Activation of p38 mitogen-activated protein kinase promotes peritoneal fibrosis by regulating fibrocytes

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Running title
p38MAPK in peritoneal fibrosis

ABSTRACT

Background: Peritoneal fibrosis is a serious complication of long-term peritoneal dialysis, yet the precise pathogenic mechanisms of peritoneal fibrosis remain unknown. Fibrocytes participate in tissue fibrosis and express chemokine receptors that are necessary for their migration. The p38 mitogen-activated protein kinase (MAPK) pathway regulates the production of chemokines, and it has been demonstrated to contribute to the pathogenesis of different fibrotic conditions. Accordingly, we examined the dependency of p38MAPK signaling in fibrocytes using an experimental mouse model of peritoneal fibrosis.

Methods: Peritoneal fibrosis was induced in mice by the injection of 0.1% chlorhexidine gluconate (CG) into the abdominal cavity. Mice were treated with FR167653, a specific inhibitor of p38MAPK, and immunohistochemical studies were performed to detect fibrocytes as well as phosphorylated p38MAPK-positive cells. The involvement of p38MAPK in the activation of fibrocytes also was investigated *in vitro*.

Results: Fibrocytes infiltrated in the peritoneum in response to CG, and this response was accompanied by progressive peritoneal fibrosis. The phosphorylation of p38MAPK was detected both in peritoneal mesothelial cells and in fibrocytes, as defined by CD45-positive spindle-shaped cells. The level of peritoneal expression of CCL2, which is a chemoattractant for fibrocytes, was up-regulated by CG injection, and treatment with FR167653 reduced the number of phosphorylated p38MAPK-positive cells, peritoneal CCL2 expression, and the extent of peritoneal fibrosis. Pre-treatment with FR167653 inhibited transforming growth factor-β1-induced expression of pro-collagen type I α1.

Conclusions: These results suggest that p38MAPK signaling contributes to peritoneal fibrosis by regulating fibrocyte function.

Key words

Chemokine, fibrocytes; fibrosis; p38MAPK; peritoneum

INTRODUCTION

Peritoneal dialysis (PD) is a beneficial treatment for patients with end-stage renal diseases. Long-term PD treatment causes histopathological alterations in the peritoneum, including fibrosis, which is associated with ultrafiltration failure and loss of the dialytic capacity in the peritoneum [1,2]. Encapsulating peritoneal sclerosis (EPS) also can develop, this causes an ileus and is associated with high mortality [3]. The precise pathogenic mechanisms that underlie the development of progressive peritoneal fibrosis remain unknown.

Circulating fibrocytes have been reported to be involved in various fibrotic diseases such as idiopathic pulmonary fibrosis, renal fibrosis, and hypertrophic scarring [4-7]. Fibrocytes comprise a minor fraction of the circulating pool of leukocytes and share markers of myeloid (e.g., CD45 and CD34) and mesenchymal (e.g., type I collagen (Col I) and fibronectin) cells [4-8]. Additionally, fibrocytes express chemokine receptors (e.g., CCR2, CCR5, CXCR4, and CCR7) that are involved in the recruitment of fibrocytes to sites of fibrosis [4-7,9]. To date, the potential involvement of fibrocytes in peritoneal fibrosis has not been investigated.

The p38 mitogen-activated protein kinase (MAPK) mediates an important intracellular signal transduction pathway by which signals from environmental stimuli are transmitted to the nucleus. At least three distinct groups of MAPKs have been identified; extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38MAPK [10]. The activation of p38MAPK is involved in apoptosis, stress responses, and inflammation [10]. Recent studies have revealed that p38MAPK phosphorylation is essential for the production of various chemokines including monocyte chemoattractant protein (MCP)-1/CCL2, and for the signal transduction of chemokine receptor such as CCR2, which is a cognate receptor for CCL2 [11-16]. In addition, p38MAPK has been demonstrated to contribute to the pathogenesis of fibrotic conditions [17-21]. Taken together, these findings prompted us to examine the involvement of p38MAPK signaling in fibrocyte function and the pathogenesis of peritoneal fibrosis. We evaluated peritoneal fibrosis induced in mice by intraperitoneal injection of chlorhexidine gluconate (CG), which is a well-known model of peritoneal fibrosis [22]. We report herein that p38 MAPK signaling mediates fibrocyte function in the pathogenesis of peritoneal fibrosis and that blockade of p38MAPK signaling may be a beneficial approach for the treatment of progressive, peritoneal fibrosis.

SUBJECTS AND METHODS

Peritoneal fibrosis in murine model

Inbred male C57BL/6 mice (8 weeks, 20-25g) were obtained from Charles River Japan (Atsugi, Kanagawa, Japan). Peritoneal fibrosis was induced by intraperitoneal injection of 0.1% CG dissolved in 15% ethanol/saline every other day, over a period of 21 days as described previously [22,23]. A specific p38MAPK inhibitor, FR167653 (16 and 32 mg/kg per day), dissolved in drinking water, was orally administrated from 2 days before CG injection [15,16,24]. Mice in each group (Day 0; n=5, CG mice; n=9, CG+FR mice; n=10) were sacrificed 21 days after CG injection. All procedures used in the animal experiments complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University.

Tissue preparation

One portion of the peritoneal tissue was fixed in 10% buffered formalin (pH 7.2), embedded in paraffin, cut at 4 μm, stained with hematoxylin and eosin or Mallory-Azan and observed under a light microscope. The thickness of the submesothelial collagenous zone above the abdominal muscle layer in cross-sections of the abdominal wall was defined as peritoneal thickness, as described previously [22]. In each image, peritoneal thickness was measured at 10 different points. Peritoneal cross-sections were observed by two investigators and averaged to determine the peritoneal thickness. Measurement of the peritoneal thickness was performed by image analysis using Mac Scope version 6.02 (Mitani shoji Co., Fukui, Japan).

Detection of fibrocytes in the peritoneum by immunohistochemical studies

Fibrocytes were identified in tissue samples by dual immunohistochemical techniques using specific antibodies against CD45 and Col I as previously described [5]. For this analysis, formalin-fixed, paraffin-embedded sections were prepared as described previously. Briefly, tissue sections were incubated with rat anti-mouse CD45 polyclonal antibodies (R&D Systems Inc., Minneapolis, USA) and rabbit anti-mouse Col I polyclonal antibodies (CHEMICON International, Inc., Temecula, California, USA). CD45 was visualized by incubating sections with fluorescein isothianate (FITC)-conjugated donkey anti-rat IgG antibodies (Jackson Immunoreseach Laboratory, Inc. West Grove, Pennsylvania, USA). Col I was visualized with Cy3-conjugated donkey anti-rabbit IgG antibodies (Jackson Immunoreseach Laboratory). The number of fibrocytes in the peritoneum was counted in all fields of

the submesothelial zone, and expressed as the mean number ± standard error (SEM)/mm^2. In addition, dual-immunostainings for CCR2 and Col I was performed to characterize fibrocytes in the peritoneum. The tissue sections were incubated with goat anti-mouse CCR2 antibody (GeneTex, Inc., Irvine, California, USA) and rabbit anti-mouse Col I polyclonal antibodies (CHEMICON International, Inc). The number of CCR2-/Col I-dual positive fibrocytes in the peritoneum was counted in all fields of the submesothelial zone and expressed as the mean number ± standard error (SEM)/mm^2.

Detection of phosphorylated p38MAPK-positive cells

Immunohistochemistry for phosphorylated p38MAPK (p-p38MAPK) was performed to clarify the localization of p-p38MAPK-positive cells. Formalin-fixed and paraffin-embedded sections were incubated with rabbit anti-mouse p-p38MAPK polyclonal antibodies (Cell Signaling Technology Inc. Danvers, Massachusetts, USA). The number of p-p38MAPK-positive mesothelial cells was counted and expressed as the percentages of total mesothelial cells. In addition, dual-immunostaining for CD45 and p-p38MAPK was performed to characterize the p-p38MAPK positive cells. Of these dual-positive cells, CD45- and p-p38MAPK-dual positive spindle-shaped cells were regarded as p-p38MAPK-positive fibrocytes. The number of these cells was counted in all fields of the submesothelial zone, and expressed as the mean number ± standard error (SEM)/mm^2.

Detection of CCL2-positive cells

Immunohistochemistry for CCL2 was performed to clarify the localization of CCL2-positive cells. Formalin-fixed and paraffin-embedded sections were incubated with rat anti-mouse CCL2 polyclonal antibodies (Hycult Biotechnology B.V. Uden, The Netherlands).

Isolation of human fibrocytes from peripheral blood

Fibrocytes were harvested and cultured as previously reported [7]. Briefly, total peripheral blood mononuclear cells (PBMCs) were isolated from venous blood drawn from healthy donors (n=3) by centrifugation on a Ficoll-Metrizonate density gradient (d=1.077 g/mL; Lymphoprep; Nycomed, Oslo, Norway) following the manufacture's protocol. After 2 days of culture on tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL Life Tech., Carlsbad, California, USA) supplemented 20% heat-inactivated fetal calf serum (FCS) (Gibco BRL Life Tech.), 100

U/mL penicillin (Gibco BRL Life Tech.), and 100 μg/mL streptomycin (Gibco BRL Life Tech.), nonadherent cells were removed by gentle aspiration and media were replaced. After 10-12 days, adherent cells were lifted by incubation in ice-cold 0.05% EDTA in PBS. The crude fibrocyte preparations then were depleted by immunomagnetic selection of contaminating T cells, B cells, and monocytes using pan-T, anti-CD2; pan-B, anti-CD19; and anti-CD14 monoclonal antibody coated with microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell purity was verified by flow cytometry using both mouse anti-human CD45 monoclonal antibody (Becton Dickinson, PharMingen) and rabbit anti-human Col I polyclonal antibodies (CHEMICON international, Inc.).

Culture condition of fibrocytes

Purified fibrocytes were incubated with transforming growth factor (TGF)- β_1 (R&D Systems Inc.) in a time dependent manner. Briefly, purified human fibrocytes ($1x10^6$/mL) were cultured in 12-well plates (Corning Inc., Corning, New York) in DMEM supplemented with 0.5% heat-inactivated FCS (Gibco, BRL) at 37C in a humidified atmosphere with 5% CO_2 for 0-24 h, and in the presence of TGF-β_1 (10 ng/mL). In parallel experiments, fibrocytes were pretreated with FR167653 (32mg/L) and then exposed to TGF-β_1.

Real-time RT-PCR

To determine transcripts for the α1 chain of pro-collagen type I (COLIAI) and CCL2 in the peritoneum, total RNA was extracted from the whole peritoneum. Similarly, total RNA was extracted from cultured human fibrocytes and was analyzed for the detection of transcripts of COLIAI. Quantitative real-time PCR was performed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, California, USA) as previously described [5]. Murine beta-glucuronidase and human glyceraldehydes-3-phosphate dehydrogenase were used for PCR controls.

Statistical analysis

The mean and SEM were calculated on all the parameters determined in this study. Statistical analyses were performed using Wilcoxon rank-sum test, Kruskal-Wallis test and ANOVA test. A P value less than 0.05 was accepted as statistically significant.

RESULTS

Effect of p38MAPK on peritoneal fibrosis

Morphologic changes were assessed by hematoxylin and eosin staining or Mallory-Azan staining. In the normal mouse, the peritoneal tissue consisted of a peritoneal mesothelial monolayer and an exiguity of connective tissues under the mesothelial layer (Figure 1a). At day 21, the peritoneal samples of wild-type mice injected with CG showed marked thickening of the submesothelial compact zone and the presence of numerous cells (Figure 1b). In contrast, the thickness of the submesothelial zone as well as the number of infiltrating cells in mice treated with FR167653 (CG+FR mice) was significantly less than those of wild-type mice (CG mice) (Figure 1c). The mean peritoneal thickness determined by computer analysis was reduced in CG+FR mice as compared with CG mice (Figure 1d). The effect of FR167653 at a dose of 32 mg/kg per day was more marked than that observed in mice treated with FR of 16 mg/kg per day. Therefore, we adopted the data at dose of 32 mg/kg per day in the following examinations. The expression of COL1A1 mRNA in the peritoneum also was reduced by the treatment with FR167653 (Figure 1e).

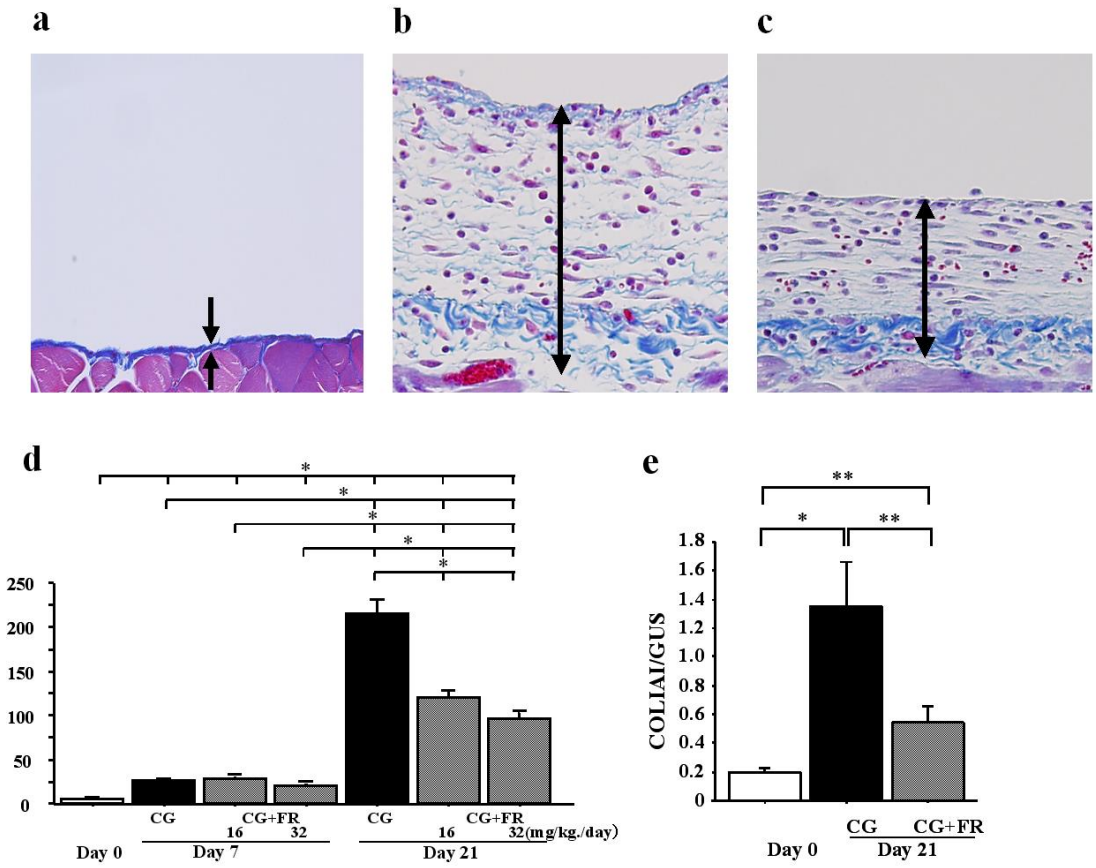

Figure 1. Peritoneal thickness and pro-collagen type I α1 (COLIAI) expression in the murine peritoneum.

a: Normal control at day 0 did not show evidence of peritoneal thickness. b: Peritoneal thickness at 21 days after CG injection (CG mice). c: Peritoneal thickness at 21day treated with FR167653 (CG+FR mice). d: CG injection caused peritoneal thickness in CG mice. In contrast, peritoneal thickness was reduced in CG+FR mice as compared with CG mice . e: The up-regulation of COLIAI mRNA was induced by CG injection and treatment with FR167653 reduced the level of COLIAI expression. Values are mean ±SEM. * P<0.01. **P<0.05. Original magnifications, X200.

CD45/Col I-dual positive fibrocytes infiltrate the peritoneum

To examine the presence of fibrocytes in the peritoneum, dual-immunostaining for CD45 and Col I was performed. As originally reported, one of the unique characteristics of fibrocytes is their simultaneous expression of both CD45 and Col I [4-9]. Accordingly, these cells can be identified in tissue samples by double immunohistochemistry using specific antibodies against CD45 and Col I. CD45/Col

I-dual positive fibrocytes infiltrated the peritoneum after CG injection (Figure 2a-c). The number of infiltrated fibrocytes increased in accordance with the progression of fibrosis after CG injection, and was reduced in CG+FR mice (231.6±27.9/mm^2) as compared with that in CG mice (616.0±123.0/mm^2) (Figure 2d).

CCR2-expressing fibrocytes and peritoneal CCL2 expression in the peritoneum

To better characterize infiltrated fibrocytes in the peritoneum, dual-immunostaining for CCR2 and Col I was performed. Numerous CCR2-positive fibrocytes were detected in the peritoneum after CG injection (Figure 3a-c). The number of CCR2-positive fibrocytes increased in accordance with the progression of fibrosis after CG injection, and was reduced in CG+FR mice (150.9±13.3/mm^2) as compared with that in CG mice (406.7±39.8/mm^2) (Figure 3d). To elucidate the effect of p-p38MAPK signaling on peritoneal chemotactic molecules, the expression of CCL2 was examined. CCL2 protein was induced by CG injection in peritoneal mesothelial cells as well as interstitial cells (Figure 3e). The mRNA transcripts for CCL2 were up-regulated in the peritoneum after CG injection, while the levels of CCL2 were reduced in CG+FR mice (Figure 3f).

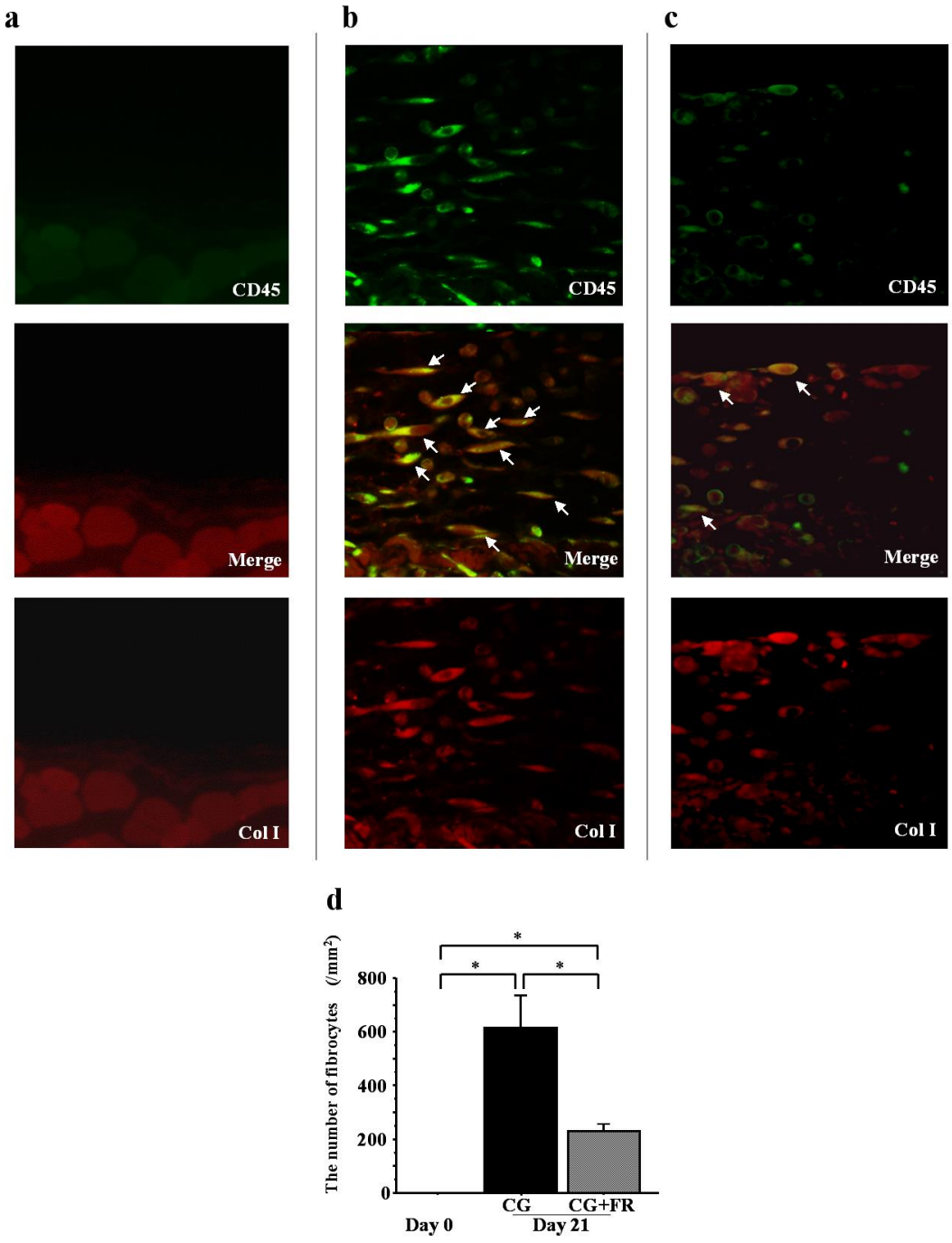

Figure 2. Fibrocyte infiltration in the peritoneum.

Dual-immunostainings for CD45/Col I were performed (green: CD45, yellow: dual positive fibrocyte(arrows), red: Col I). CD45/ Col I-dual positive fibrocytes infiltrated in the peritoneum after CG injection (a: Normal control at day 0, b: CG mice, c: CG+FR mice). Arrows indicate dual positive cells for CD45 and Col I. d: The number of infiltrated

fibrocytes was reduced in mice treated with FR167653. Values are mean ±SEM. * P<0.01. Original magnifications, X200.

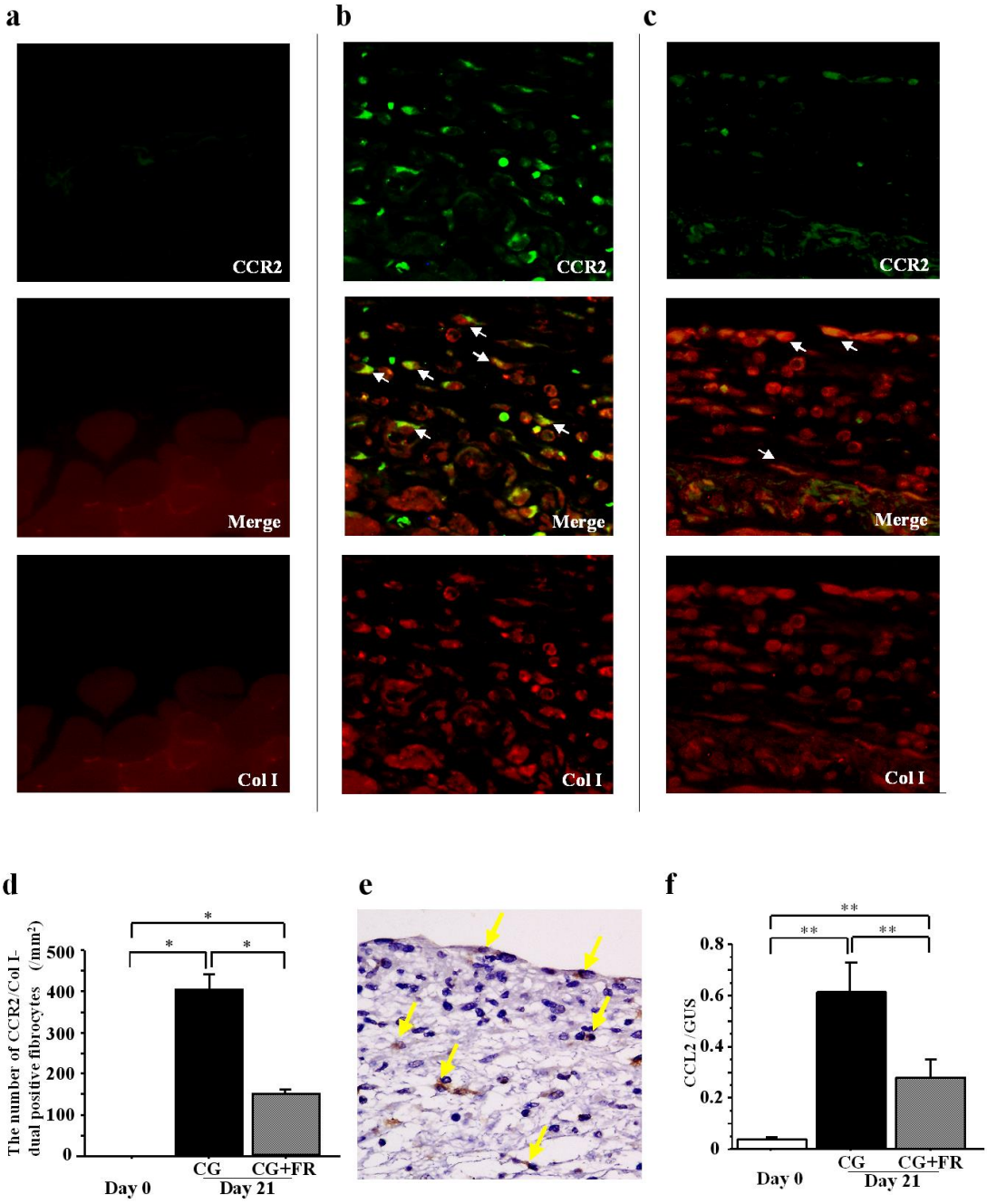

Figure 3. CCR2-expressing fibrocytes and CCL2 production in the peritoneum. Dual-immunostainings for CCR2/Col I were performed (green: CCR2, yellow: CCR2-positive fibrocyte(arrows), red: Col I). CCR2-positive fibrocytes were detected in the peritoneum after CG injection (a: Normal control at day 0, b: CG mice, c: CG+FR mice). White arrows

indicate dual positive cells for CCR2 and Col I. d: The number of infiltrated CCR2-positive fibrocytes was reduced in mice treated with FR167653. e: The production of CCL2 protein was induced by CG injection in peritoneal mesothelial cells as well as interstitial cells. Yellow arrows indicate CCL2-positive cells. f: mRNA transcripts for CCL2 in the peritoneum after CG injection were up-regulated, while the levels of CCL2 were reduced in mice treated with FR167653. Values are mean ±SEM. * P<0.01. **P<0.05. Original magnifications, X200.

Phosphorylated p38MAPK-positive cells in the peritoneum

Immunohistochemical studies were performed in order to clarify the localization of p-p38MAPK-positive cells in the peritoneum. p-p38MAPK-positive cells were detected in peritoneal mesothelial cells as well as interstitial cells in thickened peritoneum after CG injection (Figure 4a). The percentages of p-p38MAPK-positive mesothelial cells in CG mice were higher than that in CG+FR mice (Figure 4b). In addition, the number of interstitial p-p38MAPK-positive cells in the peritoneum was reduced in CG+FR mice compared with that in CG mice (Figure 4c).

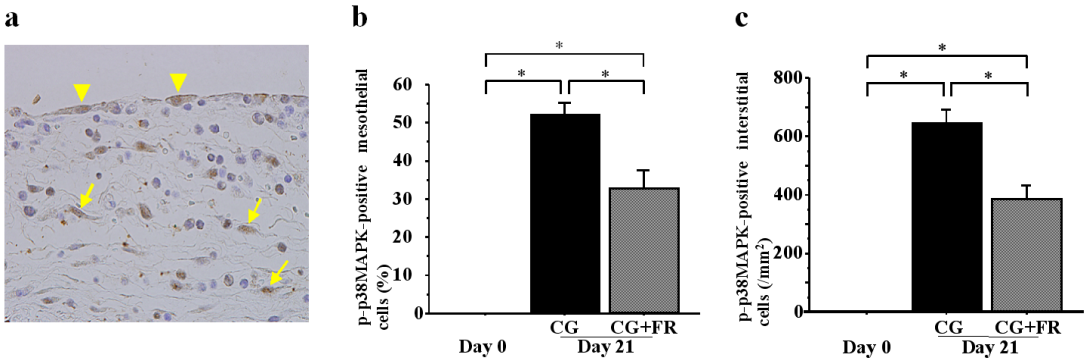

Figure 4. The presence of p-p38MAPK positive cells in the peritoneum.

a: Arrows indicate p-p38MAPK positive mesothelial cells. Arrowheads indicate p-p38MAPK-positive spindle-shaped cells. b: The percentage of p-p38MAPK-positive mesothelial cells in peritoneum was decreased in mice treated with FR167653. c: The number of interstitial p38MAPK-positive cells in the peritoneum was significantly reduced in mice treated with FR167653. Values are mean ±SEM. * P<0.01. Original magnifications, X200.

CD45/phosphorylated p38MAPK-dual positive cells in the peritoneum

To confirm the identity of peritoneal p-p38 MAPK-positive cells, dual immunostaining for CD45 and p-p38MAPK was performed. A portion of the

CD45/p-p38MAPK-dual positive cells comprised spindle-shaped cells, suggesting the presence of p-p38MAPK-positive fibrocytes (Figure 5a-c). The number of CD45/p-p38MAPK-dual positive spindle-shaped cells in the peritoneum was reduced in CG+FR mice compared with that in CG mice (Figure 5d).

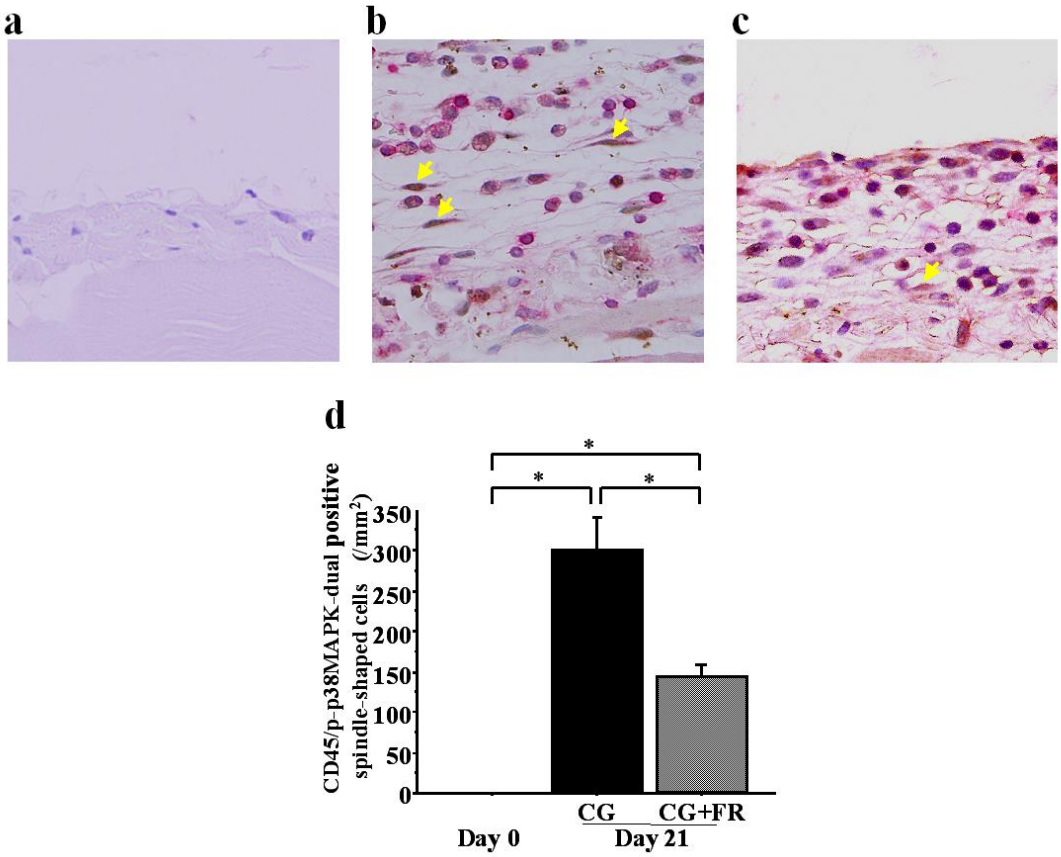

Figure 5. The characterization of peritoneal p-p38MAPK-positive cells.
Dual-immunostainings for CD45/p-p38MAPK were performed (brown: p-p38MAPK, red: CD45). Arrows indicate CD45/p-p38MAPK-dual positive spindle-shaped cells (a: Normal control at day 0, b: CG mice, c: CG+FR mice). d: The number of CD45/p-p38MAPK-dual positive spindle-shaped cells was reduced in mice treated with FR167653. Values are mean ±SEM. * P<0.01. **P<0.05. Original magnifications, X200.

The production of COLIAI mRNA in isolated fibrocyte was reduced by the treatment with FR167653

The impact of p38MAPK signaling on COLIAI expression in isolated fibrocytes was examined *in vitro*. Circulating fibrocytes were isolated from human peripheral blood (>97% pure population of cells coexpressing CD45 and Col I), cultured in

DMEM/0.5% heat-inactivated FCS for 24 hours, and then stimulated with TGF-β□ (10ng/mL) for 24 hours. Stimulation with TGF-β$_1$ increased the expression of COLIAI while pretreatment with FR167653 abrogated TGF-β$_1$-induced expression of COLIAI (Figure 6).

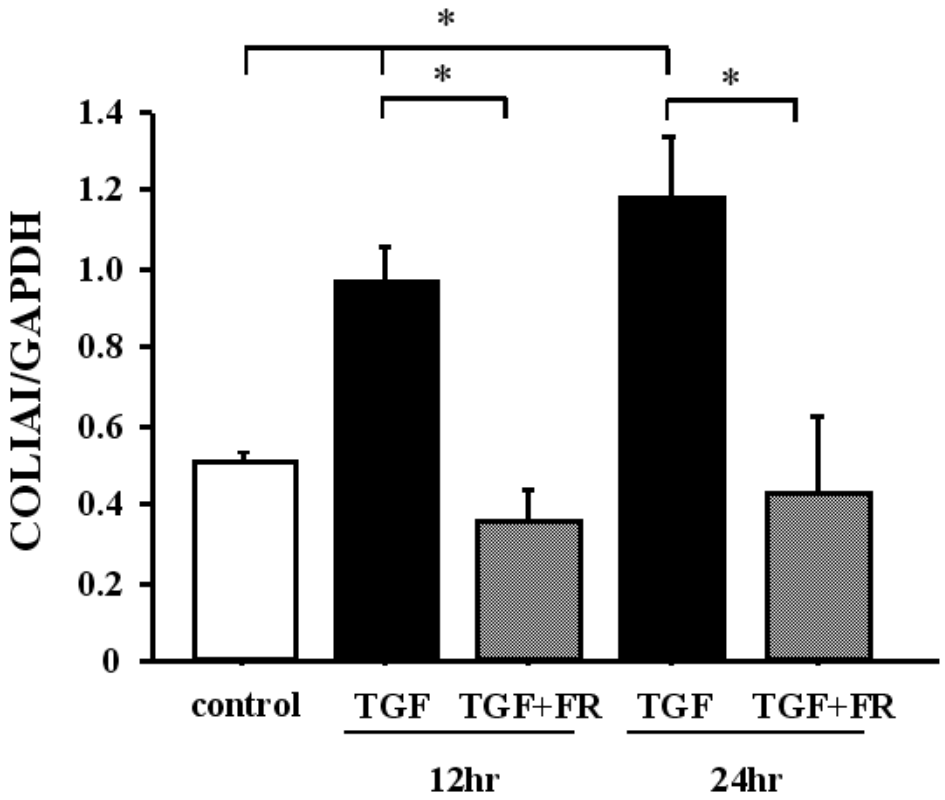

Figure 6. The effect of p38MAPK on the production of procollagen typeI α1 (COLIAI). The production of COLIAI mRNA in isolated fibrocytes was induced by the stimulation of TGFβ$_1$, and was reduced by the treatment with FR167653. Values are mean ±SEM. * P<0.05.

DISCUSSION

In this study, we provide evidence for the infiltration of fibrocytes in the peritoneum in a murine peritoneal fibrosis model. The number of fibrocytes in the peritoneum increased in accordance with the progression of peritoneal fibrosis. The infiltrated fibrocytes expressed the chemokine receptor CCR2 and an increase in the

phosphorylation of p38MAPK was detected in peritoneal mesothelial cells and interstitial cells including fibrocytes. The number of p-p38MAPK-positive cells as well as the level of peritoneal CCL2 expression was associated with the severity of fibrosis. Pharmacologic inhibition of p38MAPK signaling reduced the extent of fibrosis, the number of fibrocytes and p-p38MAPK-positive cells, and the peritoneal expression of peritoneal CCL2. Furthermore, inhibition of p38MAPK signaling attenuated the expression of COLIAI induced by the stimulation with TGF-β_1 in cultured fibrocytes. These results suggest that the p38MAPK pathway contribute to the pathogenesis of peritoneal fibrosis through the infiltration and activation of fibrocytes.

The activation of p38MAPK signaling is involved in the pathogenesis of renal and pulmonary fibrosis via the up-regulation of pro-inflammatory mediators [10, 11,17,18,20,21,25]. The phosphorylation of p38MAPK has been demonstrated in resident cells including renal tubular epithelial cells (RTECs) both in an experimental renal fibrosis model and human renal diseases [20,21]. The present study also revealed the phosphorylation of p38MAPK in peritoneal mesothelial cells. RTECs as well as peritoneal mesothelial cells have been reported to be capable of producing chemokines via p38MAPK signaling cascades [26,27]. The number of p-p38MAPK-positive cells as well as the level of CCL2 expression in mesothelial cells also increased concomitantly with disease progression, while the treatment with FR167653 reduced those changes. From these findings, p38MAPK may contribute to the production of CCL2 in peritoneal mesothelial cells, thereby promoting the pathogenesis of peritoneal fibrosis.

The infiltration of fibrocytes into diseased organs has been reported to be regulated by chemokine-chemokine receptor systems, including CCL2 and its receptor CCR2 [4-6,9,28]. In addition, the inhibition of CCR2/CCL2 system has been shown to ameliorate the infiltration of fibrocytes and the severity of fibrosis in a murine pulmonary fibrosis model [9]. In the present study, the blockade of p38MAPK signaling reduced the number of CCR2-expressing fibrocytes in accordance with the levels of CCL2 expression as well as the intensity of peritoneal fibrosis, suggesting that the CCR2/CCL2 system is dependent on p38MAPK. The potential roles of other chemokine systems including CCR7/CCL21 and CXCR4/CXCL12 in the pathogenesis of peritoneal fibrosis remain to be determined.

Finally, we observed that the expression of COLIAI in cultured fibrocytes was observed to be enhanced by the stimulation with TGF-β_1, and pre-treatment with FR167653 abrogated the expression of COLIAI. p38MAPK signaling thus may

contribute additionally to the pathogenesis of peritoneal fibrosis by directly regulating the production of collagen in fibrocytes. Such a pathway has previously been suggested in studies of pro-collagen type I and fibronectin expression by scleroderma fibroblasts [29]. Furthermore, CCR2-expressing fibrocytes have been shown to express type I pro-collagen in response to stimulation with CCL2 in a murine pulmonary fibrosis model [9].

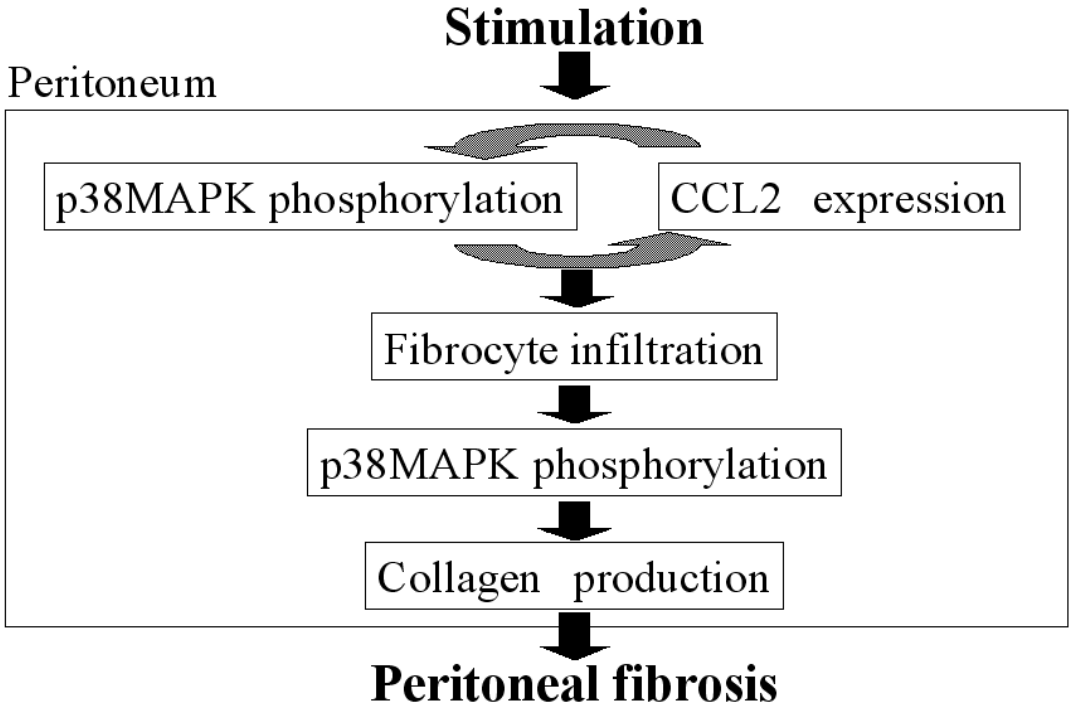

Figure 7. Proposed schema for p38MAPK signaling in the pathogenesis of peritoneal fibrosis through (1) the regulation of fibrocytes infiltration via CCL2 production and (2) production of collagen in fibrocytes.

CONCLUSION

Our results suggest that p38MAPK signaling is involved in the pathogenesis of peritoneal fibrosis through the regulation of fibrocyte infiltration and collagen production (Figure7). Inhibition of the recruitment and activation of fibrocytes with p38MAPK signal transduction could provide a novel therapeutic approach for combating organ fibrosis.

ACKNOWLEDGEMENTS

We are grateful to Professor Richard Bucala (Yale University) for careful review of the manuscript. This work was supported by a Grant-in-aid from the Ministry of Education, Science, Sport and Culture of Japan (TW, NS), Takeda Science Foundation (TW) and Japan Baxter PD Fund (NS).

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