Fibrocytes are involved in the pathogenesis of human chronic kidney disease

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Norihiko Sakai, M.D., PhD*, Kengo Furuichi, M.D., PhD*, Yasuyuki Shinozaki, M.D.**, Hiroyuki Yamauchi, M.D.**, Tadashi Toyama, M.D.**, Shinji Kitajima, M.D.**, Toshiya Okumura, M.D.**, Satoshi Kokubo, M.D.**, Motoo Kobayashi, M.D.**, Kazuya Takasawa, M.D., PhD†, Shin-ichi Takeda, M.D., PhD††, Mitsuhiro Yoshimura, M.D., PhD †††, Shuichi Kaneko, M.D., PhD**, Takashi Wada, M.D., PhD***

*Division of Blood Purification, Kanazawa University Hospital, Kanazawa, Japan.
**Department of Disease Control and Homeostasis, and ***Department of Laboratory Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Faculty of Medicine, Kanazawa University, Kanazawa, Japan.
†Department of Internal Medicine, Public Central Hospital of Matto Ishikawa, Hakusan, Japan.
††Department of Internal Medicine, Kurobe Municipal Hospital, Kurobe, Japan.
†††Department of Internal Medicine, Kanazawa Medical Center, Kanazawa, Japan.

Reprint request to: Dr. Takashi Wada,
Department of Laboratory Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Faculty of Medicine, Kanazawa University, Kanazawa, Japan.
13-1 Takara-machi, Kanazawa 920-8641, Japan
tel +81-76-265-2000 (ext 2850), fax +81-76-234-4250, e-mail: twada@m-kanazawa.jp

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Summary

The presence of chronic kidney disease in humans is associated with a risk of kidney function loss as well as the development of cardiovascular disease. Fibrocytes have been shown to contribute to organ fibrosis. In this study, the presence of fibrocytes was investigated immunohistochemically in kidney biopsy specimens from 100 patients with chronic kidney disease. In addition, 6 patients with thin basement membrane disease were studied as a disease control. In patients with chronic kidney disease, the infiltration of fibrocytes was observed mainly in the interstitium. The number of interstitial fibrocytes in patients with chronic kidney disease was higher than that in patients with thin basement membrane disease. The number of infiltrated fibrocytes in the interstitium correlated well with the severity of tubulointerstitial lesions, such as interstitial fibrosis, in patients with chronic kidney disease. In addition, there were significant correlations between the number of interstitial fibrocytes and the number of CD68-positive macrophages in the interstitium as well as urinary monocyte chemoattractant protein-1/CCL2 levels. In particular, there was an inverse correlation between the number of interstitial fibrocytes and kidney function at the time of biopsy. Finally, the numbers of interstitial fibrocytes and macrophages as well as urinary CCL2 levels were significantly decreased during convalescence induced by glucocorticoid therapy. These results suggest that fibrocytes may be involved in the pathogenesis of chronic kidney disease through the interaction with macrophages as well as CCL2.

Introduction

The presence of chronic kidney disease (CKD), manifested by low glomerular filtration rates (GFR) and/or urinary abnormalities, is associated with risk of kidney function loss leading to end-stage renal disease (ESRD). Recently, CKD has also been recognized as an independent risk factor of cardiovascular disease (CVD) [1]. Therefore, it is important to elucidate the pathophysiology of CKD. Despite varied etiologies, CKD progresses to ESRD through common pathological findings, including glomerulosclerosis and interstitial fibrosis. Especially, the severity of interstitial fibrosis has been reported to determine the prognosis of kidney function [2]. To date, resident fibroblasts, epithelial-mesenchymal transition (EMT)-derived fibroblasts/myofibroblasts, and monocytes/macrophages have been suggested to be involved in the progression of kidney diseases [3,4]. However, the precise pathogenic mechanisms of tubulointerstitial lesions, especially related to interstitial fibrosis, in patients with CKD remain to be determined.

Recently, a circulating bone marrow-derived population of fibroblast-like cells, termed fibrocytes, has been proposed to be a new cellular participant in the pathogenesis of organ fibrosis [5 – 9]. Fibrocytes express markers of leukocytes (*e.g.*, CD45 and CD34) and have the ability to produce extracellular matrix proteins (*e.g.*, type I collagen and fibronectin) [10,11]. The presence of fibrocytes has been demonstrated in experimental fibrosis associated with various conditions, such as lung, kidney, and liver fibrosis, as well as skin wounds [5 – 9]. Fibrocytes are also detected in human fibrosing disorders, including nephrogenic systemic fibrosis, bronchial asthma, and burns [12 – 14]. Fibrocytes have been reported to express chemokine receptors such as CCR2,

CCR7 and CXCR4 [5,6,15,16]. In addition, fibrocytes are capable of producing various chemokines including monocyte chemoattractant protein (MCP)-1/CCL2 as well as fibrogenic cytokines (*e.g.*, transforming growth factor (TGF)-β_1) [10]. Recent studies demonstrated that chemokine/chemokine receptor systems are required for the recruitment of fibrocytes to sites of fibrosis [5,6,8,16]. However, the involvement of fibrocytes in the pathogenesis of human CKD has not been fully investigated.

From these findings, we hypothesized that fibrocytes dependent on chemokine/chemokine receptor systems are involved in the pathogenesis of human CKD. To achieve this goal, we determined the presence of fibrocytes in patients with CKD. We also examined the association of fibrocytes infiltration with pathological findings as well as urinary levels of chemokines.

Subjects and methods

Subjects

One hundred patients (41 men and 59 women; median age, 50.2 years) with CKD were evaluated in this study (Table 1). CKD was defined as urinary abnormalities or GFR < 60 mL/min/1.73 m^2 for 3 months or more irrespective of cause, as described previously [17]. Twenty-seven patients (10 men and 17 women; median age, 62.5 years) had crescentic glomerulonephritis (CreGN) with more than 50% of the total crescents (cellular, fibrocellular, and fibrous) of all glomeruli showing rapidly progressive glomerulonephritis (RPGN) clinically. Twenty-six patients (2 men and 24 women; median age, 37.2 years) had lupus nephritis (LN) and were classified based on the glomerular appearance according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification (8 in Class II, 5 in Class III, 9 in Class IV, 4 in Class V) as described previously [18]. Twenty-three patients with type II diabetes mellitus (14 men and 9 women; median age, 55.8 years) had diabetic nephropathy (DN). In addition, other kidney diseases without crescents, including IgA nephropathy (IgA-N), obesity-related glomerulopathy (ORG), benign nephrosclerosis (BNS), and membranous nephropathy (MN), were also examined in this study. Six patients with thin basement membrane disease (TBMD; 4 men and 2 women; median age, 39.1 years) were included as disease controls. The patients in this study were chosen consecutively from May 1988 to July 2008 at Kanazawa University Hospital or its affiliated hospitals. All diagnoses were verified by kidney biopsy. Whenever possible, patients did not receive immunosuppressive agents before sample collection. Patients with CreGN, LN, or IgA-N in a clinically active state were treated with

glucocorticoids including methylprednisolone pulse therapy (500 – 1000 mg/day, 3 days) during this study. Specimens from second biopsies were obtained from 16 patients with CreGN ($n = 7$), LN ($n = 7$), and IgA-N ($n = 2$) after glucocorticoid therapy. Estimated glomerular filtration rate (eGFR) was determined as reported previously [19]. All kidney biopsies and this study were performed with approval from the Institutional Review Board and on receipt of informed consent from the patients.

Pathological studies

One hundred twenty-two kidney specimens were obtained by kidney biopsy. Each specimen contained 10 or more glomeruli. Kidney specimens were fixed in 10% buffered formalin (pH 7.2), embedded in paraffin, cut into sections 4 μm thick, and stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) reagent, periodic acid silver methenamine (PAM), and Mallory-Azan. Two observers with no knowledge of the patients' clinical course examined the kidney specimens by light microscopy to establish the diagnosis. For patients with CreGN, the percentages of crescent formation were evaluated. For DN patients, the severity of the diffuse lesions of glomeruli was graded on a scale of 0 to IV as described previously [20]. For LN patients, ISN/RPS 2003 classification was used for light microscopic classification of LN. For all patients, the mean interstitial fibrotic area (blue on Mallory-Azan staining) was determined and expressed as percentage per square millimeter of the field using Mac Scope version 6.02 (Mitani Shoji Co., Ltd., Fukui, Japan). Similarly, for evaluation of glomerulosclerosis, mean mesangial area (black on PAM staining) per glomerulus was examined from more

than 10 glomeruli using Mac Scope version 6.02 (Mitani Shoji Co., Ltd.) as reported previously [20].

Immunohistochemical studies

Fibrocytes were identified by dual immunohistochemical techniques on formalin-fixed, paraffin-embedded tissue specimens using specific antibodies against CD45 and type I pro-collagen α_1 as described previously [13]. Briefly, CD45 was detected by the indirect avidin-biotinylated alkaline phosphatase method using a murine anti-human CD45 monoclonal antibody (DAKO, Glostrup, Denmark). Type I pro-collagen α_1 (proCOLI; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was detected using the indirect avidin-biotinylated peroxidase complex method. In this dual immunostaining, the color of CD45-positive cells was red, while type I pro-collagen α_1-positive cells were brown. CD68-positive macrophages were also detected immunohistochemically on formalin-fixed, paraffin-embedded tissue sections by the indirect avidin-biotinylated alkaline phosphatase complex method using a murine anti-human macrophage CD68 monoclonal antibody (clone KP1; DAKO). As a positive control, resected tonsils from patients with tonsillitis were used. Mean numbers of interstitial CD45/type I pro-collagen α_1 dual-positive fibrocytes as well as CD68-positive macrophages were counted from more than 10 randomly chosen fields under high-power magnification (×200). Two independent observers also examined the immunohistochemical findings without prior knowledge of chemokine levels or the patients' clinical courses.

Measurements of urinary chemokines

Spontaneously voided midstream urine catches were collected on the morning of kidney biopsy. Samples of 10 mL of the each urine specimen were spun at 200 × *g* for 5 minutes at room temperature to remove cells and precipitate. The urinary supernatants were kept frozen at –70°C until measurement. Urinary CCL2 levels were determined by enzyme-linked immunosorbent assay (ELISA), using a specific murine monoclonal anti-human CCL2 antibody (clone ME69) for capture and a rabbit anti-CCL2 polyclonal antibody as the second antibody, as described previously [21]. The recovery rate was confirmed to be more than 95% up to 3 ng/mL in these ELISA systems. The urinary levels of stromal cell-derived factor (SDF)-1α/CXCL12, a ligand for CXCR4, as well as secondary lymphoid tissue chemokine (SLC)/CCL21, a ligand for CCR7, were also determined using ELISA kits (R&D Systems, Minneapolis, MN). All assays were performed in duplicate. The detection limits of this ELISA system were 40 pg/mL for CCL2, 18 pg/mL for CXCL12, and 9.9 pg/mL for CCL21. Urinary levels of these chemokines were standardized by the amount of creatinine in the urine. All kidney biopsies were performed with the informed consent of the patients. Urinary tract infections were excluded in all cases by bacterial culture, microscopic findings, or both, because urinary tract infection is associated with increased urinary CCL2 levels (data not shown).

Statistics

Statistical significance was analyzed using paired or unpaired Student's *t* test, ANOVA, and Spearman's and Pearson's correlation coefficients for analyses of nonparametric and parametric data. $P < 0.05$ was considered to indicate statistical significance.

Results

Fibrocytes infiltrating the interstitium in patients with CKD

To determine the presence of fibrocytes, kidney samples from 100 patients with CKD, including CreGN, LN, DN, IgA-N, ORG, MN, and BNS, and 6 patients with TBMD were examined by immunohistochemical analyses. In patients with CKD, CD45/proCOLI dual-positive fibrocytes were observed mainly in the interstitium (Fig. 1A). The number of interstitial fibrocytes in patients with CKD was higher than that in patients with TBMD (8.1 ± 0.7 *vs.* 0.9 ± 0.4/visual field; $P < 0.01$, Fig. 1B). In contrast, fibrocytes in glomeruli were hardly observed in patients with either CKD or TBMD. The degree of fibrocyte infiltration in patients with CreGN (13.3 ± 1.8/visual field), LN (6.8 ± 1.4/visual field), DN (7.6 ± 0.9/visual field), IgA-N (3.9 ± 0.9/visual field) as well as BNS (4.6 ± 1.1/visual field) was more severe than that in TBMD patients (0.9 ± 0.4/visual field) (Table 1). In addition, the number of interstitial fibrocytes in patients with CreGN was higher than those in both TBMD and other forms of CKD (Table 1).

Correlation of fibrocyte infiltration with the pathological findings

The number of infiltrated fibrocytes in the interstitium correlated well with the severity of tubulointerstitial lesions, such as mean interstitial fibrotic area in patients with CKD ($r = 0.374$, $P < 0.01$, Table 2). In addition, there was a significant correlation between the number of interstitial fibrocytes and the number of CD68-positive macrophages in the interstitium ($r = 0.386$, $P < 0.05$, Table 2). On the other hand, fibrocyte infiltration was not significantly correlated with the extent of

glomerulosclerosis in CKD patients (Table 2). In patients with CreGN, LN, and DN, the number of interstitial fibrocytes increased with the extent of interstitial fibrosis (r = 0.401, $P < 0.05$; $r = 0.545$, $P < 0.05$; $r = 0.421$, $P < 0.05$, respectively). In glomerular lesions, the percentages of fibrocellular/fibrous crescents correlated well with the number of interstitial fibrocytes in CreGN ($r = 0.453$, $P < 0.05$). In patients with LN, the number of fibrocytes in Class IV was higher than in other forms of LN (11.1 ± 2.7 *vs*. 5.1 ± 1.3/visual field; $P < 0.05$, Fig. 2A). Furthermore, the number of interstitial fibrocytes increased in accordance with the severity of glomerular diffuse lesions (grade I – II, 6.0 ± 1.1; grade III – IV, 10.2 ± 1.0; $P < 0.05$, Fig. 2B).

Correlations between the number of fibrocytes and clinical parameters

In patients with CKD, the number of interstitial fibrocytes increased in accordance with the levels of serum creatinine at the time of biopsy ($r = 0.331$, $P < 0.05$, Table 3). In addition, there was an inverse correlation between the number of interstitial fibrocytes and kidney function determined by creatinine clearance and eGFR at the time of biopsy ($r = –0.451$, $P < 0.05$; $r = –0.352$, $P < 0.05$, Table 3). The levels of CRP also correlated well with the extent of fibrocyte infiltration ($r = 0.317$, $P < 0.05$, Table 3). Moreover, the levels of urinary CCL2 were significantly correlated with the number of interstitial fibrocytes in patients with CKD ($r = 0.637$, $P < 0.05$). On the other hand, there were no correlations between the number of fibrocytes and the extent of proteinuria, hemoglobin A_1c levels, or the levels of urinary CXCL12. Urinary CCL21 protein was not detected in any cases in the present study.

Effects of glucocorticoid therapy on infiltration of fibrocytes and urinary CCL2 levels in patients with CKD

Sixteen of fifty-two patients treated with glucocorticoid therapy received second renal biopsy (CreGN, $n = 7$; LN, $n = 7$; IgA-N, $n = 2$). The number of interstitial fibrocytes decreased significantly during convalescence induced by glucocorticoid therapy including methylprednisolone pulse therapy (before therapy 8.3 ± 1.3 *vs*. after therapy 5.0 ± 1.0, $P < 0.05$, Fig. 3A). In addition, the number of CD68-positive macrophages in the interstitium also decreased after glucocorticoid therapy (before therapy 11.2 ± 2.8 *vs*. after therapy 5.8 ± 1.1, $P < 0.05$). Similarly, elevated urinary CCL2 levels significantly decreased during remission induced by glucocorticoid therapy in the patients examined (before therapy 10.7 ± 1.4 *vs*. after therapy 3.1 ± 0.5 pg/mg·creatinine, $P < 0.05$, Fig. 3B).

Discussion

In the present study, we investigated whether fibrocytes participate in the pathogenesis of human CKD. CD45/proCOLI dual-positive fibrocytes were detected in the interstitium in patients with CKD. In addition, the number of interstitial fibrocytes in CKD patients was higher than that in patients with TBMD examined as a disease control. The infiltrated fibrocytes number was well correlated with the extent of interstitial fibrosis. In particular, there were inverse correlations between the number of infiltrated fibrocytes in the interstitium and parameters of kidney function, such as serum creatinine levels, Ccr, and eGFR at the time of biopsy. Taken together, these results suggest that the infiltration of fibrocytes into diseased kidneys may be involved in the pathogenesis of human CKD, especially tubulointerstitial lesions.

Recently, the presence of fibrocytes has been reported in various human fibrotic diseases, including skin, lung, and eye diseases [13,22 – 24]. In addition, the extent of fibrocyte infiltration into the bronchial mucosa increases in accordance with the severity of persistent airflow obstruction [23]. In fibrotic conditions, the signaling of fibrogenic cytokines such as TGF-β_1 has been shown to be involved in the pathogenesis of organ fibrosis, including human kidney disease [25]. Fibrocytes have been demonstrated to produce collagen on stimulation with TGF-β_1 [8,13 – 14]. In this study, the number of interstitial fibrocytes was significantly correlated with the extent of interstitial fibrosis as well as kidney function. These findings suggested that fibrocytes may be involved in the pathogenesis of tubulointerstitial lesions in CKD patients, at least in part, *via* collagen production. On the other hand, TGF-β_1 is a well-characterized

inducer of EMT in tubular epithelial cells, while bone morphogenic protein-7 (BMP-7) counteracts TGF-β_1-induced EMT, resulting in improvement of interstitial fibrosis and kidney function in experimental progressive kidney diseases [3]. Recent studies revealed that fibrocytes are capable of producing TGF-β_1 under fibrotic conditions [10,15]. Therefore, fibrocytes may regulate EMT through the production of TGF-β_1, thereby contributing to the pathogenesis of CKD, in addition to direct synthesis of collagen.

Chemokines are a superfamily of small proteins that are important in recruiting and activating leukocytes during inflammation. Chemokines have been demonstrated to play important roles in the mechanisms of leukocyte entry into the kidneys and the activation of leukocytes in diseased kidneys [26]. CCL2, a member of the CC chemokine family, has been reported to play a significant role in the pathogenesis of human and experimental kidney diseases, especially interstitial fibrosis through recruitment and activation of macrophages [26 – 27]. In addition, fibrocytes are capable of producing CCL2 [10]. Recent studies also revealed that fibrocytes express various chemokine receptors, including CCR2, a cognate receptor for CCL2 [16]. Furthermore, the expression of type I collagen has been shown to be upregulated by stimulation with CCL2 in cultured fibrocytes [16]. In this study, we demonstrated that the number of interstitial fibrocytes was well correlated with urinary CCL2 levels as well as the number of interstitial CD68-positive macrophages in patients with CKD. Moreover, glucocorticoid therapy induced reductions in number of fibrocytes and CD68-positive macrophages as well as urinary CCL2 levels. Taken together, these observations suggest that the CCL2-dependent amplification loop for activation and

recruitment of fibrocytes and macrophages may be involved in the pathogenesis of human CKD.

This study demonstrated no association between fibrocyte infiltration and the levels of urinary CXCL12. The CXCL12/CXCR4 pathway has been reported to be involved in the pathogenesis of pulmonary fibrosis through infiltration of fibrocytes [5]. Recently, CXCR4 has been demonstrated to be expressed not only on fibrocytes but also on various cell types, including mesenchymal stem cells [28]. Therefore, CXCL12/CXCR4 signaling may contribute to the mechanisms of disease progression as well as tissue repair. Further studies are required to elucidate the involvement of CCL12/CXCR4 in the pathogenesis of human kidney diseases. Furthermore, urinary CCL21 protein was not detected in any sample in this study. CCL21/CCR7 signaling has been reported to contribute to the migration of fibrocytes leading to tissue fibrosis, including that in the kidney [6,8]. CCL21 is a unique chemokine expressed mainly on vessels, thereby contributing to the infiltration of CCR7-positive cells, such as fibrocytes and dendritic cells [6]. Therefore, it is suggested that CCL21 is hardly secreted into the urine. Further studies of CCL21 expression in human kidney tissues are required.

In this study, the number of interstitial fibrocytes was correlated well with the extent of interstitial fibrosis in patients with CreGN, LN, and DN. Recent studies revealed that mitogen-activated protein kinases (MAPKs) contribute to the pathogenesis of tubulointerstitial lesions in progressive kidney diseases, such as CreGN, LN, and DN [29 – 31]. To date, at least three distinct groups of MAPKs have been identified: extracellular signal-regulated kinases (ERK), p38MAPK, and c-Jun NH2-

terminal kinase/stress-activated protein kinase (JNK/SAPK). Especially, p38MAPK is phosphorylated in response to hyperosmolarity, oxidative stress, and inflammatory cytokines, thereby contributing to the activation of nuclear transcription factors, including nuclear factor (NF)-κB, which principally regulates the gene expression of various chemokines, such as CCL2 [31]. In addition, the expression of type I collagen is also regulated by the MAPK family in cultured fibrocytes [32]. Taken together, these observations suggest that fibrocytes regulated by MAPKs may be involved in the pathogenesis of CKD. Further studies are needed to elucidate the effects of MAPKs on fibrocytes in CKD. In this study, little infiltration of fibrocytes into glomeruli was detected. In addition, the number of interstitial fibrocytes was not correlated with the index of glomerulosclerosis in all patients with CKD. However, the extent of fibrocyte infiltration in the interstitium was correlated with the percentages of fibrocellular/fibrous crescents in CreGN patients. The number of interstitial fibrocytes also increased in accordance with the progression of glomerular lesions in LN and DN patients. These findings suggest that fibrocytes are not directly involved in the pathogenesis of glomerular injury in patients with CKD, while fibrocyte infiltration in the interstitium may reflect the activity and phase of glomerular lesions in CreGN, LN, and DN.

The clinical management of CKD remains to be investigated due to a lack of effective treatment or accurate indicators of disease progression. Previous clinical studies have shown that the renin-angiotensin system (RAS) is a major pathway involved in the pathogenesis of cardiovascular diseases as well as CKD [33,34]. A recent study indicated the expression of two isoforms of angiotensin II receptor (AT1

and AT2) and the effects of RAS on the expression of COLAI as well as TGF-β_1 in cultured fibrocytes [15]. Therefore, the effects of RAS on the pathogenesis of CKD may be mediated, in part, by regulation of fibrocytes. Further studies are required to elucidate the interaction of fibrocytes with RAS in CKD. In this study, there was an inverse correlation between the number of interstitial fibrocytes and kidney function. In addition, fibrocytes numbers in peripheral blood have been reported to predict early mortality in patients with idiopathic pulmonary fibrosis and increase in accordance with the severity of asthma [23,35]. Taken together, these observations suggest that the number of fibrocytes in clinical samples may be useful as a biomarker for the progression of organ fibrosis. However, additional investigations are needed to determine the role of fibrocytes in CKD.

In summary, our results suggested that the pathogenesis of human CKD may be closely related to fibrocyte infiltration, which may be regulated by CCL2. In addition, fibrocytes may be a novel therapeutic target for human CKD.

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Legends

Table 1. Patient Profiles

Table 2. Correlation of the number of interstitial fibrocytes with pathological findings in CKD

Table 3. Correlations between the number of interstitial fibrocytes and clinical parameters in CKD

Fig. 1. Immunohistochemical examination of fibrocytes in a representative patient with CKD. (A) CD45/proCOLI dual-positive fibrocytes were detected mainly in the interstitium (arrows). CD45 (red) and proCOLI (brown). (B) The number of interstitial CD45/proCOLI dual-positive fibrocytes in patients with CKD was higher than that in TBMD patients. Bars indicate means ± SEM.

Fig. 2. Increased fibrocytes in the interstitium reflect glomerular lesions in patients with LN and DN. (A) The number of interstitial fibrocytes in LN Class IV was higher than that in LN non-Class IV. (B) The number of interstitial fibrocytes increased in accordance with the severity of glomerular diffuse lesions in DN patients. Bars indicate means ± SEM.

Fig. 3. Changes in the number of fibrocytes in the interstitium (A) and the levels of urinary CCL2 levels (B) following glucocorticoid therapy in patients with CKD. Bars indicate means ± SEM.