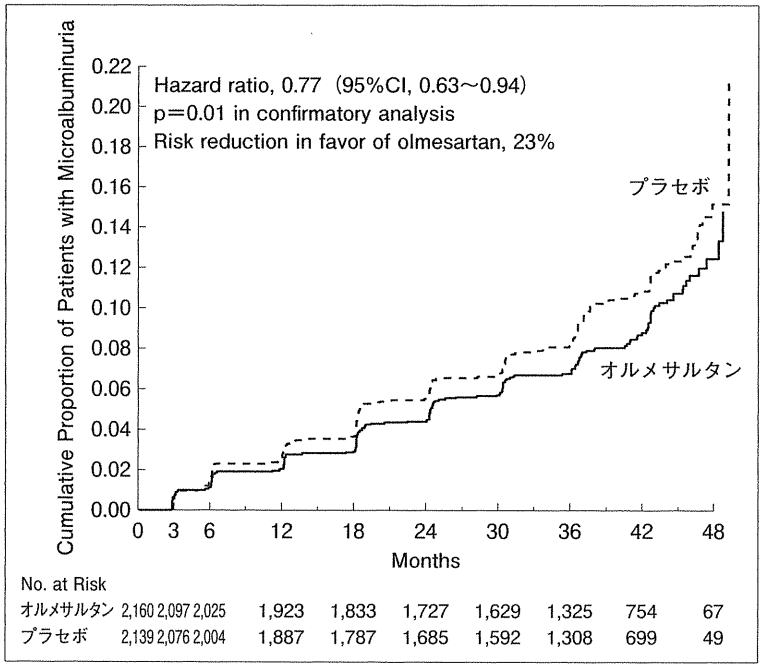


図
オルメサルタン服用によりアルブミン尿出現が抑制される
〔参考文献10）より引用改変〕

在する．例えば，「正常アルブミン尿で推算糸球体濾過量（estimated glomerular filtration rate：eGFR）が60mL/分/1.73m²未満の症例」は腎症病期分類では第1期（腎症前期）に，CKDステージ分類ではステージ3以降に分類される．一方，「顕性タンパク尿でeGFRが60mL/分/1.73m²以上の症例」は，腎症病期分類では第3期（顕性腎症期），CKDステージ分類ではステージ1あるいは2となる．このような症例は臨床的にもまれではなく，JDDMは，①正常アルブミン尿の11.4%，微量アルブミン尿の14.9%が，GFR 60mL/分/1.73m²未満（CKDステージ3以降）であること，②顕性タンパク尿の52.7%がGFR 60mL/分/1.73m²以上（CKDステージ1あるいは2）であることを報告している[9]．目下のところ，本邦におけるこのような症例の臨床病理学的な病態は不明なところが多く，アルブミン尿（タンパク尿），腎機能と腎予後，生命予後，心血管事故との関連は十分検討されていない．海外においては，2型糖尿病患者10,640例を対象としたAction in Diabetes and Vascular disease：preterAx and diamicroN-MR Controlled Evaluation（ADVANCE）試験のサブ解析において，eGFR 60mL/分/1.73m²未満・正常アルブミン尿例（尿中アルブミン/クレアチニン排泄率 30mg/g未満）のハザード比（vs. eGFR 90mL/分/1.73m²以上・正常アルブミン尿例，多変量調整後）は，腎死で3.95倍（95%信頼区間 1.38～11.34），心血管疾患（cardiovascular disease：CVD）発症で1.33倍（1.02～1.75），CVD死亡で1.85倍（1.17～2.92）といずれも有意に上昇し，eGFRとアルブミン尿が腎・心血管イベントのそれぞれ独立した危険因子であることが示されている（図）[10),11)]．本邦においても，JDCP study，J-DOIT3，さらに腎臓病総合レジストリーであるJDNCSによる，多施設による臨床研究が進行中であり今後の展開が期待される．なお，本邦の1型と2型糖尿病を比較すると，2型糖尿病において，アルブミン尿陽性，CKDステージ3以上の若年者例がより多いという報告がなされた[12)]．病態を考えるうえで興味深い．

Ⅳ 糖尿病性腎症の治療の進歩

1 糖尿病性腎症は予防できるか？

現在，腎症の治療は生活習慣の修正に加えて，高血糖，全身血圧ならびに糸球体高血圧のコントロールが基本であり，寛解（remission）と退縮（regression）を目指して血清脂質の管理などが集約的に行われる

1. 生活習慣の改善
   減量，運動，タンパク質・食塩・アルコール制限，禁煙
2. 高血糖の是正：厳格な血糖コントロール（HbA1c値<6.5%）
3. 糸球体高血圧の是正：
   ・レニン・アンジオテンシン系阻害薬（ACE阻害薬，アンジオテンシンⅡ受容体拮抗薬）の使用
   ・全身血圧の管理：目標血圧値<130/80mmHg
   （長時間作用型Ca拮抗薬，利尿薬を併用）
4. 血清脂質の管理（スタチン）
5. タンパク質制限食（0.8g/kg/日）

表2
糖尿病性腎症の集約的治療
〔参考文献13）より引用改変〕

（表2）[13]．一方，予防に勝る治療法はないとも指摘されている．最近になり，糖尿病の発症そのものがピオグリタゾン投与により抑制されるという報告がなされた[14]．このACT NOW研究では，48カ月の観察期間で，プラセボでは7.6%であった年率発症が，ピオグリタゾン投与により2.1%にまで低下することが示されている．さらに，ROADMAP研究では2型糖尿病患者に対して，アンジオテンシンⅡ受容体拮抗薬（ARB）であるオルメサルタン40mg投与により約3.2年の観察期間においてアルブミン尿の発症遅延もしくは予防効果を示している[10]．130/80mmHg未満の目標血圧にはオルメサルタン群で約80%，プラセボ群で71%が達成したが，随時血圧はオルメサルタン群で3.1/1.9mmHgで低かった．アルブミン尿はプラセボ群で2,139例中210例（9.8%）にみられたのに対し，オルメサルタン群では2,160例中178例（8.2%）であり，23%のrisk reductionであった（図）．しかしながら，ことに冠動脈疾患の既往例を含めて致死的な心血管イベントの増加がみられることが示されている．

2　糖尿病性腎症の寛解（remission）と退縮（regression）

最近になり，アルブミン尿から正常アルブミン尿への改善あるいは顕性タンパク尿から正常もしくはアルブミン尿への改善が報告され，いったん発症した糖尿病性腎症も寛解と退縮を目指す包括的な治療へと考え方が変化している．本邦でも，2型糖尿病の早期腎症例が寛解する知見が得られている．例を挙げると，JDCSでは軽度のアルブミン尿から正常アルブミン尿への寛解が，452例中137例（30.3%）に認められたと報告している[15]．さらに，Arakiらは寛解に関与する因子として，①微量アルブミン尿の出現期間が短いこと，②レニン・アンジオテンシン系（renin-angiotensin system：RAS）阻害薬を使用していること，③血糖コントロールが良好なこと，④収縮期血圧が低いことが重要としている[16]．さらに，同グループでは2年間の経過観察を追加したところ，アルブミン尿の寛解もしくは50%以上の減少を認めた例では腎機能低下速度の抑制，心血管病変抑制がみられたと報告している[17]．一方，海外では1型糖尿病例の顕性腎症[18]あるいは1型，2型ともにネフローゼ症候群[19),20)]の寛解・退縮も報告されている．元来，退縮は病理学的な用語であり，今後，このような症例では病理学的な評価が求められる．特に膜性腎症など他の糸球体疾患の合併も念頭において精査が必要であると考える．

Ⅴ　まとめ

糖尿病性腎症の疫学，今後の課題となる病態の解明，治療を中心に概説した．治療効果を期待させる報告がなされる一方，2型糖尿病を中心に治療が必要な症例の未受診率が高い現実もある．今後も糖尿病性腎症の発症予防，予後改善に向けてより総合的な取り組みが求められている．

参考文献

1）猪俣茂樹，羽田勝計，守屋達美ほか：糖尿病性腎症の新しい早期診断基準．糖尿病 48：757-759, 2005
2）糖尿病性腎症に関する合同委員会：糖尿病性腎症病期分類厚生省案の改定について．糖尿病 44：623, 2001
3）Yokoyama H, Kawai K, Kobayashi M；Japan Diabetes Clinical Data Management Study Group：Microalbuminuria is common in Japanese type 2 diabetic patients：a nationwide

survey from the Japan Diabetes Clinical Data Management Study Group (JDDM 10). Diabetes Care **30**：989-992, 2007
4) 横山　仁：厚生労働科学研究費補助金 腎疾患対策研究事業 糖尿病性腎症の病態解明と新規治療法確立のための評価法の開発（研究代表者 和田隆志）平成22年度総括・分担研究報告書. 34-38, 2010
5) Akimoto T, Ito C, Takahashi H, et al：Microscopic hematuria and diabetic glomerulosclerosis-clinicopathological analysis of type 2 diabetic patients associated with overt proteinuria. Nephron Clin Pract **109**：c119-126, 2008
6) 大久典子，吉田克己，賀来満夫ほか：糖尿病患者における尿潜血反応陽性の意義. 日腎会誌 **48**：664-668, 2006
7) 日本腎臓学会編：CKD診療ガイド2009. 東京医学社，東京，2009
8) 日本腎臓学会編：エビデンスに基づくCKD診療ガイドライン2009. 東京医学社，東京，2009
9) Yokoyama H, Sone H, Oishi M, et al：Prevalence of albuminuria and renal insufficiency and associated clinical factors in type 2 diabetes：the Japan Diabetes Clinical Data Management study (JDDM15). Nephrol Dial Transplant **24**：1212-1219, 2009
10) Haller H, Ito S, Izzo JL, et al：Olmesartan for the delay or prevention of microalbuminuria in type 2 diabetes. N Engl J Med **364**：907-917, 2011
11) Ninomiya T, Perkovic V, de Galan BE, et al：Albuminuria and kidney function independently predict cardiovascular and renal outcomes in diabetes. J Am Soc Nephrol **20**：1813-1821, 2009
12) Ohata M, Babazono T, Uchigata Y, et al：Comparison of the prevalence of chronic kidney disease in Japanese patients with type 1 and type 2 diabetes. Diabet Med **27**：1017-1023, 2010
13) 羽田勝計：糖尿病性腎症. 初学者から専門医までの腎臓学入門 改定第2版. p129-137，東京医学社，東京，2009
14) DeFronzo RA, Tripathy D, Schwenke DC, et al：Pioglitazone for diabetes prevention in impaired glucose tolerance. N Engl J Med **364**：1104-1115, 2011
15) Katayama S, Moriya T, Tanaka S, et al：Low transition rate from normo- and low microalbuminuria to proteinuria in Japanese type 2 diabetic individuals：the Japan Diabetes Complications Study (JDCS). Diabetologia **54**：1025-1031, 2011
16) Araki S, Haneda M, Sugimoto T, et al：Factors associated with frequent remission of microalbuminuria in patients with type 2 diabetes. Diabetes **54**：2983-2987, 2005
17) Araki S, Haneda M, Koya D, et al：Reduction in microalbuminuria as an integrated indicator for renal and cardiovascular risk reduction in patients with type 2 diabetes. Diabetes **56**：1727-1730, 2007
18) Hovind P, Rossing P, Tarnow L, et al：Remission and regression in the nephropathy of type 1 diabetes when blood pressure is controlled aggressively. Kidney Int **60**：277-283, 2001
19) Hovind P, Rossing P, Tarnow L, et al：Remmision of nephrotic-range albuminuria in type 1 diabetic patients. Diabetes Care **24**：1972-1977, 2001
20) Rossing K, Christensen PK, Hovind P, et al：Remission of nephrotic-range albuminuria reduces risk of end-stage renal disease and improves survival in type 2 diabetic patients. Diabetologia **48**：2241-2247, 2005

Hypertension Research (2011) 34, 747–752

www.nature.com/hr

ORIGINAL ARTICLE

Fractalkine and its receptor, CX3CR1, promote hypertensive interstitial fibrosis in the kidney

Kazuaki Shimizu[1], Kengo Furuichi[1], Norihiko Sakai[1], Kiyoki Kitagawa[1], Kouji Matsushima[2], Naofumi Mukaida[3], Shuichi Kaneko[1] and Takashi Wada[4]

Hypertension promotes and escalates kidney injury, including kidney fibrosis. Fractalkine/CX3CL1 is a unique chemokine that works as a leukocyte chemoattractant and an adhesion molecule. Recently, fractalkine/CX3CL1 has been reported to promote tissue fibrosis via its cognate receptor, CX3CR1. However, the involvement of the fractalkine-CX3CR1 axis in the pathogenesis of hypertensive kidney fibrosis remains unclear. The impacts of the fractalkine-CX3CR1 axis on hypertensive kidney fibrosis were investigated in a deoxycorticosterone acetate (DOCA)-salt hypertensive model in CX3CR1-deficient mice, which were sacrificed on day 28. The blood pressure levels were similarly elevated in both CX3CR1−/− C57BL/6 and wild-type C57BL/6 mice. Fractalkine and CX3CR1 were upregulated in kidneys that were damaged by hypertension. Deficiency in CX3CR1 inhibited kidney fibrosis, as evidenced by a decrease in the presence of interstitial fibrotic area detected by type I collagen in Mallory–Azan staining, concomitant with the downregulation of transforming growth factor (TGF)-β_1 and type I procollagen mRNA expression in damaged kidneys. The CX3CR1 blockade also decreased the number of infiltrating F4/80-positive macrophages in damaged kidneys. These results suggest that the fractalkine-CX3CR1 axis contributes to kidney fibrosis in a hypertensive mouse model, possibly by the upregulation of macrophage infiltration and the expression of TGF-β_1 and type I collagen.
Hypertension Research (2011) **34**, 747–752; doi:10.1038/hr.2011.23; published online 31 March 2011

Keywords: CX3CR1; fibrosis; fractalkine; kidney

INTRODUCTION

Kidney fibrosis is a characteristic finding of progressive kidney injury that results in organ failure, and it is accepted as the major determinant of the prognosis of kidney diseases. Accumulating evidence suggests that chemokines and their cognate receptors, such as monocyte chemoattractant protein-1/macrophage chemotactic and activating factor/CCL2 and its cognate receptor, CCR2, contribute to chronic kidney inflammation, leading to kidney fibrosis.[1–4] Additionally, secondary lymphatic chemokine/CCL21 and its receptor, CCR7, have a role in kidney fibrosis.[5] Recently, fractalkine/CX3CL1, a CX3C chemokine, was found to regulate kidney interstitial fibrosis via its cognate receptor, CX3CR1, after ischemia reperfusion injury in mice.[6] To support this notion, patients with systemic sclerosis exhibited high levels of serum fractalkine, concomitantly with the presence of CX3CR1-positive macrophages and T cells in fibrotic skin and lungs.[7] Originally, fractalkine/CX3CL1 was reported to be a potent chemoattractant for macrophages, natural killer cells, T cells, mast cells and platelets, and it uniquely serves as an adhesion molecule expressed on endothelial cells.[8] Recently, pathophysiological roles of the fractalkine-CX3CR1 axis were noted in the bactericidal host defense during septic peritonitis[9] and corneal neovascularization.[10] Additionally, fractalkine/CX3CL1 was reported to be involved in the progression of human glomerulopathy, including crescentic glomerulonephritis.[11] Collectively, these data suggest that the fractalkine-CX3CR1 axis may promote tissue injury through immune competent cell infiltration and fibrogenesis. However, the impacts of the fractalkine-CX3CR1 axis on progressive interstitial lesions, including kidney fibrosis associated with hypertension, are poorly understood.

Hypertensive kidney disease, including interstitial fibrosis, is often present in patients with long-term hypertension.[12] However, the precise underlying mechanisms involved in hypertensive kidney damage remain unclear. Several lines of evidence suggest that the inflammatory processes may have a key role in the pathogenesis of hypertensive kidney damage. Tian *et al.*[13] reported that an increase of oxidant stress in hypertensive kidney disease activated nuclear factor (NF)-κ B, which eventually induced the expression of proinflammatory cytokines and chemokines in the kidney. Moreover, anti-oxidant treatment decreased kidney inflammatory cytokines and chemokines, kidney immune competent cells and NF-κ B, thereby improving kidney function and damage. Thus, the inflammatory process may be a key to interstitial fibrosis in hypertensive kidney.

[1]Department of Disease Control and Homeostasis, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan; [2]Department of Molecular Preventive Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; [3]Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, Kanazawa, Japan and [4]Department of Laboratory Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan
Correspondence: Dr K Shimizu, Department of Disease Control and Homeostasis, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan.
E-mail: ts243133@ton21.ne.jp
Received 12 August 2010; revised 20 November 2010; accepted 13 January 2011; published online 31 March 2011

These findings prompted us to examine whether fractalkine/CX3CL1 and its cognate receptor, CX3CR1, contribute to progressive kidney injury associated with hypertension. In the present study, we tested this hypothesis in a deoxycorticosterone acetate (DOCA)-salt hypertensive model in CX3CR1-deficient mice. In this study, we report that fractalkine-CX3CR1 axis promotes kidney fibrosis with concomitant increases in transforming growth factor (TGF)-β_1 and type I collagen in damaged kidneys.

METHODS

Animals

Inbred male C57BL/6 mice aged 30 weeks were purchased from Charles River Japan (Yokohama, Japan). The male CX3CR1-deficient mice were on an outbred C57BL/6 genetic background ($n>8$ generations), and were used at 30 weeks of age.[9,10] Five mice were used in each experiment. All animal experiments were performed at the Institute for Experimental Animals (Kanazawa University Advanced Science Research Center, Kanazawa, Japan), complied with the standards set out in the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University, and were approved by the Committee on Animal Experimentation of Kanazawa University.

Hypertensive kidney injury model

CX3CR1-deficient and wild-type mice were anesthetized with diethyl ether and pentobarbital sodium. A flank incision and left unilateral nephrectomy were made. Then, 21-day-release DOCA pellets containing 50 mg of DOCA (Innovative research of America, Sarasota, FL, USA) were implanted subcutaneously by incision of the right flank. The sham operation was performed in a similar manner, except for unilateral nephrectomy and DOCA implantation for control mice. The DOCA animals received isotonic saline for 28 days starting with the administration of DOCA. Blood pressure was measured using the tail cuff method twice a week until the day of sacrifice. For pathological examination, the remaining right kidney was harvested on day 28.

Tissue preparation

One portion of the kidney tissue was fixed in 10% buffered formalin (pH 7.2), embedded in paraffin, cut at 4 μm, stained with hematoxylin and eosin, periodic acid Schiff's reagent or Mallory–Azan and observed under a light microscope. Two independent observers with no prior knowledge of the experimental design evaluated each section. The mean interstitial fibrotic area, expressed as blue coloration in the Mallory–Azan staining, was evaluated from the whole area of the cortex and outer medulla in the individual complete sagittal kidney section and expressed as a percentage of the field using Mac Scope version 6.02 (Mitani Shoji, Fukui, Japan).

Immunohistochemical studies

The other portion of fresh renal tissue, which was embedded in the OCT compound (Sakura Finetek USA Inc, Torrance, CA, USA) and snap-frozen in *n*-hexane cooled with a mixture of dry ice and acetone, was cut at 6 μm on a cryostat (Tissue-Tek systems; Miles, Naperville, IL, USA). The presence of F4/80-positive macrophages was detected immunohistochemically using the rat anti-mouse F4/80 monoclonal antibody (clone: A3-1; BMA Biomedicals AG, Augst, Switzerland). The number of interstitial infiltrated F4/80-positive macrophages was counted in the whole area of the cortex and outer medulla where cell migration was maximal, and it was expressed as the mean number ± standard error per mm^2. The presence of TGF-β_1 protein was demonstrated immunohistochemically on formalin-fixed, paraffin-embedded kidney tissue specimens using the indirect avidin-biotinylated peroxidase complex method with rabbit anti-mouse TGF-β_1 polyclonal antibodies (clone: sc-146; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antigen was retrieved with Target Retrieval Solution (DAKO, Glostrup, Denmark). The presence of type I collagen was also demonstrated immunohistochemically on paraffin-embedded kidney tissue with rabbit anti-mouse type I collagen polyclonal antibodies (CHEMICON International, Temecula, CA, USA). The expression of TGF-β_1 and type I collagen was evaluated from the whole area of cortex and outer medulla in the individual complete sagittal kidney section, and expressed as a percentage using Mac Scope version 6.02.

The presence of CX3CL1 protein was also demonstrated immunohistochemically on paraffin-embedded kidney tissue with goat anti-rat CX3CL1 polyclonal antibodies (R&D Systems, Minneapolis, MN, USA).[9]

Detection of type I procollagen, CX3CL1 and CX3CR1 transcripts in kidneys by real-time reverse transcription PCR

To determine the transcripts of type I procollagen, CX3CL1 and CX3CR1, total RNA was extracted from the whole kidneys. The complimentary DNA was reverse transcribed from 1 μg of total RNA using a SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using the following parameters: 10 min at 25 °C, 30 min at 48 °C and 5 min at 95 °C. For all PCR experiments, the Light Cycler (Roche Diagnostics, Basel, Switzerland) was used. Quantitative real-time reverse transcription PCRs were performed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using 384-well microtiter plates. The reactions were performed in a total volume of 20 μl, containing 1 μl of complimentary DNA sample, TaqMan Gene Expression Assays (Applied Biosystems) and Taqman Universal PCR Master Mix (Applied Biosystems) using the universal temperature cycles: 10 min at 94 °C followed by 40 two-temperature cycles (15 s at 94 °C and 1 min at 60 °C). Assay IDs of TaqMan Gene Expression Assays were Mm00801666 for mouse procollagen type 1, Mm00436454_m1 for mouse CX3CL1, Mm00438354_m1 for mouse CX3CR1 and Mm00446953_m1 for murine β-glucuronidase. The mRNA expression in each sample was finally described after correction with β-glucuronidase expression.

Statistical analysis

The mean number ± standard error. were calculated on all parameters determined in this study. Statistical analyses were performed using the analysis of variance test. A value of $P<0.05$ was accepted as statistically significant.

RESULTS

DOCA mice with unilateral nephrectomy showed the elevation of blood pressure, but a CX3CR1 deficiency hardly affected blood pressure

To determine the impact of the deficiency of CX3CR1 on blood pressure, blood pressure was measured in unilateral nephrectomized mice with or without DOCA administration. Unilateral nephrectomy alone did not increase the blood pressure levels in any mice examined in this study. In contrast, DOCA administration elevated blood pressure to similar extents in both wild-type and CX3CR1-deficient mice (Figure 1).

Fractalkine-CX3CR1 expression in damaged kidneys

To determine the impact of CX3CR1 deficiency on fractalkine-CX3CR1 expression in the kidney, the presence of transcripts of fractalkine/CX3CL1 and CX3CR1 was evaluated. Fractalkine/CX3CL1 expression was upregulated especially in DOCA-administered mice, which was ameliorated by CX3CR1 deficiency (Figure 2a). The CX3CR1 mRNA expression was upregulated in unilateral nephrectomized mice, which was augmented by DOCA administration (Figure 2b).

Furthermore, the fractalkine/CX3CL1 protein was expressed mainly on proximal tubular epithelial cells, peritubular capillaries and vascular endothelial cells in DOCA-administered B6 mice, similar to that reported previously[6] (Figures 2e and f). Fractalkine/CX3CL1 protein expression was not upregulated in B6 control mice (Figure 2c), and DOCA-administered CX3CR1−/− mice (Figure 2d).

CX3CR1 deficiency ameliorated kidney interstitial fibrosis

To determine the impact of CX3CR1 on progressive kidney fibrosis, the fibrotic area (indicated in blue on the Mallory–Azan staining) was examined. Untreated mice (Figures 3a and d), sham-operated mice

(data not shown) or CX3CR1-deficient mice (Figures 3a and e) exhibited little or no fibrosis. Interstitial fibrosis was observed in damaged kidneys in unilateral nephrectomized mice (Figure 3a). The DOCA treatment further aggravated kidney fibrosis induced by unilateral nephrectomy (Figures 3a and f). Concomitantly, type I collagen protein and mRNA expression levels were upregulated in uninephrectomized mice with DOCA treatment (Figures 3b, c and h).

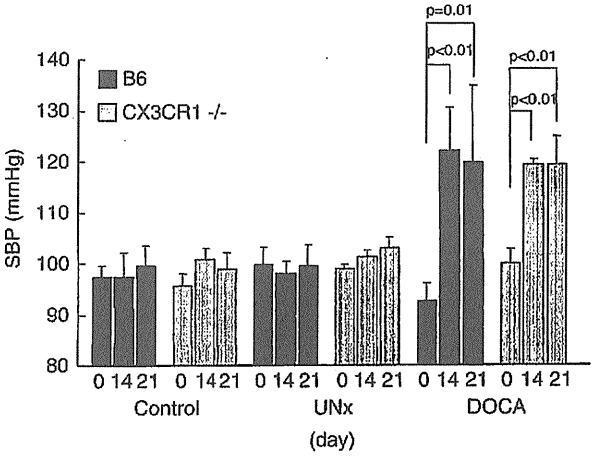


Figure 1 Deoxycorticosterone acetate (DOCA)-treated uninephrectomized mice developed hypertension. Systolic blood pressure measured by the tail cuff method in the mice of each experimental group. Control, sham-operated control mice; UNx, uninephrectomized mice; DOCA, DOCA-treated uninephrectomized mice; B6, C57BL/6; CX3CR1−/−, CX3CR1-deficient mice. Systolic blood pressure was increased in all mice 14 days and 21 days after DOCA treatment in both B6 and CX3CR1−/− mice. There was no statistical difference in blood pressure between B6 and CX3CR1−/− mice. n=5 in each experiment. Values are expressed as the mean number ± standard error.

It is important to note that a CX3CR1 deficiency markedly attenuated kidney fibrosis in DOCA-administered mice (Figures 3a and g) and reduced type I collagen in mRNA and protein levels (Figures 3b, c and i).

F4/80-positive macrophages were decreased in number in the CX3CR1-deficient mice

Because fractalkine/CX3CL1 is a potent chemoattractant for macrophages, we examined whether CX3CR1 deficiency impacts interstitial cell infiltration. The F4/80-positive macrophages infiltrated mainly into the outer medulla of damaged kidneys. The number of infiltrating F4/80-positive macrophages in the interstitium of unilateral nephrectomized mice was increased with DOCA administration. On the contrary, CX3CR1 deficiency reduced the number of infiltrated F4/80-positive macrophages in damaged kidneys (Figure 4). Therefore, fractalkine via CX3CR1 affected macrophage infiltration in damaged kidneys in this model.

Kidney TGF-β_1 expression was decreased by CX3CR1 deficiency

To clarify the molecular mechanisms involved in the increase in fibrogenesis caused by CX3CR1 deficiency, the expression of TGF-β_1, a potent fibrogenic molecule, was examined. Unilateral nephrectomy enhanced the expression of TGF-β_1 protein detected mainly in renal tubular epithelial cells and infiltrating cells in unilateral nephrectomized mice (Figure 5a), compared with normal C57BL/6 mice (Figure 5b) or sham-operated C57BL/6 mice (data not shown). DOCA treatment marginally enhanced TGF-β_1 expression induced by unilateral nephrectomy, and it should be noted that CX3CR1 deficiency downregulated TGF-β_1 immunoreactivity in damaged kidneys (Figure 5c).

DISCUSSION

In the present study, we examined the impact of CX3CR1 signaling on kidney fibrosis in a DOCA-salt hypertensive model using mice that were genetically deficient in CX3CR1. Inhibition of CX3CR1 signaling attenuated progressive kidney fibrosis, concomitant with the down-

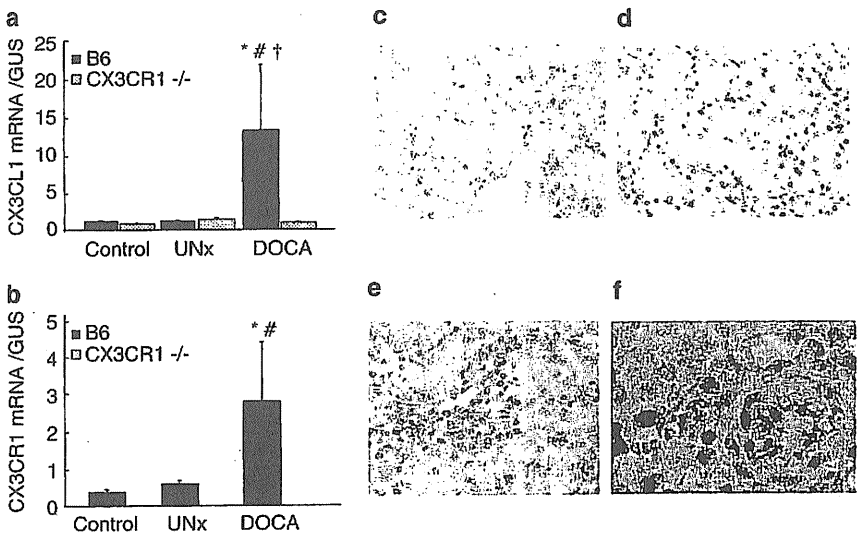


Figure 2 Enhanced mRNA expression of CX3CL1 and CX3CR1 in whole kidney by deoxycorticosterone acetate (DOCA) treatment. **(a)** Transcripts of CX3CL1 were upregulated after DOCA treatment in C57BL/6 mice detected by real-time reverse transcription PCR, which was reduced by CX3CR1 deficiency. **(b)** Transcripts of CX3CR1 were upregulated after DOCA treatment in C57BL/6 mice detected by real-time reverse transcription PCR. CX3CL1 protein was not detected immunohistochemically in untreated C57BL/6 mice **(c)** and DOCA-administered CX3CR1−/− mice **(d)**. CX3CL1 was expressed in tubular epithelial cells **(e)** and vascular endothelial cells **(f)**. n=5 in each experiment. Values are the mean number ± standard error. $^*P<0.05$ compared with the control group, $^\#P<0.05$ compared with the uninephrectomized group, $^\dagger P<0.05$ compared with CX3CR1-deficient mice. Original magnification is ×200 **(c, d** and **e)** and ×400 **(f)**. A full color version of this figure is available at the *Hypertension Research* journal online.

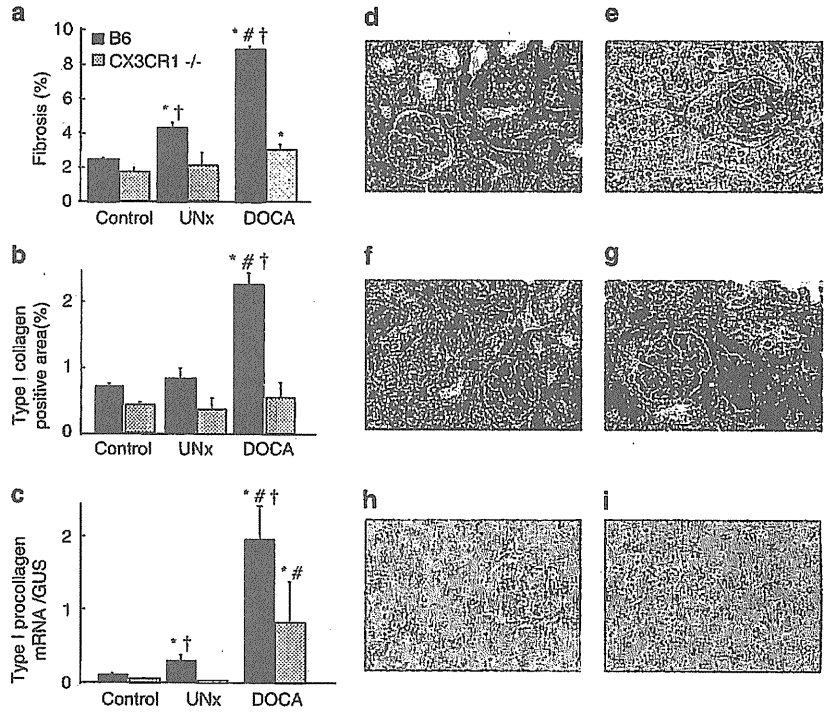


Figure 3 Deficiency in CX3CR1 reduced kidney interstitial fibrosis. Kidney interstitial fibrosis, expressed as a percentage involvement of the field, was increased in deoxycorticosterone acetate (DOCA)-administered mice (a, f) compared with the DOCA-untreated C57BL/6 mice (a, d) and CX3CR1-deficient mice (a, e). In contrast, the mean interstitial fibrosis was reduced in DOCA-administered CX3CR1-deficient mice (a, g). Type I collagen expression was upregulated in C57BL/6 mice after DOCA treatment (b, h). In contrast, the CX3CR1-deficient mice that received DOCA treatment had a reduced expression of type I collagen (b, i). There was no statistical difference in expression levels between sham-operated C57BL/6 mice and sham-operated CX3CR1-deficient mice (b). Similarly, the mRNA expression of type I procollagen was reduced in CX3CR1-deficient mice compared with that of C57Bl/6 mice (c). n=5 in each experiment. Values are the mean number ± standard error. $^{*}P<0.05$ compared with DOCA-untreated mice, $^{\#}P<0.05$ compared with uninephrectomized group, $^{\dagger}P<0.05$ compared with CX3CR1-deficient mice. Original magnification is ×400. A full color version of this figure is available at the *Hypertension Research* journal online.

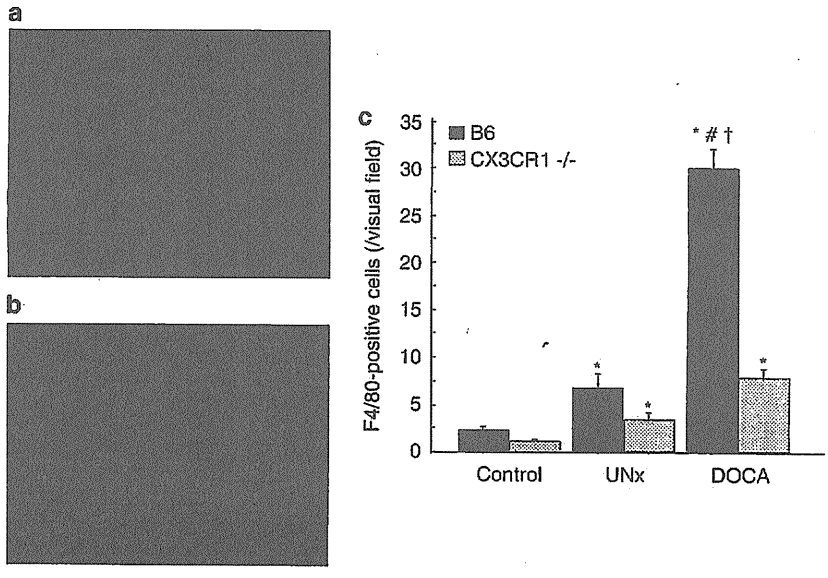


Figure 4 Infiltration of F4/80-positive cells by deoxycorticosterone acetate (DOCA) treatment was reduced in CX3CR1-deficient mice. F4/80-positive cells were detected using immunofluorescence imaging. The number of F4/80-positive cells was reduced in CX3CR1-deficient mice (a, c) compared with those in wild-type mice (b, c) after DOCA treatment. $^{*}P<0.05$ compared with the control group, $^{\#}P<0.05$ compared with the uninephrectomized group, $^{\dagger}P<0.05$ compared with CX3CR1-deficient mice. n=5 in each experiment. A full color version of this figure is available at the *Hypertension Research* journal online.

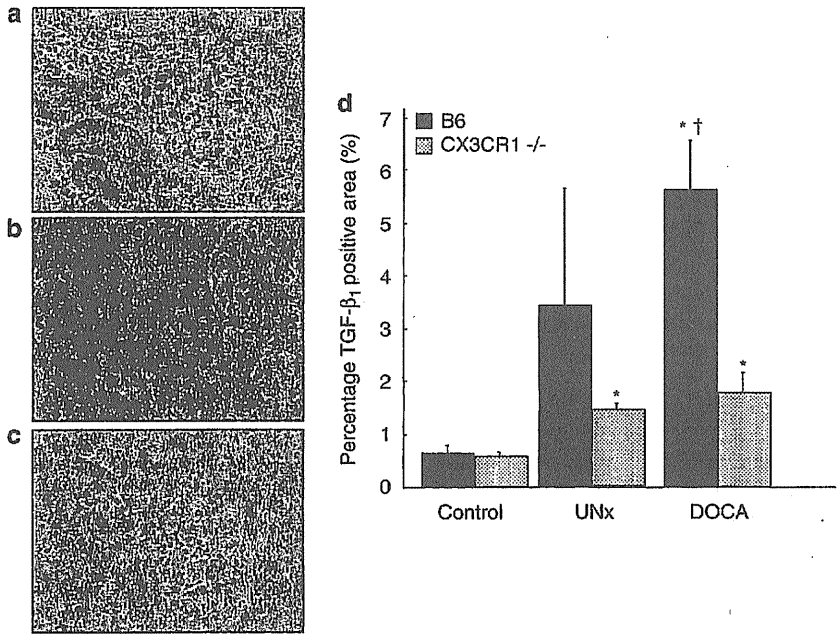


Figure 5 CX3CR1 deletion reduced transforming growth factor (TGF)-β_1 expression. (a) TGF-β_1 was detected by immunohistochemical staining. Upregulation of TGF-β_1 was detected mainly in tubular epithelial cells in C57BL/6 mice after deoxycorticosterone acetate (DOCA) treatment as compared with DOCA-untreated C57BL/6 mice (c). In contrast, the CX3CR1-deficient mice that received DOCA treatment had reduced expression of TGF-β_1 (b). (d) TGF-β_1 expression was reduced in CX3CR1-deficient mice. n=5 in each experiment. Values are the mean number ± standard error. *P<0.05 compared with the control group, †P<0.05 compared with CX3CR1-deficient mice. Original magnification is ×400. A full color version of this figure is available at the *Hypertension Research* journal online.

regulation of TGF-β_1 and type I procollagen, and a decrease in macrophage infiltration in damaged kidneys. It was also noted that a blockade of CX3CR1 signaling hardly affected the elevation of blood pressure. Taken together, these results suggest that the fractalkine-CX3CR1 axis is required for fibrotic processes involved in progressive hypertensive kidney damage via increasing the expression of type I collagen and TGF-β_1 and the infiltration of macrophages.

Fractalkine/CX3CL1 contains a CX3C motif and exists as a membrane-bound glycoprotein with a chemokine domain atop an extended mucin-like stalk.[14] Fractalkine/CX3CL1 can be induced on endothelium by inflammatory cytokines, including interleukin-1 and tumor necrosis factor-α. In turn, fractalkine/CX3CL1 has the capacity to be chemotactic to CX3CR1-expressing inflammatory cells, such as macrophages. Macrophage infiltration was observed predominantly in the outer medulla, which is similar to those in other models, such as ischemia-reperfusion,[6] kidney fibrosis[5] and human kidney disease.[15] This result is partly because high endothelial venules (HEVs) that express MECA-79 affect infiltrating cells into kidneys and could be detected in outer medulla;[16] however, we did not examine whether HEVs-like vessels in the outer medulla expressed fractalkine/CX3CL1 in this particular model. Recently, fractalkine/CX3CL1 and CX3CR1 have been reported to contribute to fibrogenesis.[6] In that report, a CX3CR1 deficiency or blockade attenuated macrophage infiltration and early platelet-derived growth factor-β expression, as well as late-phase interstitial fibrosis and kidney dysfunction after ischemia reperfusion injury. In the present study, the fractalkine-CX3CR1 axis-dependent macrophage infiltration in the damaged kidneys may have a role in the pathogenesis of kidney fibrosis in unilateral nephrectomized mice with DOCA administration. This result might be partly explained by the decrease in TGF-β_1 expression in macrophages and/or tubular epithelial cells, which was activated by fractalkine-CX3CR1 pathways.

Endothelial cells are equipped with numerous receptors that allow them to detect and respond to the mechanical forces generated by pressure and shear stress. The cytoskeleton and other structural components have an established role in the mechanotransduction to be able to transmit and modulate tension within the cell via adhesion sites, integrins, cellular junctions and the extracellular matrix. Mechanical forces also initiate complex signal transduction cascades, including the NF-κ B and mitogen-activated protein kinase pathways. Transcription factor NF-κ B in endothelial cells is activated following the exposure to shear stress.[17] NF-κ B induces the transcription of a large range of genes implicated in inflammation, including fractalkine/CX3CL1.[18] Supporting this notion, dexamethasone suppressed the expression of fractalkine/CX3CL1.[19] Alternatively, fractalkine/CX3CL1 has a role in vascular remodeling in pulmonary hypertension.[20] A recent report described that fractalkine/CX3CL1 stimulates the phosphorylation of the mitogen-activated protein kinases p38, c-Jun N-terminal kinase and extracellular-regulated kinase 1/2 as well as the serine-threonine kinase Akt at Ser 473 and Thr 308.[21] These results may be supportive because mitogen-activated protein kinases have been reported to be involved kidney fibrosis.[22] Although this remodeling process remains to be investigated, it would be reasonable to speculate that fractalkine/CX3CL1 induced by hypertensive endothelial cells injury might, in turn, promote vascular remodeling in damaged kidneys. Further study will be required to test this hypothesis.

The expression of fractalkine/CX3CL1 was reduced by the fractalkine-CX3CR1 blockade. This finding may be explained by some speculations. First, this reduction may be dependent on the decrease in the number of macrophages in damaged kidneys. In support of this finding, it has been reported that fractalkine/CX3CL1 is expressed on infiltrating mononuclear cells, such as endothelial cells.[23] Second, the interaction of endothelial cells and macrophages may be important.

Activated macrophages expressing CX3CR1 might produce proinflammatory cytokines and chemokines, which activate endothelial cells and subsequently promote the inflammatory process. A fractalkine-CX3CR1 blockade may inhibit these processes, leading to anti-inflammatory responses, thereby possibly decreasing the expression of fractalkine/CX3CL1 in kidneys.

In a recent study, it was found that direct mineralocorticoid effects and high blood pressure contributes to fibrosis of the kidney in DOCA-salt hypertension.[24] Therefore, in this study, kidney fibrosis might be induced by the mineralocorticoid effect of DOCA and by hypertension. However, DOCA alone did not induce obvious kidney fibrosis in C57BL/6 mice without unilateral nephrectomy in the previously mentioned study.[24] Collectively, although the possible direct effect of DOCA should not be neglected, kidney fibrosis in this particular model may be mostly due to systemic hypertension.

In conclusion, the fractalkine-CX3CR1 axis may be involved in the pathogenesis of progressive kidney fibrosis in a hypertensive model, possibly due to the upregulation of TGF-β_1 and type I collagen. Thus, blockade of the fractalkine-CX3CR1 axis may be useful in anti-fibrotic therapeutic strategies in progressive kidney fibrosis.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan. There is no conflict of interest in this manuscript.

1 Wada T, Matsushima K, Kaneko S. Chemokines and the kidney. In: Linkes WP (ed). *Progress in Chemokine Research*. Nova Science Publishers: Hauppauge, 2007, pp 179–186.
2 Wada T, Yokoyama H, Furuichi K, Kobayashi KI, Harada K, Naruto M, Su SB, Akiyama M, Mukaida N, Matsushima K. Intervention of crescentic glomerulonephritis by antibodies to monocyte chemotactic and activating factor (MCAF/MCP-1). *FASEB J* 1996; **10**: 1418–1425.
3 Kitagawa K, Wada T, Furuichi K, Hashimoto H, Ishiwata Y, Asano M, Takeya M, Kuziel WA, Matsushima K, Mukaida N, Yokoyama H. Blockade of CCR2 ameliorates progressive fibrosis in kidney. *Am J Pathol* 2004; **165**: 237–246.
4 Wada T, Furuichi K, Sakai N, Iwata Y, Kitagawa K, Ishida Y, Kondo T, Hashimoto H, Ishiwata Y, Mukaida N, Tomosugi N, Matsushima K, Egashira K, Yokoyama H. Gene therapy via blockade of monocyte chemoattractant protein-1 for renal fibrosis. *J Am Soc Nephrol* 2004; **15**: 940–948.
5 Sakai N, Wada T, Yokoyama H, Lipp M, Ueha S, Matsushima K, Kaneko S. Secondary lymphoid tissue chemokine (SLC/CCL21)/CCR7 signaling regulates fibrocytes in renal fibrosis. *Proc Natl Acad Sci USA* 2006; **103**: 14098–14103.
6 Furuichi K, Gao JL, Murphy PM. Chemokine receptor CX3CR1 regulates renal interstitial fibrosis after ischemia-reperfusion injury. *Am J Pathol* 2006; **169**: 372–387.
7 Hasegawa M, Sato S, Echigo T, Hamaguchi Y, Yasui M, Takehara K. Up-regulated expression of fractalkine/CX3CL1 and CX3CR1 in patients with systemic sclerosis. *Ann Rheum Dis* 2005; **64**: 21–28.
8 Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, Kakizaki M, Takagi S, Nomiyama H, Schall TJ, Yoshie O. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell* 1997; **91**: 521–530.
9 Ishida Y, Hayashi T, Goto T, Kimura A, Akimoto S, Mukaida N, Kondo T. Essential involvement of CX3CR1-mediated signals in the bactericidal host defense during septic peritonitis. *J Immunol* 2008; **181**: 4208–4218.
10 Lu P, Li L, Kuno K, Wu Y, Baba T, Li YY, Zhang X, Mukaida N. Protective roles of fractalkine/CX3CL1-CX3CR1 interactions in alkali-induced corneal neovasculization through enhanced antiangiogenic factor expression. *J Immunol* 2008; **180**: 4283–4291.
11 Furuichi K, Wada T, Iwata Y, Sakai N, Yoshimoto K, Shimizu M, Kobayashi K, Takasawa K, Kida H, Takeda S, Matsushima K, Yokoyama H. Upregulation of fractalkine in human crescentic glomerulonephritis. *Nephron* 2001; **87**: 312–320.
12 Kaplan NM. *Kaplan's Clinical Hypertension*, 9th edn. Lippincott Willams&Wilkins: Philadelphia, 2006, pp 122–137.
13 Tian N, Moore RS, Braddy S, Rose RA, Gu JW, Hughson MD, Manning Jr RD. Interactions between oxidative stress and inflammation in salt-sensitive hypertension. *Am J Physiol Heart Circ Physiol* 2007; **293**: H3388–H3395.
14 Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik A, Schall TJ. A new class of membrane-bound chemokine with a CX3C motif. *Nature* 1997; **385**: 640–644.
15 Takaeda M, Yokoyama H, Segawa-Takaeda C, Wada T, Kobayashi K. High endothelial-venule-like vessels in the interstitial lesions of human glomerulonephritis. *Am J Nephrol* 2002; **22**: 48–57.
16 Segawa C, Wada T, Takaeda M, Furuichi K, Matsuda I, Hisada Y, Ohta S, Takasawa K, Takeda S, Kobayashi K, Yokoyama H. *In situ* expression and soluble form of P-selectin in human glomerulonephritis. *Kidney Int* 1997; **52**: 1054–1063.
17 Lan QX, Mercurius KO, Davies PF. Stimulation of transcription factors NFκB and AP1 in endothelial cells subjected to shear stress. *Biochem Biophys Res Communi* 1994; **201**: 950–956.
18 Bhabsar OK, Sukkar MB, Khorasani N, Lee KY, Chung KF. Glucocorticoid suppression of CX3CL1(fractalkine) by reduced gene promoter recruitment of NF-kappa B. *FASEB J* 2008; **22**: 1807–1816.
19 Yoshida T, Ishikawa I, Omo Y, Imai T, Suzuki R, Yoshie O. An activation-responsive element in single C motif-1/lymphotactin promoter is a site of constitutive and inducible DNA-protein interactions involving nuclear factor of activated T cell. *J Immunol* 1999; **163**: 3295–3303.
20 Schober A, Zernecke A. Chemokines in vascular remodeling. *Thromb Haemost* 2007; **97**: 730–737.
21 Klosowska K, Volin MV, Huynh N, Chong KK, Halloran MM, Woods JM. Fractalkine functions as a chemoattractant for osteoarthritis synovial fibroblasts and stimulates phosphorylation of mitogen-activated protein kinases and Akt. *Clin Exp Immunol* 2009; **156**: 312–319.
22 Wada T, Furuichi K, Sakai N, Hisada Y, Kobayashi K, Mukaida N, Tomosugi N, Matsushima K, Yokoyama H. Involvement of p38 mitogen-activated protein kinase followed by chemokine expression in crescentic glomerulonephritis. *Am J Kidney Dis* 2001; **38**: 1169–1177.
23 Suzuki F, Nanki T, Imai T, Kikuchi H, Hirohata S, Kohsaka H, Miyasaka N. Inhibition of CX3CL1(fractalkine) improves experimental autoimmune myositis in SJL/J mice. *J immunol* 2005; **175**: 6987–6996.
24 Klanke B, Cordasic N, Hartner A, Schmieder RE, Veelken R, Hilgers KF. Blood pressure versus mineralocorticoid effects on kidney inflammation and fibrosis in DOCA-salt hypertension. *Nephrol Dial Transplant* 2008; **23**: 3456–3463.

Clin Exp Nephrol
DOI 10.1007/s10157-011-0508-z

REVIEW ARTICLE

Clinical impact of albuminuria in diabetic nephropathy

Takashi Wada · Miho Shimizu · Tadashi Toyama · Akinori Hara · Shuichi Kaneko · Kengo Furuichi

Received: 10 May 2011 / Accepted: 14 July 2011

Abstract Patients suffering from diabetic nephropathy, resulting in end-stage renal failure, are increasing in number. The pathophysiology of diabetic nephropathy remains to be fully investigated. In the clinical setting, the presence of albuminuria/overt proteinuria and a low glomerular filtration rate may predict poor renal prognosis, but the prognosis of the normoalbuminuric renally insufficient diabetic patient remains controversial. In addition to the measurement of urinary albumin excretion, biomarker studies to detect diabetic nephropathy more specifically at the early stage have been performed worldwide. There is a growing body of evidence for remission and/or regression of diabetic nephropathy, which may be an indicator for cardiovascular and renal risk reduction. Deeper insights into the pathological characteristics as well as the clinical impact of albuminuria on renal and cardiovascular outcome are required.

Keywords Diabetic nephropathy · Albuminuria · Proteinuria · Glomerular filtration rate · Cardiovascular disease · Renal outcome

T. Wada (✉)
Division of Nephrology, Department of Laboratory Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Faculty of Medicine, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan
e-mail: twada@m-kanazawa.jp

M. Shimizu · T. Toyama · A. Hara
Division of Nephrology, Department of Disease Control and Homeostasis, Institute of Medical, Pharmaceutical and Health Sciences, Faculty of Medicine, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan

S. Kaneko
Department of Disease Control and Homeostasis, Institute of Medical, Pharmaceutical and Health Sciences, Faculty of Medicine, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan

K. Furuichi
Division of Blood purification, Kanazawa University Hospital, 13-1 Takara-machi, Kanazawa 920-8641, Japan

K. Furuichi
Division of Nephrology, Kanazawa University Hospital, 13-1 Takara-machi, Kanazawa 920-8641, Japan

Introduction

Based on the annual report of the Japanese Society for Dialysis Therapy (JSDT), diabetic nephropathy is a leading cause of end-stage renal failure in Japan [1]. The number of dialysis patients had increased to 297,126 by the end of 2010. According to the annual report of the JSDT, diabetic nephropathy has been a leading primary disease of new patients who have been started on dialysis since 1998 [1]: the number of such patients with diabetic nephropathy has increased to 43.5%. In addition, cardiovascular diseases and deaths in patients with diabetes and underlying renal disease before and after dialysis has increased [2, 3]. Therefore, preventing and halting the progression of diabetic nephropathy is important if we are to prolong the survival of such patients.

Characteristic pathologic changes associated with diabetic nephropathy are accumulation of extracellular matrix (ECM) and the infiltration of inflammatory cells into glomeruli and tubulointerstitial regions [4, 5]. These pathologic abnormalities are induced by alterations in ECM production or degradation [6]. Generally speaking, the occurrence of albuminuria is a reflection of increased matrix deposition, leading to glomerular and tubulointerstitial lesions. Diabetic nephropathy is a clinical entity in

Published online: 10 August 2011

which the presence of persistent albuminuria and declines in renal function and glomerular filtration rate (GFR) are the major characteristic findings, which are closely associated with end-stage renal diseases, enhanced cardiovascular morbidity and eventual mortality [7]. The incidence of albuminuria, which currently contributes to the diagnosis of diabetic nephropathy, is well correlated with a decrease in GFR and the incidence of cardiovascular diseases.

Here, we focus on the clinical impact of albuminuria along with GFR levels on the progression of diabetic nephropathy and the incidence of cardiovascular diseases, which is closely related to the mortality of patients with diabetic nephropathy in this manuscript.

Albuminuria in the diagnosis of diabetic nephropathy

The definitive diagnosis of diabetic nephropathy is based on pathological findings such as the presence of diffuse mesangial lesions and nodular lesions. However, renal biopsy is not performed for all patients with diabetic nephropathy. In the clinical setting, the presence of persistent proteinuria as well as other complications such as diabetic retinopathy and renal dysfunction is important in the diagnosis of diabetic nephropathy. However, early detection of the presence of diabetic nephropathy is clinically required for the best prognosis. The measurement of urinary albumin excretion is currently crucial to the detection of early diabetic nephropathy. The increased excretion of albumin (albuminuria) is an early diagnostic indicator of diabetic nephropathy. Thus, Mogensen et al. [8] proposed a classification of diabetic nephropathy in patients with type 1 diabetes based on increased urinary albumin excretion once diabetic nephropathy was diagnosed. Diabetic nephropathy is also staged in Japan [9, 10], and the staging was described by Yokoyama et al. [11] as follows: stage I: urinary albumin-to-creatinine ratio (ACR) <30 mg/g creatinine; stage II: ACR $\geq$ 30 and <300 mg/g creatinine (i.e., albuminuria); stage III: ACR $\geq$ 300 mg/g creatinine and/or persistent proteinuria with serum concentration of creatinine <2 mg/dl; stage IV: serum concentration of creatinine $\geq$2 mg/dl with proteinuria; and stage V: being treated with dialysis. The Japan Diabetes Clinical Data Management Study Group (JDDM) reported that the prevalence of albuminuria (stage II) in Japanese type 2 diabetic patients was 32%, which is very similar to the 39% observed in the DEMAND study [12]. These results suggest that albuminuria is common, and that 76% of patients with diabetic nephropathy are categorized as stage II, as evidenced by the presence of albuminuria. Further, 58% of the patients enrolled were at stage I, 7% were at stage III, 2.6% were at stage IV, and 0.4% were at stage V [11]. A very recent study from the Japan Diabetes Complications Study (JDCS) revealed that the annual transition rate to proteinuria (ACR $\geq$ 300 mg/g creatinine) was 0.67%, and that this was substantially higher for the low-albuminuric group (defined as a urinary ACR of 30–150 mg/g creatinine) than for the normoalbuminuric group (defined as a urinary ACR of <30 mg/g creatinine) [13]. In this sense, UKPDS 64 reported that the progression to albuminuria occurred at 2.0% per year, and from albuminuria to macroalbuminuria at 2.8% per year [14]. However, about 40% of the diabetic patients had no urinary albumin excretion measurements, regardless of the recommendation for the JDDM cohort [11]. Therefore, the measurement of urinary albumin excretion is required for the early detection of diabetic nephropathy in Japan.

Biomarkers for diabetic nephropathy and disease progression

Further studies to detect diabetic nephropathy more specifically at the early stage in addition to urinary albumin excretion are needed. In this sense, biomarker studies to identify the presence and predict the progression of diabetic nephropathy have been performed worldwide [15]. Recently, Kamijo-Ikemori et al. [16] reported that urinary levels of liver-type fatty acid-binding protein (L-FABP) accurately reflected the severity of diabetic nephropathy in type 2 diabetes. Importantly, urinary L-FABP levels were high in patients with normoalbuminuria, suggesting its usefulness for detecting early nephropathy in these patients. Further, an increase in urinary Smad1—a key transcriptional factor for mesangial matrix expansion in diabetic nephropathy—at the early stage was correlated with subsequent development of glomerulosclerosis in experimental rodent models [17]. Regarding renal function, serum cystatin C was reported to be a good marker for nephropathy [18]. Notably, cases of early renal dysfunction, defined by a loss of cystatin C GFR exceeding −3.3%/year, occurred in 9% of the normoalbuminuria group and 31% of the albuminuria group [19].

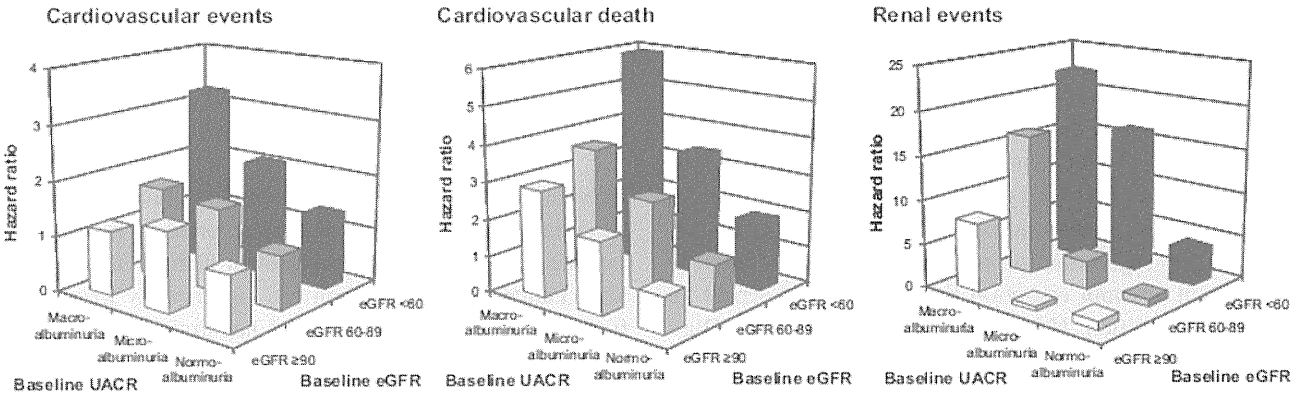
Prevalence of albuminuria and low GFR in type 2 diabetic patients in Japan

As previously described, diabetic nephropathy is diagnosed through the detection of albuminuria. Recently, Kidney Disease: Improving Global Outcomes (KDIGO) reported the definition, classification and prognosis of chronic kidney disease based on both estimated GFR and urinary levels of albumin excretion [20]. In this sense, there are diabetic patients with decreases in GFR and normoalbuminuria. Is diabetic nephropathy observed in such patients? In fact, the percentage of diabetic patients with normoalbuminuria and low estimated GFR is believed to be relatively high. Importantly, Yokoyama et al. [21] described

that the proportion of subjects with low estimated GFR (<60 ml/min/1.73 m^2) and normoalbuminuria was 11.4% of the type 2 diabetic patients examined (262/2298). In this manuscript, 63.4% of the 262 patients studied had neither diabetic retinopathy nor neuropathy. On the other hand, these patients were older and included a higher proportion of women and patients with hypertension, hyperlipidemia and cardiovascular disease, as well as fewer smokers compared with those with normoalbuminuria and preserved GFR. In contrast, the proportion of type 2 diabetic patients with preserved GFR but albuminuria or overt proteinuria was 27% (755/2791). Most importantly, the lack of histologically proven diabetic nephropathy should be discussed. In type 1 diabetes patients with normoalbuminuria and low GFR, renal biopsy specimens revealed more advanced diabetic glomerular lesions. It is worth noting that a reduced GFR was found much more often among female patients, particularly if retinopathy and/or hypertension were also present [22]. Deep insight into the prevalence and prognoses of these patients with proven pathological characteristics and grading is required to understand the pathophysiology of diabetic nephropathy in greater depth, together with future perspectives.

Clinical impacts of albuminuria and GFR on the prognoses of diabetic patients

Obviously, diabetic patients who had both albuminuria/overt proteinuria and low GFR were at risk of adverse outcomes, including cardiovascular events, cardiovascular death, and renal events, as reported by the Action in Diabetes and Vascular Disease: Preterax and DiamicroN MR Controlled Evaluation (ADVANCE) study [23] (Fig. 1). Do normoalbuminuric renally insufficient diabetic patients have a poor prognosis? Rigalleau et al. [24] reported that the risks of renal progression and death in these patients with type 1 or type 2 diabetes are lower. Concomitantly, in type 2 diabetic patients, the Casale Monferrato study revealed that macroalbuminuira was the main predictor of mortality, independently of both estimated GFR and cardiovascular risk factors, whereas the estimated GFR provided no further information on all-cause mortality and cardiovascular mortality in normoalbuminuric patients [25]. Supporting this notion, regarding renal end-points, there was also a progressive increase in risk associated with declined renal function, which was mainly observed in the albuminuric group in Chinese type 2 diabetic patients [26]. Interestingly, those with a reduced estimated GFR were at high risk of developing cardiovascular end-points (cardiovascular death, new admissions due to angina, myocardial infarction, stroke, revascularization or heart failure) and all-cause mortality, independent of albuminuria [26]. In contrast, as previously described, in the ADVANCE study, patients with normoalbuminuria and estimated GFR <60 ml/min per 1.73 m^2 had a 3.95-fold higher risk of renal events, a 1.33-fold higher risk of cardiovascular events, and a 1.85-fold higher risk of cardiovascular death [23] (Fig. 1). Moreover, Vlek et al. reported that an estimated GFR <60 ml/min/1.73 m^2 without albuminuria was



Cardiovascular events	Baseline eGFR (ml/min/1.73 m^2)		
Baseline UACR	GFR ≥90	GFR 60-89	GFR <60
Normoalbuminuria	1.00 (Reference)	0.98 (0.78-1.22)	1.33 (1.02-1.75)
Microalbuminuria	1.48 (1.09-2.01)	1.54 (1.20-1.96)	2.04 (1.54-2.69)
Macroalbuminuria	1.18 (0.52-2.69)	1.67 (1.09-2.57)	3.23 (2.20-4.73)

Cardiovascular death	Baseline eGFR (ml/min/1.73 m^2)		
Baseline UACR	GFR ≥90	GFR 60-89	GFR <60
Normoalbuminuria	1.00 (Reference)	1.22 (0.81-1.84)	1.85 (1.17-2.92)
Microalbuminuria	1.96 (1.16-3.32)	2.52 (1.65-3.84)	3.37 (2.15-5.30)
Macroalbuminuria	2.87 (1.01-8.18)	3.61 (2.02-6.43)	5.93 (3.45-10.20)

Renal events	Baseline eGFR (ml/min/1.73 m^2)		
Baseline UACR	GFR ≥90	GFR 60-89	GFR <60
Normoalbuminuria	1.00 (Reference)	0.89 (0.31-2.58)	3.95 (1.38-11.34)
Microalbuminuria	0.45 (0.05-3.83)	3.17 (1.15-8.74)	16.19 (6.16-42.54)
Macroalbuminuria	7.82 (1.51-40.53)	16.13 (5.49-47.42)	22.20 (7.62-64.72)

Fig. 1 Combined effects of albuminuria and eGFR levels at baseline on the risk of an adverse outcome. The estimates are adjusted for baseline covariates, including age, gender, duration of diabetes, SBP, history of currently treated hypertension, history of macrovascular disease, HbA1c, LDL cholesterol, HDL cholesterol, log-transformed triglycerides, BMI, electrocardiogram abnormalities, current smoking, and current drinking. (From Ref. [23] reproduced with permission from the American Society of Nephrology)

the strongest risk factor in the occurrence of vascular events (hazard ratio 1.50; 1.05–2.15) [27]. Recently, the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study revealed that normoalbuminuric patients with eGFR 30–59 ml/min per 1.73 m^2 were at higher risk of a cardiovascular event, cardiovascular death, noncoronary heart disease death, and death from any cause than normoalbuminuric patients with eGFR ≥60 ml/min per 1.73 m^2 [28]. Interestingly, high normal levels of albuminuria (≥5 μg/min) predicted the development of micro- and macroalbuminuria and increased mortality in Brazilian type 2 diabetic patients [29]. Furthermore, in Japanese patients with type 1 and type 2 diabetes, even within the normal range (≤30 mg/g), ACR ≥10 mg/g in women and ≥5 mg/g in men was associated with a significantly greater rate of decline in eGFR relative to subjects with ACR ≤5 mg/g [30]. It is of interest that the risk of cardiovascular events in individuals with diabetes increases with the ACR, starting well below the microalbumin cutoff [31]. Taken together, an evaluation of the clinical impact of albuminuria along with an evaluation of the effect of GFR on the prognoses of diabetic patients is required.

Remission/regression of albuminuria in patients with diabetic nephropathy

Fioretto et al. [32] reported that pancreas transplantation reversed the lesions of diabetic nephropathy in patients with type 1 diabetes mellitus, but that reversal required more than 5 years of normoglycemia. A growing body of evidence since then has pointed to the possibility of remission and/or regression of diabetic nephropathy, especially in patients treated with renin-angiotensin system blockade drugs. However, there is a lack of data on pathological findings in these patients. In the clinical setting, Perkins et al. [33] stated that regression of albuminuria was frequent in patients with type 1 diabetes mellitus, with a 6-year cumulative incidence of 58%. In this context, the definition of regression of microalbuminuria is a 50% reduction in albumin excretion from one 2-year period to the next. In addition, Hovind et al. [34] at the Steno Diabetes Center reported that the total number of patients who obtained remission was 92 (31%), with a duration of remission of 3.4 years, and regression occurred in 67 (22%) of 301 consecutive type 1 diabetic patients with diabetic nephropathy. Remission was defined as albuminuria <200 μg/min sustained for at least 1 year and a decrease of at least 30% from pre-remission levels, and regression as a rate of decline in GFR equal to the natural aging process: ≤1 ml/min/year during the investigation period in this report. Moreover, remission of nephrotic-range albuminuria in type 1 diabetic patients was also reported at the Steno Diabetes Center [35]. In this report, remission was induced in 28 of 126 (22%) patients; 21 were predominantly treated with angiotensin-converting enzyme (ACE) inhibitors, and 7 with non-ACE inhibitor medications. Remission lasted 3.6 years. In particular, more women (37%) than men (16%) obtained remission. In addition to type 1 diabetic patients, recent studies have revealed that remission is induced in type 2 diabetic patients. Araki et al. [36] reported that a reduction in urinary albumin excretion rate was frequent, with a 6-year cumulative incidence of 51% for remission, defined as a shift to normoalbuminuria, and 54% for regression, defined as a 50% reduction in the urinary albumin excretion rate. Interestingly, in this particular study, the frequency of progression to overt proteinuria was 28%, and albuminuria of short duration, the use of renin-angiotensin system-blocking drugs, and lower titers for HbA1c and systolic blood pressure were independently associated with remission or regression. More recently, JDCS revealed that a return from low microalbuminuria to normoalbuminuria was observed in 137 out of 452 patients (30.3%) [13].

Further, the clinical impact of remission/regression on renal outcome and cardiovascular events is still to be fully investigated. Importantly, Araki et al. [37] have reported that a reduction in albuminuria in patients with type 2 diabetes is an indicator of cardiovascular and renal risk reduction. In this study, the cumulative incidence of mortality from and hospitalization for renal and cardiovascular events was significantly lower in patients with a 50% reduction. Collectively, remission/regression in patients with diabetic nephropathy is relatively frequent, and insight into the pathological characteristics as well as the clinical impact on renal and cardiovascular outcomes when remission/regression is induced is needed.

Hematuria in diabetic nephropathy

Hematuria, the other major characteristic finding aside from albuminuria/overt proteinuria, was reported in 14 out of 34 Japanese patients with biopsy-proven diabetic nephropathy [38]. Patients with hematuria had significantly lower renal function, and the prevalences of nephrotic syndrome and retinopathy were significantly higher than in patients without hematuria. Interestingly, based on a logistic regression analysis, the presence of nephrotic syndrome and a known duration of diabetes were identified as significant predictors for hematuria with diabetic nephropathy.

Concluding remarks and future directions

Deep insights into the onset and progression of albuminuria along with GFR may elucidate the pathogenesis of

progressive kidney complications and associated cardiovascular diseases. Further studies of the clinical characteristics and the pathological findings of kidney involvement in patients with diabetes are required for a better understanding of diabetic nephropathy and the benefits of therapy for it.

Acknowledgments This study was supported in part by a Grant-in-Aid for Diabetic Nephropathy Research from the Ministry of Health, Labour and Welfare of Japan.

References

1. Nakai S, Suzuki K, Masakane I, Wada A, Itami N, Ogata S, et al. Overview of regular dialysis treatment in Japan (as of 31 December 2008). Ther Apher Dial. 2010;14:505–40.
2. Nakayama M, Sato T, Sato H, Yamaguchi Y, Obara K, Kurihara I, et al. Different clinical outcomes for cardiovascular events and mortality in chronic kidney disease according to underlying renal disease: the Gonryo study. Clin Exp Nephrol. 2010;14:333–9.
3. Foley RN, Culleton BF, Parfrey PS, Harnett JD, Kent GM, Murray DC, et al. Cardiac diseases in diabetic end-stage renal disease. Diabetologia. 1997;40:1307–12.
4. Saito Y, Kida H, Takeda S, Yoshimura M, Yokoyama H, Koshino Y, et al. Mesangiolysis in diabetic glomeruli: its role in the formation of nodular lesions. Kidney Int. 1988;34:389–96.
5. Wada T, Furuichi K, Sakai N, Iwata Y, Yoshimoto K, Shimizu M, et al. Up-regulation of monocyte chemoattractant protein-1 in tubulointerstitial lesions in human diabetic nephropathy. Kidney Int. 2000;58:1492–9.
6. Furuichi K, Hisada Y, Shimizu M, Okumura T, Kitagawa K, Yoshimoto K et al. Matrix metalloproteinase-2 (MMP-2) and membrane-type 1 MMP (MT1-MMP) affect the remodeling of glomerulosclerosis in diabetic OLETF rat. Nephrol Dial Transplant 2011 (Epub ahead of print).
7. Parving HH. Diabetic nephropathy: prevention and treatment. Kidney Int. 2001;60:2041–55.
8. Mogensen CE, Christensen CK, Vittinghus E. The stages in diabetic renal disease. With emphasis on the stage of incipient diabetic nephropathy. Diabetes. 1983;32(Suppl 2):64–78.
9. Shigeta Y, Kikkawa R. Diabetic nephropathy in Japan. Diabetes Res Clin Pract. 1994;24(Suppl):S191–7.
10. Guideline Committee of the Japan Diabetes Society. Japan Diabetes Society guideline for the management of diabetes based on scientific evidence. Tokyo: Japan Diabetes Society; 2004.
11. Yokoyama H, Kawai K, Kobayashi M, Japan Diabetes Clinical Data Management Study Group. Microalbuminuria is common in Japanese type 2 diabetic patients: a nationwide survey from the Japan Diabetes Clinical Data Management Study Group (JDDM 10). Diabetes Care. 2007;30:989–92.
12. Parving HH, Lewis JB, Ravid M, Remuzzi G, Hunsicker LG, DEMAND investigators. Prevalence and risk factors for microalbuminuria in a referred cohort of type II diabetic patients: a global perspective. Kidney Int. 2006;69:2057–63.
13. Katayama S, Moriya T, Tanaka S, Tanaka Y, Yajima H, Sone S, et al. Low transition rate from normo- and low microalbuminuria to proteinuria in Japanese type 2 diabetic individuals: the Japan Diabetes Complications Study (JDCS). Diabetologia. 2011;54:1025–31.
14. Adler AI, Stevens RJ, Manley SE, Bilous RW, Cull CA, Holman RR, UKPDS GROUP. Development and progression of nephropathy in type 2 diabetes: The United Kingdom Prospective Diabetes Study (UKPDS 64). Kidney Int. 2003;63:225–32.
15. Valk EJ, Bruijn JA, Bajema IM. Diabetic nephropathy in humans: pathologic diversity. Curr Opin Nephrol Hypertens. 2011;20:285–9.
16. Kamijo-Ikemori A, Sugaya T, Yasuda T, Kawata T, Ota A, Tatsunami S, et al. Clinical significance of urinary liver-type fatty acid-binding protein in diabetic nephropathy of type 2 diabetic patients. Diabetes Care. 2011;34:691–6.
17. Mima A, Arai H, Matsubara T, Abe H, Nagai K, Tamura Y, et al. Urinary Smad1 is a novel marker to predict later onset of mesangial matrix expansion in diabetic nephropathy. Diabetes. 2008;57:1712–22.
18. Kimura T, Ikeda H, Fujikawa J, Nomura K, Aoyama T, Wada Y, et al. Usefulness of serum cystatin C in Japanese patients with type 2 diabetes mellitus and nephropathy. Diabetes Res Clin Pract. 2009;83:e58–61.
19. Perkins BA, Ficociello LH, Ostrander BE, Silva KH, Weinberg J, Warram JH, et al. Microalbuminuria and the risk for early progressive renal function decline in type 1 diabetes. J Am Soc Nephrol. 2007;18:1353–61.
20. Levey AS, de Jong PE, Coresh J, Nahas ME, Astor BC, Matsushita K, et al. The definition, classification and prognosis of chronic kidney disease: a KDIGO Controversies Conference report. Kidney Int. 2011;80:17–28.
21. Yokoyama H, Sone H, Oishi M, Kawai K, Fukumoto Y, Kobayashi M, Japan Diabetes Clinical Data Management Study Group. Prevalence of albuminuria and renal insufficiency and associated clinical factors in type 2 diabetes: the Japan Diabetes Clinical Data Management study (JDDM15). Nephrol Dial Transplant. 2009;24:1212–9.
22. Caramori ML, Floretto P, Mauer M. Low glomerular filtration rate in normoalbuminuric type 1 diabetic patients: an indicator of more advanced glomerular lesions. Diabetes. 2003;52:1036–40.
23. Ninomiya T, Perkovic V, de Galan BE, Zoungas S, Pillai A, Jardine M, et al. Albuminuria and kidney function independently predict cardiovascular and renal outcomes in diabetes. J Am Soc Nephrol. 2009;20:1813–21.
24. Rigalleau V, Lasseur C, Raffaitin C, Beauvieux MC, Barthe N, Chauveau P, et al. Normoalbuminuric renal-insufficient diabetic patients: a lower-risk group. Diabetes Care. 2007;30:2034–9.
25. Bruno G, Merletti F, Bargero G, Novelli G, Melis D, Soddu A, et al. Estimated glomerular filtration rate, albuminuria and mortality in type 2 diabetes: the Casale Monferrato study. Diabetologia. 2007;50:941–8.
26. So WY, Kong AP, Ma RC, Ozaki R, Szeto CC, Chan NN, et al. Glomerular filtration rate, cardiorenal end points, and all-cause mortality in type 2 diabetic patients. Diabetes Care. 2006;29:2046–52.
27. Vlek AL, van der Graaf Y, Spiering W, Algra A, Visseren FL, SMART study group. Cardiovascular events and all-cause mortality by albuminuria and decreased glomerular filtration rate in patients with vascular disease. J Intern Med. 2008;264:351–60.
28. Drury PL, Zannino TD, Ehnholm C, Flack J, Whiting M, Fassett R, et al. Estimated glomerular filtration rate and albuminuria are independent predictors of cardiovascular events and death in type 2 diabetes mellitus: the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study. Diabetologia. 2011;54:32–43.
29. Murussi M, Campagnolo N, Beck MO, Gross JL, Silveiro SP. High-normal levels of albuminuria predict the development of micro- and macroalbuminuria and increased mortality in Brazilian type 2 diabetic patients: an 8-year follow-up study. Diabetes Med. 2007;24:1136–42.
30. Babazono T, Nyumura I, Toya K, Hayashi T, Ohta M, Suzuki K, et al. Higher levels of urinary albumin excretion within the normal range predict faster decline in glomerular filtration rate in diabetic patients. Diabetes Care. 2009;32:1518–20.

31. Gerstein HC, Mann JF, Yi Q, Zinman B, Dinneen SF, Hoogwerf B, et al. Albuminuria and risk of cardiovascular events, death, and heart failure in diabetic and nondiabetic individuals. JAMA. 2001;286:421–6.
32. Fioretto P, Steffes MW, Sutherland DE, Goetz FC, Mauer M. Reversal of lesions diabetic nephropathy after pancreas transplantation. N Engl J Med. 1998;339:69–75.
33. Perkins BA, Ficociello LH, Silva KH, Finkelstein DM, Warram JH, Krolewski AS. Regression of microalbuminuria in type 1 diabetes. N Engl J Med. 2003;348:2285–93.
34. Hovind P, Rossing P, Tarnow L, Smidt UM, Parving HH. Remission and regression in the nephropathy of type 1 diabetes when blood pressure is controlled aggressively. Kidney Int. 2001;60:277–83.
35. Hovind P, Rossing P, Tarnow L, Toft H, Parving J, Parving HH. Remission of nephrotic-range albuminuria in type 1 diabetic patients. Diabetes Care. 2001;24:1972–7.
36. Araki S, Haneda M, Sugimoto T, Isono M, Isshiki K, Kashiwagi A, et al. Factors associated with frequent remission of microalbuminuria I patients with type 2 diabetes. Diabetes. 2005;54:2983–7.
37. Araki S, Haneda M, Koya D, Hidaka H, Sugimoto T, Isono M, et al. Reduction in microalbuminuria as an integrated indicator for renal and cardiovascular risk reduction in patients with type 2 diabetes. Diabetes. 2007;56:1727–30.
38. Akimoto T, Ito C, Saito O, Takahashi H, Takeda S, Ando Y, et al. Microscopic hematuria and diabetic glomerulosclerosis—clinicopathological analysis of type 2 diabetic patients associated with overt proteinuria. Nephron Clin Pract. 2008;109:c119–26.

SHORT REPORT

Association between coefficients of variation of the R-R intervals on electrocardiograms and post-challenge hyperglycemia in patients with newly diagnosed type 2 diabetes

Yusuke Nakade[1], Toshinari Takamura[2]*, Masaru Sakurai[3], Hirofumi Misu[2], Mitsuko Nagata[1], Yuko Nanbu[1], Hiroyasu Oe[1], Toshiji Takamura[1], Yoshio Sakai[1], Shuichi Kaneko[2], Takashi Wada[1]

ABSTRACT

The aim of the present study was to examine whether there is a relationship between autonomic function and post-challenge hyperglycemia in patients with type 2 diabetes. Subjects included 122 Japanese patients newly diagnosed with type 2 diabetes. Autonomic nerve function was assessed using coefficients of variation of the R-R intervals on electrocardiograms (CVRR). Unlike anthropometry, insulin secretion and insulin resistance, age ($r = -0.209$, $P < 0.021$) and post-challenge plasma glucose at 120 min (PG120; $r = -0.219$, $P < 0.015$) were the only variables significantly correlated with CVRR. Age was not significantly correlated with PG120. In multiple regression analyses, CVRR *Z*-score, but not age, was significantly correlated with PG120. The present results suggest that autonomic function affects post-challenge blood glucose levels independently of age. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00098.x, 2011)

KEY WORDS: Type 2 diabetes mellitus, Hyperglycemia, Autonomic function

INTRODUCTION

Diabetic autonomic neuropathy is a major complication in patients with diabetes; it decreases quality of life and increases mortality. Long-term hyperglycemia is a primary cause of diabetic neuropathy[1]. Conversely, autonomic function might affect glycemic control through gastrointestinal peristalsis. In addition, results from animal experiments have suggested that the autonomic nervous system contributes to glucose homeostasis by mediating interorgan networks[2–4]. Matsuhisa *et al.* have reported that the vagus nerve, which in part controls the liver, plays an important role in regulating postprandial glucose levels[5,6]. In addition, the intestinal–brain–liver neuronal axis has been reported to be involved in liver gluconeogenesis[4]. However, evidence showing that these pathways and autonomic function play a role in glycemic control is lacking in humans. The aim of the present study was to examine whether there is a relationship between autonomic function, evaluated by coefficient of variation of the R-R interval on electrocardiograms (ECG; CVRR), and post-challenge hyperglycemia in patients with newly diagnosed type 2 diabetes.

[1]Department of Clinical Laboratory, Kanazawa University Hospital, and [2]Department of Disease Control and Homeostasis, Kanazawa University Graduate School of Medical Science, and [3]Department of Epidemiology and Public Health, Kanazawa Medical University, Kanazawa, Japan
*Corresponding author. Toshinari Takamura Tel.: +81-76-265-2233
Fax: +81-76-234-4250 E-mail address: ttakamura@m-kanazawa.jp
Received 25 April 2010; revised 27 October 2010; accepted 29 November 2010

MATERIAL AND METHODS

Subjects

A total of 122 Japanese patients newly diagnosed with type 2 diabetes mellitus between January 2008 and December 2009, in the Division of Endocrinology and Metabolism at Kanazawa University Hospital, were recruited for the present study. None of the patients were treated with an oral hypoglycemic agent or insulin. Table 1 shows the demographic, clinical and physical characteristics of the subjects.

Written informed consent was obtained from all patients before initiation of the study. The study was approved by the ethics committee established at the Kanazawa University Hospital (Approval No. 729) and was carried out in accordance with the Declaration of Helsinki.

Laboratory Data

HbA_{1c} was described in the Japan Diabetes Society (JDS) value. Oral glucose tolerance tests using 75 g of glucose (75-g OGTT) were carried out for all patients. The plasma glucose concentration was measured using an automated glucose analyzer (model GA08; ATWiLL M.I., Kanazawa, Japan). The plasma insulin concentration was measured by immunoassay (AIA-1800ST; Tosoh, Tokyo, Japan).

Insulin resistance in the liver was evaluated using the liver insulin resistance index (L-IR) as reported by Muhammad *et al.*[7]. Insulin resistance in muscle was evaluated using the muscle

Table 1 | Clinical characteristics of the 74 newly diagnosed type 2 diabetic patients

Clinical parameters		Value
Age (years)		60.1 ± 11.1
Sex (male/female)		82/40
Body mass index (kg/m^2)		25.1 ± 4.7
Fasting plasma glucose (mg/dL)		139.9 ± 31.8
HbA_{1c} (%) (JDS)		7.9 ± 2.0
Diabetic complications	*n*	Prevalence (%)
Retinopathy (Fukuda†)		
0	88/112	79
A1	7/112	6
A2	6/112	5
A3	4/112	3
B1	2/112	2
B4	5/112	4
Nephropathy		
Stage 1	90/120	75
Stage 2	19/120	16
Stage 3a	7/120	6
Stage 3b	3/120	3
Stage 4	1/120	1
Somatic polyneuropathy‡	17/95	18

Data are means ± SD. JDS, Japan Diabetes Society. †Retinopathy stages were diagnosed according to Fukuda's classification (Fukuda M. Classification and treatment of diabetic retinopathy. *Diabetes Research and Clinical Practice.* 1994; 24 Suppl: s171–s176) as follows: Stage 0, patients without retinopathy; Stage A1, mild to moderate simple retinopathy; Stage A2, severe simple retinopathy; Stage A3, mild to moderate interrupted proliferative retinopathy; Stage A4, 5, severe interrupted proliferative retinopathy; Stage B1, pre-proliferative diabetic retinopathy; Stage B2, early proliferative diabetic retinopathy; Stage B3, advanced proliferative diabetic retinopathy; Stage B4, end-stage proliferative diabetic retinopathy. ‡Somatic polyneuropathy was defined as below mean-2SD in nerve conduction velocity in two or more of six sites in the healthy subject.

insulin resistance index (M-IR) as reported by Matsuda *et al.*[8]. To evaluate initial insulin secretion, the insulinogenic index was calculated by dividing the change in immunoreactive insulin (IRI) over 30 min by the change in plasma glucose (PG) over 30 min: ΔIRI (30′ – 0′) μU/mL/ΔPG (30′ – 0′) mg/dL.

Evaluation of Autonomic Nerve Function

The CVRR was used to evaluate diabetic autonomic neuropathy[9]. After the patient had rested in the supine position for at least 10 min, a standard 12-lead ECG was recorded (Cardio Star FCP-7541; Nihon Kohden, Tokyo, Japan). The R-R intervals were measured for 3 min, and the CVRR was obtained by dividing the standard deviations (SD) by the means (M): CV (%) = (SD/M) × 100. Because CVRR is influenced by age, we also used CVRR *Z*-score calculated by (CVRR-mean CVRR)/SD-CVRR in each age category to the age-specific normal value of CVRR (Table S2).

Statistical Analysis

For statistical analyses, SPSS II for Windows (SPSS, Chicago, IL, USA) was used. Single regression analysis and multiple regression analysis were used to examine associations between CVRR and clinical parameters. All data are presented as means ± SD.

RESULTS

Clinical characteristics of the 122 newly diagnosed type 2 diabetic patients are shown in Table 1. The prevalence of retinopathy and nephropathy was 21% and 25% of the subjects, respectively. When somatic polyneuropathy is defined as below mean-2SD in nerve conduction velocity in more than two of six sites in the healthy subjects, its prevalence was 18%.

The results for single linear regression analyses between CVRR and each clinical parameter are shown in Table 2. CVRR was significantly correlated with age ($r = -0.209$, $P < 0.021$) and PG120 ($r = -0.219$, $P < 0.015$; Table 2), but was not correlated with body mass index, HbA_{1c}, insulinogenic index, PG0-60 and IRI0-120, L-IR, and M-IR ($P > 0.05$; Table 2). Although CVRR diminishes with age, CVRR, but not age, significantly correlated to PG120 (Table S1). In a multiple regression analysis for PG 120 as a dependent variable, CVRR was still significantly correlated with PG120 ($P = 0.004$), even after adjustment for age (Table 3, upper panel). When we used CVRR *Z*-scores in the analysis, CVRR Z score, but not age, was significantly correlated with PG120 in a multiple regression model (Table 3, lower panel).

Table 2 | Single linear regression analyses between coefficients of variation of the R-R intervals on electrocardiograms and clinical parameters

Variable	*r*-value	*P*-value
Age	−0.209	0.021*
BMI	0.188	0.075
HbA_{1c} (JDS)	0.057	0.536
Insulinogenic index	0.014	0.902
PG0	−0.054	0.554
PG30	0.041	0.650
PG60	−0.108	0.236
PG120	−0.219	0.015*
IRI0	−0.114	0.211
IRI30	−0.021	0.817
IRI60	0.002	0.978
IRI120	0.024	0.794
L-IR	0.042	0.718
M-IR	−0.182	0.121

*$P < 0.05$. PG0, plasma glucose concentration before load; PG30, plasma glucose concentrations at 30 min after load; PG60, plasma glucose concentrations at 60 min after load; PG120, plasma glucose concentrations at 120 min after load; IRI0, immunoreactive insulin before load; IRI30, immunoreactive insulin at 30 min after load; IRI60, immunoreactive insulin at 60 min after load; IRI120, immunoreactive insulin at 120 min after load; L-IR, liver insulin resistance index; M-IR, muscle insulin resistance index.

Table 3 | Multiple regression analysis for plasma glucose concentrations at 120 min after load as a dependent variable and clinical parameters as independent variables

	Partial regression coefficient β	*t*-value	*P*-value
Sex	−12.061	−1.135	0.259
Age	−1.109	−1.994	0.048*
CVRR	−16.879	−2.901	0.004*
Sex	−12.091	−1.143	0.255
Age	−0.534	−0.924	0.357
CVRR *Z*-score	−18.123	−2.935	0.004*

CVRR, coefficients of variation of the R-R intervals on electrocardiograms. *$P < 0.05$; $n = 121$.

DISCUSSION

The preset results suggest that autonomic nerve dysfunction, specifically parasympathetic nerve dysfunction, increases post-challenge glucose levels without affecting insulin secretion or insulin sensitivity. One possible mechanism underlying autonomic dysfunction-associated post-challenge hyperglycemia is gastrointestinal peristalsis that affects absorption of nutrients[10].

The failure of liver gluconeogenesis, regulated by the vagus nerve, has been shown to contribute to diabetic autonomic neuropathy-related hyperglycemia. Wang *et al.*[4] reported that lipids in the upper intestine activate the intestine–brain–liver neural axis to inhibit glucose production. This mechanism, which inhibits liver gluconeogenesis after food consumption, is mediated through the vagus nerve, which innervates the small intestine from the cerebrum. We suspect that dysfunction of this mechanism caused by diabetic autonomic neuropathy might contribute to hyperglycemia.

The neuronal pathway might mediate the action of incretin hormones secreted by the gut, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent gastric inhibitory peptide (GIP). Incretin hormones enhance glucose-mediated insulin secretion and suppress exaggerated glucagon secretion[11–14], thereby regulating postprandial plasma glucose levels.

Diabetic autonomic neuropathy might also cause the dysfunction of cardiovascular, gastrointestinal, genitourinary, sudomotor or ocular organs[1]. Postprandial and post-challenge hyperglycemia is an independent risk factor for macrovascular disease[15–17] as well as many other complications, such as diabetic retinopathy[18], increased carotid intima-media thickness[19], increased oxidative stress[20], decreased myocardial blood volume and myocardial blood flow[21], increased risk for cancer[22], and impaired cognitive function[23]. Further studies are needed to clarify whether diabetic autonomic neuropathy increases the risks for these complications.

In conclusion, the present results suggest that autonomic function affects post-challenge blood glucose levels independently of age in patients with type 2 diabetes.

ACKNOWLEDGEMENTS

We thank the staff of the Clinical Laboratory at Kanazawa University Hospital for their technical assistance in this study. We have no financial support and relationship with conflicts of interest.

REFERENCES

1. American Diabetes Association American Academy of Neurology. Consensus statement: report and recommendations of the San Antonio conference on diabetic neuropathy. *Diabetes Care* 1988; 11: 592–597.
2. Uno K, Katagiri H, Yamada T, *et al.* Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity. *Science* 2006; 312: 1656–1659.
3. Imai J, Katagiri H, Yamada T, *et al.* Regulation of pancreatic beta cell mass by neuronal signals from the liver. *Science* 2008; 322: 1250–1254.
4. Wang PY, Caspi L, Lam CK, *et al.* Upper intestinal lipids trigger a gut-brain-liver axis to regulate glucose production. *Nature* 2008; 452: 1012–1016.
5. Matsuhisa M, Yamasaki Y, Shiba Y, *et al.* Important role of the hepatic vagus nerve in glucose uptake and production by the liver. *Metabolism* 2000; 49: 11–16.
6. Nordback I, Harju E. Immediate effect of vagotomy on pancreatic insulin secretion. *Gut* 1991; 32: 303–305.
7. Abdul-Ghani MA, Matsuda M, Balas B, *et al.* Muscle and liver insulin resistance indexes derived from the oral glucose tolerance test. *Diabetes Care* 2007; 30: 89–94.
8. Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 1999; 22: 1462–1470.
9. Hayano J, Sakakibara Y, Yamada A, *et al.* Accuracy of assessment of cardiac vagal tone by heart rate variability in normal subjects. *Am J Cardiol* 1991; 67: 199–204.
10. Camilleri M. Clinical practice. Diabetic gastroparesis. *N Engl J Med* 2007; 356: 820–829.
11. Elliott RM, Morgan LM, Tredger JA, *et al.* Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. *J Endocrinol* 1993; 138: 159–166.
12. Orskov C, Wettergren A, Holst JJ. Secretion of the incretin hormones glucagon-like peptide-1 and gastric inhibitory polypeptide correlates with insulin secretion in normal man throughout the day. *Scand J Gastroenterol* 1996; 31: 665–670.
13. Holst JJ, Vilsboll T, Deacon CF. The incretin system and its role in type 2 diabetes mellitus. *Mol Cell Endocrinol* 2009; 297: 127–136.
14. Dunning BE, Foley JE, Ahren B. Alpha cell function in health and disease: influence of glucagon-like peptide-1. *Diabetologia* 2005; 48: 1700–1713.

15. DECODE Study Group, the European Diabetes Epidemiology Group. Glucose tolerance and cardiovascular mortality: comparison of fasting and 2-hour diagnostic criteria. *Arch Intern Med* 2001; 161: 397–405.
16. Nakagami T, Qiao Q, Tuomilehto J, *et al.* Screen-detected diabetes, hypertension and hypercholesterolemia as predictors of cardiovascular mortality in five populations of Asian origin: the DECODA study. *Eur J Cardiovasc Prev Rehabil* 2006; 13: 555–561.
17. Levitan EB, Song Y, Ford ES, *et al.* Is nondiabetic hyperglycemia a risk factor for cardiovascular disease? A meta-analysis of prospective studies. *Arch Intern Med* 2004; 164: 2147–2155.
18. Shiraiwa T, Kaneto H, Miyatsuka T, *et al.* Post-prandial hyperglycemia is an important predictor of the incidence of diabetic microangiopathy in Japanese type 2 diabetic patients. *Biochem Biophys Res Commun* 2005; 336: 339–345.
19. Hanefeld M, Koehler C, Schaper F, *et al.* Postprandial plasma glucose is an independent risk factor for increased carotid intima-media thickness in non-diabetic individuals. *Atherosclerosis* 1999; 144: 229–235.
20. Monnier L, Mas E, Ginet C, *et al.* Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes. *JAMA* 2006; 295: 1681–1687.
21. Scognamiglio R, Negut C, De Kreutzenberg SV, *et al.* Postprandial myocardial perfusion in healthy subjects and in type 2 diabetic patients. *Circulation* 2005; 112: 179–184.
22. Gapstur SM, Gann PH, Lowe W, *et al.* Abnormal glucose metabolism and pancreatic cancer mortality. *JAMA* 2000; 283: 2552–2558.
23. Abbatecola AM, Rizzo MR, Barbieri M, *et al.* Postprandial plasma glucose excursions and cognitive functioning in aged type 2 diabetics. *Neurology* 2006; 67: 235–240.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 | Single linear regression analyses between PG120 and clinical parameters
Table S2 | Age-specific normal values of the coefficients of variation of the R-R interval on electrocardiograms (CVRR)

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Peritoneal Dialysis International, Vol. 32, pp. 10–19
doi: 10.3747/pdi.2010.00200

0896-8608/12 $3.00 + .00

ORIGINAL ARTICLES

ACTIVATION OF p38 MITOGEN-ACTIVATED PROTEIN KINASE PROMOTES PERITONEAL FIBROSIS BY REGULATING FIBROCYTES

Satoshi Kokubo,[1] Norihiko Sakai,[2] Kengo Furuichi,[2] Tadashi Toyama,[1] Shinji Kitajima,[1] Toshiya Okumura,[1] Kouji Matsushima,[3] Shuichi Kaneko,[1] and Takashi Wada[4]

Department of Disease Control and Homeostasis,[1] Institute of Medical, Pharmaceutical and Health Sciences, Faculty of Medicine, and Division of Blood Purification,[2] Kanazawa University Hospital, Kanazawa University, Kanazawa; Department of Molecular Preventive Medicine,[3] Graduate School of Medicine, The University of Tokyo, Tokyo; and Department of Laboratory Medicine,[4] Institute of Medical, Pharmaceutical and Health Sciences, Faculty of Medicine, Kanazawa University, Kanazawa, Japan

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

♦ ***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 and cells positive for phosphorylated p38MAPK. The involvement of p38MAPK in the activation of fibrocytes also was also investigated *in vitro*.**

♦ ***Results:*** **Fibrocytes infiltrated peritoneum in response to CG, and that response was accompanied by progressive peritoneal fibrosis. The phosphorylation of p38MAPK, as defined by CD45+ spindle-shaped cells, was detected both in peritoneal mesothelial cells and in fibrocytes. The level of peritoneal expression of CCL2, a chemoattractant for fibrocytes, was upregulated by CG injection, and treatment with FR167653 reduced the number of cells positive for phosphorylated p38MAPK, the peritoneal expression of CCL2, and the extent of peritoneal fibrosis. Pretreatment with FR167653 inhibited the expression of procollagen type I α1 induced by transforming growth factor-β1.**

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

Perit Dial Int **2012; 32(1):10-19 www.PDIConnect.com**
epub ahead of print: 30 Jun 2011 doi:10.3747/pdi.2010.00200

KEYWORDS: Chemokine; fibrocytes; fibrosis; p38MAPK; peritoneum.

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

Circulating fibrocytes, which constitute a small fraction of the circulating pool of leukocytes, have been reported to be involved in various fibrotic diseases such as idiopathic pulmonary fibrosis, renal fibrosis, and hypertrophic

Correspondence to: S. Kokubo, Department of Disease Control and Homeostasis, Institute of Medical, Pharmaceutical and Health Sciences, Faculty of Medicine, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641 Japan.
kokkii1973@hotmail.com
Received 23 January 2011; accepted 7 February 2011