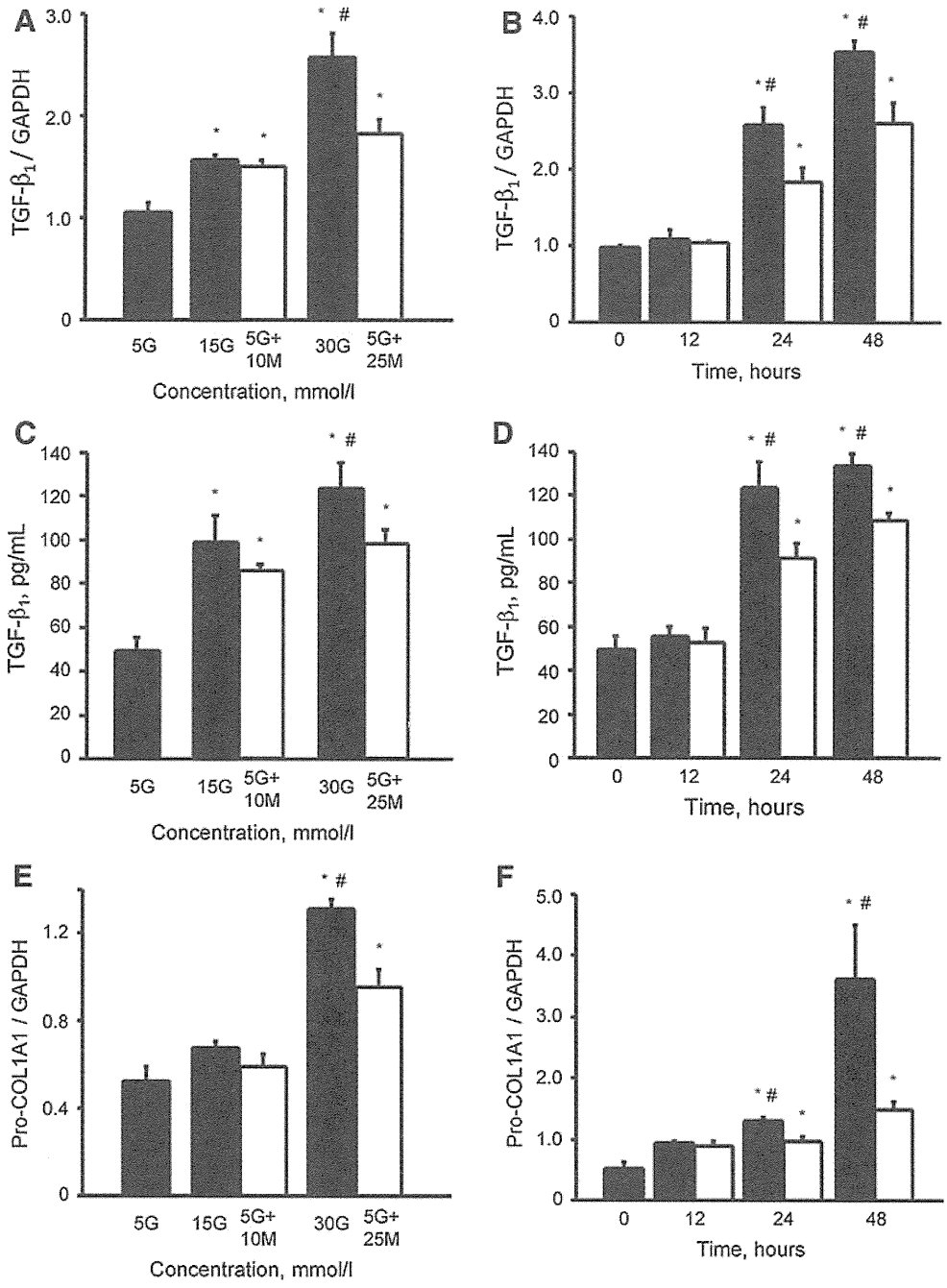

Fig. 1 Effect of increasing concentrations of D-glucose on the expression of TGF-β_1 and pro-COL1A1 in human isolated $CD45^+/Col1^+$ cells. Dose effect of increasing concentrations of D-glucose (*filled square*) or D-mannitol (*open square*) on the induction of TGF-β_1 mRNA (**a**), TGF-β_1 protein (**c**) and pro-COL1A1 mRNA (**e**) synthesis. Data presented are the mean ± SEM from three separate experiments performed with $CD45^+/Col1^+$ cells isolated from different donors. 5G, 5 mmol/l D-glucose; 15G, 15 mmol/l D-glucose; 30G, 30 mmol/l D-glucose; 5G + 10 M, 5 mmol/l D-glucose + 10 mmol/l D-mannitol; 5G + 25 M, 5 mmol/l D-glucose + 25 mmol/l D-mannitol. $^*p < 0.05$ compared with 5 mmol/l D-glucose. $^\#p < 0.05$ compared with 25 mmol/l D-mannitol with 5 mmol/l D-glucose. Time course of 30 mmol/l D-glucose (*filled square*) or 25 mmol/l D-mannitol with 5 mmol/l D-glucose (*open square*)-mediated induction of TGF-β_1 mRNA (**b**), TGF-β_1 protein (**d**) and pro-COL1A1 mRNA (**f**) synthesis. Data presented are the mean ± SEM from three separate experiments performed with $CD45^+/Col1^+$ cells isolated from different donors. $^*p < 0.05$ compared with the 0 h time point, $^\#p < 0.05$ compared with 25 mmol/l D-mannitol with 5 mmol/l D-glucose

protein in the supernatant was decreased in the presence of cytochalasin B or NAC (Fig. 3d). The levels of TGF-β_1 mRNA and protein and pro-COL1A1 mRNA detected in the presence of cytochalasin B were not significantly different to those induced by 25 mmol/l D-mannitol with 5 mmol/l D-glucose (Fig. 3b, d, f). In parallel experiments, the D-mannitol-induced increase in the expression of TGF-β_1 mRNA and protein and pro-COL1A1 mRNA was not changed by the simultaneous presence of cytochalasin B or NAC (Fig. 3c, e, g).

Effect of inhibition of glucose transport or ROS on the expression of CCL2

Similarly, to examine the effect of inhibition of glucose transport and oxidative stress on the expression of CCL2 in human $CD45^+/Col1^+$ cells, the cells were incubated for 24 h with or without 5 μmol/l of cytochalasin B or 2 mmol/l of NAC under high glucose concentrations or increased osmolality. The high glucose-induced increase in the expression of CCL2 mRNA and protein was significantly

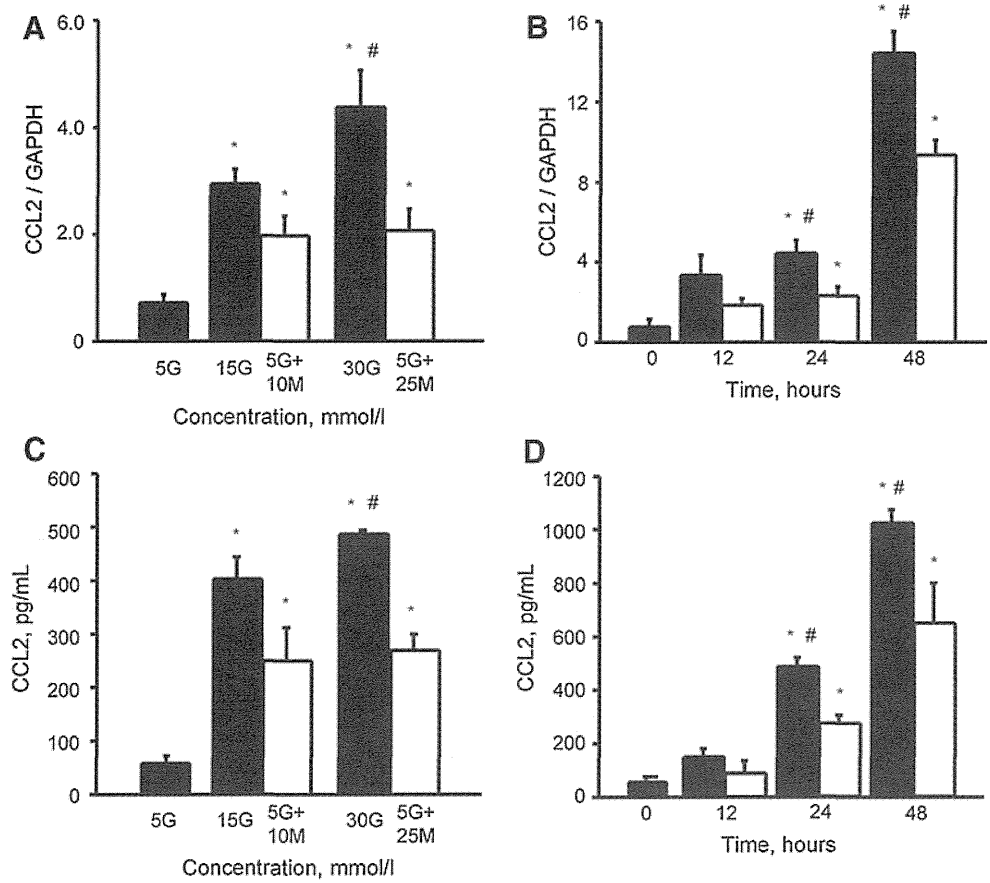

Fig. 2 Effect of increasing concentrations of D-glucose on the expression of CCL2 in human isolated $CD45^+/Col1^+$ cells. Dose effect of increasing concentrations of D-glucose (*filled square*) or D-mannitol (*open square*) on the induction of CCL2 mRNA (**a**) and protein (**c**) synthesis. Data presented are the mean ± SEM from three separate experiments performed with $CD45^+/Col1^+$ cells isolated from different donors. 5G, 5 mmol/l D-glucose; 15G, 15 mmol/l D-glucose; 30G, 30 mmol/l D-glucose; 5G + 10 M, 5 mmol/l D-glucose + 10 mmol/l D-mannitol; 5G + 25 M, 5 mmol/l D-glucose + 25 mmol/l D-mannitol. $^*p < 0.05$ compared with 5 mmol/l D-glucose. $^\#p < 0.05$ compared with 25 mmol/l D-mannitol with 5 mmol/l D-glucose. Time course of 30 mmol/l D-glucose (filled square) or 25 mmol/l D-mannitol with 5 mmol/l D-glucose (open square)-mediated induction of CCL2 mRNA (**b**) and protein (**d**) synthesis. Data presented are the mean ± SEM from three separate experiments performed with $CD45^+/Col1^+$ cells isolated from different donors. $^*p < 0.05$ compared with 0 h time point, $\#p < 0.05$ compared with 25 mmol/l D-mannitol with 5 mmol/l D-glucose

reduced in the presence of cytochalasin B or NAC (Fig. 4a, c). CCL2 mRNA and protein levels in the presence of cytochalasin B or NAC were not significantly different to those induced by 25 mmol/l D-mannitol with 5 mmol/l D-glucose (Fig. 4a, c). On the other hand, the D-mannitol-induced increase in the expression of CCL2 mRNA and protein was not significantly reduced by the simultaneous presence of cytochalasin B or NAC (Fig. 4b, d).

Effect of CCL2 and CCR2 inhibition on the expression of TGF-β_1, pro-COL1A1 and CCL2 and cell migration under high glucose concentrations

To examine the expression of CCR2 on $CD45^+/Col1^+$ cells under diabetic conditions, isolated $CD45^+/Col1^+$ cells first were cultured for 48 h under high glucose concentrations. 30 mmol/l D-glucose led to an increase in the expression of CCR2 mRNA, but this finding was also observed in the osmotic control (Fig. 5a). Secondly, to investigate the impact of CCL2 as well as high glucose on the expression of TGF-β_1, pro-COL1A1 and CCL2 and on the extent of migration of the cells, isolated $CD45^+/Col1^+$ cells were cultured under the same conditions as described above for 48 h but with CCL2. The stimulation with CCL2 synergistically enhanced mRNA level of TGF-β_1, pro-COL1A1 and CCL2 (Fig. 5b, d, e) and the migration rate of the cells (Fig. 5f). With regard to the expression of TGF-β_1, its protein level in the supernatant was also increased by stimulation with CCL2 (Fig. 5c). Finally, to determine whether the CCL2-induced up-regulation of these molecules and increase in cell migration rate are dependent on CCR2, propagermanium and RS-504393, which are inhibitors of CCL2/CCR2 signaling, were applied [21, 22]. The up-regulated expression of

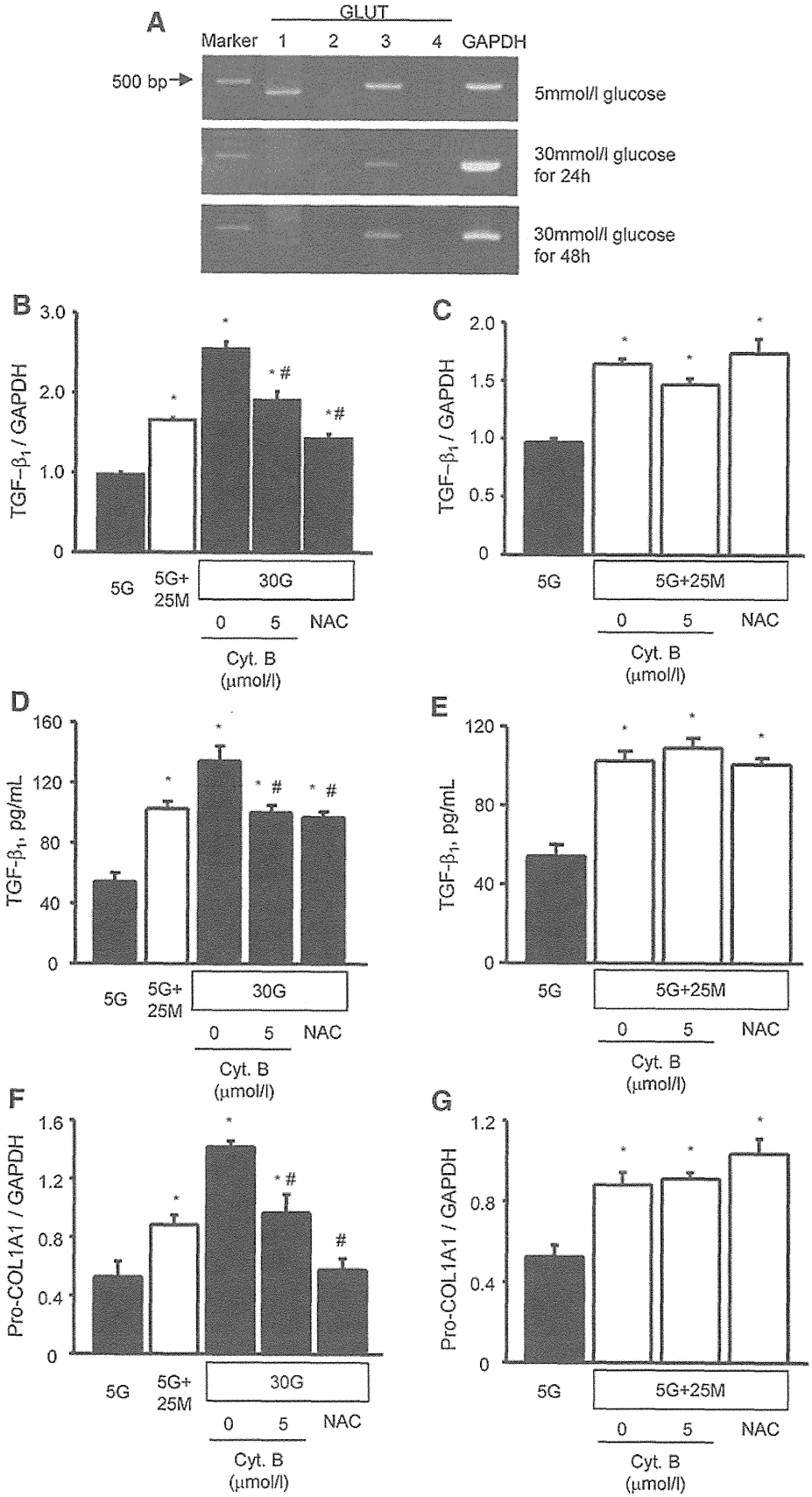

Fig. 3 Expression of GLUT isoforms and effect of cytochalasin B and N-acetylcysteine on the production of TGF-β_1 and pro-COL1A1 in human isolated $CD45^+/Col1^+$ cells. The expression of *GLUT* isoforms on human $CD45^+/Col1^+$ cells under normal or high glucose concentrations was assessed by RT-PCR (**a**). Effect of cytochalasin B and N-acetylcysteine on 30 mmol/l D-glucose-induced TGF-β_1 mRNA (**b**), TGF-β_1 protein (**d**) and pro-COL1A1 mRNA (**f**) synthesis in human $CD45^+/Col1^+$ cells. Data presented are the mean ± SEM from three separate experiments performed with $CD45^+/Col1^+$ cells isolated from different donors. 5G, 5 mmol/l D-glucose; 30G, 30 mmol/l D-glucose; 5G + 25 M, 5 mmol/l D-glucose + 25 mmol/l D-mannitol; *Cyt. B* cytochalasin B; *NAC* N-acetylcysteine. *$p < 0.05$ compared with 5 mmol/D-glucose. #$p < 0.05$ compared with 30 mmol/l D-glucose alone. Effect of cytochalasin B on induction of TGF-β_1 mRNA (**c**), TGF-β_1 protein (**e**) and pro-COL1A1 mRNA (G) synthesis by 25 mmol/l D-mannitol with 5 mmol/l D-glucose in human $CD45^+/Col1^+$ cells. Data presented are the mean ± SEM from three separate experiments performed with $CD45^+/Col1^+$ cells isolated from different donors. 5G, 5 mmol/l D-glucose; 30G, 30 mmol/l D-glucose; 5G + 25 M, 5 mmol/l D-glucose + 25 mmol/l D-mannitol; *Cyt. B* cytochalasin B; *NAC* N-acetylcysteine. *$p < 0.05$ compared with 5 mmol/D-glucose. None of the differences are statistically significant between 25 mmol/l D-mannitol with 5 mmol/l D-glucose and 25 mmol/l D-mannitol with 5 mmol/l D-glucose and cytochalasin B or N-acetylcysteine

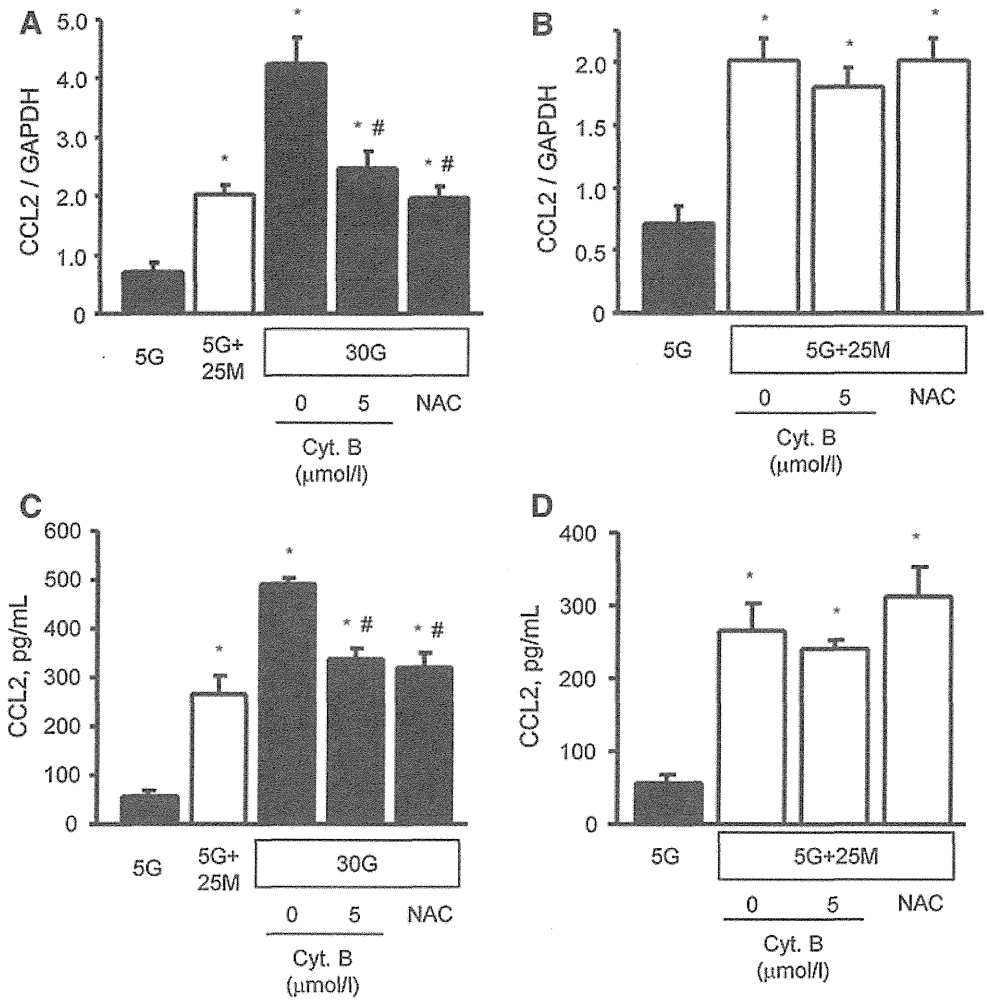

Fig. 4 Effect of cytochalasin B and N-acetylcysteine on the production of CCL2 in human isolated $CD45^+/Col1^+$ cells. Effect of cytochalasin B and N-acetylcysteine on 30 mmol/l D-glucose-induced CCL2 mRNA (**a**) and protein (**c**) synthesis in human $CD45^+/Col1^+$ cells. Data presented are the mean ± SEM from three separate experiments performed with $CD45^+/Col1^+$ cells isolated from different donors. 5G, 5 mmol/l D-glucose; 30G, 30 mmol/l D-glucose; 5G + 25 M, 5 mmol/l D-glucose + 25 mmol/l D-mannitol; *Cyt. B* cytochalasin B; *NAC* N-acetylcysteine. $^*p < 0.05$ compared with 5 mmol/D-glucose, $^\#p < 0.05$ compared with 30 mmol/l D-glucose alone. Effect of cytochalasin B and N-acetylcysteine on induction of CCL2 mRNA (**b**) and protein (**d**) synthesis by 25 mmol/l D-mannitol with 5 mmol/l D-glucose in human $CD45^+/Col1^+$ cells. Data presented are the mean ± SEM from three separate experiments performed with $CD45^+/Col1^+$ cells isolated from different donors. 5G, 5 mmol/l D-glucose; 30G, 30 mmol/l D-glucose; 5G + 25 M, 5 mmol/l D-glucose + 25 mmol/l D-mannitol; *Cyt. B* cytochalasin B; *NAC* N-acetylcysteine. $^*p < 0.05$ compared with 5 mmol/D-glucose. None of the differences are statistically significant between 25 mmol/l D-mannitol with 5 mmol/l D-glucose and 25 mmol/l D-mannitol with 5 mmol/l D-glucose and cytochalasin B or N-acetylcysteine

TGF-β_1, pro-COL1A1 and CCL2 mRNA and TGF-β_1 protein were reduced by pretreatment with propagermanium or RS-504393 using <30 mmol/l D-glucose, although reduction of the expression of these molecules using <25 mmol/l D-mannitol with 5 mmol/l D-glucose was not statistically significant (Fig. 5b–e). The increase in percent migration of the cells was also inhibited by pretreatment with 3 μg/ml of propagermanium (Fig. 5f). These findings suggest that the CCL2-dependent migration and fibrogenic response in $CD45^+/Col1^+$ cells under high glucose concentrations are regulated, in part, by CCR2 signaling.

Discussion

The present study demonstrates that stimulation with high glucose concentrations and CCL2 increased the expression of TGF-β_1, pro-Col1, and CCL2 and migration rate, while inhibition of glucose transport, ROS or CCR2 decreased the levels of these molecules and cell migration in isolated human $CD45^+/Col1^+$ cells. These results suggest that the function of $CD45^+/Col1^+$ cells is regulated by glucose and CCL2/CCR2 signaling.

Fibrocytes, identified by dual positivity of CD45 and pro-Col1, are now recognized to be involved in the

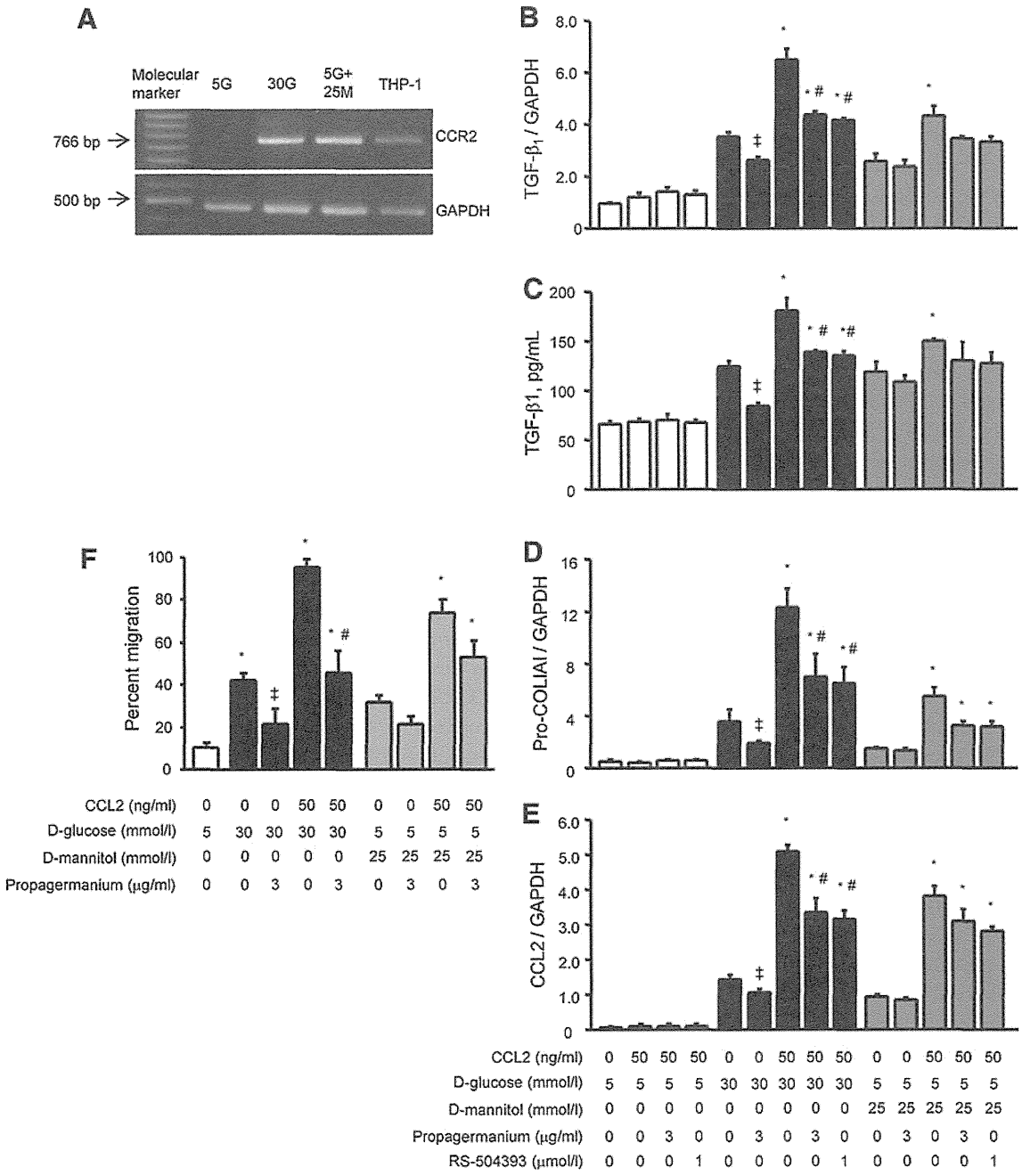

Fig. 5 Effect of CCL2/CCR2 signaling on the expression of TGF-β_1, pro-COL1A1 and CCL2 and on the extent of migration in isolated human $CD45^+/Col1^+$ cells. Human $CD45^+/Col1^+$ cells express CCR2 mRNA in response to high glucose concentrations (**a**). Figures are representative of three experiments. Stimulation of $CD45^+/Col1^+$ cells with CCL2 for 48 h under high glucose enhanced the expression of TGF-β_1 mRNA (**b**) and protein (**c**), pro-COL1A1 mRNA (**d**) and CCL2 mRNA (**e**). Pretreatment with propagermanium or RS-504393 attenuated high glucose- and CCL2-induced expression of TGF-β_1, pro-COL1A1 and CCL2. Values are the mean ± SEM. $*p < 0.05$ compared with 30 mmol/D-glucose or 25 mmol/l D-mannitol with 5 mmol/l D-glucose, $‡p <$ compared with 30 mmol/l D-glucose alone. $\#p < 0.05$ compared with 30 mmol/l D-glucose to which CCL2 was added. Effect of high glucose and CCL2 on the cell migration (**f**). Values are the mean ± SEM. $*p < 0.05$ compared with 30 mmol/ D-glucose or 25 mmol/l D-mannitol with 5 mmol/l D-glucose, $‡p <$ compared with 30 mmol/l D-glucose alone, $\#p < 0.05$ compared with 30 mmol/l D-glucose to which CCL2 was added

pathogenesis of a wide variety of focal and diffuse fibrosing disorders including those localized to the skin, lungs, liver, kidney, pancreas, and bladder, and a more diffuse involvement as seen in atherosclerosis and in tumors [13, 25]. Fibrocytes express a number of chemokine receptors including CCR2, and specific chemokine/chemokine receptor signals are critical for the recruitment of fibrocytes to sites of tissue injury [17, 25]. In our previous study,

$CD45^+/Col1^+$ cells were present in human diabetic kidneys, and the number of the cells in kidney correlated well with the severity of tubulointerstitial lesions, the number of CD68-positive macrophages, and urinary CCL2 levels [15]. These findings prompted us to examine the direct effect of high glucose concentrations on the activation of human fibrocytes in vitro. The stimulation of isolated $CD45^+/Col1^+$ cells with high glucose concentrations enhanced the expression of pro-COL1A1, TGF-β_1, and CCL2, although this effect was mediated partly by increased osmolality. In white blood cells, the increase in glucose utilization is a prominent feature during the immune response, and this effect depends on the function of specific GLUT isoforms [26]. Among GLUT isoforms, a recent study demonstrated that GLUT1, GLUT3, and GLUT4 were expressed on the plasma membrane of resting and activated white blood cells [27]. The present study revealed that $CD45^+/Col1^+$ cells also expressed GLUT1 and GLUT3 and that excessive glucose transport across the plasma membrane and the following pathological processes in this particular cell were blocked by cytochalasin B and NAC, respectively. A number of studies have suggested a role for glucose and oxidative stress in modulating cellular inflammatory responses [6]. Incubation of endothelial cells, monocytes/macrophages, mesangial cells in kidney, or peritoneal mesothelial cells with high concentrations of glucose leads to induction of expression of inflammatory cytokine/chemokine genes including TNF-α, interleukin-1β, TGF-β, and CCL2 and generation of ROS [28–32]. Urinary and serum levels of CCL2 were higher in patients with diabetes than in normal subjects, and serum CCL2 levels correlated with plasma glucose [4, 33]. With regard to oxidative stress, serum 8-hydroxy-guanine levels or urinary reactive carbonyl derivates in diabetic patients with nephropathy were higher than in healthy controls [34, 35]. These basic and clinical findings suggest that $CD45^+/Col1^+$ cells may interact with these cells and participate in inducing and augmenting inflammatory and fibrosing processes by producing these cytokines and chemokines under diabetic conditions.

In experiments where human $CD45^+/Col1^+$ cells were exposed to mannitol, there were also time- and dose-dependent increases in the expression of pro-COL1A1, TGF-β_1, and CCL2, although the level was lower than that induced by equivalent D-glucose concentrations. These data suggest that while D-glucose-driven stimuli significantly contribute to expression of these molecules in $CD45^+/Col1^+$ cells, osmolality-driven events also contribute to $CD45^+/Col1^+$ cells activation, as previously observed in other cell types [30, 32]. The exact mechanism by which hyperosmolality activates cellular function of $CD45^+/Col1^+$ cells has not been elucidated. In other cell systems, there are a variety of molecular signals that respond to alterations in cell volume and osmosensors or volume sensors responding to these signals, as described elsewhere [36]. The early signals of volume perturbation include integrins, the cytoskeleton, receptor tyrosine kinases, and transient receptor potential channels [36]. These processes are clearly involved in cell activation, although whether all of these or similar mechanisms are activated in $CD45^+/Col1^+$ cells by osmolality requires further study.

Following high glucose stimulation of human $CD45^+/Col1^+$ cells in vitro, an induction of CCR2 mRNA and a CCR2-mediated increase in the production of pro-COL1A1, TGF-β_1, and CCL2 were detected. These findings are consistent with a previous study, which demonstrated that undifferentiated human fibrocytes do not express CCR2 without specific treatment [37]. The up-regulation of CCR2 expression under high glucose concentrations was associated with a further increase in the production of pro-COL1A1, TGF-β_1 and CCL2 and in the extent of cell migration by exogenous CCL2 than by high glucose alone, suggesting that fibrocytes may contribute directly to the pathogenesis of organ fibrosis under diabetes through the CCL2/CCR2 signaling pathway.

In conclusion, $CD45^+/Col1^+$ cells are directly involved in the fibrogenesis under diabetic conditions via a CCL2/CCR2 -dependent amplification mechanisms. Within the context of the role of the CCL2/CCR2 system in the induction of monocyte/macrophage infiltration and the activation of constituent cells in various organs including the kidney [11, 38], it should be noted that similar trafficking and activation phenomena occur in atherosclerosis [39], which is another major complication of diabetes. Taken together, these findings suggest that pharmacologic CCR2 inhibition may be a potential therapy for diabetic complications including nephropathy.

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Conflict of interest The authors have declared that no conflict of interest exists.

References

1. Wada T, Shimizu M, Toyama T, Hara A, Kaneko S, Furuichi K. Clinical impact of albuminuria in diabetic nephropathy. Clin Ext Nephrol. 2012; 16(1): 96–101
2. Parving HH, Mauer M, Ritz E. Diabetic nephropathy. In: Brenner BM editor. The kidney, 7th ed. W. B. Saunders Company, Philadelphia, 2004. pp. 1777–1818.
3. Qian Y, Feldman E, Pennathur S, Kretzler M, Brosius FC. From fibrosis to sclerosis. Mechanisms of glomerulosclerosis in diabetic nephropathy. Diabetes. 2008;57:1439–45.

4. Wada T, Furuichi K, Sakai N, Iwata Y, Yoshimoto K, Shimizu M, Takeda SI, Takasawa K, Yoshimura M, Kida H, Kobayashi KI, Mukaida N, Naito T, Matsushima K, Yokoyama H. Up-regulation of MCP-1 in tubulointerstitial lesions of human diabetic nephropathy. Kidney Int. 2000;58:1492–9.
5. Cooper ME. Pathogenesis, prevention, and treatment of diabetic nephropathy. Lancet. 1998;352:213–9.
6. Navarro-gonzalez JF, Mora-Fernandez C, Muros de Fuentes M, Garcia-Perez J. Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy. Nat Rev Nephrol. 2011;7:327–40.
7. Sakai N, Wada T, Furuichi K, Iwata Y, Yoshimoto K, Kitagawa K, Kokubo S, Kobayashi M, Hara A, Yamahana J, Okumura T, Takasawa K, Takeda S, Yoshimura M, Kida H, Yokoyama H. Involvement of extracellular signal-regulated kinase and p38 in human diabetic nephropathy. Am J Kidney Dis. 2005;45:54–65.
8. Wada T, Yokoyama H, Furuichi K, Kobayashi KI, Harada K, Naruto M, Su SB, Akiyama H, Mukaida N, Matsushima K. Intervention of crescentic glomerulonephritis by antibodies to monocyte chemotactic and activating factor (MCAF/MCP-1). FASEB J. 1996;10:1418–25.
9. Wada T, Matsushima K, Kaneko S. The role of chemokines in glomerulonephritis. Front Biosci. 2008;13:3966–74.
10. Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med. 1994;1:71–81.
11. Wada T, Sakai N, Sakai Y, Matsushima K, Kaneko S, Furuichi K. Involvement of bone-marrow-derived cells in kidney fibrosis. Clin Exp Nephrol. 2011;15:8–13.
12. Moore BB, Kolodsick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, Wilke CA, Toews GB. CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. Am J Pathol. 2005;166:675–84.
13. Wada T, Sakai N, Matsushima K, Kaneko S. Fibrocytes: a new insight into kidney fibrosis. Kidney Int. 2007;72:269–73.
14. Sakai N, Wada T, Yokoyama H, Lipp M, Ueha S, Matsushima K, Kaneko S. Secondary lymphoid tissues chemokine (SLC/CCL21)/CCR7 signaling regulates fibrocytes in renal fibrosis. Proc Natl Acad Sci USA. 2006;103:14098–103.
15. Sakai N, Furuichi K, Shinozaki Y, Yamauchi H, Toyama T, Kitajima S, Okumura T, Kokubo S, Kobayashi M, Takasawa K, Takeda S, Yoshimura M, Kaneko S, Wada T. Fibrocytes are involved in the pathogenesis of human chronic kidney disease. Hum Pathol. 2010;41:672–8.
16. Pilling D, Fan T, Huang D, Kaul B, Gomer RH. Identification of markers that distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts. PLoS One. 2009;4:e7475.
17. Reilkoff RA, Bucala R, Herzog EL. Fibrocytes: emerging effector cells in chronic inflammation. Nat Rev Immunol. 2011;11:427–35.
18. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediates fibrosis. J Clin Invest. 2004;114:438–46.
19. Sakai N, Wada T, Matsushima K, Bucala R, Iwai M, Horiuchi M, Kaneko S. The renin-angiotensin system contributes to renal fibrosis through regulation of fibrocytes. J Hypertens. 2008;26:780–90.
20. Lachaal M, Rampal AL, Lee W, Shi Y, Jung CY. GLUT1 transmembrane glucose pathway. Affinity labeling with a transportable D-glucose diazirine. J Biol Chem. 1996;271:5225–30.
21. Ito A, Suganami T, Yamauchi A, Degawa-Yamauchi M, Tanaka M, Kouyama R, Kobayashi Y, Nitta N, Yasuda K, Hirata Y, Kuziel WA, Takeya M, Kanegasaki S, Kamei Y, Ogawa Y. Role of CC chemokine receptor 2 in bone marrow cells in the recruitment of macrophages into obese adipose tissue. J Biol Chem. 2008;283:35715–23.
22. Sakai N, Wada T, Furuichi K, Shimizu K, Kokubo S, Hara A, Yamahana J, Okumura T, Matsushima K, Yokoyama H, Kaneko S. MCP-1/CCR2-dependent loop for fibrogenesis in human peripheral CD14-positive monocytes. J Leukoc Biol. 2006;79:555–63.
23. 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;165:237–46.
24. Schröppel B, Fischereder M, Wiese P, Segerer S, Huber S, Kretzler M, Heiss P, Sitter T, Schlöndorff D. Expression of glucose transporters in human peritoneal mesothelial cells. Kidney Int. 1998;53:1278–87.
25. Strieter RM, Keeley EC, Hughes MA, Burdick MD, Mehrad B. The role of circulating mesenchymal progenitor cells (fibrocytes) in the pathogenesis of pulmonary fibrosis. J Leukoc Biol. 2009;86:1111–8.
26. Calder PC, Dimitriadis G, Newsholme P. Glucose metabolism in lymphoid and inflammatory cells and tissues. Curr Opin Clin Nutr Metab Care. 2007;10:531–40.
27. Maratou E, Dimitriadis G, Kollias A, Boutati E, Lambadiari V, Mitrou P, Raptis SA. Glucose transporter expression on the plasma membrane of resting and activated white blood cells. Eur J Clin Invest. 2007;37:282–90.
28. Takaishi H, Taniguchi T, Takahashi A, Ishikawa Y, Yokoyama M. High glucose accelerates MCP-1 production via p38 MAPK in vascular endothelial cells. Biochem Biophys Res Commun. 2003;305:122–8.
29. Shanmugam N, Reddy MA, Guha M, Natarajan R. High glucose-induced expression of proinflammatory cytokine and chemokine genes in monocytic cells. Diabetes. 2003;52:1256–64.
30. Morohoshi M, Fujisawa K, Uchimura I, Numano F. Glucose-dependent interleukin-6 and tumor necrosis factor production by human peripheral blood monocytes in vitro. Diabetes. 1996;45:954–9.
31. Janssen U, Sowa E, Marchand P, Floege J, Phillips AO, Radeke HH. Differential expression of MCP-1 and its receptor CCR2 in glucose primed human mesangial cells. Nephron. 2002;92:797–806.
32. Wong TYH, Phillips AO, Witowski J, Topley N. Glucose-mediated induction of TGF-β1 and MCP-1 in mesothelial cells in vitro is osmolality and polyol pathway dependent. Kidney Int. 2003;62:1404–16.
33. Mine S, Okada Y, Tanikawa T, Kawahara C, Tabata T, Tanaka Y. Increased expression levels of monocyte CCR2 and monocyte chemoattractant protein-1 in patients with diabetes mellitus. Biochem Biophys Res Commun. 2006;344:780–5.
34. Shin CS, Moon BS, Park KS, Kim SY, Park SJ, Chung MH, Lee HK. Serum 8-hydroxy-guanine levels are increased in diabetic patients. Diabetes Care. 2001;24:733–7.
35. Cvetković T, Mitić B, Lazarević G, Vlahović P, Antić S, Stefanović V. Oxidative parameters as possible urine markers in patients with diabetic nephropathy. J Diabetes Complicat. 2009;23:337–42.
36. Pedersen SF, Kapus A, Hoffmann EK. Osmosensory mechanisms in cellular and systemic volume regulation. J Am Soc Nephrol. 2011;22:1587–97.
37. Hong KM, Burdick MD, Phillips RJ, Heber D, Strieter RM. Characterization of human fibrocytes as circulating adipocyte progenitors and the formation of human adipose tissue in SCID mice. FASEB J. 2005;19:2029–31.

38. Tarabra E, Giunti S, Barutta F, Salvidio G, Burt D, Deferrari G, Gambino R, Vergola D, Pinach S, Perin PC, Camussi G, Gruden G. Effect of the monocyte chemoattractant protein-1/CC chemokine receptor 2 system on nephrin expression in streptozotocin-treated mice and human cultured podocytes. Diabetes. 2009;58:2109–18.

39. Bellini A, Mattoli S. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. Lab Invest. 2007;87:858–70.

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ORIGINAL ARTICLE

Japan Diabetic Nephropathy Cohort Study: study design, methods, and implementation

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Abstract
Background Diabetic nephropathy, leading to end-stage renal disease, has a considerable impact on public health and the social economy. However, there are few national registries of diabetic nephropathy in Japan. The aims of this prospective cohort study are to obtain clinical data and urine samples for revising the clinical staging of diabetic nephropathy, and developing new diagnostic markers for early diabetic nephropathy.
Methods The Japanese Society of Nephrology established a nationwide, web-based, and prospective registry system. On the system, there are two basic registries; the Japan Renal Biopsy Registry (JRBR), and the Japan Kidney Disease Registry (JKDR). In addition to the two basic registries, we established a new prospective registry to the system; the Japan Diabetic Nephropathy Cohort Study (JDNCS), which collected physical and laboratory data.
Results We analyzed the data of 321 participants (106 female, 215 male; average age 65 years) in the JDNCS. Systolic and diastolic blood pressure was 130.1 and 72.3 mmHg, respectively. Median estimated glomerular filtration rate (eGFR) was 33.3 ml/min/1.73 m^2. Proteinuria was 1.8 g/gCr, and serum levels of albumin were 3.6 g/dl. The majority of the JDNCS patients presented with preserved eGFR and low albuminuria or low eGFR and advanced proteinuria. In the JRBR and JKDR registries, 484

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and 125 participants, respectively, were enrolled as having diabetes mellitus. In comparison with the JRBR and JKDR registries, the JDNCS was characterized by diabetic patients presenting with low proteinuria with moderately preserved eGFR.

Conclusions There are few national registries of diabetic nephropathy to evaluate prognosis in Japan. Future analysis of the JDNCS will provide clinical insights into the epidemiology and renal and cardiovascular outcomes of type 2 diabetic patients in Japan.

Keywords Diabetic nephropathy · Cohort study · Estimated glomerular filtration rate · Japan Diabetic Nephropathy Cohort Study · Japan Renal Biopsy Registry · Japan Kidney Disease Registry

Introduction

The most serious issue in clinical nephrology is the relentless and progressive increase in patients with end-stage renal disease in Japan [1, 2]. Today, diabetic nephropathy has a considerable impact on society in the fields of public health and social economy; many physician scientists are involved in research to elucidate the pathogenesis of diabetic nephropathy and the prevention and cure of the disease.

However, there are few national registries of diabetic nephropathy in Japan. The Committee for the Working Group for Renal Biopsy Database in the Japanese Society of Nephrology established a nationwide, web-based, and prospective registry system, with or without renal biopsy—the Japan Renal Biopsy Registry (JRBR) and the Japan Kidney Disease Registry (JKDR) respectively, from 2007 [3]. However, these two registries have no follow-up data. Therefore, we have established a nationwide prospective diabetic nephropathy cohort—the Japan Diabetic Nephropathy Cohort Study (JDNCS). The aims of this prospective cohort study are to obtain clinical data and urine samples for revising the clinical staging of diabetic nephropathy, and developing new diagnostic markers for early diabetic nephropathy. The JDNCS is now prospectively collecting clinical data annually.

The aim of the current study was to compare baseline characteristics of JDNCS patients with the diabetic patients on the JRBR and the JKDR. Long-term follow-up of the JDNCS patients will provide clinical insights into the epidemiology and renal and cardiovascular outcomes of diabetic nephropathy.

Subjects and methods

Registry system

The researchers on the Committee for the Diabetic Nephropathy Research, which was supported by the Ministry of Health, Labour and Welfare of Japan, participated in this study. The report includes data from patients on the JRBR and JKDR, registered prospectively from January 2007. Patient data including age, gender, laboratory data, and clinical and pathological diagnoses were electronically recorded at each institution and registered on the web page of the JRBR and JKDR utilizing the system of Internet Data and Information Center for Medical Research (INDICE) in the University Hospital Medical Information Network (UMIN). JDNCS patient data were also electronically recorded at each institution and registered on the web page of the INDICE system in UMIN. The ethical committee of the Kanazawa University and the Japanese Society of Nephrology

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comprehensively approved the study, and a local committee of participating centers and their affiliated hospitals individually approved the study. Written informed consent was obtained from the patients at the time of biopsy or before participation in the study. The JRBR is registered to the Clinical Trial Registry of UMIN (registered number UMIN000000618) and is available in Japanese and English.

Screening and enrollment

Entry criteria to the JDNCS were adult type 2 diabetic patients with early to advanced nephropathy. Exclusion criteria to the JDNCS were patients <20 years at entry, patients with type 1 diabetes, secondary diabetes, and/or overt primary kidney diseases. Baseline information and laboratory data of eligible patients were collected.

Clinical information and laboratory data

Diagnosis of diabetes mellitus (DM) was performed by the attending physician, and recorded on the database. Whole blood and serum were collected for measurement of hemoglobin, creatinine, protein, albumin, plasma glucose, HbA1c, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, low-density lipoprotein (LDL) cholesterol. HbA1c measured by the Japanese Diabetes Society (JDS) method was corrected to the A1C value measured by the National Glycohemoglobin Standardization Program (NGSP) method by adding 0.4 % as determined by the JDS. Estimated glomerular filtration rate (eGFR) was calculated using the following equation: eGFR (ml/min/1.73 m^2) = 194 × $Cr^{-1.094}$ × $Age^{-0.287}$ (for men) and eGFR (ml/min/1.73 m^2) = 194 × $Cr^{-1.094}$ × $Age^{-0.287}$ × 0.739 (for women) [4]. Spot urine samples were collected for measurement of albuminuria, proteinuria, and urine creatinine. In the JDNCS, data of both albuminuria and proteinuria were collected, but only data of proteinuria were collected in the JRBR and JKDR. Urine samples were collected and stocked in cases with agreement for future biomarker analysis.

Statistics

Statistical analyses were performed using the SPSS Statistics software program, version 19 (SPSS Inc., NY, USA). Comparisons of categorical variables among groups of different indications or diagnoses were performed using Fisher's exact test. Kolmogoov–Smirnov test was used to evaluate normal or non-normal distribution. Continuous variables were compared using the Student's t test for parametric data and Wilcoxon's signed rank test or the Kruskal–Wallis test for non-parametric data. P values of <0.05 (obtained by two-tailed testing) were considered to indicate statistical significant difference. Normal distribution data were expressed by mean ± SD, and non-normal distribution data were expressed by median and interquartile range (IQR).

Results

Characteristics of entry data to the JDNCS

Data for the JDNCS were collected from 321 patients (106 female, 215 male) with a median age of 65 years (IQR 59.0–74.0). Systolic and diastolic blood pressure, HbA1c (NGSP) levels, eGFR, proteinuria, serum levels of albumin, and serum levels of total cholesterol in JDNCS patients were 130.1 ± 17.3, 72.3 ± 11.6 mmHg, 6.8 (IQR 5.8–7.1) %, 33.3 (IQR 17.1–58.2) ml/min/1.73 m^2, 1.8 (IQR 0.3–4.9) g/gCr, 3.6 (IQR 3.2–4.1) g/dl, and 181.0 (IQR 52.0–208.8) mg/dl, respectively. 36.8 % of patients were treated by insulin, 11.8 % used angiotensin-converting enzyme inhibitors, 61.4 % used angiotensin receptor blockers, and 41.1 % used statins.

Distribution of eGFR and albuminuria in the JDNCS

When categorized by degree of eGFR and albuminuria, the majority of patients presented with preserved eGFR and low albuminuria, or low eGFR and advanced proteinuria (Table 1). The proportion of patients with an eGFR ≥60 ml/min/1.73 m^2 and albuminuria <30 mg/gCr was approximately 30 %, and the proportion with an eGFR <30 ml/min/1.73 m^2 and albuminuria ≥300 mg/gCr was approximately 20 % in the JDNCS. However, the proportion of patients with low eGFR (<30 ml/min/1.73 m^2) and low albuminuria (<30 mg/gCr), or preserved eGFR (≥60 ml/min/1.73 m^2) and advanced albuminuria (≥300 mg/gCr) was approximately 1 and 6 %, respectively.

Characteristics of JDNCS entry data in GFR stages

JDNCS entry data for eGFR is shown in Table 2. Duration of DM was prolonged in proportion to advanced GFR stage. Systolic blood pressure increased in association with decreasing eGFR, but diastolic blood pressure was not significantly different. Serum levels of total protein and albumin decreased in proportion to advanced GFR stage. There was no statistically significant difference among GFR stages in LDL and HDL cholesterol, and triglycerides. Although there is no statistically significant difference, albuminuria tended to increase in proportion to advanced

Table 1 Distribution of eGFR and albuminuria in JDNCS patients at entry

JDNCS ($N = 259$)		Albuminuria (mg/gCr)					Total (%)
		<10	10–29	30–299	300–1999	≥2000	
GFR (ml/min/1.73 m^2)	≥90	1.9	3.9	2.7	0.4	0.8	9.7
	60–89	13.9	11.2	7.7	3.5	1.5	37.8
	45–59	2.3	4.2	5.0	4.2	2.7	18.4
	30–44	1.2	1.5	0.8	2.7	5.4	11.6
	15–29	0.4	0.0	0.8	3.1	7.3	11.6
	<15	0.8	0.0	0.0	3.5	6.6	10.9
Total (%)		20.5	20.8	17.0	17.4	24.3	100.0

GFR stage. Hb and HbA1c decreased in proportion to advanced GFR stage.

Characteristics of JDNCS entry data in albuminuria stages

In addition to GFR stages, JDNCS entry data was shown in terms of albuminuria stages (Table 3). Systolic blood pressure increased in proportion to advanced albuminuria stage, but diastolic blood pressure was not significantly different. Serum levels of total protein and albumin decreased in proportion to advanced albuminuria stage. Triglycerides increased in proportion to advanced albuminuria stage, but total cholesterol (Tcho), LDL and HDL cholesterol was not significantly different. eGFR was significantly decreased in Stage A3.

Comparison of JDNCS patients with diabetic patients in JRBR and JKDR

The JRBR and JKDR contained 484 patients (143 female, 341 male) and 125 patients (31 female, 94 male), respectively (Table 4; Fig. 1). HbA1c levels (JDS) were similar among the three groups [JDNCS 6.3 (IQR 5.8–7.1) %, JRBR 6.2 (IQR 5.5–7.0) %, JKDR 6.1 (IQR 5.7–6.9) %]. The patients in the JDNCS (65.0; IQR 59.0–74.0 years) were older than kidney biopsy-proven diabetic patients in the JRBR (61.0; IQR 53.0–66.0 years). Moreover, the JDNCS patients showed lower levels of proteinuria and serum levels of total cholesterol, and higher serum levels of albumin [1.8 (IQR 0.3–4.9) g/gCr, 181.0 (IQR 152.0–208.8) mg/dl, 3.6 (IQR 3.2–4.1) g/dl, respectively] than the JRBR patients [4.0 (IQR 1.4–8.1) g/gCr, 210.0 (IQR 173.8–255.3) mg/dl, 3.1 (IQR 2.3–3.8) g/dl, respectively]. eGFR was higher in JDNCS patients [33.3 (IQR 17.1–58.2) ml/min/1.73 m^2] than JKDR patients [13.6 (IQR 5.3–31.5) ml/min/1.73 m^2]. Systolic and diastolic blood pressure was lower in JDNCS patients (130.1 ± 17.3, 72.3 ± 11.6 mmHg, respectively) than JRBR patients (145.9 ± 21.5, 79.6 ± 14.1 mmHg, respectively).

Discussion

The JDNCS aimed to evaluate the epidemiology and long-term renal and cardiovascular outcomes of diabetic nephropathy. The JDNCS enrolled 321 Japanese diabetic patients with early to advanced nephropathy. Median eGFR was 33.3 (IQR 17.1–58.2) ml/min/1.73 m^2, and proteinuria was 1.8 (IQR 0.3–4.9) g/gCr at entry. The majority of JDNCS patients presented with preserved eGFR and low albuminuria, or low eGFR and advanced proteinuria. In comparison with JDNCS patients, JRBR diabetic patients showed lower serum albumin levels with advanced proteinuria, while JKDR diabetic patients showed lower eGFR with advanced proteinuria.

The JDNCS study enrolled Japanese diabetic patients with preserved to low eGFR and normoalbuminuria to massive proteinuria. eGFR and proteinuria are clinically important prognostic factors for adverse outcomes, including renal and cardiovascular events, and death [5]. Moreover, macroalbuminuira was the main predictor of mortality, independently of both eGFR and cardiovascular risk factors [6, 7]. However, in patients with normoalbuminuria, eGFR provided no further information for all-cause mortality and cardiovascular mortality [6]. There has been a nationwide and yearly statistical survey of chronic dialysis patients since 1968, conducted by the Japanese Society for Dialysis Therapy in Japan [8]. The combined data of the three registries with this dialysis registry will allow us to evaluate the long-term outcome of patients with diabetic nephropathy in the near future. Moreover, JDNCS is prospectively collecting clinical data annually. Therefore, the JDNCS will provide prognostic data of diabetic patients in Japan.

Although the majority of JDNCS patients presented with preserved eGFR and low albuminuria, or low eGFR and advanced proteinuria at entry, approximately 10 % of JDNCS patients showed an eGFR <60 ml/min/1.73 m^2 and albuminuria <30 mg/gCr. Yokoyama et al. [9] also reported that the proportion of subjects with low eGFR (<60 ml/min/1.73 m^2) and normoalbuminuria was 11.4 % of type 2

Table 2 Characteristic of JDNCS entry data for GFR stage

eGFR stage	Stage G1		Stage G2		Stage G3A		Stage G3B		Stage G4		Stage G5		*P* value
	Mean/ median	SD/IQR	Mean/ median	SD/IQR	Mean/ median	SD/IQR	Mean/ median	SD/IQR	Mean/ median	SD/IQR	Mean/ median	SD/IQR	
Age	57.5	10.5	66.2	9.0	67.3	11.6	68.4	11.4	67.2	9.7	68.1	10.3	0.000
Duration	9.5	8.7	13.2	9.2	16.3	10.3	14.5	9.1	15.4	11.9	19.1	10.0	0.001
HT	163.1	8.7	160.0	8.9	159.1	9.8	159.9	9.3	162.2	8.6	159.9	8.5	0.409
BW	68.9	18.7	64.8	13.8	65.0	14.9	66.2	12.9	62.8	11.1	63.8	19.6	0.492
BMI	26.0	5.7	25.0	4.3	25.8	4.4	25.9	4.5	23.9	3.7	25.0	7.4	0.109
SBP	127.2	16.9	126.8	15.5	128.9	16.4	127.1	15.6	137.6	21.1	144.2	14.9	0.000
DBP	72.3	8.8	73.6	10.3	70.4	11.8	71.1	12.2	74.1	14.9	71.2	13.9	0.514
sCr	0.56	0.10	0.76	0.12	1.01	0.20	1.45	0.25	2.35	0.46	4.73	1.67	0.000
sTPro	7.2	0.6	7.1	0.6	7.0	0.8	6.7	0.9	6.6	1.0	6.4	0.8	0.000
Alb	4.1	0.5	4.1	0.5	3.8	0.7	3.7	0.7	3.3	0.6	3.4	0.6	0.000
PG	166.1	74.0	154.5	59.1	156.5	51.1	139.3	56.7	133.4	46.3	129.6	61.8	0.003
Tcho	170.1	42.5	189.9	39.6	174.5	43.8	174.4	44.4	204.2	54.5	187.5	64.4	0.034
LDL	96.0	24.4	101.4	31.6	98.0	28.2	88.6	36.1	115.1	43.5	95.8	45.2	0.149
HDL	51.2	19.0	51.9	18.9	46.4	12.5	48.2	15.6	50.5	18.9	51.8	28.0	0.784
TG	97.5	62.0–151.3	116.5	84.3–172.3	108.0	73.0–143.0	84.0	61.0–161.5	125.0	101.0–232.5	141.5	87.8–215.5	0.212
HbA1c	7.7	6.0–9.3	6.7	6.3–7.9	6.7	5.9–7.4	6.3	6.0–7.4	5.8	5.0–8.0	7.1	6.2–7.4	0.003
Hb	13.5	2.2	13.7	1.5	13.0	1.7	11.9	2.0	11.0	1.6	9.7	1.8	0.000
ACR	22.9	6.9–107.0	21.3	9.7–68.8	90.6	20.8–205.9	17.7	5.5–219.4	250.0	37.0–1354.6	166.5	1.1–504.8	0.083
eGFR	103.6	12.0	72.4	8.4	52.4	4.6	36.2	4.4	22.5	4.0	10.7	2.8	0.000

Kruskal–Wallis test was used for analysis. TG, HbA1c, and ACR were expressed by median and interquartile range (IQR), and the others were expressed by mean and SD

Duration duration of DM, *HT* height, *BW* body weight, *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, hemoglobin, *sCr* serum levels of creatinine, *sTPro* serum levels of total protein, *Alb* albumin, *PG* plasma glucose, *Tcho* total cholesterol, *LDL* LDL cholesterol, HDL cholesterol, *TG* triglyceride, *ACR* urinary albumin-creatinine ratio

Table 3 Characteristics of JDNCS entry data in albuminuria stages

Albuminuria stages	Stage A1		Stage A2		Stage A3		*P* value
	Mean/median	SD/IQR	Mean/median	SD/IQR	Mean/median	SD/IQR	
Age	64.4	10.1	68.2	9.8	64.8	11.1	0.131
Duration	13.2	9.8	15.2	9.4	15.4	10.6	0.222
HT	159.8	9.6	160.7	8.6	161.5	9.0	0.430
BW	65.5	14.6	63.2	13.1	65.2	15.9	0.584
BMI	25.0	23.2–27.6	25.5	21.4–28.0	24.4	23.8–31.0	0.129
SBP	126.1	15.7	131.1	17.8	135.7	18.6	0.000
DBP	71.9	11.5	72.7	10.2	72.7	12.4	0.803
sCr	0.84	0.69–1.01	0.86	0.69–1.11	1.08	0.75–1.93	0.000
sTPro	7.1	0.4	7.3	0.6	6.5	1.0	0.000
Alb	4.1	3.8–4.4	4.2	3.8–4.4	4.0	3.5–4.27	0.000
PG	134.0	110.3–168.5	172.0	114.0–218.0	162.5	146.8–199.3	0.022
Tcho	180.0	159.8–204.3	187.0	157.0–203.0	171.0	143.3–199.3	0.948
LDL	96.0	78.8–105.0	96.0	77.0–115.0	102.5	76.5–122.8	0.544
HDL	50.9	16.5	47.4	12.6	51.3	22.0	0.469
TG	93.0	72.8–152.3	125.0	103.0-173.0	120.0	87.5–164.8	0.045
HbA1c	6.5	6.0–7.3	7.1	6.4–8.4	6.8	5.9–7.4	0.001
Hb	13.4	1.6	13.6	1.9	11.5	2.2	0.000
ACR	10.6	5.5–17.9	72.8	44.4–124.2	618.5	376.9–1104.7	0.000
eGFR	69.4	20.6	70.4	25.7	35.4	23.7	0.000

Kruskal–Wallis test was used for analysis. BMI, sCr, Alb, PG, Tcho, LDL, TG, HbA1c, and ACR were expressed by median and interquartile range (IQR), and the others were expressed by mean and SD

Duration duration of DM, *HT* height, *BW* body weight, *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, hemoglobin, *sCr* serum levels of creatinine, *sTPro* serum levels of total protein, *Alb* albumin, *PG* plasma glucose, *Tcho* total cholesterol, *LDL* LDL cholesterol, HDL cholesterol, *TG* triglyceride, *ACR* urinary albumin–creatinine ratio

Table 4 Comparison of JDNCS with JRBR and JKDR

	JDNCS			JRBR			JKDR				
	n	Mean/ median	SD/IQR	*n*	Mean/ median	SD/IQR	*n*	Mean/ median	SD/IQR	JDNCS vs. JRBR	JDNCS vs. JKDR
Gender	321	F 106	M 215	484	F 143	M 341	125	F 31	M 94		
Age	321	65.0	59.0–74.0	484	61.0	53.0–66.0	125	66.0	60.0–73.0	<0.001	0.548
BW	321	65.0	14.8	452	65.6	14.3	125	63.0	121.1	0.589	0.168
BMI	300	23.9	21.8–27.3	484	24.4	21.2–27.2	125	23.3	21.4–25.9	<0.001	0.021
SBP	312	130.1	17.3	327	145.9	21.5	122	142.1	24.2	<0.001	<0.001
DBP	321	72.4	11.6	326	79.6	14.1	122	73.0	13.7	<0.001	0.605
HbA1c	321	6.3	5.8–7.1	322	6.2	5.5–7.0	117	6.1	5.7–6.9	0.329	0.007
eGFR	312	33.3	17.1–58.2	484	43.6	30.5–61.5	125	13.6	5.3–31.5	<0.001	<0.001
Alb	318	3.6	3.2–4.1	475	3.1	2.3–3.8	125	3.6	3.0–4.0	<0.001	0.001
Tcho	266	181.0	152.0–208.8	456	210.0	173.8–255.3	125	180.5	154.8–221.0	<0.001	0.012
U–p/cr	148	1.8	0.3–4.9	253	4.0	1.4–8.1	92	3.4	1.2–6.5	<0.001	0.006

BW body weight, *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *Alb* albumin, *Tcho* total cholesterol, *U-P/Cr* urinary protein-creatinine ratio

diabetic patients examined. These clinical characteristics were more common among female patients, particularly if retinopathy and/or hypertension were also present [10]. Although patients with advanced proteinuria and low eGFR are major and high risk for end-stage kidney disease, patients with normoalbuminuria and low eGFR show a

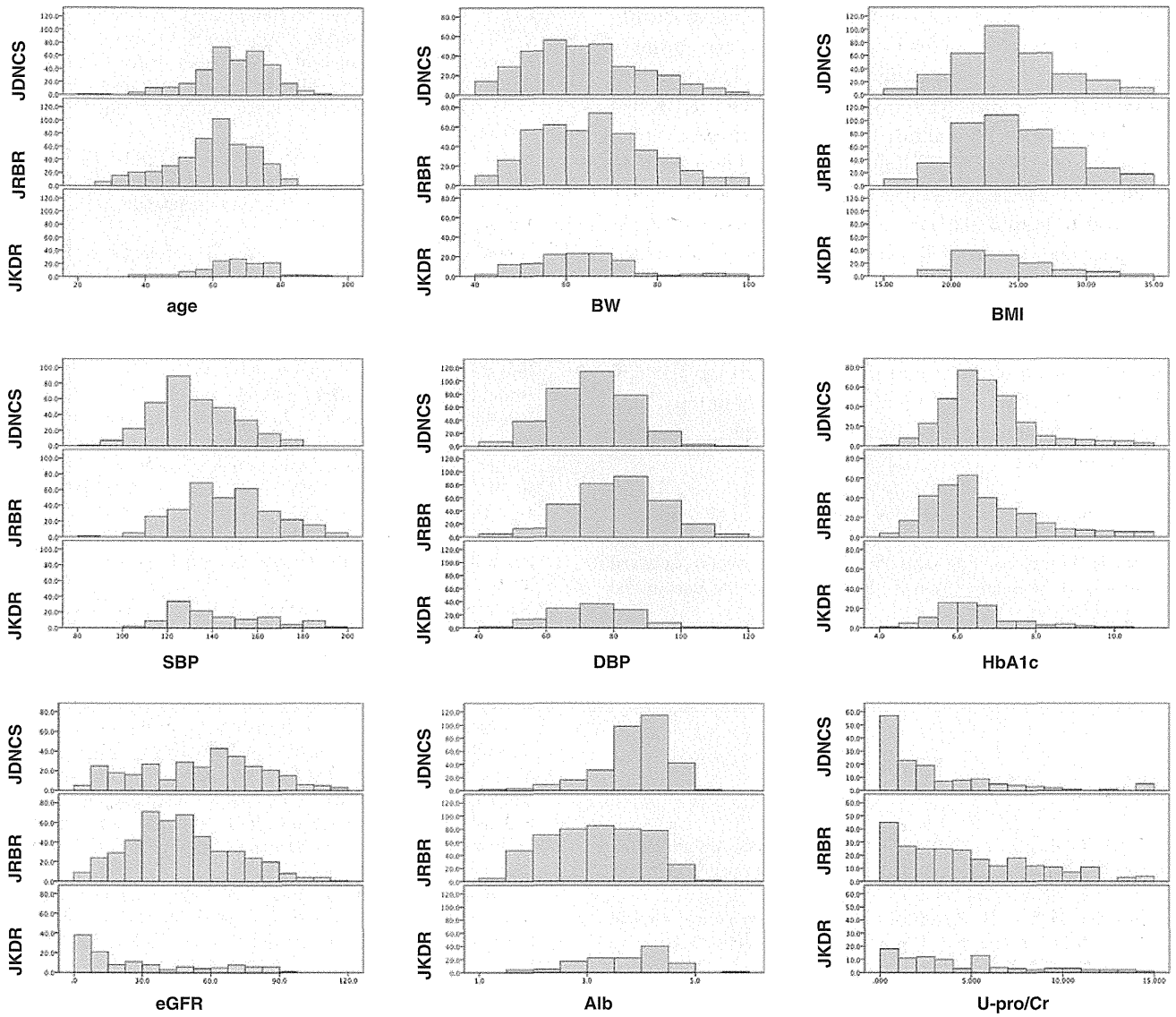

Fig. 1 Distribution of age, body weight (BW), body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA1c, estimated glomerular filtration ratio (eGFR), serum levels of albumin, and urinary levels of protein and creatinine ratio (U-pro/Cr) in JDNCS, JRBR, and JKDR

unique characteristic kidney outcome. Moreover, pathological manifestations of these normoalbuminuria and low eGFR patients were so far unclear. Pathological characteristics and grading would be required to understand the pathophysiology of diabetic nephropathy in more depth together with future perspectives. Kidney biopsy samples are essential to evaluate the relationship between histological findings and clinical manifestation or kidney outcome; however, kidney biopsy is rarely performed in diabetic nephropathy patients. Therefore, the 484 kidney biopsy samples of the JRBR are certainly valuable. Accordingly, clinical long-term follow-up data from the JDNCS together with the JRBR biopsy samples will be useful for evaluating clinical and pathological characteristics of this typical subgroup in future.

In this study, we compared data from two registries and one cohort study. The main objectives of the registry were to establish the frequency of kidney disease based on the histopathological findings (JRBR), or clinical diagnosis (JKDR). In addition to the frequency of diabetic kidney disease in kidney biopsy or clinical diagnosis, this study revealed that entry data of the diabetic patients in these three registries were characteristically different. The basic data from these three registries will be important for evaluating the results from each registry relatively. Although, overt primary kidney diseases were excluded

from these registries and cohort, it is difficult to clearly distinguish between diabetic nephropathy and primary kidney disease in a general clinical setting. This is a limitation of these studies. Moreover, the JRBR and JKDR had no follow-up data. In contrast to these two cross-sectional registries, the JDNCS is a prospective cohort study to evaluate cardiovascular events and progression of kidney dysfunction. Future analysis of data from these two registries and one cohort will provide valuable clinical and pathological information of type 2 diabetes in Japan.

In conclusion, there are few national registries of diabetic nephropathy to evaluate prognosis in Japan. Future analysis of prospective cohort studies, such as the JDNCS, will provide clinical information on epidemiology, and renal and cardiovascular outcomes of type 2 diabetic patients in Japan.

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Conflict of interest The authors have declared that no conflict of interest exists.

References

1. Wada T, Shimizu M, Toyama T, Hara A, Kaneko S, Furuichi K. Clinical impact of albuminuria in diabetic nephropathy. Clin Exp Nephrol. 2012;16:96–101.
2. Wada J, Makino H. Historical chronology of basic and clinical research in diabetic nephropathy and contributions of Japanese scientists. Clin Exp Nephrol. 2009;13:405–14.
3. Sugiyama H, Yokoyama H, Sato H, Saito T, Kohda Y, Nishi S, Tsuruya K, Kiyomoto H, Iida H, Sasaki T, Higuchi M, Hattori M, Oka K, Kagami S, Nagata M, Kawamura T, Honda M, Fukasawa Y, Fukatsu A, Morozumi K, Yoshikawa N, Yuzawa Y, Matsuo S, Kiyohara Y, Joh K, Taguchi T, Makino H. Japan Renal Biopsy Registry: the first nationwide, web-based, and prospective registry system of renal biopsies in Japan. Clin Exp Nephrol. 2011;15:493–503.
4. Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, Yamagata K, Tomino Y, Yokoyama H, Hishida A. Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis. 2009;53:982–92.
5. Ninomiya T, Perkovic V, de Galan BE, Zoungas S, Pillai A, Jardine M, Patel A, Cass A, Neal B, Poulter N, Mogensen CE, Cooper M, Marre M, Williams B, Hamet P, Mancia G, Woodward M, Macmahon S, Chalmers J. Albuminuria and kidney function independently predict cardiovascular and renal outcomes in diabetes. J Am Soc Nephrol. 2009;20:1813–21.
6. Bruno G, Merletti F, Bargero G, Novelli G, Melis D, Soddu A, Perotto M, Pagano G, Cavallo-Perin P. Estimated glomerular filtration rate, albuminuria and mortality in type 2 diabetes: the Casale Monferrato study. Diabetologia. 2007;50:941–8.
7. So WY, Kong AP, Ma RC, Ozaki R, Szeto CC, Chan NN, Ng V, Ho CS, Lam CW, Chow CC, Cockram CS, Chan JC, Tong PC. Glomerular filtration rate, cardiorenal end points, and all-cause mortality in type 2 diabetic patients. Diabetes Care. 2006;29:2046–52.
8. Nakai S, Masakane I, Shigematsu T, Hamano T, Yamagata K, Watanabe Y, Itami N, Ogata S, Kimata N, Shinoda T, Syouji T, Suzuki K, Taniguchi M, Tsuchida K, Nakamoto H, Nishi S, Nishi H, Hashimoto S, Hasegawa T, Hanafusa N, Fujii N, Marubayashi S, Morita O, Wakai K, Wada A, Iseki K, Tsubakihara Y. An overview of regular dialysis treatment in Japan (as of 31 December 2007). Ther Apher Dial. 2009;13:457–504.
9. Yokoyama H, Sone H, Oishi M, Kawai K, Fukumoto Y, Kobayashi M. 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.
10. Caramori ML, Fioretto 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.

Long-term Outcomes of Japanese Type 2 Diabetic Patients With Biopsy-Proven Diabetic Nephropathy

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OBJECTIVE—We evaluated the structural-functional relationships and the prognostic factors for renal events, cardiovascular events, and all-cause mortality in type 2 diabetic patients with biopsy-proven diabetic nephropathy.

RESEARCH DESIGN AND METHODS—Japanese type 2 diabetic patients with biopsy-proven diabetic nephropathy (n = 260) were enrolled. Patients were stratified by albuminuria (proteinuria) and estimated glomerular filtration rate (eGFR) at the time of renal biopsy. The outcomes were the first occurrence of renal events (requirement of dialysis or a 50% decline in eGFR from baseline), cardiovascular events (cardiovascular death, nonfatal myocardial infarction, coronary interventions, or nonfatal stroke), and all-cause mortality.

RESULTS—The factors associated with albuminuria (proteinuria) regardless of eGFR were hematuria, diabetic retinopathy, low hemoglobin, and glomerular lesions. The factors associated with low eGFR regardless of albuminuria (proteinuria) were age and diffuse, nodular, tubulointerstitial, and vascular lesions. The glomerular, tubulointerstitial, and vascular lesions in patients with normoalbuminuria (normal proteinuria) and low eGFR were more advanced compared to those in patients with normoalbuminuria (normal proteinuria) and maintained eGFR. In addition, compared to patients with micro-/macroalbuminuria (mild/severe proteinuria) and low eGFR, their tubulointerstitial and vascular lesions were similar or more advanced in contrast to glomerular lesions. The mean follow-up period was 8.1 years. There were 118 renal events, 62 cardiovascular events, and 45 deaths. The pathological determinants were glomerular lesions, interstitial fibrosis and tubular atrophy (IFTA), and arteriosclerosis for renal events, arteriosclerosis for cardiovascular events, and IFTA for all-cause mortality. The major clinical determinant for renal events and all-cause mortality was macroalbuminuria (severe proteinuria).

CONCLUSIONS—Our study suggests that the characteristic pathological lesions as well as macroalbuminuria (severe proteinuria) were closely related to the long-term outcomes of biopsy-proven diabetic nephropathy in type 2 diabetes.

Diabetic nephropathy occurs in 20–40% of patients with diabetes (1). The prevalence of diabetic nephropathy is increasing in proportion to the increase in prevalence of diabetes, and it has been predicted to continue to increase in future (2). Diabetes is a risk factor of cardiovascular disease and death, and diabetic nephropathy further increases these risks (3). In addition, diabetic nephropathy is the leading cause of end-stage renal disease requiring dialysis or transplantation in developed countries (4–6).

In recent years, many clinical studies have suggested strict glycemic control and blood pressure management by use of appropriate medication to suppress the onset and progression of diabetic nephropathy. Thus, it is important to identify patients at risk in the early stages to improve prognosis in patients with diabetic nephropathy (1). Albuminuria and glomerular filtration rate (GFR) are recommended for use as clinical markers of diabetic nephropathy (1,7–9). On the other hand, selection of pathological markers is complicated because a variety of renal lesions can be found in diabetic nephropathy in addition to factors such as obesity, hypertension, dyslipidemia, and aging, which are frequently complicated in type 2 diabetes, causing a wide variety of pathological changes (10).

We previously reported on the clinical factors related to the development and progression of renal lesions in diabetic nephropathy by the evaluation of serial renal biopsies or autopsy (11). In this report, we demonstrated a significant relationship between the progression of diabetic glomerulosclerosis and clinical factors such as the control of blood glucose, type of diabetes, age at onset, type of treatment, and degree of obesity.

After this study, we conducted a long-term retrospective study to evaluate the structural-functional relationships and the predictive impacts of clinicopathological parameters for renal events, cardiovascular events, and all-cause mortality among Japanese patients with biopsy-proven diabetic nephropathy in type 2 diabetes.

RESEARCH DESIGN AND METHODS

RESEARCH DESIGN AND METHODS—A total of 260 patients who were diagnosed with diabetic nephropathy in type 2 diabetes at Kanazawa University Hospital or Kanazawa Medical Center between 1985 and 2010 were

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included in this study. The diagnosis of diabetes was based on the criteria of the Japanese Diabetic Society (12). The diagnosis of diabetic nephropathy was confirmed by histological characteristics, such as glomerular hypertrophy, thickened capillary basement membranes, diffuse mesangial expansion (sclerosis), nodular mesangial sclerosis, exudative lesions such as capsular drop or fibrin cap, mesangiolysis, capillary microaneurysm, or hyalinosis of afferent and efferent arterioles, using appropriate standards for renal biopsy including light microscopy, electron microscopy, and immunofluorescence examination. Patients with other glomerular diseases concomitant with diabetic nephropathy were excluded from this study. Renal biopsy was performed for precise diagnosis of renal lesions with the consent of each patient. The study protocol was approved by the medical ethics committee of Kanazawa University and Kanazawa Medical Center.

Clinical examinations

Age, sex, 24-h urinary albumin excretion, 24-h urinary protein excretion, urine dipstick test results (proteinuria and hematuria), serum creatinine, estimated GFR (eGFR), duration of diabetes, presence of diabetic retinopathy, HbA_{1c}, BMI, systolic blood pressure, diastolic blood pressure, total cholesterol, and hemoglobin were used as baseline clinical parameters at the time of renal biopsy. eGFR for Japanese patients was calculated using the following equation: eGFR (mL/min/1.73 m^2) = $194 \times \text{serum creatinine}^{-1.094} \times \text{age}^{-0.287}$ (if female, $\times 0.739$) (13). HbA_{1c} levels were presented as National Glycohemoglobin Standardization Program values according to the recommendations of the Japanese Diabetic Society (12) and International Federation of Clinical Chemistry values.

Based on the new classification of chronic kidney disease, albuminuria at baseline was categorized as normoalbuminuria (<30 mg/day [category A1]), microalbuminuria (≥30 and <300 mg/day [category A2]), and macroalbuminuria (≥ 300 mg/day [category A3]) (7,8). We classified proteinuria among patients for whom albuminuria was not evaluated as normal proteinuria (<0.15 g/day or urine dipstick negative or trace [category A1]), mild proteinuria (≥0.15 and <0.5 g/day or urine dipstick + [category A2]), and severe proteinuria (≥0.5 g/day or urine dipstick ≥2+ [category A3]) (7,8). When results were inconsistent, we gave priority to 24-h urinary albumin excretion, 24-h urinary protein excretion, and urine dipstick test results—in that order. In addition, eGFR at baseline was categorized as ≥60 mL/min/1.73 m^2 (categories G1–2) and <60 mL/min/1.73 m^2 (categories G3a-5) for categorical analyses comparing risks.

Outcomes

The outcomes for this study were the first occurrence of renal events (requirement of dialysis or a 50% decline in eGFR from baseline), cardiovascular events (cardiovascular death, nonfatal myocardial infarction, coronary interventions, or nonfatal stroke), and all-cause mortality. The patients were followed up until the end of 2011 or death.

Pathological examinations

For light microscopic examination, renal biopsy specimens were fixed in 10% phosphate-buffered formalin (pH 7.2), embedded in paraffin, and sliced into sections 4 μm thick. These specimens were stained with periodic acid Schiff (PAS) reagent, periodic acid silver methenamine, hematoxylin-eosin, and Mallory-Azan and examined by light microscopy. The severity of diffuse lesions of glomeruli was graded on a scale of 0 to 4 according to the description by Gellman et al. (14) as follows: grade 0, all glomeruli appear normal; grade 1, local lesions present within each glomerulus and focal lesions present within the kidney; grade 2, mesangial thickening is diffuse within the glomerulus and generalized throughout the kidney; grade 3, capillary lumina are narrowed and obliterated only locally; and grade 4, the lumen is generally narrowed and the entire glomerulus is ischemic and appears to be hyalinized (14–17) (Supplementary Fig. 1*A–D*). Nodular lesions, exudative lesions, and mesangiolysis were simply shown as their presence or absence in each specimen (15–17) (Supplementary Fig. 1*E–G*). The severity of interstitial fibrosis and tubular atrophy (IFTA) and interstitial inflammation was scored according to the description by Tervaert et al. (18). The severity of IFTA was evaluated and graded on a scale from 0 to 3: grade 0, no IFTA; grade 1, <25%; grade 2, 25–50%; and grade 3, >50% (18). The severity of interstitial inflammation was evaluated and graded on a scale from 0 to 2: grade 0, absent; grade 1, infiltration only in relation to IFTA; and grade 2, infiltration in areas without IFTA (18). The severity of arteriolar hyalinosis was evaluated and graded on a scale from 0 to 3 according to the description by Takazakura et al. (11) as follows: grade 0, normal appearance without PAS-positive deposit; grade 1, a light PAS-positive thickening is observed but at less than half the circumference of the arteriole in many arterioles; grade 2, most vessel walls are moderately thickened with PAS-positive deposition without apparent luminal narrowing; and grade 3, a heavy thickening of the majority of the vessel walls is seen with luminal narrowing or obliteration (Supplementary Fig. 1*H–J*). The severity of arteriosclerosis was evaluated and graded on a scale from 0 to 2 according to the description by Tervaert et al. (18) as follows: grade 0, no intimal thickening; grade 1, intimal thickening less than thickness of media; and grade 2, intimal thickening greater than thickness of media (Supplementary Fig. 1*K* and *L*). Renal tissue specimens were examined by four nephrologists.

Statistical analysis

Data are expressed as means ± SD. Comparisons of continuous variables among groups were performed using the Mann-Whitney *U* test for nonparametric data. Comparisons of categorical variables among groups were performed using χ^2 test. The survival curves were obtained using the Kaplan-Meier method and compared by log-rank test. The influence of different categories of albuminuria (proteinuria) and eGFR on each outcome was evaluated with the use of the Cox proportional hazards model after adjustment for age and sex. The results are presented as hazard ratios (HRs) and 95% CI. Patients with normoalbuminuria (normal proteinuria) and eGFR ≥60 mL/min/1.73 m^2 were served as the reference group in the analyses. A multivariate Cox proportional hazard regression model was used to select factors that significantly affected the incidence of each outcome and to estimate the risks. The following variables were incorporated as covariates: age, sex, microalbuminuria (mild proteinuria), macroalbuminuria (severe proteinuria), eGFR, duration of diabetes, presence of diabetic retinopathy, HbA_{1c}, BMI, systolic blood pressure, total cholesterol, and hemoglobin as clinical covariates or diffuse lesions, nodular lesions, exudative lesions, mesangiolysis, IFTA, interstitial inflammation, arteriolar hyalinosis, and arteriosclerosis as pathological covariates. All analyses were carried out using SPSS, version 19 (SPSS, Tokyo, Japan). Two-sided $P < 0.05$

was considered indicative of statistical significance.

RESULTS

Baseline characteristics

The baseline characteristics of 260 patients are shown in Table 1. In the clinical parameters, the mean age was 58.2 years, and 63.1% of the patients were male. Among the 95 patients for whom daily urinary albumin excretion measurements were available, 10 (10.5%) showed normoalbuminuria (A1), 31 (32.6%) showed microalbuminuria (A2), and 54 (56.8%) showed macroalbuminuria (A3). Among the 231 patients for whom daily urinary protein excretion measurements were available, 31 (13.4%) showed normal proteinuria (A1), 44 (19.0%) showed mild proteinuria (A2), and 156 (67.5%) showed severe proteinuria (A3). Among the 256 patients for whom urinary dipstick protein test results were available, 53 (20.7%) showed negative (A1), 19 (7.4%) showed trace (A1), 42 (16.4%) showed + (A2), 63 (26.4%) showed 2+ (A3), and 79 (30.9%) showed ≥3+ (A3). The mean serum creatinine was 1.4 mg/dL, and the mean eGFR was 58.0 mL/min/1.73 m^2. The proportions with eGFR ≥90 (G1), 60–89 (G2), 45–59 (G3a), 30–44 (G3b), 15–29 (G4), and <15 (G5) mL/min/1.73 m^2 were 15.0%, 25.8%, 21.9%, 18.5%, 12.7%, and 6.2%, respectively.

The proportions of patients stratified by albuminuria (proteinuria) and eGFR categories are demonstrated in Supplementary Table 1. The proportions of patients with normoalbuminuria (normal proteinuria), microalbuminuria (mild proteinuria), and macroalbuminuria (severe proteinuria) were 16.5% (43 of 260), 21.2% (55 of 260), and 62.3% (162 of 260), respectively. The proportions of patients with eGFR ≥60 and <60 mL/min/1.73 m^2 were 40.8% (106 of 260) and 59.2% (154 of 260), respectively. The proportions of patients with normoalbuminuria (normal proteinuria), microalbuminuria (mild proteinuria), and macroalbuminuria (severe proteinuria) among those with eGFR ≥60 mL/min/1.73 m^2 were 26.4% (28 of 106), 29.2% (31 of 106), and 44.3% (47 of 106), respectively. The proportions of patients with normoalbuminuria (normal proteinuria), microalbuminuria (mild proteinuria), and macroalbuminuria (severe proteinuria) among those with eGFR <60 mL/min/1.73 m^2 were 9.7% (15 of 154), 15.6% (24 of 154), and 74.7% (115 of 154), respectively.

Table 1—*Clinical characteristics of patients at the time of renal biopsy (n = 260)*

Age (years)	58.2 ± 11.4
Male	164 (63.1)
Kidney-related parameters	
Urine albumin category (mg/day), *n* = 95	
Normoalbuminuria (<30)	10 (10.5)
Microalbuminuria (30–299)	31 (32.6)
Macroalbuminuria (≥300)	54 (56.8)
Urine protein category (g/day), *n* = 231	
Normal proteinuria (<0.15)	31 (13.4)
Mild proteinuria (0.15–0.49)	44 (19.0)
Severe proteinuria (≥0.5)	156 (67.5)
Dipstick test results, *n* = 256	
–	53 (20.7)
±	19 (7.4)
+	42 (16.4)
2+	63 (24.6)
≥3+	79 (30.9)
Serum creatinine (mg/dL)	1.4 ± 1.3
eGFR (mL/min/1.73 m^2)	58.0 ± 31.7
≥90	39 (15.0)
60–89	67 (25.8)
45–59	57 (21.9)
30–44	48 (18.5)
<30	49 (18.8)
Hematuria (%)	39.1
Diabetes parameters	
Diabetes duration (years)	11.2 ± 8.1
Diabetic retinopathy (%)	79.5
HbA_{1c} (%)	8.2 ± 2.3
HbA_{1c} (mmol/mol)	61.6 ± 24.7
Other major risk factors	
BMI (kg/m^2)	23.2 ± 3.7
Systolic blood pressure (mmHg)	142.1 ± 21.4
Diastolic blood pressure (mmHg)	76.8 ± 12.0
Total cholesterol (mg/dL)	218.9 ± 83.2
Hemoglobin (g/dL)	12.1 ± 2.4

Data are means ± SD or *n* (%).

Clinical and pathological features associated with albuminuria (proteinuria) and low eGFR

The baseline clinical and pathological features were compared among subgroups stratified by albuminuria (proteinuria) and eGFR categories (Table 2). Clinical and pathological factors associated with micro-/macroalbuminuria (mild/severe proteinuria) regardless of eGFR categories were hematuria, diabetic retinopathy, low hemoglobin, and glomerular lesions. On the other hand, clinical and pathological factors associated with low eGFR regardless of albuminuria (proteinuria) categories were age, diffuse lesions, nodular lesions, tubulointerstitial lesions, and vascular lesions. Glomerular lesions in patients with normoalbuminuria (normal proteinuria) were less advanced for both eGFR ≥60 and eGFR <60 mL/min/1.73 m^2 categories. On the other hand, as to tubulointerstitial and vascular lesions in patients with normoalbuminuria (normal proteinuria), there were different trends between eGFR ≥60 and eGFR <60 mL/min/1.73 m^2 categories. In the eGFR ≥60 ml/min/1.73 m^2 category, tubulointerstitial and vascular lesions in patients with normoalbuminuria (normal proteinuria) were less advanced compared with those in patients with micro-/macroalbuminuria (mild/severe proteinuria). However, in the eGFR <60 ml/min/1.73 m^2 category, tubulointerstitial and vascular lesions in patients with normoalbuminuria (normal proteinuria) were similar or more advanced compared with those in patients with micro-/macroalbuminuria (mild/severe

Table 2—*Baseline clinical and pathological features of patients stratified by albuminuria (proteinuria) and eGFR categories*

	Normoalbuminuria (normal proteinuria)		Micro-/macroalbuminuria (mild/severe proteinuria)		*P* for normo (normal) vs. micro (mild)/macro (severe)	
	eGFR ≥60 mL/min/1.73 m^2	eGFR <60 mL/min/1.73 m^2	eGFR ≥60 mL/min/1.73 m^2	eGFR <60 mL/min/1.73 m^2	eGFR ≥60 mL/min/1.73 m^2	eGFR <60 mL/min/1.73 m^2
n	28	15	78	139		
Clinical parameters						
Age (years)	48.8 ± 13.8	62.5 ± 6.2**	53.8 ± 10.8	62.1 ± 9.6††	0.10	0.74
Male	53.6	46.7	65.4	65.5	0.27	0.15
Serum creatinine (mg/dL)	0.7 ± 0.1	1.2 ± 0.4**	0.7 ± 0.2	1.9 ± 1.5††	0.95	<0.01
eGFR (mL/min/1.73 m^2)	86.0 ± 16.4	46.0 ± 10.6**	89.0 ± 26.4	36.4 ± 15.5††	0.81	<0.05
Hematuria	4.2	7.7	32.8	51.9†	<0.01	<0.01
Diabetes duration (years)	8.0 ± 7.4	7.4 ± 6.4	9.6 ± 6.6	13.1 ± 8.8††	0.21	<0.05
Diabetic retinopathy	41.7	50.0	81.7	87.9	<0.01	<0.01
HbA_{1c} (%)	8.5 ± 2.4	8.3 ± 2.2	8.4 ± 2.5	7.4 ± 2.0††	0.53	0.35
HbA_{1c} (mmol/mol)	65.0 ± 26.2	64.1 ± 25.1	68.8 ± 27.1	57.1 ± 22.3††	0.53	0.35
BMI (kg/m^2)	23.5 ± 2.2	22.2 ± 2.2	22.9 ± 4.8	23.5 ± 3.4†	0.07	0.34
Systolic blood pressure (mmHg)	132.0 ± 17.1	129.3 ± 14.3	138.8 ± 22.1	146.9 ± 21.0††	0.31	<0.01
Diastolic blood pressure (mmHg)	75.0 ± 11.2	75.6 ± 10.0	77.6 ± 12.0	76.8 ± 12.4	0.26	0.91
Total cholesterol (mg/dL)	192.7 ± 37.4	196.6 ± 51.5	215.0 ± 54.4	227.6 ± 100.6	0.07	0.31
Hemoglobin (g/dL)	14.2 ± 1.6	13.0 ± 1.8	13.2 ± 2.1	11.1 ± 2.2††	<0.05	<0.01
Pathological parameters						
Diffuse lesion (0–4)	0.9 ± 0.6	1.5 ± 0.9*	2.0 ± 0.9	2.4 ± 0.8†	<0.01	<0.01
Nodular lesion	0.0	20.0*	44.7	65.1††	<0.01	<0.01
Exudative lesion	0.0	6.7	25.0	44.1††	<0.01	<0.01
Mesangiolysis	0.0	0.0	29.6	30.2	<0.01	<0.05
IFTA (0–3)	1.1 ± 1.0	2.3 ± 0.7**	1.6 ± 0.9	2.1 ± 0.9††	<0.05	0.41
Interstitial inflammation (0–2)	0.9 ± 0.8	1.5 ± 0.5**	0.9 ± 0.5	1.2 ± 0.5††	0.61	<0.05
Arteriolar hyalinosis (0–3)	1.4 ± 1.1	2.4 ± 0.8**	1.8 ± 1.0	2.2 ± 0.8††	0.07	0.33
Arteriosclerosis (0–2)	0.7 ± 0.6	1.9 ± 0.4**	1.2 ± 0.7	1.5 ± 0.5††	<0.01	<0.05

Data are means ± SD or % unless otherwise indicated. Differences among albuminuria (proteinuria) and eGFR categories are compared by Mann-Whitney *U* test for continuous variables and χ^2 test for categorical variables. micro, microalbuminuria; macro, macroalbuminuria; normo, normoalbuminuria. $^{**}P < 0.01$ vs. normoalbuminuria (normal proteinuria) and eGFR ≥60 mL/min/1.73 m^2 group. $^{††}P < 0.01$ vs. micro-/macroalbuminuria (mild/severe proteinuria) and eGFR ≥60 mL/min/1.73 m^2 group. $^{*}P < 0.05$ vs. normoalbuminuria (normal proteinuria) and eGFR ≥60 mL/min/1.73 m^2 group. $^{†}P < 0.05$ vs. micro-/macroalbuminuria (mild/severe proteinuria) and eGFR ≥60 mL/min/1.73 m^2 group.

proteinuria) in contrast to glomerular lesions (Supplementary Fig. 2*A–C*).

Prognosis of renal events, cardiovascular events, and all-cause mortality

Follow-up data were available for renal events in 229 patients and for cardiovascular events and all-cause mortality in 233 patients. The mean duration of follow-up was 8.1 years (range 5–9,739 days) during 1985–2011. There were a total of 118 renal events, 62 cardiovascular events, and 45 deaths (Supplementary Table 2). Event-free rate of renal events in patients with macroalbuminuria (severe proteinuria) was significantly lower than in those with normoalbuminuria (normal proteinuria) or microalbuminuria (mild proteinuria) for both eGFR ≥60 and <60 mL/min/1.73 m^2 categories (vs. normoalbuminuria [normal proteinuria] and eGFR ≥60 mL/min/1.73 m^2, $P < 0.01$; vs. microalbuminuria [mild proteinuria] and eGFR ≥60 mL/min/1.73 m^2, $P < 0.01$; vs. normoalbuminuria [normal proteinuria] and eGFR <60 mL/min/1.73 m^2, $P < 0.01$; and vs. microalbuminuria [mild proteinuria] and eGFR <60 mL/min/1.73 m^2, $P < 0.01$) (Fig. 1*A* and *B*). Event-free rate of cardiovascular events showed no significant differences between albuminuria (proteinuria) categories for both eGFR ≥60 and <60 mL/min/1.73 m^2 categories. Event-free rate of all-cause mortality in patients with macroalbuminuria (severe proteinuria) was significantly lower than in those with normoalbuminuria (normal proteinuria) or microalbuminuria (mild proteinuria) in the eGFR <60 mL/min/1.73 m^2 category (vs. normoalbuminuria [normal proteinuria] and eGFR <60 mL/min/1.73 m^2, $P < 0.05$; vs. microalbuminuria [mild proteinuria] and eGFR <60 mL/min/1.73 m^2, $P < 0.01$) (Fig. 1*C* and *D*). Event-free rates of renal events, cardiovascular events, and all-cause mortality in patients with eGFR <60 mL/min/1.73 m^2 were significantly lower than in those with eGFR ≥60 mL/min/1.73 m^2 only among patients with macroalbuminuria (severe proteinuria) (renal events $P < 0.01$, cardiovascular events $P < 0.05$, all-cause mortality $P < 0.01$).

Risks of renal events, cardiovascular events, and all-cause mortality stratified by albuminuria (proteinuria) and eGFR categories

HRs of renal events, cardiovascular events, and all-cause mortality were calculated in subgroups of patients stratified