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ORIGINAL ARTICLE

CCL2/CCR2 augments the production of transforming growth factor-beta1, type 1 collagen and CCL2 by human CD45-/collagen 1-positive cells under high glucose concentrations

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Abstract

Background The migration and activation of circulating profibrotic cells including fibrocytes by the action of the chemokine/chemokine receptor system has been implicated in pathological fibrogenesis. In the present study, the involvement of collagen 1 (Col1)-producing cells, CD45-positive/collagen-1-positive (CD45$^+$/Col1$^+$) cells originally named as fibrocytes via CC chemokine receptor 2 (CCR2), a cognate receptor of CCL2/monocyte chemoattractant protein, was examined in diabetic conditions.

Methods Human CD45$^+$/Col1$^+$ cells originating from the peripheral blood of healthy volunteers were incubated with high concentrations of D-glucose or D-mannitol as an osmotic control for 12, 24 or 48 h. In addition, these cells were preincubated with CCL2 under high glucose concentrations. We also examined the effects of the inhibitors of glucose transporters (GLUTs), reactive oxygen species or CCR2 on the expression of transforming growth factor beta1 (TGF-β_1), pro-α1 chain of Col1 (COL1A1), and CCL2.

Results Stimulation of CD45$^+$/Col1$^+$ cells with high glucose concentrations increased the mRNA and protein levels of TGF-β_1 and CCL2 and those of pro-COL1A1, and this effect was mediated in part by increased osmolality. Preincubation of the cells with cytochalasin B (a GLUT inhibitor) or *N*-acetylcysteine (an antioxidant) blocked the stimulatory effect of high glucose concentrations on these profibrotic molecules. In addition, preincubation of the cells with CCL2 enhanced the high glucose-induced upregulation of TGF-β_1, pro-COL1A1 and CCL2 and migration of the cells, and this effect was partly inhibited by treatment with CCR2 inhibitors.

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Conclusion These results suggest that $CD45^+/Col1^+$ cells may be directly involved, in part through CCL2/CCR2 signaling, in the fibrotic process under diabetic conditions.

Keywords Diabetes mellitus · CCR2 · Fibrosis · Bone marrow-derived cells · Fibrocyte · CD45

Introduction

Diabetic nephropathy is one of the major microvascular complications of diabetes. It is by far the most common cause of end-stage kidney failure and is epidemic worldwide [1, 2]. In the course of the disease progression, the accumulation of matrix proteins resulting in glomerulosclerosis and interstitial fibrosis is a prominent feature of the disease [2]. In this histopathological picture, the infiltration of bone marrow-derived inflammatory cells such as monocytes/macrophages into the diseased kidneys is a hallmark of the progression of diabetic nephropathy [3, 4]. Infiltrated monocytes/macrophages release lysosomal enzymes, nitric oxide, and transforming growth factor beta (TGF-β), which have an essential role in kidney damage [2, 5]. Recent studies have uncovered that CCL2, also termed monocyte chemoattractant protein-1, plays an important role through its cognate receptor, CCR2 and its downstream signal transduction system in the pathogenesis of diabetic nephropathy as well as glomerulonephritis [6–9].

In recent years, fibrocytes, a precursor to several mature cell lineages in monocyte fraction of peripheral blood, have been shown to be involved in the pathogenesis of organ fibrosis through distinct chemokine/chemokine receptor systems [10–13]. These cells are associated with fibrotic conditions such as lung and kidney fibrosis, bronchial asthma, and skin wounds [12–15]. Fibrocytes are identified by the coexpression of the stem cell marker CD34, the leukocyte common antigen CD45, and extracellular matrix proteins such as type 1 collagen (Col1) [10]. Although a recent study revealed that markers of CD45RO, 25F9 and S100A8/A9 distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts [16], until now fibrocytes have been identified by dual positivity of CD34 or CD45 and Col1 or pro-collagen 1 (pro-Col1) [17]. Furthermore, fibrocytes express chemokine receptors such as CCR2, CCR7 and CXCR4, and the function and differentiation of fibrocytes are influenced by various mediators such as TGF-β [13, 17]. While CD45 and pro-Col1 dual-positive ($CD45^+/pro-Col1^+$) cells may be found in human diabetic nephropathy and may have a role in the progression of the disease [15], the specific cellular and molecular mechanisms involved in the development and/or progression of diabetic complications, especially in the kidney, remains to be investigated.

In the present study, we examined the direct involvement of $CD45^+/Col1^+$ cells in the development and/or progression of the profibrotic process under high glucose with or without the costimulation of CCL2. We report here that high glucose concentrations stimulate the production of TGF-β_1, pro-Col1 and CCL2, and cell migration, which are augmented partly by CCL2/CCR2 signaling, in human $CD45^+/Col1^+$ cells in vitro.

Materials and methods

Differentiation and isolation of human $CD45^+/Col1^+$ cells originating from peripheral blood

$CD45^+/Col1^+$ cells were harvested and cultured as previously reported [18, 19]. Briefly, total peripheral blood mononuclear cells were isolated from venous blood drawn from healthy donors by centrifugation on a Ficoll-Metrizoate density gradient ($d = 1.077$ g/ml; Lymphoprep; Nycomed, Oslo, Norway) following the manufacturer's protocol. After 2 days of culture in a tissue culture flask using Dulbecco's Modified Eagle's Medium (DMEM) containing 4500 mg/L D-glucose (Gibco BRL) supplemented with 20 % heat-inactivated fetal calf serum (FCS) (Gibco BRL), 100 U/ml penicillin (Gibco BRL), and 100 μg/ml streptomycin (Gibco BRL), non-adherent cells were removed by gentle aspiration, and media were replaced. After 10–12 days, adherent cells were lifted by incubation in ice-cold 0.05 % ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS). The crude cell preparations were then depleted by immunomagnetic selection of contaminating T cells, B cells, and $CD14^+$ monocytes using pan-T, anti-CD2; pan-B, anti-CD19; and anti-CD14 monoclonal antibody coated with microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) [19]. Cell purity was examined by flow cytometry using both fluorescein isothiocyanate-conjugated mouse anti-human CD45 monoclonal antibody (Becton Dickinson/Pharmingen, San Diego, CA, USA) and R-phycoerythrin-labeled rabbit antihuman type 1 collagen polyclonal antibodies (Millipore, Temecula, CA, USA) and confirmed as >92 % pure population of cells coexpressing CD45 and type 1 collagen, as described previously [19]. With regard to ethical considerations, the medical ethics committee of Kanazawa University approved the experiments which were conducted with the understanding and the consent of each participant.

Culture conditions

To examine the effect of glucose and CCL2 on function of $CD45^+/Col1^+$ cells, $CD45^+/Col1^+$ cells were incubated

with D-glucose (Wako Chemicals Inc., Tokyo, Japan) and/or CCL2 in a dose- and time-dependent manner. Briefly, $CD45^+/Col1^+$ cells (1 × 10^6/ml) were cultured in plastic tissue culture plates in DMEM supplemented with 0.5 % heat-inactivated FCS (Gibco BRL) at 37 °C in a humidified atmosphere with 5 % CO_2 for 24 h after isolation in the presence of increasing concentrations (5–30 mmol/l) of D-glucose using D-mannitol (Wako Chemicals Inc.) as osmotic controls. In addition, $CD45^+/Col1^+$ cells were cultured for an increasing period of time (0–48 h) in DMEM supplemented with 0.5 % heat-inactivated FCS in the presence of 30 mmol/l D-glucose or 5 mmol/l D-glucose with 25 mmol/l D-mannitol. In additional experiments to explore the role of glucose-specific signaling pathways in the production of TGF-β_1, pro-Col1 and CCL2, $CD45^+/Col1^+$ cells were treated with D-glucose or D-mannitol in the presence of cytochalasin B (Sigma-Aldrich, Inc., St. Louis, MO, USA), a well-known glucose transporter (GLUT) inhibitor [20], at 5 µmol/l for 24 h. According to earlier dosing studies in our laboratory, this concentration of cytochalasin B did not influence the viability of cultured $CD45^+/Col1^+$ cells as assessed by Trypan blue dye exclusion (data not shown). In addition, to investigate the role of reactive oxygen species (ROS) in this pathological process, N-acetylcysteine (NAC) (Sigma-Aldrich, Inc.) was used as an antioxidant at 2 mmol/l for 24 h. Furthermore, to examine whether CCL2/CCR2 is responsible for the production of TGF-β_1, pro-Col1 and CCL2, propagermanium (kindly provided by Sanwa Kagaku) and RS-504393 (Tocris Bioscience, Bristol, UK) [21] inhibitors of CCR2 signaling were used [21, 22]. $CD45^+ Col1^+$ cells (1 × 10^6/ml) were preincubated in DMEM supplemented with 0.5 % heat-inactivated FCS by adding propagermanium (3 µg/ml) or RS-504393 (1 µM) into each culture medium for 30 min. Subsequently, $CD45^+/Col1^+$ cells were incubated in the presence of recombinant human CCL2 (50 ng/ml) (R&D Systems Inc., MN, USA) for 48 h.

Analysis of GLUT isoforms and CCR2 by reverse transcription-polymerase chain reaction (RT-PCR)

Transcripts of GLUT isoforms were detected using RT-PCR. In brief, total RNA was extracted from cultured human $CD45^+/Col1^+$ cells under normal or high concentrations of glucose, and complementary DNA (cDNA) was reverse-transcribed from 1 µg total RNA using a RT-PCR kit (Takara Shuzo, Tokyo, Japan) as previously described [14, 23]. The cDNA product was amplified by PCR as follows—incubation for 3 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C and a final extension for 7 min at 72 °C [24]. Primers for human GLUT1, GLUT2, GLUT3, and GLUT4 were used to examine expression of GLUT1, GLUT2, GLUT3, and

Table 1 Oligonucleotide primer pairs used for RT-PCR

Gene		Oligonucleotide primers 5'-3'	Product size
GLUT1	S	TCCACGAGCATCTTCGAGA	393
	AS	ATACTGGAAGCACATGCCC	
GLUT2	S	CACTGATGCTGCATGTGGC	522
	AS	ATGTGAACAGGGTAAAGGCC	
GLUT3	S	TTCAAGAGCCCATCTATGCC	458
	AS	GGTCTCAGGGACTTTGAAGA	
GLUT4	S	GGCATGTGTGGCTGTGCCATC	414
	AS	GGGTTTCACCTCCTGCTCTAA	
CCR2	S	GGATTGAACAAGGACGCATT	766
	AS	TCTCACTGCCCTATGCCTCT	
GAPDH	S	CCCATCACCATCTTCCAGGAGC	473
	AS	CCAGTGAGCTTCCCGTTCAGC	

Sense (S) and antisense (AS) primer pairs specific for facilitative glucose transporter isoforms (GLUT1-4), and glycerophosphate dehydrogenase (GAPDH). Nucleotide numbers refer to positions within the published sequence

GLUT4, respectively (Table 1) [24]. Similarly, to determine CCR2 transcripts, the cDNA products from total RNA were amplified by semiquantitative RT-PCR using that from a human monocyte cell line, THP-1 (DS Pharma Biomedical, Osaka, Japan), as a positive control. Primers for CCR2 were used to detect CCR2 transcripts (Table 1) [22]. The amplification profile for CCR2 was described previously [22]. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for PCR controls (Table 1) [24]. Photographs of DNA-stained agarose gels were evaluated by band intensity comparison of GAPDH expression versus GLUT expression using computer image analysis [22].

Analysis of gene expression by real-time quantitative PCR

To determine transcripts of the pro-α1 chain of Col1 (pro-COL1A1), TGF-β_1, and CCL2, total RNA was extracted from cultured human $CD45^+/Col1^+$ cells as described above. Quantitative real-time RT-PCR was performed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as previously described [19, 22]. Real-time PCR was performed in a total volume of 20 ml, containing 1 ml cDNA 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 Hs00234140 for CCL2, Hs00171257 for TGF-β_1, Hs00164004 for COL1A1 and Hs99999905 for GAPDH.

Enzyme-linked immunosorbent assay (ELISA)

To determine the protein levels of CCL2 and TGF-β_1, supernatants of the samples which were prepared by centrifuging at 1,500 rpm for 10 min and then storing 1 ml at −80 °C until use, were evaluated using the commercial Quantikine Human CCL2 and TGF-β_1 ELISA kit, respectively, in accordance with the protocol specified by the manufacturer (R&D Systems Inc.).

Cell migration assay

To examine the degree to which cultured human CD45+/Col1+ cells migrate in vitro in response to high glucose concentrations and CCL2/CCR2 signaling, cell migration assay was performed using the Cultrex 96 Well Cell Migration Assay Kit in accordance with the protocol specified by the manufacturer (Trevigen, Inc., Gaithersburg, MD, USA).

Statistical analysis

The mean and SEM were calculated on all of the parameters determined in this study. Statistical analyses were performed using Kruskal–Wallis test and analysis of variance. $P < 0.05$ was accepted as statistically significant.

Results

Effect of high glucose concentrations on expression of TGF-β_1 and pro-COL1A1 by isolated CD45+/Col1+ cells

The impact of high glucose concentrations on isolated human CD45+/Col1+ cells was examined in vitro. The exposure of human CD45+/Col1+ cells to D-glucose resulted in the induction of TGF-β_1 and pro-COL1A1 mRNA in a time- and dose-dependent manner (Fig. 1a, b, e, f). In addition to mRNA up-regulation of TGF-β_1, the concentration of the TGF-β_1 protein in the supernatant was increased by stimulation with high glucose concentrations (Fig. 1c, d). When human CD45+/Col1+ cells were incubated with D-mannitol, there was a similar, albeit lower magnitude, dose- and time-dependent increase in mRNA and protein levels of TGF-β_1 and pro-COL1A1 mRNA was observed (Fig. 1a–f). After 24 h, mRNA and protein levels of TGF-β_1 and pro-COL1A1 mRNA were significantly higher in D-glucose-treated human CD45+/Col1+ cells compared with those treated with D-mannitol (Fig. 1b, d, f).

Effect of high glucose concentrations on expression of CCL2 by isolated CD45+/Col1+ cells

To examine the effect of high glucose concentrations on the expression of CCL2 in human CD45+/Col1+ cells, the cells were incubated for 24 h with high glucose concentrations. Stimulation with high glucose enhanced the levels of CCL2 mRNA as well as protein in a dose-dependent manner (Fig. 2a, c). In addition, human CD45+/Col1+ cells were cultured in 30 mmol/l D-glucose or 25 mmol/l D-mannitol with 5 mmol/l D-glucose for up to 48 h. Enhanced mRNA levels of CCL2 were observed in a time-dependent manner, which were followed by the up-regulation of CCL2 protein (Fig. 2b, d). As well as the expression of TGF-β_1 and pro-COL1A1, high osmolality also increased mRNA and protein levels of CCL2 (Fig. 2a–d). After 24 h, mRNA and protein levels of CCL2 were significantly higher in D-glucose-treated human CD45+/Col1+ cells compared with those treated with D-mannitol (Fig. 2b, d).

Expression of mRNAs for GLUTs

The expression of GLUT isoforms in human CD45+/Col1+ cells cultured with 5 or 30 mmol/l D-glucose was examined by RT-PCR. The expression of GLUT1 and GLUT3 mRNA was detectable under normal glucose concentrations, and their expression was downregulated at 24 h after incubation with high glucose concentrations (Fig. 3a). Transcripts for the mRNA for GLUT2 and GLUT4 were not detected by this qualitative analysis (Fig. 3a).

Effect of inhibition of glucose transport or ROS on the expression of TGF-β_1 and pro-COL1A1

Cytochalasin B, an antioxidant and an inhibitor of GLUT and NAC, was used to determine the contribution of glucose-specific mechanisms and their downstream pathways leading to the expression of TGF-β_1 and pro-COL1A1 in human CD45+/Col1+ cells. Human CD45+/Col1+ cells were incubated for 24 h with control medium (5 mmol/l D-glucose), 30 mmol/l D-glucose, or 25 mmol/l D-mannitol with 5 mmol/l D-glucose in the presence or absence of 5 μmol/l of cytochalasin B or 2 mmol/l of NAC. D-glucose and mannitol significantly increased mRNA and protein levels of TGF-β_1 and pro-COL1A1 mRNA, when compared to the control condition (Fig. 3b, d, f). In the presence of cytochalasin B or NAC, the D-glucose-induced increase in the expression of TGF-β_1 and pro-COL1A1 mRNA was significantly reduced (Fig. 3b, f). Similarly, the concentration of the TGF-β_1
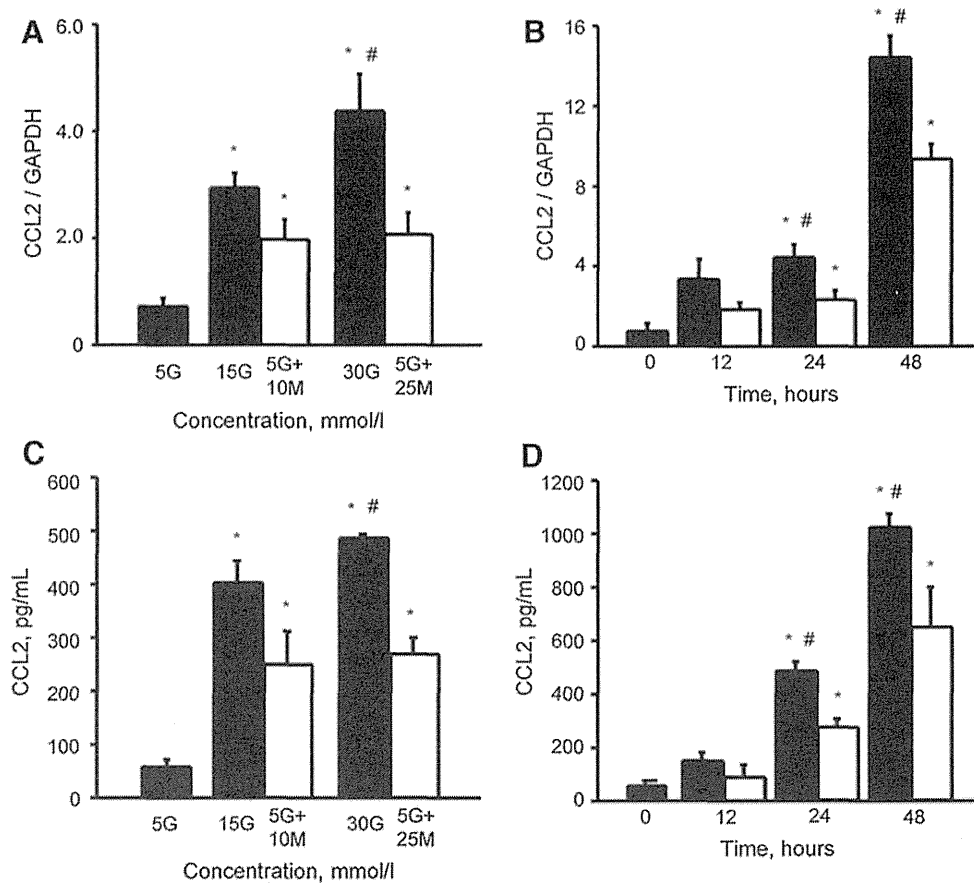
Fig. 1 Effect of increasing concentrations of D-glucose on the expression of TGF-β_1 and pro-COL1A1 in human isolated CD45$^+$/Col1$^+$ cells. Dose effect of increasing concentrations of D-glucose (*filled square*) or D-mannitol (*open square*) on the induction of TGF-β_1 mRNA (**a**), TGF-β_1 protein (**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
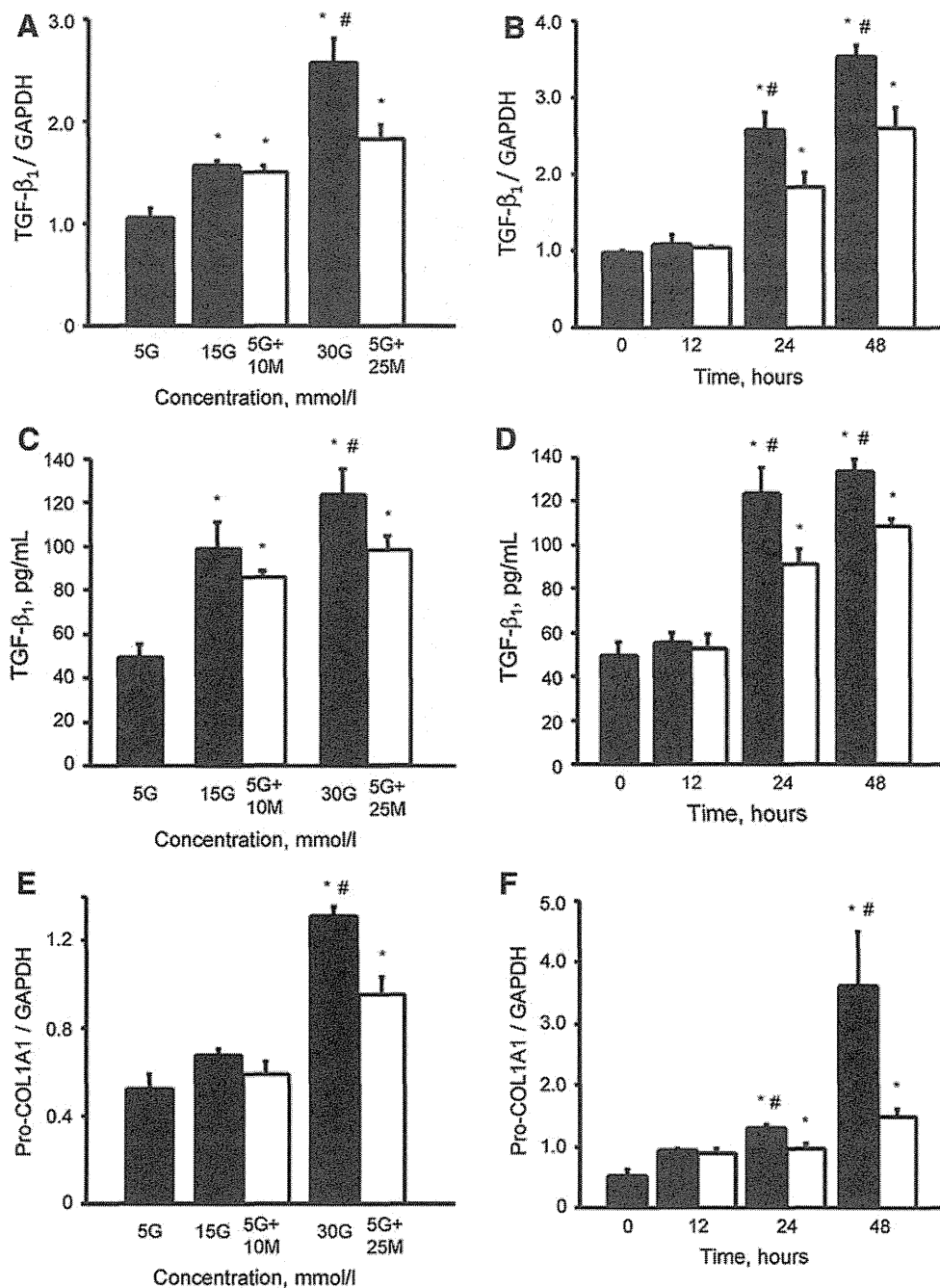
protein in the supernatant was decreased in the presence of cytochalasin B or NAC (Fig. 3d). The levels of TGF-β_1 mRNA and protein and pro-COL1A1 mRNA detected in the presence of cytochalasin B were not significantly different to those induced by 25 mmol/l D-mannitol with 5 mmol/l D-glucose (Fig. 3b, 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-β₁, 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-β₁, 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-β₁, pro-COL1A1 and CCL2 (Fig. 5b, d, e) and the migration rate of the cells (Fig. 5f). With regard to the expression of TGF-β₁, 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
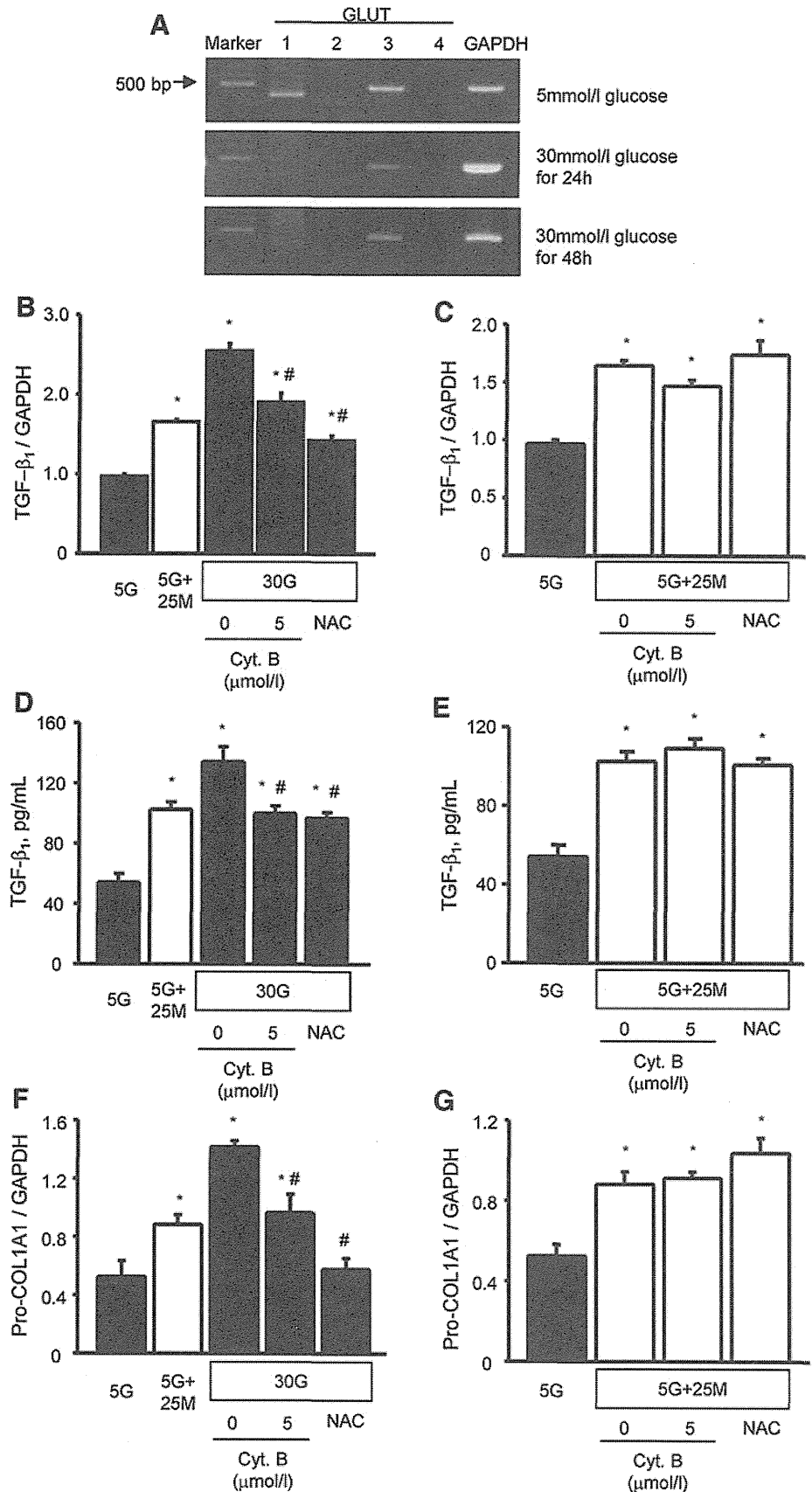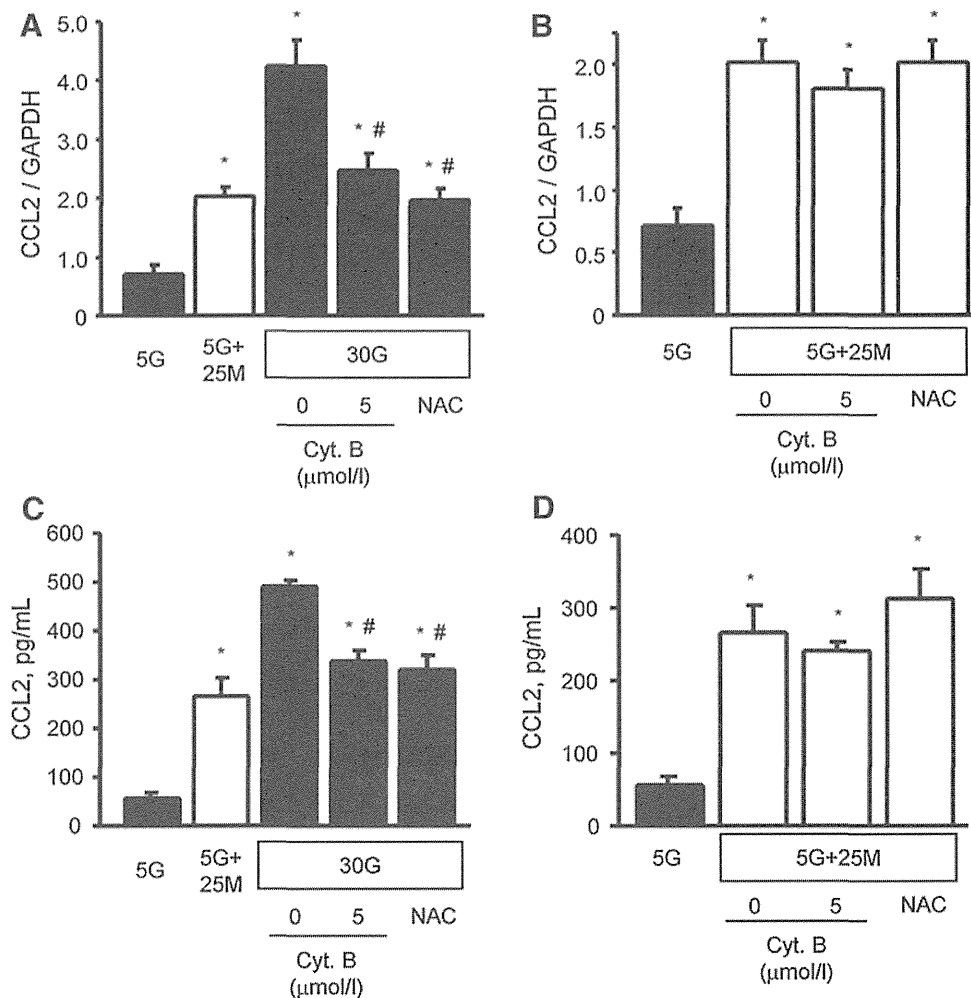
Fig. 4 Effect of cytochalasin B and N-acetylcysteine on the production of CCL2 in human isolated CD45$^+$/Col1$^+$ cells. Effect of cytochalasin B and N-acetylcysteine on 30 mmol/l D-glucose-induced CCL2 mRNA (**a**) and protein (**c**) synthesis in human CD45$^+$/Col1$^+$ cells. Data presented are the 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
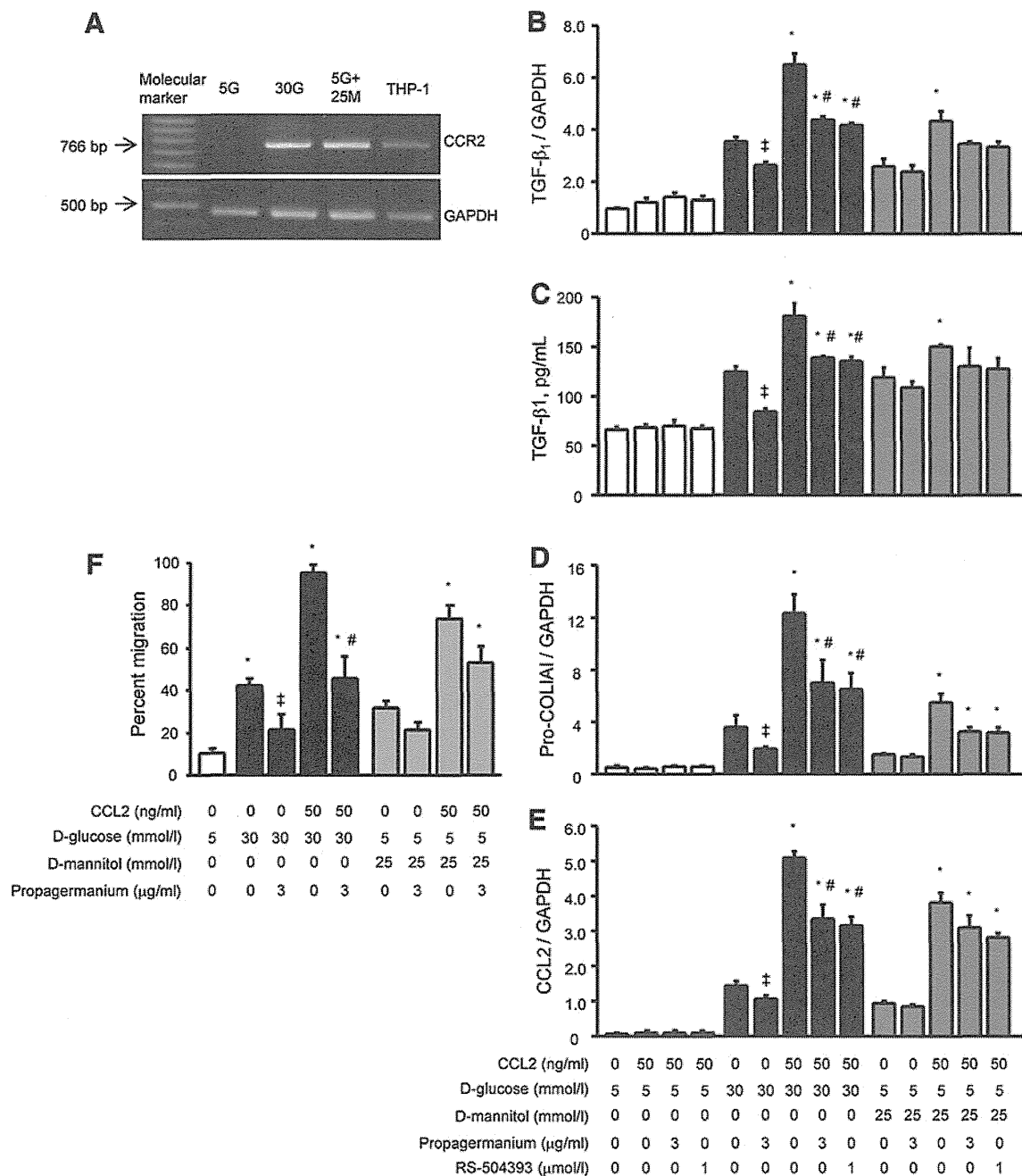
Fig. 5 Effect of CCL2/CCR2 signaling on the expression of TGF-β₁, pro-COL1A1 and CCL2 and on the extent of migration in isolated human CD45⁺/Col1⁺ cells. Human CD45⁺/Col1⁺ cells express CCR2 mRNA in response to high glucose concentrations (**a**). Figures are representative of three experiments. Stimulation of CD45⁺/Col1⁺ cells with CCL2 for 48 h under high glucose enhanced the expression of TGF-β₁ 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-β₁,

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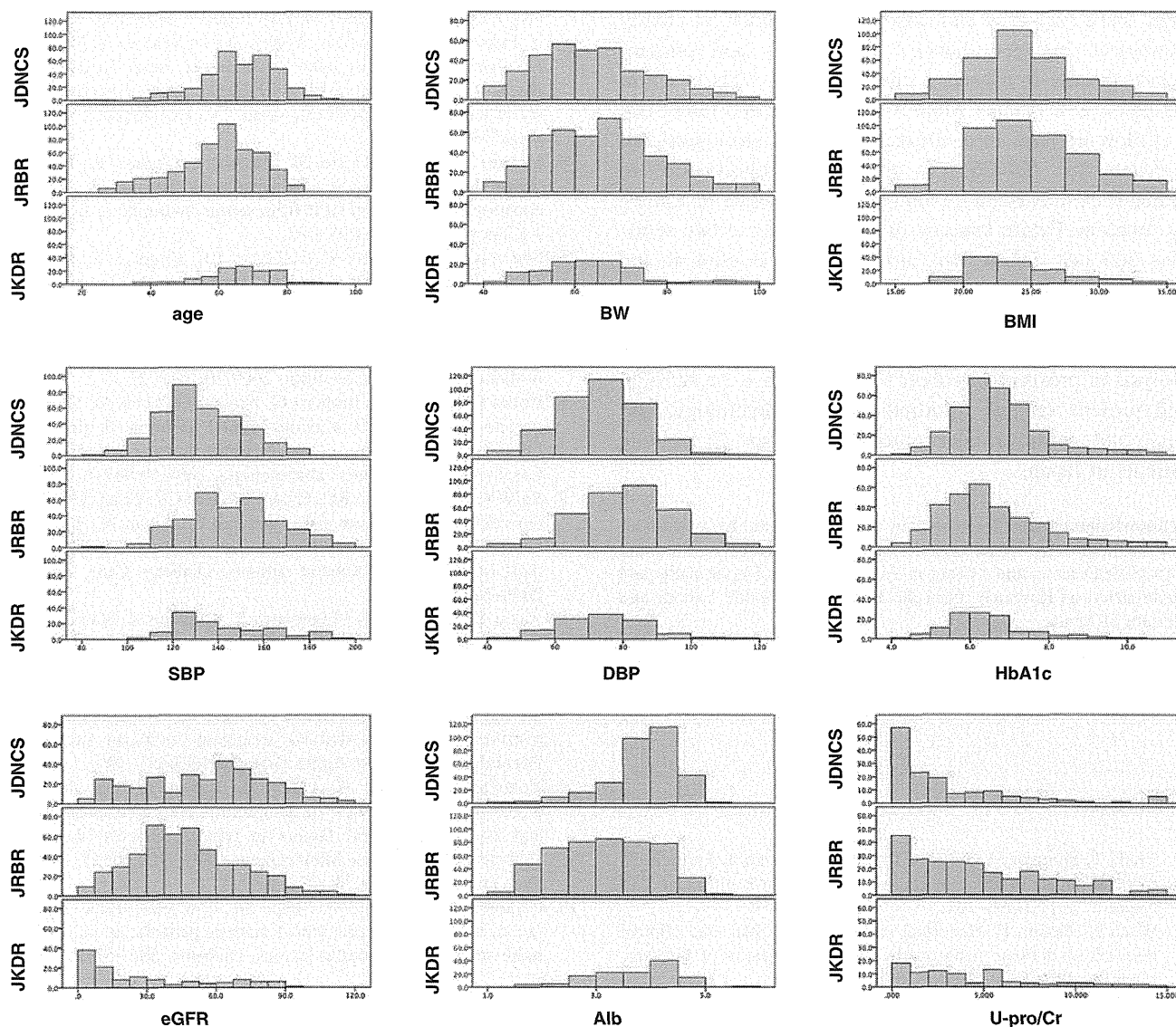
Fig. 1 Distribution of age, body weight (BW), body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA1c, estimated glomerular filtration ratio (eGFR), serum levels of albumin, and urinary levels of protein and creatinine ratio (U-pro/Cr) in JDNCS, JRBR, and JKDR

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