure in pediatric Crohn's disease and murine colitis. *Inflamm Bowel Dis*. 2010;16:856–869.
- Zareie M, Singh PK, Irvine EJ, Sherman PM, McKay DM, Perdue MH. Monocyte/macrophage activation by normal bacteria and bacterial products: implications for altered epithelial function in Crohn's disease. *Am J Pathol*. 2001;158:1101–1109.
- Johnson LA, Luke A, Sauder K, Moons DS, Horowitz JC, Higgins PD. Intestinal fibrosis is reduced by early elimination of inflammation in a mouse model of IBD: impact of a "Top-Down" approach to intestinal fibrosis in mice. *Inflamm Bowel Dis*. 2012;18:460–471.