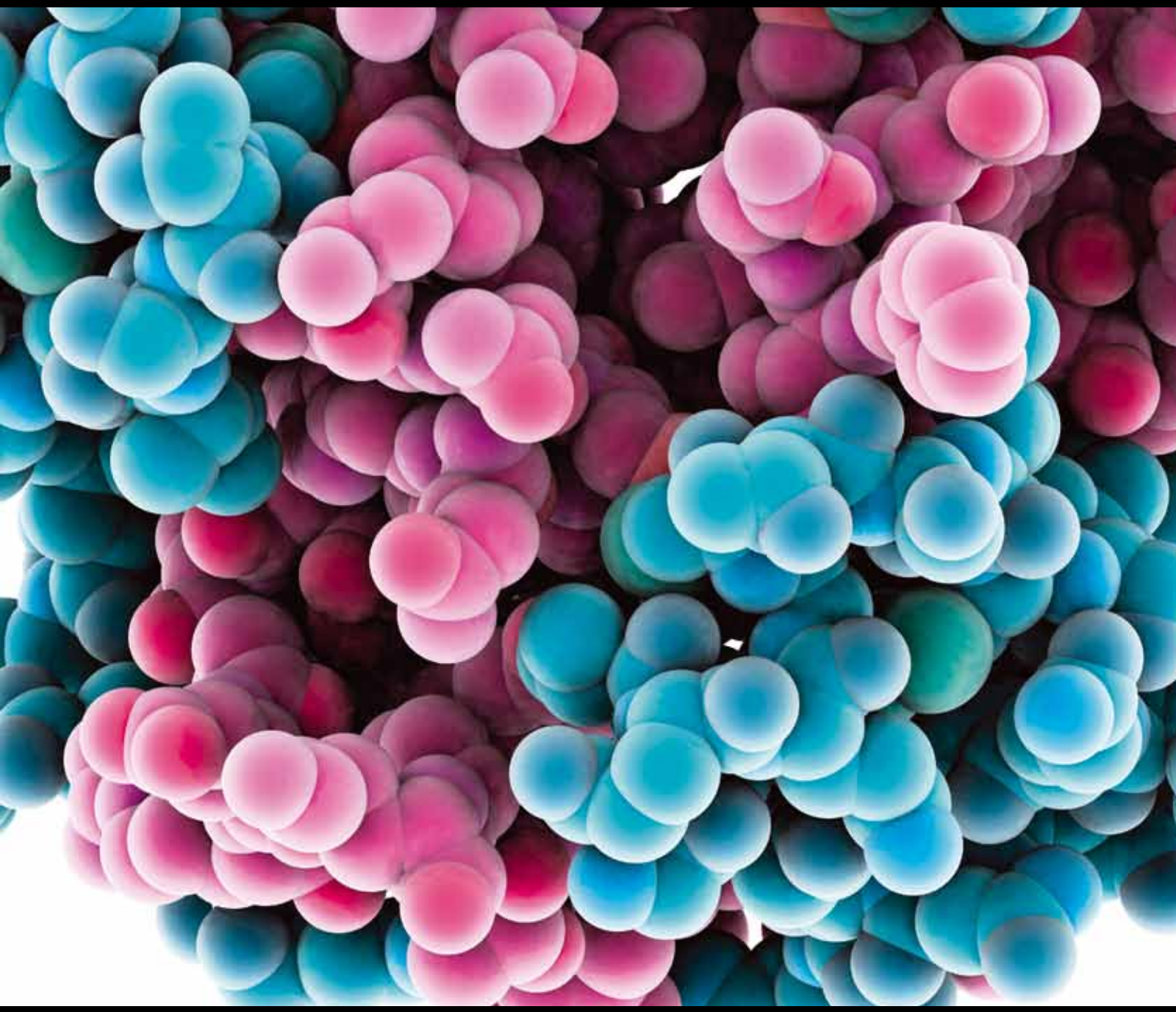
Journal of Diabetes Research

Combat Diabetic Nephropathy: From Pathogenesis to Treatment

Guest Editors: Tomohito Gohda, Akira Mima, Ju-Young Moon, and Keizo Kanasaki



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Contents

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Editorial

Combat Diabetic Nephropathy: From Pathogenesis to Treatment

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Diabetic nephropathy (DN) is the most common microvascular complication of diabetes and remains one of the leading causes of end-stage renal disease worldwide. The pathogenesis of DN was thought of as very simplistic up to several decades ago. Basically, the main risk factors contributing to the development and/or progression of DN are hyperglycemia, hypertension, dyslipidemia, and so on. In fact, Steno-2 study demonstrated that multifactorial therapies in patients with type 2 diabetes (T2D) successfully delayed the onset and retarded macrovascular and microvascular complications. However, the prevalence of nephropathy among patients with T2D has not fully decreased at this moment. Understanding other molecular mechanisms involved in DN is urgently needed for provision of better care for the patients.

In this special issue, original as well as review articles regarding the pathophysiologic mechanisms which are involved in the development and/or progression of DN were invited. Finally, we accepted six papers including three excellent review articles for the publication of this special issue after careful consideration.

In a research article of biomarker research, F.-q. Chen et al. (China Medical University, Shenyang, China) performed the multiple regression analysis and principle component analysis in patients with various stages of Chinese type 2 diabetes (112 normo-, 93 micro-, and 56 macroalbuminuric patients) to examine the predictive value of albuminuria as the dependent variable. They concluded principle component for hs-CRP, serum TNFα, serum amyloid-A (SAA) and urinary MCP-1 was determinant for prediction of albuminuria in addition to HbA1c.

In another biomarker research, N. A. Seman et al. (Karolinska University Hospital, Stockholm, Sweden) measured the plasma pentraxin 3 (PTX3) levels in Malay type 2 diabetic patients with ($n = 102$) and without nephropathy ($n = 91$). They clearly demonstrated the levels of PTX3 differ according to the presence or absence of nephropathy, obesity, and sex.

C. Gao et al. (affiliated Hospital of Luzhou Medical College, Sichuan, China) have tested the efficacy of MG132, a ubiquitin protease inhibitor in the treatment of DN using experimental type 1 diabetes. They showed that this drug might alleviate the kidney damage by inhibiting Smad7 ubiquitin degradation and TGF-β activation.

Finally, three review articles are especially exceptional. K. Yamahara et al. (Shiga University of Medical science, Shiga, Japan) proposed that autophagy insufficiency has some potential role in the progression of DN although it remains unclear whether autophagy activation is beneficial in all stages of DN. T. Nakagawa et al. (Kyoto University Graduate School of Medicine, Kyoto, Japan) proposed the importance of appropriate VEGF for normal renal physiology especially in diabetic condition. They emphasized that the coupling of VEGF-NO axis (both appropriate VEGF and NO levels) is required to maintain normal angiogenesis. S. Hagiwara et al.

(Baker IDI Heart and Diabetes Institute, Melbourne, Australia) wrote a nice review of the literature about the role of microRNA in DN, especially focused on renin-angiotensin system, AGE/RAGE signaling, and oxidative stress. Exciting data demonstrating the potential to attenuate or even reverse renal disease in experimental diabetes by restoring the expression of a dysregulated miRNA provide impetus for further studies in this area.

In summary, we still have a long way to go before resolution of combat DN but believe this special issue provides some clues to combat DN in the clinical setting. We would be most grateful if this special issue has been helpful for not only nephrologists and endocrinologists but also all internal physicians.

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Research Article

MG132 Ameliorates Kidney Lesions by Inhibiting the Degradation of Smad7 in Streptozotocin-Induced Diabetic Nephropathy

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Background. Smad7 is the main negative regulatory protein in the transforming growth factor-β (TGF-β) downstream signaling pathway, which plays an important role in diabetic nephropathy (DN) and may be related to the ubiquitin proteasome pathway (UPP). *Aim*. We investigated the role of UPP in regulating TGF-β/SMAD signaling and explored the therapeutic effect of the ubiquitin proteasome inhibitor MG132 on DN. *Methods*. Wistar rats were randomly divided into a diabetes group and a normal control group. Rats in the diabetes group were injected intraperitoneally with streptozotocin. Diabetic rats were then randomly divided into a diabetic nephropathy group (DN group), an MG132 high concentration (MH) group, and an MG132 low concentration (ML) group. After 8 weeks of treatment, 24-hour urinary microalbumin (UAlb), urinary protein/urinary creatinine (Up/Ucr) values, ALT, AST, Bcr, kidney damage, TGF-β, Smad7, fibronectin (FN), and Smurf2 were detected. *Results*. The body mass and Smad7 protein expression decreased in DN group, but kidney weight, kidney weight index, UAlb, Up/Ucr, FN and Smurf2 mRNA expression, and TGF-β protein expression increased. However, these changes diminished following treatment with MG132, and a more pronounced effect was evident in MH group compared to ML group. *Conclusion*. MG132 alleviates kidney damage by inhibiting Smad7 ubiquitin degradation and TGF-β activation in DN.

1. Introduction

Diabetic nephropathy (DN) is one of the most prevalent and serious microvascular complications of diabetes mellitus (DM) [1]. Early pathological characteristics are basement membrane thickening, increase in mesangial matrix, and extracellular matrix accumulation, followed by development of glomerulosclerosis and tubulointerstitial fibrosis, eventually leading to irreversible renal damage [2–5]. The exact pathogenesis of diabetic nephropathy has not yet been completely clarified.

Smad7 is the main negative regulatory protein and antifibrotic factor in the transforming growth factor β (TGF-β) downstream signaling pathway [6] and can compete with Smad2/3 for binding to the type I TGF-β receptor, blocking Smad2/3 activation. Smad7 can also be transferred to the cell membrane for degradation of Smad2/3 and TGF-β receptor complexes, as well as inhibition of TGF-β signal activation after binding to the Smad ubiquitin regulatory factor 2 (Smurf2). Activation of TGF-β plays an important role in the pathological progress of diabetic nephropathy [7], which involves increased expression of many cytokines, inflammatory cytokines and adhesion molecules, induction of fibronectin (FN) expression [8, 9], and involvement in actual development of diabetic nephropathy [10, 11].

The ubiquitin proteasome pathway (UPP) is the main mechanism for intracellular protein degradation, and can degrade specific proteins, and regulate cell differentiation and transcription; Smurf is a ubiquitin ligase, which belongs to the E3 ligase family, and can specifically degrade Smad proteins. It has been determined that Smad proteins are degraded by a ubiquitin mechanism [12]. The Smurf ligase family includes Smurf1 and Smurf2. The function of Smurf2 is carried out via binding with the TGF-β receptor complex through Smad7,

leading to ubiquitin degradation of Smad7, which weakens the inhibitory effect of Smad7 on the TGF-β receptor [6].

However, whether the UPP is activated or involved in the development of diabetic nephropathy in kidneys remains unclear. Research has shown that MG132 has therapeutic effects on diabetic nephropathy [13–15], but the mechanism by which it acts is unclear. The possibility that MG132 is able to inhibit activation of the TGF-β signaling pathway through blocking ubiquitin degradation of Smad7 in diabetic nephropathy has not been studied. Therefore, additional research to understand the relationship between the UPP and the TGF-β signaling pathway and the mechanism of action of MG132 in diabetic nephropathy is necessary. In this study, we established a rat model of diabetic nephropathy by using STZ and selected MG132 as the specific ubiquitin proteasome inhibitor for blocking the TGF-β/SMAD signaling pathway, in order to explore the relationship between the UPP and the TGF-β/SMAD signaling pathway in diabetic nephropathy.

2. Materials and Methods

2.1. Animal Model. A total of 45 male Wistar rats weighing 200 g were obtained from the Biotechnology Corporation of Teng Xing, ChongQing (China). Rats were kept in a special room with a stable ambient temperature of 18°C–22°C and housed in wire cages with free access to a standard diet and tap water for 7 days before the experiment. Blood glucose levels of all rats were measured prior to the experiment.

Rats were divided into two groups, namely, a control group (NC group, $n = 10$) and an experimental group ($n = 35$); diabetic rats in the experimental group were rendered diabetic by intraperitoneal injection of streptozotocin (Sigma-Aldrich, USA) at a dose of 60 mg/kg. Streptozotocin was dissolved in 0.1 M citrate buffer at pH 4.5. Meanwhile the rats in the NC group received, by intraperitoneal injection, the same volume of citrate buffer. After 3 days following the STZ injection, fasting glycemic measurements were performed in blood samples from tail veins; rats with a blood glucose level of ⩾16.7 mmol/L were confirmed as "diabetic," and 4 weeks later, diabetic rats presented with mild microalbuminuria (an early sign of DN) and were included in the study. Diabetic rats were then further divided into three groups: diabetic nephropathy group (diabetic control, $n = 10$). ML group (treated with 0.05 mg/kg MG132 every day (CALBIOCHEM, USA), $n = 10$), and MH group (treated with 0.1 mg/kg MG132 every day, $n = 10$), Meanwhile, the NC and diabetic nephropathy groups received intraperitoneal injections of the same volume of citrate buffer every day.

2.2. Sample Collection and Body Weight and Kidney Weight Determination. All rats were weighed and 24-hour urine was collected every day. After 8 weeks of injections, rats were sacrificed after anesthetizing with pentobarbital (50 mg/kg, 1% concentration). Blood biochemistry was analyzed from collection of heart blood. Both kidneys were weighed and cut along the coronal plane; the upper poles of the right kidneys were used for pathology analysis, and the remaining parts of the right kidneys were used for transmission electron microscope analysis. Left kidneys were dissected for the assessment of biochemical parameters. Renal tissues were preserved at −80°C until required for analysis.

2.3. Biochemical Measurements. Measurements of 24-hour urinary microalbumin (UAlb), urine creatinine concentration, urine protein/urine creatinine (Up/Ucr) concentration and ALT, AST, TP, ALB, BUN, Crea, and GLU of blood were measured by an automatic biochemistry analyzer.

2.4. Kidney Pathology. The upper poles of the right kidneys were rapidly removed, fixed in 10% formaldehyde, dehydrated by gradient ethanol, embedded in paraffin, and sectioned at 4 μm thickness. Renal sections were stained with HE and Masson staining. All sections were evaluated using a light microscope.

2.5. Kidney Transmission Electron Microscope. Renal cortices were cut into 1 mm pieces and fixed in 2.5% glutaraldehyde for 2 hours at 4°C. After being washed three times with 0.01 M phosphate buffer, samples were post-fixed in 1% osmic acid for 3 hours at 4°C. Samples were then dehydrated by gradient acetone and embedded in propylene oxide. Ultrathin sections (60 nm) were cut, double-stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (Philips Tecnai 10, USA).

2.6. Western Blot. Renal cortices were homogenized in lysis buffer (Kaiji, Shanghai, China) on ice for 30 minutes. Western blotting was performed as previously described [13]. Immunoblot analysis was performed using TGF-β antibody (rabbit, 1 : 1000; Cell Signaling Technology (CST), USA), Smurf2 antibody (rabbit, 1 : 1000; Abcam, USA), actin antibody (rabbit, 1 : 1000; Abcam, USA), Smad7 antibody (rabbit, 1 : 500; Boster Biological Technology, China). Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit) were obtained from the Beyotime Institute of Biotechnology, China. Proteins were detected using the enhanced chemiluminescence (ECL) system and ECL Hyperfilm (Millipore, USA).

2.7. Real-Time Fluorescent Quantitative PCR. Total RNA was extracted from renal cortical homogenate using an RNA extraction kit (Tiangen Biotech, Beijing, China). PCR was performed as previously described [16]. The primer sequences were as follows: FN, forward: 5′-CATACTCCTCCAGACCTACC-3′, reverse: 5′-TGGAGGTTAGTGGGAGCATC-3′, Smad7, forward: 5′- CTGCAACCCCCATCACCTTA -3′, reverse: 5′-GCAACGCCTCCATAGTC-3′, actin, forward: 5′-TGGCATTGTCATGGACTCTG-3′ reverse: 5′-CCAGAAGAAGTTGGGAATCTGA-3′.

2.8. Statistical Analysis. All experimental data were expressed as means ± S.D. ($\overline{x} \pm s$). For statistical evaluation of the data obtained in our study, one-way analysis of variance (one-way ANOVA) was used to compare more than two groups, followed by Fisher's least significant difference (LSD) test for multiple comparisons, by using the statistical package

Table 1: Results of Body Weight, Kidney Weight, and Kidney Weight Index. Body weight was lower in the experimental groups compared with the NC group, whereas it was significantly recovered in the MH and ML groups compared with the DN group. Conversely, kidney weight and kidney weight index were greater in the experimental groups, but decreased upon MG132 treatment. Changes in the MH group were more significant than the ML group.

Group	Amount	BW (g)	KW (g)	IKW (10^{-3})
NC group	10	428.60 ± 20.74	3.04 ± 0.26	6.77 ± 0.90
DN group	10	214.40 ± 7.89$^{\blacktriangle}$	5.06 ± 2.13$^{\blacktriangle}$	45.32 ± 1.83$^{\blacktriangle}$
MH group	10	322.60 ± 25.89$^{\blacktriangle\blacksquare *}$	3.58 ± 2.31$^{\blacktriangle\bullet}$	22.55 ± 2.7$^{\blacktriangle\bullet *}$
ML group	10	271.40 ± 28.17$^{\blacktriangle\blacksquare}$	3.77 ± 1.43$^{\blacktriangle\bullet}$	27.33 ± 2.97$^{\blacktriangle\bullet}$

$^{\blacktriangle}P < 0.05$ versus NC group, $^{\blacksquare}P < 0.01$ versus DN group, $^{\bullet}P < 0.05$ versus DN group,$^{*}P < 0.01$ versus ML group.

Table 2: Results of UV, UAER, and Up/Ucr ($\overline{x} \pm s$). The values of UV, UAER, and Up/Ucr were greater in the experimental groups compared with the NC group, and were the highest in the DN group. However, these values decreased in the MH and ML groups compared with the DN group, with a more pronounced decrease in the MH group compared with the ML group.

Group	Amount	UV (mL)	UAER (mg/24 h)	UP/UCR (g/gcr)
NC group	10	38.40 ± 3.78	3.21 ± 0.97	0.13 ± 0.03
DN group	10	169.75 ± 10.724$^{\blacktriangle}$	64.23 ± 11.45$^{\blacktriangle}$	0.54 ± 0.11$^{\blacktriangle}$
MH group	10	123.25 ± 24.6$^{\blacktriangle\bullet *}$	35.75 ± 10.06$^{\blacktriangle\bullet *}$	0.29 ± 0.04$^{\blacktriangle\bullet *}$
ML group	10	139.80 ± 14.87$^{\blacktriangle\bullet}$	42.88 ± 4.43$^{\blacktriangle\bullet}$	0.36 ± 0.06$^{\blacktriangle\bullet}$

$^{\blacktriangle}P < 0.05$ versus NC group, $^{\bullet}P < 0.05$ versus DN group, $^{*}P < 0.05$ versus ML group.

SPSS 13.0; a P value of <0.05 was considered as statistically significant.

3. Results

3.1. Change in Body Weight, Kidney Weight, and Kidney Weight Index. Body weight was lower in the experimental groups compared with the NC group ($P < 0.05$), but it significantly recovered in the MH and ML groups compared with the diabetic nephropathy group ($P < 0.05$). Conversely, kidney weight and kidney weight index were greater in the experimental groups compared with the NC group ($P < 0.05$) but decreased upon MG132 treatment ($P < 0.05$). Changes in the MH group were more significant than in the ML group ($P < 0.05$) (Table 1).

3.2. Volume of Urine (UV), Urinary Albumin Excretion Rate (UAER), and Urine Protein/Urine Creatinine (Up/Ucr) Ratio. The UV, UAER, and Up/Ucr ratios were increased in the experimental groups compared with the NC group, and were the highest in the diabetic nephropathy group ($P < 0.05$). However, values were less in the MH and ML groups compared with the diabetic nephropathy group ($P < 0.05$); the MH group had even lower values than the ML group ($P < 0.05$) (Table 2). Meanwhile, there was not significant change of ALT, AST and Bcr in each group ($P > 0.05$) (Table 3).

3.3. Renal Pathology. The renal tissue volume in the experimental group is larger than in the NC group in gross appearance. After HE and Masson staining, we observed, by light microscopy, that the glomerular volume was greater in the experimental group compared to the NC group. In addition, renal tubular edema, abnormal glomerular mesangial deposition, and atrophy and degeneration of renal glomeruli were evident in the experimental groups, although changes were significantly less in the MH group (Figure 1).

Table 3: Results of Bcr, ALT, and AST. The values of Bcr, ALT, and AST do not change in each group ($P > 0.05$).

Group	Amount	Bcr (μmmol/L)	ALT (U/L)	AST (U/L)
NC group	10	53.70 ± 4.01	18.3 ± 1.47	14.6 ± 1.04
DN group	10	57.5 ± 2.3	16.0 ± 1.72	16.1 ± 1.39
MH group	10	56.4 ± 5.53	20.1 ± 2.99	17.5 ± 1.82
ML group	10	54.9 ± 3.12	19.2 ± 1.86	16.9 ± 1.67

3.4. Transmission Electron Microscopy of the Kidney Tissue. Under the transmission electron microscope, we were able to visualize hydropic endothelial cells and podocytes, endothelial pore broadening, irregular thickening and decrease in the electronic density of the basement membrane, and partial foot process fusion in the experimental groups, especially in the diabetic nephropathy group, which were found to be recovered in the MH group (Figure 2).

3.5. Western Blot. There was a decreased expression of Smad7 in the experimental group compared with the NC group, particularly evident in the diabetic nephropathy group ($P < 0.01$), but Smad7 expression increased following MG132 treatment, and the increase in the MH group was more pronounced than in the ML group ($P < 0.05$). By contrast, TGF-β expression increased in the experimental group compared with the NC group, especially in the diabetic nephropathy group ($P < 0.01$), but MG132 treatment led to a decrease in TGF-β expression ($P < 0.05$), and there was a significantly lower expression of TGF-β in the MH group compared with the ML group ($P < 0.05$) (Figures 3 and 4).

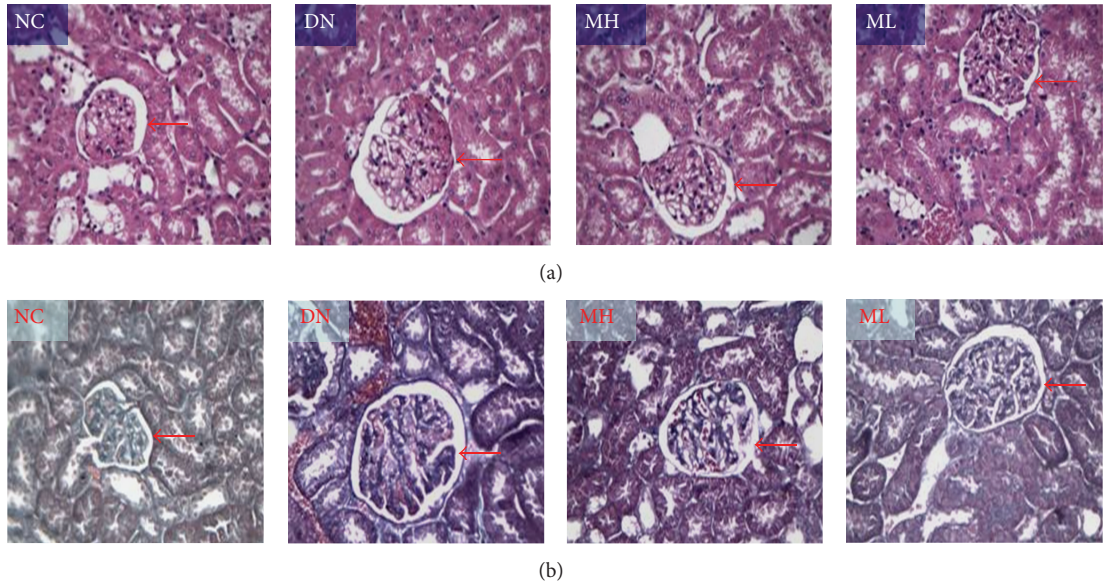

Figure 1: Changes of renal pathology observed by light microscopy. (a) HE staining (×200). (b) Masson staining (×200). The glomerular volume (red arrow) was greater in the experimental group than in the NC group, whilst renal tubular edema, abnormal glomerular mesangial deposition, and atrophy and degeneration of renal glomeruli were observed in the experimental groups, although changes were significantly less in the MH group.

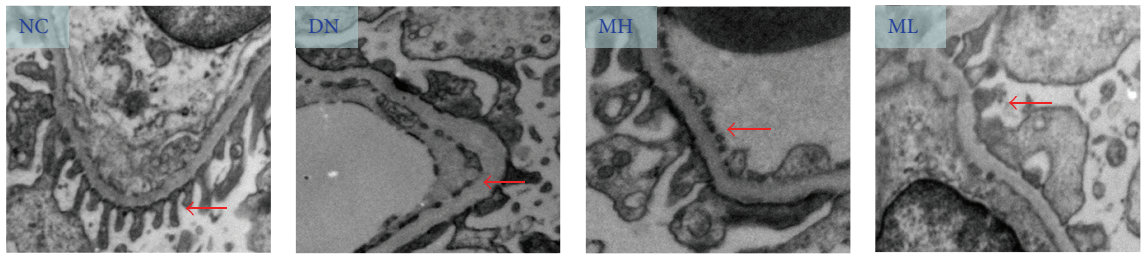

Figure 2: Transmission electron microscopy of the kidney tissue (×10,000). Hydropic endothelial cells and podocytes, endothelial pore broadening, irregular thickening and decrease in the electronic density of the basement membrane, and partial foot process fusion were visualized in the experimental groups (red arrow), especially in the diabetic nephropathy group, but were found to be recovered in the MH group.

3.6. Real-Time Fluorescent Quantitative PCR. The expression of FN mRNA and Smurf2 mRNA increased in each experimental group compared with the NC group, especially in the diabetic nephropathy group ($P < 0.01$). There was however a decrease in FN and Smurf2 mRNA expression in the MH and ML groups compared with the diabetic nephropathy group ($P < 0.05$), and this decrease was more pronounced in the MH group compared with the ML group ($P < 0.05$) (Figure 5). The mRNA expression of Smad7 do not have any significant change in each group ($P > 0.05$) (Figure 6).

4. Discussion

Currently, diabetic nephropathy has become the primary cause of end stage renal disease (ESRD) [17–19]. Previously, some studies found that a lot of cell signaling pathway could regulate the diabetic nephropathy fibrosis, such as NF-κB, MAPK, TGF-β and so on [7, 20, 21].The TGF-β signaling pathway has been recognized as an important one in diabetic nephropathy fibrosis, its main biological function being to promote renal cell hypertrophy and regulate ECM metabolism. TGF-β signaling may inhibit cell proliferation by controlling cell transformation from Gl phase to S phase, inducing cell hypertrophy, increasing matrix synthesis, and decreasing matrix degradation, so as to promote the accumulation of ECM [11].

4.1. The Regulatory Role of the UPP in the TGF-β Signaling Pathway in Early Diabetic Nephropathy and the Relationship between the UPP and DN. The ubiquitin proteasome pathway (UPP) is an important nonlysosomal protein degradation pathway, which is widespread in eukaryotic cells. It is able to efficiently degrade intracellular proteins with high selectivity, which affects cell cycle regulation, apoptosis, antigen presentation, inflammatory reactions, and gene transcription [22, 23]. In particular, the UPP can upregulate or downregulate signaling pathways by degrading the intracellular inhibiting or activating factor of each pathway [24].

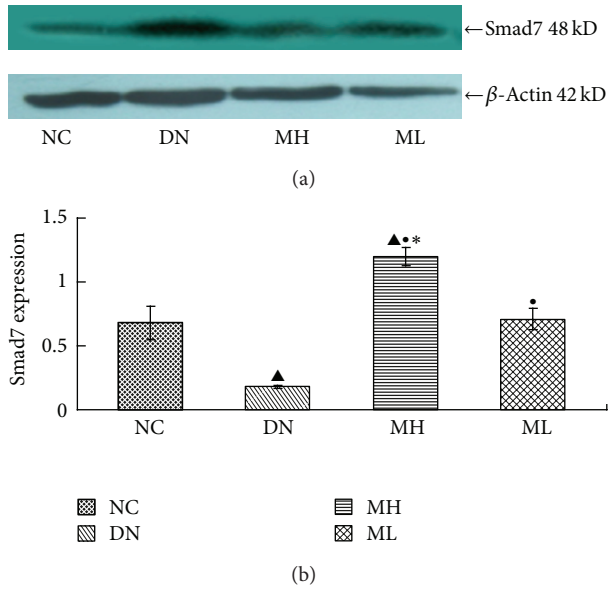

Figure 3: Smad7 protein expression in each group by Western blot. (a) Western blot strip chart. (b) The gray graph shows the relative statistical values of Smad7 for each group. Compared with the NC group, Smad7 expression decreased in the experimental groups, especially in the diabetic nephropathy group, with a subsequent increase in the MH and ML groups, and a more pronounced increase in the MH group compared with the ML group. $^{\blacktriangle}P < 0.01$ versus NC group, $^{\bullet}P < 0.01$ versus diabetic nephropathy group, and $^{*}P < 0.05$ versus ML group.

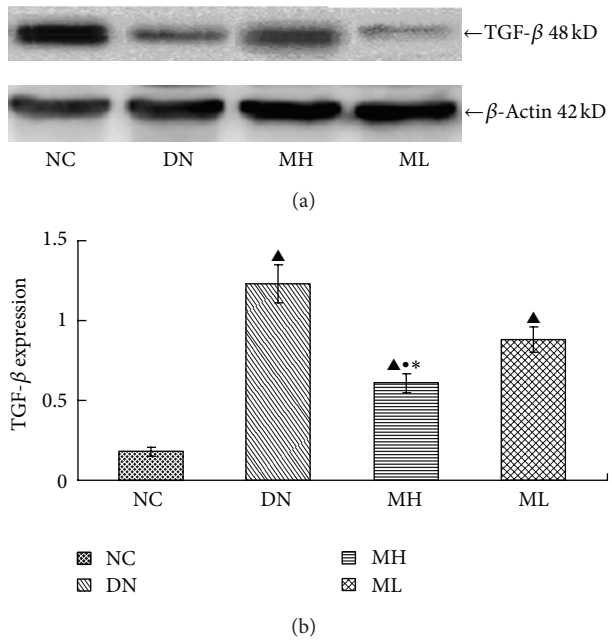

Figure 4: Expression of TGF-β protein in each group by Western blot. (a) Western blot strip chart. (b) The gray graph shows the relative statistical values of TGF-β for each group. Compared with the NC group, TGF-β expression increased in the experimental groups, especially in the diabetic nephropathy group, with a subsequent decrease upon MG132 treatment, and a more pronounced, significant decrease in the MH group.

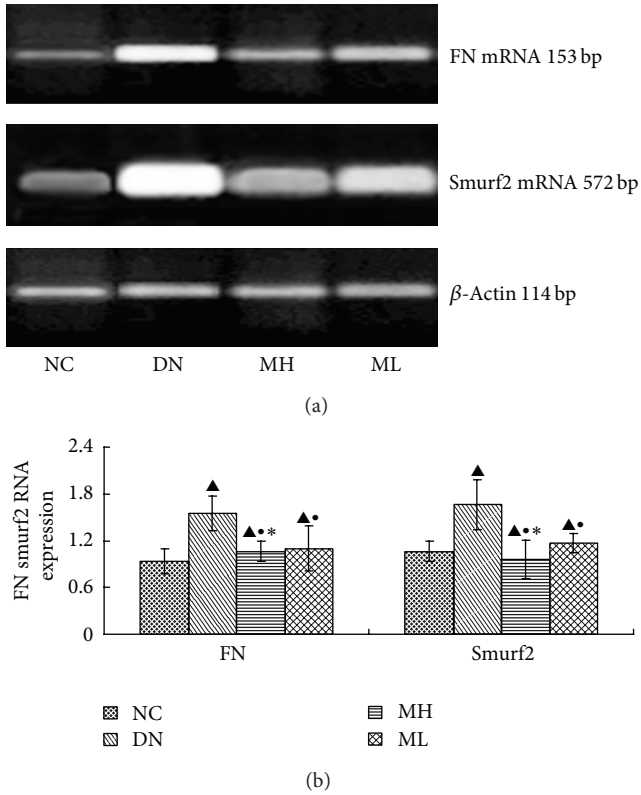

Figure 5: FN and Smurf2 mRNA levels by real-time fluorescent quantitative PCR in each group. (a) RT-PCR strip chart. (b) The gray graph shows the relative statistical values of the mRNA levels. Compared with the NC group, the levels of FN mRNA and Smurf2 mRNA increased in each experimental group, especially in the diabetic nephropathy group, but subsequently decreased in the MH and ML groups compared with the diabetic nephropathy group, with a more significant decrease in the MH group compared with the ML group.

Smad7 is the key negative regulatory protein of the TGF-β signaling pathway, is located downstream of the TGF-β signaling pathway and is regulated by UPP [25, 26]. Smad7 can bind to the Smad ubiquitin regulatory factor 2 (Smurf2) for transfer to the cell membrane, followed by degradation of Smad2 and 3 and TGF-β receptor complexes, and inhibition of the activation of the TGF-β signaling pathway. Smurf2 is a ubiquitin ligase, which belongs to the E3 ligase family. It can specifically degrade Smad7 and weaken the inhibitory effect of Smad7 on the TGF-β receptor.

TGF-β activation plays an important role in the development of diabetic nephropathy, which may involve the expression of fibronectin (FN). A recent study reported that UPP was the main method for specific intracellular protein degradation. Furthermore, ubiquitin levels were found to be increased in type 2 diabetic neuropathy compared with the control group [27], suggesting that the development of diabetic nephropathy may be due to the degradation of neuroproteins. Kaniuk et al. [28] showed that high quantities of ubiquitin were found surrounding the pancreatic β cells in diabetic rats. In recent years, studies have determined that Smurf2 expression is increased in kidney disease models,

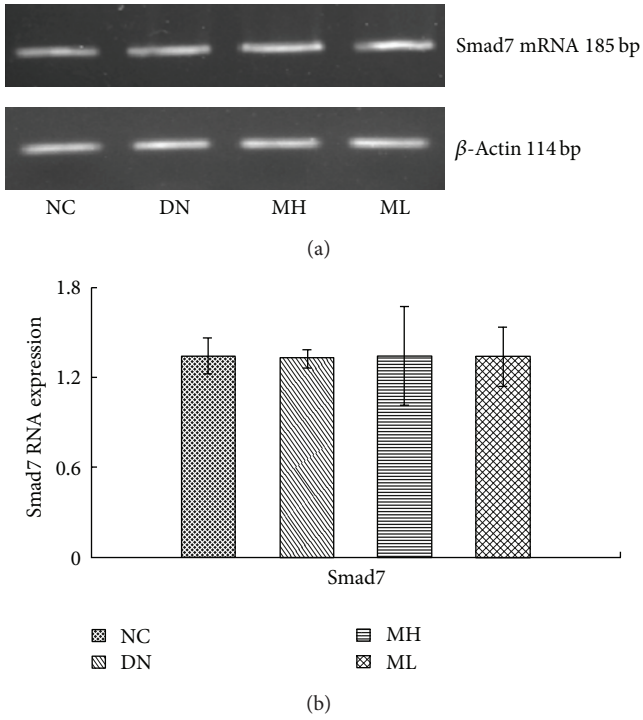

Figure 6: Smad7 mRNA levels by Real-Time fluorescent quantitative PCR in each group. (a) RT-PCR strip chart. (b) The gray graph shows the relative statistical values of the mRNA levels. There was not significant change of Smad7 mRNA levels in the different group.

for example, Smurf2 and TGF-β protein expression is increased after 7 days in the unilateral ureteral obstruction kidney, but expression is then decreased by the UPP inhibitor, MG132; at the same time, Smad7 expression is increased and fibrous degeneration is improved [29]. It has been reported that the UPP can reduce Smad7 expression in the kidney [6, 30]. These reports indicate that the UPP is involved in renal scarring.

However, whether ubiquitin degradation of Smad7, which regulates the TGF-β signaling pathway, is involved in the development of diabetic nephropathy by the UPP is unknown. In this study, we discovered that Smurf2, a member of the UPP family, increased in the diabetic nephropathy group, concomitantly with increased expression of TGF-β and FN, followed by decreased expression of Smad7. However, these effects were reduced by the UPP inhibitor, MG132. Results demonstrated that regulation of the TGF-β signaling pathway by Smad7 was involved in the development of DN. The UPP participated in the activation of the TGF-β pathway and induced the progress of diabetic nephropathy by ubiquitin degradation of Smad7.

4.2. The Therapeutic Effect of the Ubiquitin-Proteasome Inhibitor MG132 in Early Diabetic Nephropathy. Currently, the incidence of diabetic nephropathy is increasing annually, along with the increased incidence of diabetes. However, the mechanisms by which diabetic nephropathy develops are not fully elucidated, and the onset of diabetic nephropathy cannot be prevented despite strict control of blood glucose, blood pressure, and blood lipids due to monitoring dietary intake. Therefore, it is necessary to explore molecular mechanisms involved in DIABETIC NEPHROPATHY development in order to find a new therapeutic target.

A key catalytic enzyme, involved in ubiquitin degradation of target proteins in the UPP, is the 26s proteasome, which is specifically inhibited by the UPP inhibitor MG132 [11, 31–33]. Tashiro et al. discovered that this UPP inhibitor can alleviate renal interstitial fibrosis in unilateral ureteral obstruction nephrosis rats [31]. Recent studies have shown that MG132 can protect the kidney against diabetes-induced oxidative damage, inflammation, and fibrosis [13–15, 34], but the exact pathogenesis has not yet been completely clarified. Our previous study has found that MG132 could depress the activation of NF-κB inflammatory signaling through inhibiting the IκBα sumoylation and ubiquitination, and could inhibit the histone ubiquitination and induce apoptosis in rat glomerular mesangial cells induced by high glucose [13, 20] Renal fibrosis in diabetic nephropathy was induced by activation of the TGF-β signaling pathway, which mediates cell proliferation and differentiation, but whether the proteasome inhibitor could treat diabetic nephropathy by blocking ubiquitin degradation of Smad7 was not reported.

In our study, we found that administration of MG132 in diabetic nephropathy rats led to a decrease in body weight and Smad7 protein expression, while we did not observed the significant changes in the mRNA expression of Smad7. In addition, kidney weight, kidney weight index, UAER, Up/Ucr, TGF-β protein and FN mRNA levels decreased upon inhibition with MG132. The pathological changes upon observation with light and electron microscopes also reduced significantly in the MH group. Furthermore, MG132 can regulate the expression of Smurf2 mRNA in a concentration-dependent manner. This suggests that MG132, as a UPP inhibitor, can protect rat podocytes from damage, improve endothelial cell edema, maintain basement membrane permeability and reduce urinary protein. It can also inhibit Smurf2 expression, reduce ubiquitin degradation of Smad7, enhance the Smad7-induced inhibition of the TGF-β signaling pathway, and partially block TGF-β protein expression and FN mRNA to delay renal fibrosis. Moreover, we did not find it has obvious side effects on rats in our study, for example, the dysfunction of liver and kidney. Therefore, a novel mechanism in diabetic nephropathy may be the activation of the UPP, which increases ubiquitin degradation of Smad7, which is a inhibiting factor in the TGF-β/SMAD signaling pathway. MG132 can improve the early stages of diabetic nephropathy in rats by reducing diabetic renal pathological changes, improving nephropathy urine protein, partly lowering fibronectin expression and reducing renal fibrosis.

5. Conclusion

We have demonstrated that activation of the TGF-β signaling pathway is related to an increased ubiquitin degradation of the Smad7 protein by the UPP, in early DN. We have also

shown that MG132 has a therapeutic effect on early diabetic nephropathy by blocking ubiquitin degradation of Smad7 and thus inhibiting activation of the TGF-β pathway.

Conflict of Interests

The authors declare that there is no conflict of interests with the trademarks mentioned in their paper. The author Keri Aqie was the co-first author.

Acknowledgments

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Review Article

The Role of Autophagy in the Pathogenesis of Diabetic Nephropathy

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Diabetic nephropathy is a leading cause of end-stage renal disease worldwide. The multipronged drug approach targeting blood pressure and serum levels of glucose, insulin, and lipids fails to fully prevent the onset and progression of diabetic nephropathy. Therefore, a new therapeutic target to combat diabetic nephropathy is required. Autophagy is a catabolic process that degrades damaged proteins and organelles in mammalian cells and plays a critical role in maintaining cellular homeostasis. The accumulation of proteins and organelles damaged by hyperglycemia and other diabetes-related metabolic changes is highly associated with the development of diabetic nephropathy. Recent studies have suggested that autophagy activity is altered in both podocytes and proximal tubular cells under diabetic conditions. Autophagy activity is regulated by both nutrient state and intracellular stresses. Under diabetic conditions, an altered nutritional state due to nutrient excess may interfere with the autophagic response stimulated by intracellular stresses, leading to exacerbation of organelle dysfunction and diabetic nephropathy. In this review, we discuss new findings showing the relationships between autophagy and diabetic nephropathy and suggest the therapeutic potential of autophagy in diabetic nephropathy.

1. Introduction

The increasing prevalence of diabetes mellitus and its vascular complications has become a major health problem worldwide. Diabetic nephropathy is a serious complication of diabetes and is a common cause of end-stage renal disease. Diabetes induces glomerular damage, along with proteinuria, and subsequent tubulointerstitial lesions, leading to end-stage renal disease [1–3]. Initially, the patient shows hyperfiltration, represented by high glomerular filtration rates (GFRs) and occasional occurrence of microalbuminuria. Later, the patient shows a gradual decline in the GFR and persistence of microalbuminuria that comes before mild and subsequently moderate proteinuria. Urinary protein seems to be almost entirely reabsorbed in early and late proximal tubules and may induce tubulointerstitial damage [3]. Reducing proteinuria by keeping blood pressure and blood glucose levels under control is therefore a primary therapeutic goal with diabetic nephropathy [4, 5]. Unfortunately, however, some patients develop treatment-resistant proteinuria, resulting in end-stage renal disease. There is now an urgent need to identify new therapeutic target molecules or cellular processes that underlie the pathogenesis of diabetic nephropathy to establish additional therapeutic options.

Autophagy has recently been found to be a stress-responsive intracellular system, because it is likely that the disturbance of this machinery is involved in the pathogenesis of age- and diabetes-related diseases [6, 7]. Autophagy is a part of the catabolic processes that degrades damaged intracellular proteins and organelles [8]. Accumulating evidence suggests that autophagy activity declines in some organs under obesity conditions, and the functional roles of autophagy in the kidney have been gradually clarified. It has been reported that autophagy has a protective function against renal damage induced by aging [9, 10], hypoxia [11, 12], and anticancer drugs [13–15]. However, the relationship between autophagy and

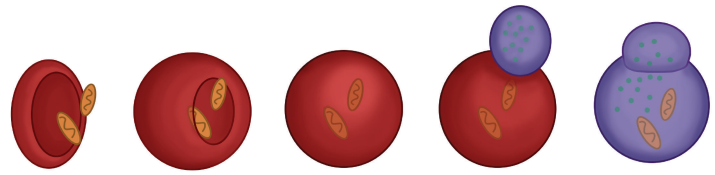

Figure 1: Steps of the autophagic pathways. Five steps in the autophagic pathways have been identified: (1) initiation, isolated membrane appears in cytosol; (2) elongation, elongation is characterized by membrane bending and an increase in the size of the phagophore; (3) closure, the autophagosome membrane wraps around the cytosolic components; (4) fusion, the fusion of the autophagosome with a lysosome to form an autolysosome; and (5) breakdown, the autolysosome is degradated by lysosomal hydrolases.

diabetic nephropathy remains to be elucidated, although several recent papers have suggested that autophagy machinery is involved in the pathogenesis of diabetic nephropathy. In this review, we summarize and discuss recent findings on the role of autophagy in diabetic nephropathy.

2. Autophagy

The term "autophagy" is derived from Greek and means self-eating. Autophagy is highly conserved from yeast to mammals. It is a bulk degradation process involved in the clearance of damaged proteins and organelles. Autophagy works to maintain cell homeostasis under various stress conditions. Three types of autophagy have been identified in cells: macroautophagy, microautophagy, and chaperone-mediated autophagy. All types differ in their mechanisms and functions [16, 17]. Of the three types, macroautophagy is the most prevalent and in this review is referred to as autophagy.

During autophagy, *de novo* isolation membranes (phagophores) elongate and fuse while engulfing a portion of the cytoplasm within double-membrane vesicles (autophagosomes). The origin of the autophagosomal membrane is likely to be the endoplasmic reticulum (ER) membrane [18]. Five major steps are involved in the formation of autophagosomes: initiation, elongation, closure, fusion, and breakdown (Figure 1). During these steps, autophagy-related genes (Atg) and proteins are involved. Autophagy is initiated by the unc-51-like kinase (Ulk) 1 (the mammalian ortholog of yeast Atg1) complex, which comprises Ulk1 Ser/Thr protein kinase, Atg13, and FIP200 (mammalian homolog of yeast Atg17) (Figure 2(a)) [19–21]. Phosphorylation of Atg13 and FIP200 by Ulk1 is essential to trigger autophagy. Phagophore nucleation is dependent on Beclin 1 (Atg6 in yeast), an hVps34 or class III phosphatidylinositol 3-kinase (PI3 K) complex, which comprises hVps34, hVps15, Beclin 1, and Atg14 (Figure 2(b)) [22, 23]. During autophagosome elongation/closure, two dependent ubiquitin-like conjugation systems are involved: Atg12 and LC3 (the mammalian ortholog of yeast Atg8) [24].

The Atg12-Atg5 conjugate, which forms the Atg12-Atg5-Atg16 complex, contributes to the stimulation and localization of the LC3 conjugation reaction. The cytosolic isoform of LC3 (LC3-I) is conjugated to phosphatidylethanolamine through two consecutive ubiquitin-like reactions catalyzed by E1-like enzyme Atg7 and the E2-like enzyme Atg3 to form LC3-II (Figure 2(c)) [25]. Thus, LC3-II formation is recognized as a marker of the existence of autophagosomes in cell or animal experiments [26–28]. After formation, autophagosomes merge with the lysosomal compartment to form autolysosomes. The protein p62, also known as sequestosome 1, is known to localize to autophagosomes via LC3 interaction and to be constantly degraded by the autophagy-lysosome system [29, 30]. The accumulation of p62 is observed in autophagy-deficient cells [29, 30].

3. Mechanisms of Autophagy Regulation

Autophagy is upregulated in response to nutrient starvation and extracellular or intracellular stress. In this section, we outline the regulatory mechanism underlying nutrient starvation- and stress-induced autophagy activation in cells.

During nutrient deprivation, autophagosome formation is dramatically induced. In both yeast and mammalian cells, two well-characterized signaling cascades that sense nutrient status, the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and AMP activated protein kinase (AMPK) pathways, are potent regulators of autophagy. Autophagy is induced by AMPK, which is a key energy sensor of AMP, and is upregulated by an increase in intracellular levels of AMP [31]. Conversely, autophagy is inhibited by mTORC1, a central cell growth regulator that integrates growth factor and hypernutrient signals [32–34].

AMPK monitors the energy condition of a cell by sensing the AMP/ATP ratio [35]. Autophagy is activated with low-glucose conditions in cultured cells [36]. Under glucose deprivation, ATP concentrations decrease and subsequently AMPK is activated in cells. There are several upstream kinases that can activate AMPK by phosphorylating a threonine residue on its catalytic α subunit, liver kinase B1, calcium/calmodulin kinase and TGF-β-activated kinase-1 [35]. AMPK can activate autophagy via two independent mechanisms: suppression of mTORC1 activity and direct control of ULK1 phosphorylation [37].

mTOR is an evolutionarily conserved protein kinase and forms two functional complexes, termed mTORC1 and mTOR complex 2 [32, 33]. mTORC1 is a rapamycin-sensitive protein kinase complex and regulates a wide array of cellular processes including cell growth, proliferation, and autophagy

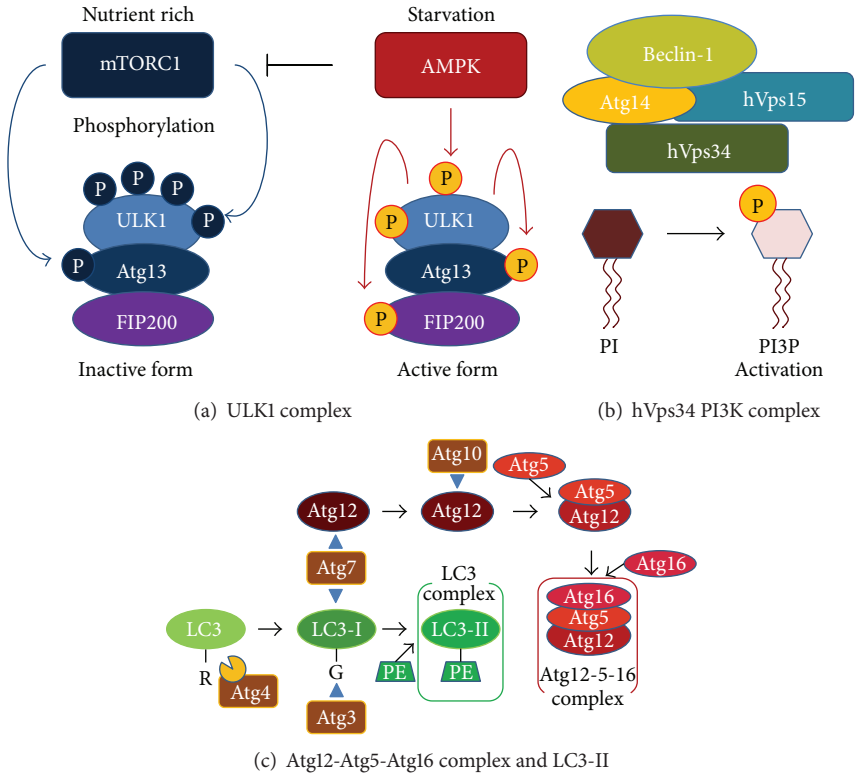

Figure 2: Autophagy regulation. (a) ULK1 protein kinase complex. ULK1 is a critical regulator of nutrient-related autophagy. mTOR-dependent phosphorylation of ULK1 (Atg1) and Atg13 under nutrient-rich conditions inhibits autophagy. In contrast, AMPK-dependent phosphorylation of ULK1 activates autophagy induction under energy-depleted condition. (b) PI3K complex. Phosphatidylinositol 3-kinase (PI3K) phosphorylates phosphatidylinositol in the membrane lipid to create phosphatidylinositol 3-phosphate. Class III PI3K comprises hVps34, hVps15, Beclin-1, and Atg14. (c) Atg12-Atg5-Atg16 complex and LC3-II. Unlike other ubiquitin-like proteins, the ubiquitin-like protein Atg12 has a C-terminal glycine, which protects it from processing. Atg12 is conjugated to the substrate Atg5 by Atg7 and Atg10. The Atg12-Atg5 conjugate forms a complex with Atg16. Self-oligomerization of Atg16 results in a multimer of the Atg12-Atg5-Atg16 complex. After the ubiquitin-like protein LC3 has had its C-terminal arginine residue cleaved by the cysteine protease Atg4, it is passed on to Atg7 and Atg3 and transferred into the head group of its substrate phosphatidylethanolamine (PE). This LC3-PE conjugate functions as part of the membrane component of the autophagosome. When LC3-PE is once again deconjugated to PE by Atg4, Atg8 is recycled.

in response to nutrients such as amino acids and growth factors [33, 38, 39]. mTORC1 activity reflects cellular nutritional status. Therefore, a better understanding of how mTORC1 regulates autophagy is of great importance because it may link nutrient signals to the regulation of autophagy. mTORC1 activity is finally and positively regulated by a lysosomal, membrane-anchored, small GTPase named Rheb [38–40].

Insulin signal phosphorylates protein kinase B (Akt) via PI3K and phosphoinositide-dependent kinase-1. Phosphorylated Akt suppresses tuberous sclerosis 2, a strong Rheb suppressor [38–40]. Therefore, an insulin signal suppresses autophagy via mTORC1 activation in cells through signal transduction.

Amino acids are also required for full activation of mTORC1 [41]; however, the mechanism of mTORC1 activation by amino acids is different from that of insulin. Recent research suggests that activation of mTORC1 by amino acids correlates with the translocation of mTORC1 from the cytoplasm to lysosomal membranes via Ras-related GTP-binding protein (Rag)-dependent system [42]. Activated mTORC1 can phosphorylate and inhibit Ulk1, which is a critical molecule in initiating autophagosome formation, leading to inhibition of autophagosome formation [20].

In addition to nutrient starvation, several intracellular stresses can induce autophagy. Reactive oxygen species (ROS) are small and highly reactive molecules that can oxidize proteins, lipids, and DNA. It has been reported that ROS induces autophagy through multiple mechanisms. Some reports have shown that exogenous hydrogen peroxide can activate PKR-like kinase (PERK), which subsequently phosphorylates eIF2a, oxidizes and activates Atg4 proteases [43], and thereby accelerates the production of proteolytic mature LC3 and inhibits mTORC1 activity. The cellular response to an increase in ROS often involves the activation of mitogen-activated protein kinases, including JNK1, which can activate autophagy [44, 45]. Furthermore, cells must remove damaged mitochondria to prevent the accumulation of ROS. This process of mitochondrial quality control is mediated by mitophagy, the selective autophagic removal of mitochondria. In response to potentially lethal stress or damage, mitochondrial membranes can be permeabilized through multiple distinct biochemical routes. The autophagic recognition of

depolarized mitochondria is mediated by a refined voltage sensor, involving mitochondrial kinase, PINK1 accumulation [46, 47].

Hypoxia also activates autophagy. In response to hypoxia, HIF1 transcription factor is activated [48, 49] and induces the transcription of BNIP3 and NIX. Their protein products compete with Beclin-1 for the binding of BCL2, thereby releasing Beclin-1 and allowing it to induce autophagy [50].

Autophagy also plays an important role in the maintenance of the structural and functional integrity of the ER. ER is not only involved in protein synthesis and maturation but may also constitute a major source/scaffold of the autophagic isolation membrane [51]. The unfolded protein response (UPR), the major ER stress pathway [52], is a potent stimulus of autophagy. Three sensors located on the membrane of the ER are responsible for monitoring ER stress and initiating the UPR: inositol requiring ER-to-nucleus signal kinase-1, PERK, and activating transcription factor-6 (ATF6). Among these, PERK and ATF6 act as autophagy inducers [53]. PERK mediates the transcriptional activation of proteins LC3 and Atg5 through the action of transcription factors ATF4 and CHOP, respectively [54]. PERK may also reduce translation of IκBa, thereby activating NF-κB, which also could contribute to autophagy. These intracellular stresses have recently been studied as a pathogenesis of diabetic nephropathy, in addition to the classical pathogenesis of diabetic nephropathy.

4. Autophagy in Podocytes under Diabetic Conditions

Podocytes are highly specialized, terminally differentiated, and unable to proliferate. Podocyte loss due to apoptosis and podocyte dysfunction contributes to proteinuria in patients with diabetic nephropathy [55–57]. Thus, maintaining podocyte cell homeostasis is regarded as a therapeutic target in diabetic nephropathy.

Autophagy is likely to play an essential role in maintaining podocyte function. Podocytes show high rates of autophagy even under nonstress conditions, suggesting that podocytes need to maintain cellular homeostasis by autophagy under basal conditions [9, 58–60]. In contrast, proximal tubular cells can proliferate and show low rates of autophagy under basal conditions [11, 14].

It has recently been reported that podocyte-specific autophagy depletion (*Atg5* gene depletion) leads to glomerulopathy in aging mice, accompanied by accumulation of oxidized and ubiquitinated proteins, ER stress, and proteinuria [9]. The role of autophagy in podocytes under diabetic conditions is still unclear. However, some reports suggest that autophagy may be involved in the pathogenesis of diabetic nephropathy. High-glucose conditions in cultured podocytes inhibit high basal autophagy by suppressing the expression of Beclin-1, Atg12-5, and LC3, and inhibition of basal autophagy impairs the filtration barrier function of podocytes [61]. Furthermore, this study reported that autophagy activity decreased in podocytes under streptozotocin (STZ)-induced type 1 diabetic conditions [61]. These results suggest that hyperglycemia reduces autophagic activity in podocytes, which may contribute to diabetes-related podocyte injury (Figure 3).

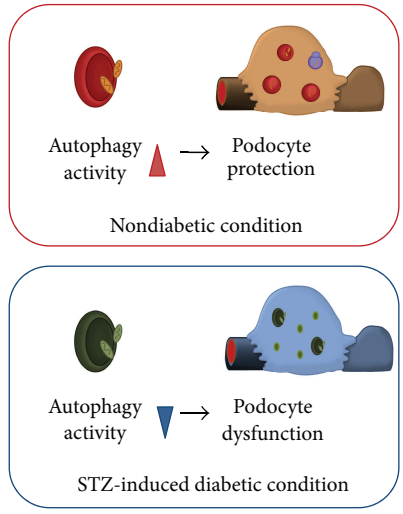

Figure 3: Autophagy in podocytes under diabetic conditions. Podocytes show high rates of autophagy even under nonstress conditions (upper panel). Autophagy activity is altered in podocytes under streptozotocin-induced type 1 diabetic conditions (lower panel). These results suggest that hyperglycemia alters autophagic activity, which may contribute to diabetes-related podocyte injury.

Autophagy activity is tightly associated with mTORC1 activity [62]. Interestingly, in podocytes of diabetic mice and patients, mTORC1 is highly activated and may be involved in the mechanisms of diabetes-related autophagy inhibition in podocytes [63]. Furthermore, podocyte hypertrophy is a predictor of renal lesion progression in patients with diabetes [64], and mTORC1 hyperactivation in the presence of hyperglycemia probably mediates a sustained hypertrophic stimulus that results in podocyte degeneration, the development of glomerulosclerosis and proteinuria [65]. These results suggest that the mTORC1-autophagy axis may be a future therapeutic target in diabetic nephropathy.

Rapamycin, a potent mTORC1 inhibitor, can ameliorate glomerular lesions in diabetic animal models [66, 67]. However, it is still not clear whether autophagy activation is involved in the mechanism underlying the rapamycin-mediated renoprotective effects in diabetes.

Some studies have reported that AMPK activation reduced podocyte permeability to albumin and podocyte dysfunction in STZ-induced diabetic mice [68]. In addition, several studies have reported that AMPK activation by AICAR or adiponectin shows podocyte protective effects against various nephrotoxic conditions. Although further evidence is required, it appears that autophagy activation is involved in AMPK-mediated podocyte protection.

5. Autophagy in Proximal Tubular Cells in Diabetic Nephropathy

The renal prognosis of diabetic patients with proteinuria is very poor compared with that of nondiabetic patients with

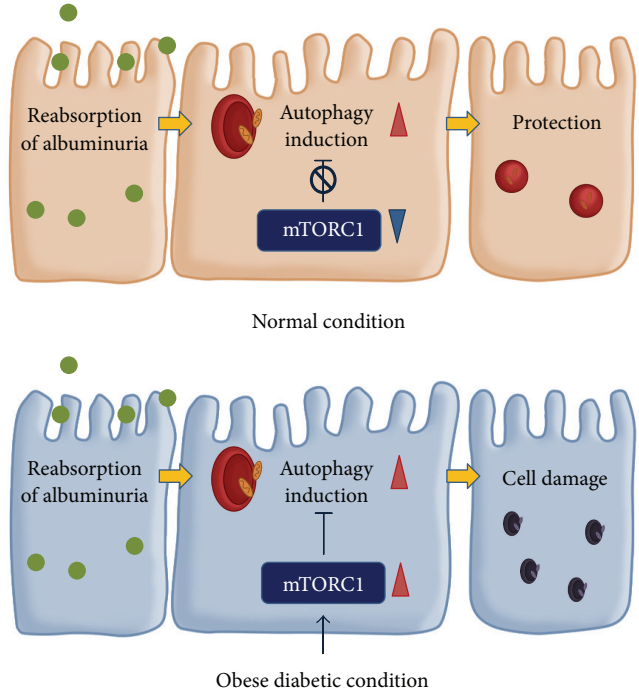

Figure 4: Autophagy in proximal tubular cells under diabetic conditions. Proximal tubular cells show low rates of autophagy under basal conditions. Proteinuria renoprotectively elicits autophagy in proximal tubular cells (upper panel). Obesity suppresses proteinuria-induced autophagy via hyperactivation of mTORC1 in proximal tubular cells, leading to obesity-mediated exacerbation of proteinuria-induced tubulointerstitial damage (lower panel).

proteinuria. Because proteinuria induces tubulointerstitial damage leading to progressive renal function decline, the diabetic condition may exacerbate proteinuria-induced proximal tubular cell damage leading to a poor renal outcome in diabetic patients with persistent proteinuria [1–3, 69]. Thus, identifying the mechanisms underlying the vulnerability of proximal tubular cells may lead to new therapies in diabetic patients.

The roles of autophagy in podocytes and proximal tubules are likely to be different. Autophagy activity in proximal tubular cells under basal conditions is very low compared with that in podocytes. Conversely, autophagy is extremely active under several nephrotoxic stresses, such as anticancer drugs and ischemia-reperfusion [11–15, 70]. Previous studies using proximal tubular cell-specific autophagy-depleted mice suggest that autophagy shows a renoprotective effect against acute kidney injury [11, 13, 14, 70]. It has also been clarified that the renoprotective effect of autophagy is not only against acute kidney injury but also chronic kidney damage, such as that with aging.

Proteinuria filtered from glomeruli has a nephrotoxic effect in proteinuric kidney diseases including diabetic nephropathy [2, 3, 69]. In an experimental mouse model that induced proteinuria-induced tubulointerstitial lesions, autophagy was activated, especially in proximal tubular cells that reabsorbed proteinuria. Of note, proteinuria-induced tubular cell damage was exacerbated in the kidneys of proximal tubular cell-specific autophagy-depleted mice, and obesity significantly suppressed proteinuria-induced autophagy. Obesity-mediated autophagy deficiency is therefore likely to be involved in the pathogenesis of the vulnerability of proximal tubular cells under diabetic conditions [71]. It appears that autophagy has a renoprotective role in proximal tubular cells under both acute and chronic conditions.

A recent study by the authors suggests that obesity significantly suppressed the renoprotective action of autophagy in proximal tubular cells, and autophagy insufficiency was confirmed in renal biopsy specimens from patients with type 2 diabetes or obesity with proteinuria [71]. In that study, we also examined the mechanisms underlying autophagy deficiency-induced proximal tubular cell damage in high-fat diet-induced obese mice and patients with type 2 diabetes or obesity with proteinuria [71]. The results suggested that hyperactivation of mTORC1 signaling in proximal tubular cells was involved in obesity-mediated autophagy suppression (Figure 4) [71]. Interestingly, obesity-mediated suppression of proteinuria-induced autophagy was recovered by diet restriction and treatment with rapamycin, a specific inhibitor of mTORC1 signaling, suggesting that obesity-mediated autophagy deficiency is a reversible phenomenon [71]. A recent study has reported that dietary restriction ameliorates diabetic nephropathy through anti-inflammatory effects and

regulation of autophagy via restoration of sirt1 in diabetic Wistar fatty (fa/fa) rats [72]. Therefore, restoration of autophagy activity may be a new therapeutic target for overt proteinuria in patients with diabetic nephropathy.

What kinds of metabolic alterations are associated with diabetes- and obesity-related autophagy insufficiency in proximal tubular cells? In diabetic conditions, hyperglycemia, hyperinsulinemia, and higher level of plasma free fatty acids are major metabolic alterations caused by the insufficient insulin actions to insulin-sensitive organs, hepatocytes, skeletal muscle cells, and adipocytes. Based on our recent study, free fatty acids caused autophagy in response to lipotoxicity-related intracellular stress [71]. It has been well known that glucose and insulin are able to inhibit autophagy in various cells. Thus, hyperglycemia and hyperinsulinemia rather than higher level of plasma fatty acids may contribute to diabetes-related suppression of autophagy in the kidney. Further examinations are needed to conclude it.

6. Drug Discovery Targeting Autophagy

Drug discovery research aimed to regulate autophagy is in progress worldwide. There are some chemical mediators that stimulate macroautophagy [73, 74]; however, no drug has yet been developed that stimulates microautophagy or chaperone-mediated autophagy. Drug research is currently focused on regulating autophagy in several degenerative and malignant diseases.

As mentioned above, autophagy activity is suppressed under diabetic conditions. We have therefore focused on a strategy for the resumption or activation of autophagy. There are two major strategies for activation of autophagy: the mTORC1-dependent pathway and the mTORC1-independent pathway.

Rapamycin is a potent activator of autophagy via the inhibition of mTORC1 [75] and appears to ameliorate mesangial expansion, glomerular basement membrane thickening, and renal macrophage recruitment in type 1 diabetic rats and to prevent proteinuria [67]. Thus, an mTOR inhibitor, such as rapamycin, has been the focus as a type of drug for that treatment of diabetic nephropathy via autophagy activation. However, other reports have suggested that complete inhibition of mTORC1 signaling by treatment with rapamycin exacerbates glomerular damage along with proteinuria in animal and human studies [76, 77]. This is a major adverse effect of mTORC1 inhibitor. Long-term mTOR inhibition may be associated with induction of malignancy as well as proteinuria, because it is well established that the mTOR pathway is necessary for activating the immune system [78]. Furthermore, recent studies suggest that mTOR inhibitor induces insulin resistance. In this respect, nonspecific mTORC1 inhibition, including rapamycin treatment, is harmful although it can activate autophagy. A mechanism specific to mTORC1-dependent autophagy suppression needs to be identified to develop safer drugs to activate autophagy via mTORC1 suppression.

As described above, AMPK activation is also a potent activator of autophagy. It has been revealed that AMPK stimulators such as metformin can work as autophagy activators [79]. There are few reports detailing its adverse effects on human and animal health [80, 81]. If pharmacological AMPK activation really acts as an autophagy activator, a drug that stimulates AMPK may be a potential therapy for diabetic nephropathy [82]. Several studies have reported that AMPK activation shows renoprotective effects in diabetic nephropathy. Autophagy may be involved in AMPK-mediated renoprotective action.

Previous reports suggest that trehalose, a disaccharide, activates autophagy in a mTORC1-independent manner [83]. Several antiepileptic and mood stabilizer drugs (e.g., LiCl, valproate, and carbamazepine) also have the ability to activate autophagy and degrade proteins via the mTORC1-independent pathway in some types of cells [84, 85]. Unfortunately, the mechanisms underlying autophagy activation with these drugs remain unknown, and it is therefore inappropriate to prescribe these drugs to patients with diabetes. Discovery for an autophagy regulator is just the beginning.

7. Discussion and Conclusion

Although the body of evidence is still small, relative autophagy insufficiency may be involved in the exacerbation of diabetic nephropathy, and several medications have the potential to activate autophagy. The authors therefore expect that autophagy activation will become a potential therapy to combat diabetic nephropathy. However, at present, there are a couple of major problems to be resolved as described below.

Further examinations are needed to conclude whether autophagy activation is a safe therapy for any kidney diseases, since some reports have suggested that autophagy activation was associated with tubular cell damages in some acute kidney injury models [86–88].

The role of autophagy in the development of diabetes is still under debate. Some investigators have shown that pancreatic β-cell-specific autophagy-deficient mouse developed glucose intolerance [89, 90], whereas others have reported that autophagy activation led to β-cell apoptosis [91]. Furthermore, recent interesting studies have shown that genetic inhibition of autophagy in adipose tissue, skeletal muscle, and liver prevented the development of high-fat diet-induced obesity [92–94]. Therefore, it remains unclear whether autophagy activation shows a health beneficial effect in all stages of diabetic diseases, from the onset of diabetes mellitus to the progression of diabetic complications.

In the past decade, several genetic links have emerged between autophagy deficiency and cancer development, providing increasing support for the concept that autophagy is a tumor suppressor pathway [95]. In contrast, several studies have shown that genetic or pharmacological inhibition of autophagy enhances cytotoxicity of cancer chemotherapeutic agents [96]. The incidence rate of malignant disorder is higher in patients with diabetes. Thus, further examinations regarding the pathogenic and therapeutic roles of autophagy in cancer biology are required.

We have no technical tool to detect autophagy activity in the kidney of humans; therefore the discovery of a biomarker

for autophagy activity with serum and/or urine samples is urgently needed. Finally, no drug has been discovered that can activate autophagy without adverse effects.

When the abovementioned problems are overcome, autophagy regulation may be an effective therapeutic target for diabetic nephropathy. We hope this review has been helpful to researchers interested in autophagy and diabetic nephropathy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

MicroRNA in Diabetic Nephropathy: Renin Angiotensin, AGE/RAGE, and Oxidative Stress Pathway

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MicroRNAs (miRNA) are a novel class of small, noncoding RNA molecules that have gained the attention of many researchers in recent years due to their ability to posttranscriptionally regulate the expression of families of genes simultaneously. Their role in normal physiology and pathobiology is intriguing and their regulation in normal and disease states is fascinating. That the cells can return to a state of homeostasis when these small molecules are perturbed is truly remarkable given the multiple cellular targets of each miRNA and that many mRNAs are targeted by multiple miRNAs. Several reviews have covered aspects of miRNA function in biology and disease. Here, we review the role of miRNA in regulating the renin-angiotensin system, AGE/RAGE signalling, and under conditions of oxidative stress in the context of diabetic nephropathy.

1. Introduction

The World Health Organization states that ~347 million people, roughly 9.5% of the adult population, were suffering from diabetes in 2008 [1]. The incidence of diabetes is rapidly increasing with estimates suggesting that this number will almost double by 2030. Diabetes mellitus is a major cause of chronic kidney disease (CKD) worldwide and is associated with enhanced morbidity and mortality, in particular accelerated cardiovascular disease [2, 3].

Diabetic nephropathy (DN) is now the most common cause of end-stage renal failure in the Western world [4]. Clinical associations that frequently precede overt DN are hypertension and poor glycaemic control [5], although a subset of patients develop nephropathy despite the proper glycemic control [6] and normal blood pressure. Once nephropathy is established, blood pressure often rises further, but glycaemic control can paradoxically improve as a result of reduced renal insulin clearance [7]. It is postulated that the interplay between metabolic and hemodynamic pathways plays an important role in the development and progression of DN [8] (Figure 1). Increased systemic and intraglomerular pressure is associated with increased albuminuria and glomerular injury. Activation of the renin-angiotensin-aldosterone system (RAAS) has been recognized as a key component of DN progression. Additionally, chronic hyperglycemia promotes the generation of advanced glycation end-products (AGEs). It is widely accepted that AGEs mediate their effects both directly and indirectly through receptor-dependent mechanisms. The receptor for AGE (RAGE) acts as a signal transduction receptor, and the RAGE-AGE interaction activates multiple intracellular signalling pathways which increase the production of growth factors, inflammatory cytokines, and oxidative stress (Figure 1).

In recent times, a novel class of non-coding RNA, microRNA (miRNA; miR), has been found to be expressed in all tissues and plays important roles in tissue homeostasis and disease progression [9, 10]. Whilst the role of miRNA in the pathogenesis of DN has been extensively reviewed by others in relation to growth factors and fibrosis in DN, the focus of this review is on the role of miRNA in the renin-angiotensin system and the AGE/RAGE signalling pathway, and their downstream the mediators such as oxidative stress and the immune response in the context of diabetic nephropathy.

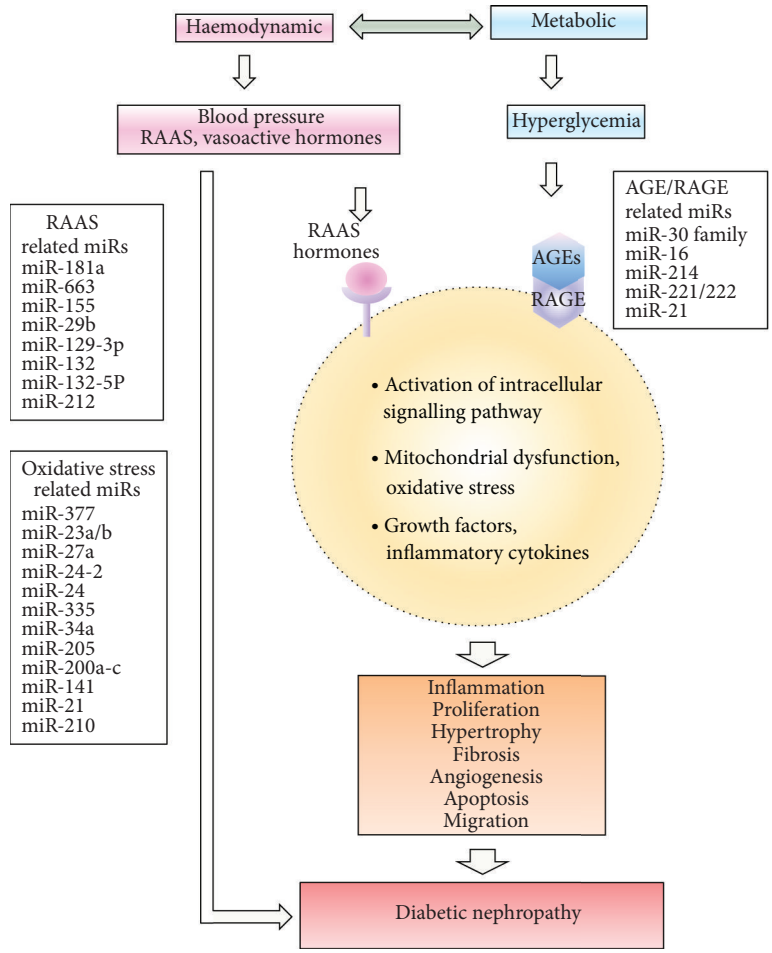

Figure 1: Schematic overview of mechanisms and microRNAs that are related to RAAS, AGE/RAGE, or oxidative stress contributing to diabetic nephropathy. AGE: advanced glycation end-product; miR: microRNA; RAAS: renin-angiotensin-aldosterone system; RAGE: receptor of AGE.

2. Biogenesis and Function of miRNA

miRNAs are a group of small (~22 nucleotide) single-stranded non-coding RNAs ubiquitously expressed in plants and animals where they act posttranscriptionally to modulate the expression of target genes [11, 12]. They were first discovered in 1993 when lin-14 protein expression was found to be regulated by the mature product of the *lin-4* gene in *Caenorhabditis Elegans (C. Elegans)* [13]. The mechanism was found to rely upon sequence specificity in the 3′-untranslated region (UTR) of lin-14 to which lin-4 bound. Whilst this occurred with only partial complementarity, the binding occurred with sufficient affinity to result in inhibition of protein translation. At the time this was considered a peculiarity in the worm. It was not until 2000 when another such regulator, let-7, was discovered in *C. Elegans* [14], and soon after came the realisation that this form of regulation was conserved across many species, representing a general mechanism for regulating the expression of several genes.

miRNAs are found in intergenic sequences or on the antisense strand of genes and may possess their own promoter and regulatory sequences [15, 16]. Other miRNAs (almost 50% in the case of human miRNA) are found within gene sequences and are together regulated with their host gene [17–19]. Approximately half are found in polycistronic units from which the mature miRNAs are processed [16]. The primary transcript (pri-miRNA) is processed by a number of proteins including ribonuclease III, Drosha, and the RNA binding protein, DiGeorge syndrome critical region gene 8 (DGCR-8) protein, into a short hairpin RNA molecule termed the precursor miRNA (pre-miRNA) which is subsequently exported from the nucleus by exportin-5 [20–22]. Once in the cytosol, further processing is mediated by another ribonuclease III, Dicer, and this is followed by the incorporation of the mature strand of the duplex miRNA into the RNA-induced silencing complex (RISC) which includes the Argonaute family of proteins [23]. The miRNA-RISC complex stabilises the miRNA against nuclease attack and miRNA direct the complex to target RNA transcripts via sequence complimentarity between the miRNA seed sequence (2–8 nucleotides) and the miRNA recognition element (MRE) in the target transcript [20]. miRNAs modulate protein synthesis by binding to the 3′ UTR of mRNAs via incomplete base pairing [24] to the complementary seed sequence in the UTR, leading to translational

repression. MREs are predominately located in the 3′ UTRs of mammalian mRNA, however; there is evidence indicating that miRNA can also mediate translational repression via binding 5′ UTRs or coding sequences [25, 26].

In plants and lower vertebrates where complete complimentarity exists between an miRNA seed sequence and the target mRNA, degradation of the target mRNA is induced. In contrast, miRNAs exercise finer control on protein expression in mammalian cells where they repress protein translation [27, 28], allowing for more comprehensive regulation of protein expression as any single miRNA can target many mRNAs, and any mRNA can be the target of several miRNAs [27]. This potential of miRNAs to regulate many genes adds significant complexity to the interpretation of many studies where the focus is on single genes. It is therefore critical to computationally detect the combination of miRNAs that target specific mRNA molecules. Such analyses often demand complex algorithms that need to be defined by high stringency levels to generate computational data that could then be assessed and validated in the wet lab. Added to this is the recent discovery of circulating miRNAs in microparticles [29] and the potential of these miRNAs to be delivered to sites distal to their generation.

3. miRNA and the Renin-Angiotensin System in Kidney Disease

Blood pressure is regulated by the renin-angiotensin-aldosterone system. Renin production is the key regulatory step that initiates an enzymatic cascade that leads to angiotensin generation and the control of blood pressure in addition to fluid and electrolyte homeostasis [30]. Renin is synthesized and released by renal juxtaglomerular (JG) cells, which are located at the entrance to the glomerulus. In early embryonic development, renin producing cells are broadly distributed along intrarenal arteries in the glomerular mesangium and in a few developing tubules. With maturation the cells become progressively restricted to the afferent arterioles. Lineage studies demonstrate that this progressive restriction is achieved by differentiation of renin cells into vascular smooth muscle cells (VSMCs), mesangial cells, and a subset of tubular cells [31].

Sequeira-Lopez et al. were the first to address the role of miRNA in the maintenance of JG cells by generating mice with a conditional deletion of Dicer (Dicer cKO mice) specifically and exclusively in renin-expressing cells, resulting in selective ablation of miRNA generation only in renin cells and their descendants [32]. Deletion of Dicer resulted in severe reduction in the number of JG cells accompanied by decreased expression of the renin *Ren1* and *Ren2* genes, decreased plasma renin concentration (PRC), decreased BP, abnormal renal function, and striking nephrovascular abnormalities including striped corticomedullary fibrosis. This study clearly showed a critical role of miRNA in the orchestration of renal function and the importance of miRNA homeostasis for specific organ functions.

The kidney has long been invoked in the etiology of essential hypertension. This could involve alterations in expression of specific genes and miRNA. Marques and colleagues conducted for the first time transcriptome-wide study of differential expression of mRNAs and miRNA in kidneys of hypertensive subjects (15 untreated hypertensive and 7 normotensive white males) by microarray technology [33]. They confirmed differences of expression for nuclear receptor subfamily 4 group A member 1 (NR4A1), NR4A2, NR4A3, period circadian protein homolog 1 (PER1), and salt-inducible kinase 1 (SIK1) mRNAs and for the miRNAs hsa-miR-638 and hsa-let-7c by real-time quantitative PCR expression in the medulla. Functional experiments confirmed the predicted binding of hsa-let-7c to the 3′ untranslated region of NR4A2 mRNA. In the renal cortex they confirmed differences in expression of apoptosis-inducing factor, mitochondrion-associated, 1 (AIFM1), alpha-1-microglobulin/bikunin precursor (AMBP), apolipoprotein E (APOE), cluster of differentiation 36 (CD36), ephrin-B1 (EFNB1), NADH dehydrogenase (ubiquinone) complex I, assembly factor 1 (NDUFAF1), peroxiredoxin 5 (PRDX5), REN, renin binding protein (RENBP), solute carrier family 13 (sodium/sulfate symporters), member 1 (SLC13A1), syntaxin 4 (STX4), and troponin T type 2 (cardiac) (TNNT2) mRNAs, and the miRNAs hsa-miR-21, hsa-miR-126, hsa-miR-181a, hsa-miR-196a, hsa-miR-451, hsa-miR-638, and hsa-miR-663. Functional experiments demonstrated that hsa-miR-663 can bind to the REN and APOE 3′ untranslated regions regulating REN and APOE mRNA levels, whereas hsa-miR-181a regulated REN and AIFM1 mRNA. A major discovery was evidence for REN mRNA regulation via binding of miRNAs hsa-miR-181a and hsa-miR-663 to its 3′ UTR as the observed downregulation of these 2 miRNAs in hypertension could explain the elevation in intrarenal renin mRNA. Due to small sampling size, these findings need to be bolstered by the acquisition of suitable samples from larger cohorts of untreated hypertensive subjects in other settings.

Chronic kidney disease (CKD) is a known cardiovascular risk factor, and most patients with CKD die of cardiovascular disease (CVD) before reaching the need for dialysis [37]. Chen and colleagues measured vascular miRNAs in blood from 90 patients with CKD and found an association between decreased circulating levels and progressive loss of eGFR by multivariate analyses [34]. Expression of vascular miRNAs was decreased in the thoracic aorta of CKD rats compared to normal rats, with concordant changes in target genes of RUNX2, AT1R, and myocardin with no alteration in DROSHA or DICER, indicating that the low levels of expression are not due to altered intracellular processing. Furthermore, the expression of miR-155 was negatively correlated with calcification of the aorta, a process known to be preceded by vascular dedifferentiation in these animals. Overexpression of miR-155 in VSMC from CKD rats inhibited AT1R expression and decreased cellular proliferation, confirming a causative role of low miR155 in VSMC transformation to a more synthetic, proliferative phenotype. However, whether downregulation of these miRNAs is the cause or consequence of the widespread vascular phenotype abnormalities in patients with CKD remains to be determined.

Jeppesen and colleagues have shown that AngII regulates five miRNAs (miR-29b, -129-3p, -132, -132-5P, and -212)

Table 1: Relevant miRNA in RAAS.

miRNAs	Tissue/organ/cell line	Source	Target	Functions	Reference
miR-181a	Kidney	Hypertensive patients	REN, AIFM1	Hypertension	[33]
miR-663			REN, APOE		
miR-155	Blood	CKD patients	AT1R	Hypertension, cardiovascular disease	[34]
miR-29b, -129-3p, -132, -132-5P and -212	HEK293N cells, cardiac fibroblasts	Human, Rat		Associated with cardiovascular disease	[35]
miR-132/212	Heart, aortic wall, and kidneys	Rat		Associated with blood pressure control	[36]
	Artery	ARB-treated patients			

AIFM1: apoptosis-inducing factor, mitochondrion-associated, 1; APOE: apolipoprotein E; AT1R: angiotensin II receptor, type 1; CKD: chronic kidney disease; HEK: human embryonic kidney cells; RAAS: renin-angiotensin-aldosterone system; REN: renin.

during *in vitro* stimulation of primary cardiac fibroblasts and of HEK293N cells overexpressing the AT1R [35]. Furthermore, Eskildsen and colleagues undertook a detailed analysis of potential miRNAs involved in AngII-mediated hypertension in rats and hypertensive patients, using miRNA microarray and qPCR analysis. miR-132 and miR-212 are highly increased in the heart, aortic wall, and kidneys of rats with hypertension (159 ± 12 mm Hg) and cardiac hypertrophy following chronic AngII infusion. In addition, activation of the endothelin receptor, another Gαq coupled receptor, also increased miR-132 and miR-212 [36]. A significant downregulation of miR-132 as well as a robust attenuation of miR-212 in human arteries from the ARB-treated patients was also observed, whereas treatment with β-blockers had no effect. In conclusion, miR-132 and miR-212 are upregulated in AngII-induced hypertension in organs associated with blood pressure control, possibly via the Gαq-dependent pathway.

Table 1 contains miRNAs that are known to be regulated by the renin-angiotensin system in the kidney and other tissues and associated with kidney disease, hypertension, and cardiovascular disease.

4. miRNAs in AGE/RAGE and Kidney Disease

Chronic hyperglycemia promotes the generation of advanced glycation end products (AGEs) as a result of sequential biochemical reactions involving nonenzymatic glycation of protein and lipids known as the Maillard reaction [8]. As a consequence of AGE formation, there is often concomitant liberation of reactive oxygen species (ROS) [38]. AGEs can induce expression of the MCP-1 in podocytes through activation of the AGE receptor (RAGE) and generation of intracellular reactive oxygen species (ROS) [39]. The induction of oxidative stress results in upregulation of nuclear factor (NF)-κB and various NF-κB-mediated proinflammatory genes, eventually leading to glomerular and tubulointerstitial injury. Therefore, AGE/RAGE and oxidative stress signalling are important in the progression of DN and targeting this axis by modulating the miRNAs that are involved is a potential new therapy.

There are very few studies examining the role of miRNA in AGE/RAGE signalling related to kidney disease. Most studies have focussed on the role of RAGE in the immune system or cancer because RAGE is a member of the immunoglobulin superfamily of cell surface molecules. When Dicer was selectively inactivated in mouse podocytes, multiple abnormalities were observed in glomeruli of mutant mice, including foot process effacement, irregular and split areas of the glomerular basement membrane, podocyte apoptosis and depletion, mesangial expansion, capillary dilation, and glomerulosclerosis [40]. Gene profiling by microarray analyses revealed upregulation of 190 genes in glomeruli isolated from mutant mice at the onset of proteinuria compared with control littermates. Target sequences for 16 miRNAs were significantly enriched in the 3′-untranslated regions of the 190 upregulated genes. Further, supporting the validity of the *in silico* analysis, 6 of the 8 top-candidate miRNAs were identified in miRNA libraries generated from podocyte cultures; these included miR-28, miR-34a, and four members of the miR-30 family, miR-30c-1, miR-30b, miR-30d, and miR-30c-2. Among the 15 upregulated target genes of the miR-30 miRNA family, RAGE, vimentin, heat-shock protein 20, and immediate early response 3 were known to be expressed in injured podocytes in experimental models and human kidney disease. RAGE and immediate early response 3 are known to mediate podocyte apoptosis, whereas vimentin and heat-shock protein 20 are involved in cytoskeletal structure [40]. The findings demonstrate the important roles of the mir-30 family in podocyte homeostasis and podocytopathies.

AGE/RAGE interaction induces inflammatory genes such as cyclooxygenase-2 (COX-2) [41]. S100b, a RAGE ligand, significantly increased COX-2 mRNA accumulation in THP-1 monocytes at 2 h via mRNA stability. S100b decreased occupancy of the DNA/RNA-binding protein, heterogeneous nuclear ribonuclear protein K (hnRNPK), at the COX-2 promoter but simultaneously increased its binding to the COX-2 3′-UTR. Additionally, S100b significantly downregulated the expression miR-16 which acts to destabilize COX-2 mRNA by binding to its 3′-UTR. hnRNPK knockdown increased miR-16 binding to COX-2 3′-UTR indicating a crosstalk between them. These results demonstrate that diabetic stimuli can

efficiently stabilize inflammatory genes via opposing actions of key RNA-binding proteins and miRNA [41].

AGEs delay spontaneous apoptosis of monocytes and contribute to the development of inflammatory responses [42]. In genome-wide miRNA expression analysis significant upregulation of miR-214 was consistently observed in THP-1 and human monocytes treated with various AGEs. A striking increase in miR-214 was also detected in monocytes from patients with chronic renal failure. PTEN was identified as a target gene of miR-214. Overexpression of pre-miR-214 led to impaired PTEN expression and delayed apoptosis of THP-1 cells, whereas knockdown of miR-214 levels largely abolished AGE-induced cell survival. These findings define a role for miR-214-targeting of PTEN in AGE-induced monocyte survival [42].

High-mobility group box 1 protein (HMGB1) is a late inflammatory cytokine that signals danger to the immune system through the RAGE and Toll-like receptor [43]. miR-221 and miR-222 are involved in cell proliferation through the inhibition of the cell cycle regulator, p27kip1, in smooth muscle cells [44] and endothelial cells (ECs) [45]. HMGB1 increases the expression of miR-221 and miR-222 in primary cultures of excised papillary lesions and in an established papillary cancer cell line and overexpression of miR-222 and miR-221 caused by HMGB1 increases growth and motility in papillary thyroid cancer cells. Recently, it was reported that miR-21 and miR-221, which are tissue inhibitors of metalloproteinases (TIMP)3-targeting miRNA, were significantly upregulated in kidneys from diabetic mice compared to control littermates and in a mesangial cell line grown in high glucose conditions [46]. Mesangial expansion is one of the main characters in DN and is mainly due to accumulation of extracellular matrix (ECM). ECM turnover is regulated by TIMPs activities. In diabetic conditions, TIMP3 expression in kidney is strongly reduced, but the causes of this reduction are still unknown. miR-221/miR-222 cluster which has been examined in several cardiovascular disorders affects the angiogenic activity of stem cell factor (SCF) by targeting the receptor c-Kit [47]. High glucose levels elevate miR-221 and this correlates with decreased expression of c-kit and reduced EC migration [48]. miR-221/222 is also considered to be important in atherosclerosis. miR-221/222 targets NO synthase and transcription factor ETS-1 which are both major contributors to the atherosclerotic process. [47, 49, 50] Taken together, these results indicate that HMGB1/RAGE and miR-221/222 axis may be activated in the kidney and vasculature resulting in ECM accumulation and atherosclerosis in DN patients.

Table 2 lists the miRNAs that are known to be modulated by the AGE/RAGE in kidney, monocytes, VSMCs, and ECs and associated with kidney disease and vascular dysfunction.

5. miRNAs in Oxidative Stress and Kidney Disease

Reactive oxygen species (ROS) is a collective term that includes a number of reactive and partially reduced oxygen (O_2) metabolites. Some of them are free radicals, such as superoxide anion (O_2^-) and hydroxyl radicals ($^\bullet OH$) that are extremely reactive molecular species with an unpaired electron in their outer orbital. A number of studies have demonstrated that both type 1 and type 2 diabetes are associated with overproduction of oxygen-derived free radicals. Increased ROS production and reduced levels of antioxidants culminate with an in increased level of oxidative stress leading to oxidative damage to cellular components [51]. Among the many enzymatic systems implicated in ROS generation in vascular tissues, enzymes of the mitochondrial respiratory chain (complexes I and III), xanthine oxidase, uncoupled nitric oxide synthase, and nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase (NOX) appear to be particularly important [52–54]. Increased NOX-mediated superoxide production has been reported in experimental models of diabetes and occurs in parallel with upregulation of NOX1 and NOX4 [55, 56]. NOX-mediated generation of superoxide is an important mediator of matrix accumulation, renal fibrosis, and podocyte injury in DN [57].

miR-377 is upregulated in spontaneous and STZ-induced mouse models of DN and in mesangial cells exposed to high glucose and TGF-β1 [58]. Stable overexpression of miR-377 in mesangial cells increased fibronectin synthesis. Furthermore, genes potentially relevant to the pathogenesis of DN were confirmed experimentally, including the cytoskeletal regulator p21-activated kinase 1 (PAK1) and superoxide dismutases (SOD1/SOD2) that catalyse ROS, which accumulates in response to hyperglycemia [58].

It has previously been shown that upregulation of the miR-23a~27a~24-2 cluster induces caspase-dependent and caspase-independent cell death in human embryonic kidney cells via c-jun N-terminal kinase (JNK) with increases in ROS and the release of proapoptotic factors such as cytochrome *c* (cyt c) in addition to apoptosis-inducing factor (AIF) from the intermembrane space of mitochondria to the cytosol [59]. In order to better understand the molecular mechanism responsible for miR-23a~27a~24-2 cluster-induced cell death, gene expression profiling was performed in control and miR-23a~27a~24-2 cluster overexpressing HEK293T cells. This revealed miR-23a~27a~24-2 cluster-induced apoptosis was associated with endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) pathways in HEK293T cells. Overexpression of the miR-23a~27a~24-2 cluster resulted in ER stress and altered mitochondrial membrane permeability and this was further established by increased intracellular and mitochondrial calcium levels in HEK293T cells [60].

There have been reports that miR-23b levels were increased in the kidneys of TGF-β1 transgenic mice and rats following subtotal nephrectomy, which was found to be localized to podocytes and tubular epithelium by *in situ* hybridization. miR-23b was also upregulated by TGF-β1 in cultured renal epithelial cells. *In vitro* gain and loss-of-function studies pointed to several miR-23b targets, including TGF-β receptor type II, SMAD3, and TGF-β1 itself, suggesting a negative feedback loop-regulating TGF-β1 signaling. Modulation of miR-23b in cultured podocytes altered expression of WT1, nephrin, and podocin and also influenced motility of cultured tubular cells [61].

Table 2: Relevant miRNA in AGE/RAGE.

miRNAs	Tissue/organ/cell line	Source	Target	Functions	Reference
miR-30 family	Podocyte	Mice	AGER, Vim, HSP20, Ier3	Podocyte homeostasis and podocytopathies	[40]
miR-16	THP-1 monocytic cells	Human	COX-2	Regulate inflammation	[41]
miR-214	THP-1 monocytic cells	Human	PTEN	Monocyte survival	[42]
miR-221/222	VSMCs	Rat	$p27^{Kip1}$ and $p57^{Kip2}$	Cell proliferation	[44]
miR-221/222	Endothelial cells	Human	$p27^{Kip1}$ and $p57^{Kip2}$	Cell proliferation	[45]
miR-21 and miR-221	MES 13 mesangial cells	Mice	Timp3	DN progression	[46]
miR-21	Kidney	Human			
miR-221/miR-222	HUVECs	Human	c-Kit	Reduced EC migration	[48]

AGE: advanced glycation end-product; AGER: advanced glycosylation end-product-specific receptor; c-kit: V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; COX: Cyclooxygenase; DN: diabetic nephropathy; EC: endothelial cells; HSP: Heat shock protein; HUVECs: human umbilical vein endothelial cells; Ier3: immediate early response 3; $p27^{Kip1}$: Cyclin-Dependent Kinase Inhibitor 1B; $p57^{Kip2}$: Cyclin-Dependent Kinase Inhibitor 1C; PTEN: phosphatase and tensin homolog; RAGE: Receptor for AGE; Timp: metallopeptidase inhibitor; Vim: Vimentin; VSMCs: vascular smooth muscle cells.

Studies have found that in the aged organs, including the kidney, expression of antioxidant enzymes such as SOD, catalase, Gpx, and peroxiredoxins is downregulated [62, 63] thus leading to reduced antioxidant capacity. From bioinformatic analysis of the miRNA expression profile of young and old rat kidneys, mitochondrial SOD2 and thioredoxin reductase 2 (Txnrd2) are potential targets of miR-335 and miR-34a, respectively, as aging mesangial cells exhibited significant upregulation of miR-335 and miR-34a and marked downregulation of SOD2 and Txnrd2. Further studies confirmed SOD2 and Txnrd2 as target genes of miR-335 and miR-34a which coincided with ROS generation [64].

Within the mitochondria, uncoupling protein 2 (UCP2) has recently been reported as a negative regulator of ROS generation [65]. Its ablation leads to marked increase of oxidative stress in several cell types [66]. In the stroke-prone spontaneously hypertensive rat (SHRsp) kidneys, severe renal damage along with increased rate of inflammation and oxidative stress was observed [67]. *UCP2* gene and protein levels were downregulated paralleled by differential expression of kidney miR-24 and -34a, which were identified to target the *UCP2* gene. The silencing of the *UCP2* gene in renal mesangial cells led to increased rate of ROS generation, increased inflammation and apoptosis, reduced cell vitality, and increased necrosis, suggesting that UCP2 is critical in preventing oxidative stress damage in renal mesangial cells [67].

Specific miRNAs, including miR-205 and the miR-200 family (miR-200a–c, miR-141, and miR-429), were shown to mediate epithelial-to-mesenchymal transition (EMT) in response to TGF-β1 in Madin-Darby canine kidney cells [68]. EMT is thought to be an important event driving renal fibrosis. Downregulation of these miRNAs relieves their cooperative repression of the mesenchymal transcription factors ZEB1 and SIP1 that, in turn, are free to inhibit E-cadherin expression promoting epithelial dedifferentiation.

Muratsu-Ikeda et al. assessed changes in miRNA expression in the cultured renal tubular cell line HK-2 under hypoxia-reoxygenation-induced oxidative stress or ER stress using miRNA microarray assay and real-time RT-PCR. Among altered miRNA expression, miR-205 was markedly decreased in both stress conditions. Functional analysis revealed that decreased miR-205 led to an increase in cell susceptibility to oxidative and ER stresses and that this increase was associated with the induction of intracellular ROS and suppression of antioxidant enzymes. Furthermore, miR-205 bound to the 3′-UTR of the prolyl hydroxylase 1 (PHD1/EGLN2) gene and suppressed the transcription level of EGLN2, which modulates both intracellular ROS level and ER stress state [69].

miRNA profiling of HUVEC treated for 8 and 24 hrs with 200 μM hydrogen peroxide (H_2O_2) showed that miR-200c and the cotranscribed miR-141 increased more than eightfold. The other miR-200 family members were also induced, albeit at a lower level [70]. miR-200c overexpression in HUVEC recapitulates many aspects of the oxidative stress-induced phenotype, since it induces cell growth arrest, apoptosis, and cellular senescence. All these effects are mediated, at least in part, by the inhibition of the target ZEB1 by the miR-200 family [70]. miR-200 family induction following H_2O_2 exposure has been confirmed in different cell lines such as human and mouse immortalized fibroblasts, colon carcinoma (CT26), mammary gland epithelial cells (NMuMG) and human cell lines, melanoma cells (MDA-MB-435S), kidney cells (293T), breast adenocarcinoma (MDA-MB-436 and BT-549), and ovarian adenocarcinoma (SKOV3). Notably, in all these cell lines, all miR-200 family members were upregulated [71]. In a recent study, an analysis of miRNAs upregulated in diabetic mouse heart compared to control was performed revealing that miR-200c and miR-141 were among the most upregulated [72]. The authors show that miR-141 targets the inner mitochondrial membrane phosphate transporter, solute carrier family 25 member 3 (Slc25a3), which provides inorganic phosphate to the mitochondrial matrix and is essential for ATP production, suggesting an important role

Table 3: Relevant miRNA in oxidative stress.

miRNAs	Tissue/organ/cell line	Source	Target	Functions	Reference
miR-377	NHMC	Human	SOD1/2, PAK1	Fibronectin synthesis	[58]
	MES 13 mesangial cells	Mouse			
	Kidney	Mouse			
miR-23a~27a~24-2	HEK293T	Human		ER stress and UPR pathways-associated apoptosis	[60]
miR-23b	Renal epithelial cells, podocyte	Mice, Rat	TGF-β receptor type II, SMAD3, TGF-β1	Negative feedback loop-regulating TGF-β1 signaling	[61]
miR-335 miR-34a	Kidney, primary mesangial cells	Rat	SOD2 Txnrd2	Renal aging	[64]
miR-24 miR-34a	Kidney, CRL 2573 mesangial cell	Rat	UCP2	Oxidative stress damage	[67]
miR-205	HK-2 renal tubular cell	Human	PHD1/EGLN2	Antioxidative and anti-ER stress	[69]
miR-200c	HUVEC	Human	ZEB1	Cell growth arrest, apoptosis, and cellular senescence	[70]
miR-200a/-141	Fibroblasts,	Mice	p38α	Enhanced oxidative stress, tumorigenesis, and chemosensitivity	[71]
	CT26 colon carcinoma,	Mice			
	NMuMG mammary gland epithelial cells,	Mice			
	MDA-MB-435S melanoma cells,	Human			
	293T kidney cells,	Human			
	MDA-MB-436 and BT-549 breast adenocarcinoma,	Human			
	SKOV3 ovarian adenocarcinoma	Human			
miR-200c/-141	Heart	Mice	Slc25a3	Decreased mitochondrial ATP production	[72]
	HEK293	Human			
miR-21	VSMCs	Rat	PDCD4	Cellular injury	[73]
miR-21	HUVECs	Human	PTEN	Increased NO production, reduced apoptosis	[74]
miR-21	Angiogenic progenitor cell	Human	SOD2	Increased intracellular ROS concentration and impaired NO bioavailability	[75]
miR-210	Kidney	Mice		Activation of VEGF Signaling pathway	[76]
	HUVECs	Human			

ATP: adenosine-triphosphate; ER: endoplasmic reticulum; HEK: human embryonic kidney cells; HUVECs: human umbilical vein endothelial cells; NF-kB: nuclear factor k-light-chain-enhancer of activated B cells; NHMC: normal human mesangial cells; NO: nitric oxide; PAK1: P21 protein (Cdc42/Rac)-activated kinase 1; PDCD4: programmed cell death 4; PHD1/EGLN2: prolyl hydroxylase 1; PTEN: phosphatase and tensin homolog; ROS: reactive oxygen; Slc25a3: solute carrier family 25 member 3; SOD: superoxide dismutase; TGF-β: transforming growth factor-β; Txnrd2: thioredoxin reductase 2; UCP2: uncoupling protein 2; UPR: unfolded protein response; VEGF: vascular endothelial growth factor; VEGF: vascular endothelial growth factor; ZEB1: zinc finger E-box-binding homeobox 1.

of miR-200 family in mitochondrial responses involved in cardiac diseases associated with diabetes and obesity.

On the contrary to the role for miR-200 family in oxidative stress, miR-200 family has been reported for antifibrotic roles in DN. The miR-200 family also plays an important role in EMT, which is considered to mediate production of renal fibroblasts, in part by targeting ZEB1/2, the transcriptional repressors of E-cad [68, 77, 78]. On the other hand, another group has demonstrated that TGF-β activated Akt in glomerular mesangial cells by inducing miR-200b and miR-200c, both of which target FOG2, an inhibitor of phosphatidylinositol 3-kinase activation [79], suggesting the role

of miR-200 family on glomerular mesangial hypertrophy in the progression of DN.

The contrasting findings above highlight the complex nature of miRNA research. Some of the differences may relate to variances in experimental models and/or conditions; however, one often overlooked explanation is that some effects of miRNA and inhibitors are likely to be indirect in nature. Our understanding of miRNA function is continually evolving. Recent evidence demonstrates regulation of gene expression via deadenylation, by altering message stability, and by effects on transcription. Bioinformatics utilising pathway analysis will be needed to better understand the crosstalk between factors that drive many downstream processes and how those processes ultimately impact the expression of individual genes.

miRNA profiling of rat VSMCs treated with 200 μM H_2O_2 for 6 hours revealed an upregulation of miR-21 [73]. This study showed that miR-21 participates in H_2O_2-mediated gene regulation via its target programmed cell death 4 (PDCD4) and transcription factor AP-1 pathway activity. Elevated miR-21 is thought to contribute to atherosclerosis by directly targeting PPARα, leading to an increased inflammatory response in ECs. Inhibition of miR-21 causes activation of AP-1 as well as upregulation of proinflammatory factors such as VCAM-1 and MCP-1 [80]. miR-21 can also act as an inhibitor of angiogenesis by reducing EC proliferation, migration, and tube formation in culture via inhibition of RhoB. miR-21 can also increase nitric oxide (NO) in HUVECs exposed to shear stress via increased phosphorylation of nitric oxide synthase (NOS) and decreased apoptosis [74]. This activity is balanced by the ability of miR-21 to repress SOD-2 which is important in antioxidant defence. Furthermore, miR-21 is elevated in angiogenic precursor cells by asymmetrical dimethylarginine (ADMA) which is a powerful NOS inhibitor, ultimately resulting in elevated intracellular reactive oxygen (ROS) species [75]. Increased miR-21 levels were recently demonstrated in renal cortex in db/db mice [81]. Knockdown of miR-21 decreased mesangial expansion, collagen I/IV, and FN1 expression and reduced macrophage infiltration and TNFα and MCP-1 expression. The gene expression changes were replicated *in vitro* in both PTC and mesangial cells (MCs) with miR-21 overexpression enhancing fibrogenesis via a mechanism which in part involved the direct targeting of SMAD7. Interestingly, miR-21 targets PTEN and also leads to decreased mesangial expansion in *db/db* mice [82]. miR-21 has also been implicated in regulation of TGF-β signalling in a number of animal models of tubulointerstitial fibrosis and associated renal dysfunction. In one such model, SMAD7 overexpression in the rat unilateral ureteral obstruction model has restored miR-21 expression to normal levels with congruent improvements in renal pathology [83]. In line with a profibrotic role for miR-21, upregulation of this miRNA is inhibited by SMAD3 deletion in an obstructive nephropathy model [84]. Furthermore, regulation of PDCD4 by miR-21 enhances podocyte apoptosis and loss in conjunction with increased tubular epithelial cells survival against growth arrest signals [85, 86].

miR-210 appears to function as master regulator of the hypoxic response as it was found to be upregulated by hypoxia in virtually all the cell types tested to date [87, 88]. Recent data demonstrate that Hif1α can block both mitochondrial respiration via the electron transport chain (ETC) through transcriptional activation of miR-210 in some cell types [87, 88]. Upregulation of miR-210 along with VEGF and VEGFR2 expression was confirmed in renal ischemia/reperfusion (I/R) injury of male Balb/c mice. Furthermore, overexpression of miR-210 in HUVEC-12 cells enhanced VEGF and VEGFR2 expression and promoted angiogenesis in matrigel *in vitro*. These results suggest that miR-210 may be involved in targeting the VEGF signaling pathway to regulate angiogenesis after renal I/R injury [76].

Table 3 summarises the miRNAs that are regulated by oxidative stress in the kidney and other tissues and associated with kidney disease and vascular dysfunction resulting from excessive ROS production.

6. Future Perspectives

Considerable progress has been made in identifying a number of important roles for miRNA in various biological processes and in disease. There is much excitement at the prospect that some miRNAs appear to be important to the regulation of several related processes in diabetes and its complications, including the modulation of the RAAS and oxidative stress pathways. The number of miRNAs relevant to these conditions is constantly increasing (Tables 1–3). It is encouraging that in some cases restoring the expression of a dysregulated miRNA can attenuate or even reverse disease. Our initial understanding of gene regulation has continued to change from simple concepts in terms of protein factors sitting on DNA to complex epigenetics involving chromatin dynamics and multiple histone and DNA modifications. Even this complexity has been superseded by the ability of miRNA to modulate the expression of multiple targets posttranscriptionally. Whilst the biology around miRNA continues to generate new and interesting findings, the challenge of the future is to translate some of the exciting experimental findings to the potential therapeutic interventions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Uncoupling of VEGF with Endothelial NO as a Potential Mechanism for Abnormal Angiogenesis in the Diabetic Nephropathy

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Abnormal angiogenesis is a well characterized complication in diabetic retinopathy and is now recognized as a feature of diabetic nephropathy. The primary growth factor driving the increased angiogenesis in diabetic retinopathy and nephropathy is vascular endothelial growth factor (VEGF). While VEGF is considered an important growth factor for maintaining glomerular capillary integrity and function, increased action of VEGF in diabetic renal disease may carry adverse consequences. Studies by our group suggest that the effects of VEGF are amplified in the setting of endothelial dysfunction and low nitric oxide (NO) levels, which are a common feature in the diabetic state. The lack of NO may amplify the effects of VEGF to induce inflammation (via effects on the macrophage) and may lead to dysregulation of the vasculature, exacerbating features of diabetic renal disease. In this review, we summarize how an "uncoupling" of the VEGF-NO axis may contribute to the pathology of the diabetic kidney.

1. Abnormal Angiogenesis Is a Characteristic Feature of Diabetic Nephropathy

The first description documenting abnormal angiogenesis in the diabetic kidney is from a 1987 study by Østerby and Nyberg [1]. These authors reported that patients with long-term type 1 diabetes showed an increase in capillaries in the renal biopsy that were both within and surrounding the glomeruli. Other investigators later demonstrated similar findings in type 2 diabetic patients with kidney disease [2, 3]. In these patients, 1–5% of glomerular capillaries were found to contain aberrant vessels. Interestingly, the abnormal vessels were also present in Bowman's capsule or in the glomerular vascular pole, presenting as an "extra efferent arteriole" [1, 4]. A Japanese research group examined human kidney samples from 94 patients with diabetes and performed detailed analyses of serial sections using computer-generated three dimensional images [5]. They reported that the abnormal vessels were often found to be anastomosed to the lobular structure of the intraglomerular capillary network, mainly to afferent branches through the widened vascular hilus, while the distal end of the vessels was connected to the peritubular capillary. Morphologically the endothelial cells were often swollen early in the disease only to become shrunken as diabetes progressed [6, 7]. Another interesting finding was that the aberrant proliferation of blood vessels was not infrequent in diabetic patients even during the first two years of disease [5], indicating that the development of these vessels could occur in the early phases of diabetic nephropathy.

Similar to human diabetic kidney disease, some diabetic animal models also developed excessive numbers of capillary vessels. For instance, Nyengaard and Rasch identified abnormal glomerular capillaries in an animal rat model induced by streptozotocin [8]. The db/db mice also exhibit an increase in endothelial cell number and an elongation of capillaries in their glomeruli [9, 10]. However, it should be noted that in the later stages of diabetic nephropathy, there is often a loss of capillaries in both human and animal models [2, 11, 12]. A

decrease in VEGF expression in advanced stage of diabetic nephropathy could account for such capillary loss [2, 11, 12].

2. VEGF Is Deleterious in Diabetic Kidney as Opposed to Nondiabetic Renal Disease

VEGF is a critical growth factor for endothelial cells, especially in the kidney. Podocytes and proximal tubular epithelial cells are likely major sources for VEGF which binds to receptors on the glomerular and peritubular endothelial cells, respectively. Under conditions in which local VEGF levels fall acutely, a loss of capillaries occurs, leading to lesions that may appear similar to a thrombotic microangiopathy. In progressive nondiabetic kidney disease, a loss of VEGF may occur more slowly, leading to a loss of capillaries in association with reduced renal function and fibrosis. Under these cases, the administration of VEGF can stimulate capillary growth and improve the kidney lesions [13–15]. Given these facts, VEGF seems to be indispensable for renal normal physiology and a loss of VEGF may play an important role in both acute and chronic kidney diseases.

In contrast, an excessive amount of VEGF is likely a contributory factor for diabetic kidney disease. This nature was first shown in a 1999 study, in which an increase in renal VEGF/VEGFR2 expression was observed in streptozotocin (STZ) induced diabetic rat [16]. Likewise, we also documented an increase in glomerular VEGF expression, which was associated with diabetic glomerular injury in the diabetic eNOSKO mice [17]. These findings were confirmed in human diabetic nephropathy, in which VEGF was found to be increased in both renal biopsies and urine [3, 18].

To determine its role in diabetic kidney disease, several investigators have attempted to inhibit the excessive VEGF. For instance, anti-VEGF antibody was the first to be tested while a pharmacological inhibitor was also used in the several types of diabetic rodents, including STZ induced diabetic rats, db/db mice, and Zucker rats [19, 20]. In general, blocking VEGF consistently demonstrated protective effects, such as a reduction in urine albumin excretion, an inhibition in glomerular matrix expansion, and podocyte protection. Likewise, Ku and colleagues utilized a molecular technology to overexpress sFlt-1 (a soluble VEGFR1) in podocytes to locally block VEGF function in STZ diabetic mice. This treatment had similar beneficial effects as systemic VEGF inhibitors [21]. While these studies unfortunately did not examine the direct effect of such therapies on the development of abnormal angiogenesis, they do provide supporting evidence that excessive VEGF expression may contribute to diabetic nephropathy.

3. Why Is VEGF Deleterious in Diabetic Nephropathy?

While VEGF is capable of producing several biological factors, one of the most important factors could be the endothelial nitric oxide (NO) because endothelial NO was found to potently protect the vasculature in several ways, including stimulating vascular relaxation and having both anticoagulation and anti-inflammatory effects. As such, it is likely that the vascular protections of VEGF might be via stimulating NO production. In contrast, a lack of NO could turn VEGF to be deleterious in vascular system. Zhao et al. reported that blocking NO production induced vascular remodeling and inflammation along with upregulation of VEGF. Importantly, blocking VEGF action resulted in ameliorating such injury, indicating that VEGF could be deleterious in vascular system in the absence of NO [22]. Thus, endothelial NO could be a key factor to regulate VEGF function.

How can NO regulate VEGF action? In 2001, Dunk and Ahmed addressed this issue with the tumor epithelial cells. They concluded that cell proliferation is mediated by VEGFR2 while VEGFR1 stimulation resulted in NO production, suggesting that these two actions are independently regulated by two different receptors. They also found that NO, which was mediated by VEGFR1 stimulation, could negatively regulate VEGFR2-mediated mitogenesis [23]. Given these facts, we hypothesized that the combination of increased VEGF with an impaired endothelial NO response might play a role in the abnormal angiogenesis observed in diabetes (Figure 1).

4. Endothelial NO Availability Is Reduced in Diabetic Condition

Is NO bioavailability reduced in the diabetic kidney? In this regard, many studies have documented that diabetes is associated with a reduction in NO bioavailability. The underlying mechanisms appear to be diverse, but many diabetes-related factors are likely involved, including hyperglycemia [24], advanced glycation end-products [25], uric acid [26], ADMA [27] and oxidative stress [28], and are actually able to reduce NO bioavailability.

Gene polymorphisms in endothelial nitric oxide synthase (eNOS) may be also a factor, which is involved in regulating NO levels [29] because eNOS is the principal enzyme producing NO in endothelial cells. Based on such assumption, eNOS gene polymorphisms have been examined in diabetic patients by several investigators. However, the role of such genetic alteration remains unclear as some [30–33], but not all, studies [34–37] documented a positive association of specific eNOS polymorphisms with diabetic nephropathy.

Alternatively, Hohenstein et al. performed immunohistochemistry to investigate eNOS expression in type 2 diabetic patients and found that eNOS expression was increased in glomeruli in patients with diabetes [38]. Similarly, STZ induced diabetic rats were found to exhibit an increase in eNOS expression in endothelial cells in both the afferent arterioles and the glomerulus [39]. While these lines of evidences do not meet our assumption, we should be aware that eNOS expression is not always correlated with its activity. In general, the production of NO requires eNOS to be "coupled" while, in turn, "uncoupled" eNOS generates superoxide as opposed to NO. Brodsky et al. found that high glucose induces uncoupling of eNOS, which causes a reduction in NO bioavailability and an increase in superoxide production [40].

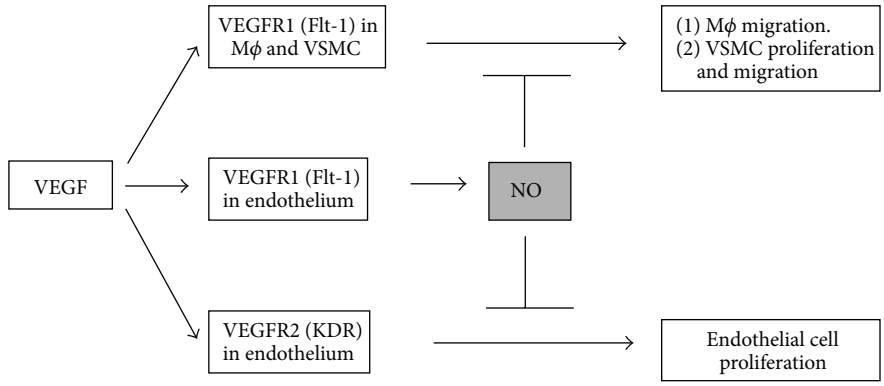

Figure 1: Central role of NO in regulating VEGF system in endothelial cells. In endothelium, VEGFR1 contributes to NO production whereas endothelial cell proliferation is regulated by VEGFR2. VEGFR1 is also expressed in macrophage as well as VSMC. In the normal setting, endothelial cells produce NO, which negatively regulates endothelial cell proliferation, macrophage migration, and VSMC activation to maintain the well balanced vascular integrity. In contrast, endothelial NO bioavailability is reduced in certain physiological conditions, such as diabetes. In the case that NO bioavailability is reduced in endothelium, a compensatory increase in VEGF expression as well as a disruption of negative regulation in vascular system in response to VEGF occurs. As a consequence, VEGF engages VEGFR2 to enhance endothelial cell proliferation while VEGFR1 on macrophage and VSMC can be activated to induce vascular injury.

Likewise, Komers et al. found that eNOS in diabetic kidney may also exist in the uncoupled form where it localized to the cytosolic fraction. Since eNOS activation also requires the translocation into plasma membrane in the coupled form [41], it is likely that the upregulated eNOS in diabetes might be an inactivated form.

5. Uncoupling of VEGF with Endothelial NO Causes Abnormal Angiogenesis in the Diabetic Kidney

Regarding such notion, our first insight came from studying STZ induced diabetes in the mouse lacking eNOS (eNOSKO) [12]. VEGF expression was increased in both diabetic wild type and diabetic eNOSKO mice. Since eNOS was genetically deleted, the kidney exhibited the condition of being upregulated. However, diabetic eNOSKO mice developed much more severe clinical manifestations that resembled overt diabetic nephropathy in humans. For example, this mouse was found to develop hypertension, massive albuminuria, and renal dysfunction [12]. This mouse model also exhibited higher mortality rates from progressive renal disease [12, 42]. Histological manifestations of diabetic eNOSKO mice also mimic those of human diabetic nephropathy. In fact, this mouse model developed not only the early manifestations, such as mesangial expansion and glomerular basement membrane thickening, but also advanced lesions including mesangiolysis, Kimmelstiel-Wilson-like nodules, arteriolar hyalinosis, and tubulointerstitial disease [12].

Importantly this mouse model demonstrated that excessive numbers of small blood vessels were induced around glomeruli where normal vessels do not normally exist. Interestingly, this is the same location where abnormal blood vessels are observed in human diabetic kidney disease [12]. In addition, an increase in endothelial cell number in both glomerular and peritubular lesions was found to exhibit a proliferative response [12], which could be a potential mechanism for the development of abnormal angiogenesis in this mouse model. These studies suggested that the combination of high VEGF and low endothelial NO levels might be responsible for the abnormal enhanced endothelial proliferation in this mouse model.

We next used the cell culture system to test our hypothesis. Here we evaluated whether a lack of NO could alter the proliferative effects of VEGF on endothelial cells [43]. Our primary finding was that blocking NO using either an NO synthase inhibitor or high glucose condition could enhance the proliferative response of endothelial cells to VEGF [43]. Next issue was to investigate the mechanism. VEGF is known to bind two different receptors, raising the question of which one might be more important in mediating these effects. Our study demonstrated that VEGFR2 was primarily responsible for the cell proliferative response in endothelial cells [43]. However, VEGFR1 was the primary receptor responsible for stimulating NO since a VEGFR2 inhibitor failed to block NO production as well as eNOS phosphorylation in response to VEGF in endothelial cells [43]. Such findings are consistent with a 2001 study demonstrating that distinct role between VEGFR1 and VEGFR2 in nonendothelial cells [23]. Taken together, VEGF likely acts on endothelial cells by two pathways; one elicits endothelial proliferation through VEGFR2 while the other the activating VEGFR1 for induction of NO, which negatively regulates the VEGFR2-mediated proliferative response.

Nonetheless, endothelial cells might not be the only target for this uncoupling condition. In our animal model, we found that an increase in macrophage infiltration was observed in glomeruli where VEGF expression was upregulated in diabetic eNOSKO mice [17], suggesting that the uncoupling condition could also mediate macrophage migration. In this regard, NO administration could fix such VEGF-NO balance, resulting in preventing macrophage migration if our assumption is correct. In cultured macrophage cell line, we

found that VEGF was able to induce macrophage migration in Boyden chamber assay while administration of NO donor alleviated such migration in response to VEGF [17]. Hence, such uncoupling theory could be also applied to macrophage infiltration in the diabetic nephropathy.

We also generated a mouse model in which we were able to test the role of NO in the VEGFR2-mediated endothelial proliferative response [44]. In this experiment, we utilized the adenoassociated virus (AAV) to overexpress a VEGF mutant (mtVEGF), which could only bind to VEGFR2. Following the injection, mice underwent uninephrectomy to amplify any renal lesions. Wild type (WT) mice were also treated in the same way as a control group. We also performed the study using mice lacking eNOS (eNOSKO) to further allow us to specifically analyze the relationship between VEGFR2 signal and endothelial NO in the kidney. Overexpression of mtVEGF resulted in increased angiogenesis and lowering of blood pressure in both types of mice whereas such effects were greater in eNOSKO than WT [44]. In addition, mtVEGF-AAV also caused severe mesangial injury with increased proliferation associated with elevated PDGF, PDGF-β receptor, and VEGFR2 in eNOSKO mice compared to similarly treated WT mice [44]. These data indicate that enhancing VEGFR2 signal could induce aberrant angiogenesis which could be further exaggerated in the absence of eNOS in the kidney.

6. Can a Low in VEGF Expression Also Be Deleterious Even in Diabetic Nephropathy?

Our studies clearly demonstrate that an elevation in VEGF may result in deleterious consequences in diabetic nephropathy, primarily by overactivation of the VEGFR2 pathway in the setting of endothelial dysfunction. However, as diabetic nephropathy continues, there may actually be a reverse situation where VEGF expression falls in association with chronic glomerular and tubular injury. For example, there are a couple of studies showing that VEGF expression is reduced in human diabetic nephropathy within sclerotic areas and in nodular lesions in the glomeruli [45, 46]. Baelde et al. documented a 2.5-fold reduction in VEGF expression late stage diabetic nephropathy in association with a loss of endothelial cells and a reduction in podocytes [11]. Such interesting concept was highlighted in an elegant study by Hohenstein and colleagues in which VEGF activity was increased only in the mildly injured glomeruli but significantly decreased in more severely injured glomeruli [2]. Given these facts, a low level of VEGF is also undesirable in diabetes and is likely to manifest as the kidney disease progresses.

Why could a lowering VEGF be also deleterious in the diabetic nephropathy? It could be because normal kidney requires a certain level of VEGF to maintain integrity of renal function. In general, normal kidney is composed of abundant vessels so that a physiological level of VEGF is required to maintain such vascular system. Perhaps, diabetic kidney does so as well. In fact, we previously demonstrated that blocking VEGF rather deteriorated tubulointerstitial injury. Importantly such injury was accompanied with a loss of peritubular capillary, indicating that blocking VEGF made its level too low so that peritubular capillary system could not be maintained. Therefore a loss of peritubular capillary seems to be a cause for deterioration of tubulointerstitial injury. Recently, this notion was tested by other researchers by using conditional mouse model. Sivaskandarajah et al. used an inducible Cre-loxP gene-targeting system that enabled genetic deletion of VEGF-A selectively from glomerular podocytes of wild type mice, and then type 1 diabetes was induced in mice using streptozotocin (STZ). Importantly, this system allowed them to reduce VEGF level which was lower than physiological level. As a consequence, a deletion of VEGF resulted in more severe kidney injury in diabetes [47]. Hence, an important message could be that if VEGF is either too low or too high, this factor could be deleterious. Rather, maintaining VEGF at physical level could be protective in the diabetic kidney.

7. How Could the Uncoupling Be Fixed?

First of all, we tested the effect of insulin therapy in this model [12]. Controlling blood sugar was found to alleviate the upregulation of VEGF and prevent the progression of diabetic glomerular injury, suggesting that such beneficial effect of insulin could be due in part to a fixation in VEGF-NO balance.

We next examined the effect of the renin-angiotensin system (RAS) blocking [48]. In contrast to diabetic wild type mice, RAS blockades failed to slow the progression of kidney injury in the diabetic eNOSKO mice. Unfortunately, we did not address the issue of VEGF-NO balance in that study. Nevertheless, similar refractoriness to ACEI/ARB in diabetic nephropathy was also reported by other investigators [49, 50]. Common nature of these three studies was that renal injury was relatively severe in the animal models. While a clear mechanism for refractoriness to ACEI/ARB remains undetermined, ACEI/ARB may be no longer protective once diabetic nephropathy progresses severely.

Finally, next compound we used was nicorandil, which has two pharmacological actions; one is to donate NO while the other action is to open K-channel dependently on ATP [51]. We expected that by donating NO, nicorandil could fix the balance of VEGF with NO. As expected, nicorandil was found to exhibit some protection as evidenced by a reduction in urinary protein excretion and slowing of the progression of glomerular injury in accompany with an increase in urinary NOx level [51]. These findings suggest that an NO donor, nicorandil, could correct the balance of VEGF with NO and be a therapeutic option to treat diabetic nephropathy in case that kidney would be under the uncoupling condition.

8. Translation of Animal Study into Clinical Medicine

It must be still immature and too preliminary to translate this concept to clinical medicine. However, it might not be impossible to apply this concept in the future study. In such case, the first step could be examining renal VEGF and endothelial

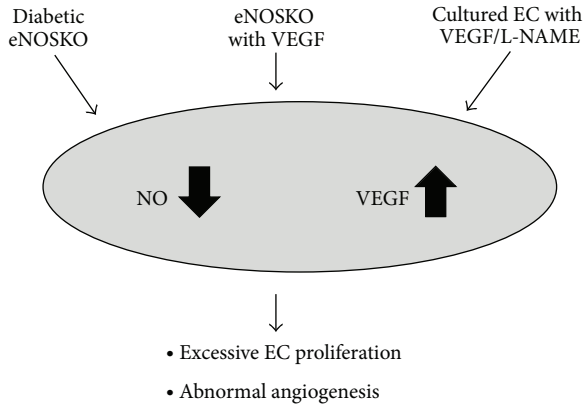

Figure 2: Uncoupling of VEGF with NO could be a pathway to induce abnormal angiogenesis. Three distinct conditions commonly cause uncoupling condition, leading to abnormal angiogenesis.

function. Urinary VEGF could be a marker of VEGF production in the kidney as its increase in urine was found to be positively associated with a degree of urinary albumin excretion in patients with type 1 diabetes [18]. In turn, endothelial function can be evaluated by several measures which have been already established in clinical medicine. For instance, urinary level of NOx could be a good marker of endothelial dysfunction as it was found to be reduced in type 2 diabetic patients with microalbuminuria compared to those with normoalbuminuria [52]. Plasma von Willebrand factor could be also used as a good marker for endothelial function in diabetic patients with proteinuria [53–55]. Alternatively, the flow mediated vasodilatation or acetylcholine-induced vasodilatation can be also clinically used to evaluate endothelial function. Nevertheless, if any measures find the combination of high VEGF with endothelial dysfunction, such patients might be under the uncoupling condition. In such cases, either donating NO or cautiously blocking of VEGF could be considered as a therapeutic option.

9. Conclusions

In summary, physiological levels of VEGF are required for the maintenance of normal renal architecture and function. In case of diabetes, VEGF expression is induced and could exhibit some deleterious effects. In particular when upregulation of VEGF is coupled with endothelial dysfunction, such combination can have a role in driving diabetic renal disease (Figure 2). High levels of VEGF may have a role in abnormal angiogenesis, macrophage activation, and even mesangial expansion. In contrast, as disease progresses, VEGF levels may fall, resulting in endothelial cell loss and capillary rarefaction.

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Research Article

Evaluation of the Association of Plasma Pentraxin 3 Levels with Type 2 Diabetes and Diabetic Nephropathy in a Malay Population

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Recent reports have demonstrated that elevated plasma long pentraxin 3 (PTX3) levels are associated with cardiovascular and chronic kidney diseases. In the current study, we investigated the plasma PTX3 levels in 296 Malay subjects including the subjects with normal glucose tolerance (NGT) and type 2 diabetes (T2DM) patients with or without DN by using an enzyme-linked immunesorbent assay. Results showed that in males, plasma PTX3 levels in T2DM patients without DN were lower than that in the subjects with NGT (2.78 versus 3.98 ng/mL; $P = 0.021$). Plasma PTX3 levels in T2DM patients with DN were decreased compared to the patients without DN (1.63 versus 2.78 ng/mL; $P = 0.013$). In females, however, no significant alteration of plasma PTX3 levels among NGT subjects and T2DM patients with and without DN was detected. Furthermore, an inverse correlation between PTX3 and body mass index was found in male subjects with NGT ($P = 0.012$; $r = -0.390$), but not in male T2DM patients, neither in all females. The current study provided the first evidence that decreased plasma PTX3 levels are associated with T2DM and DN in Malay men and also suggested that PTX3 may have different effects in DN and chronic kidney diseases.

1. Introduction

Type 2 diabetes (T2DM) and obesity have become epidemic in Malaysia. According to the latest National Health and Morbidity Surveys, 14.9% of Malaysian adults aged 30 years and above are diabetic and they are often obese or overweight [1, 2]. Moreover, diabetic nephropathy (DN) is the most common cause of end-stage renal disease (ESRD) and contributes to 57% of patients with T2DM in this country. Although T2DM represents a preventable and treatable cause of ESRD, the number of ESRD cases caused by T2DM has increased and accounts for more than 50% of incident dialysis patients [3–5]. The public burden from diabetes and DN is enormous.

Pentraxin 3 (PTX3) is an acute-phase glycoprotein and a soluble receptor acting as an opsonin. PTX3 protein is expressed in vascular endothelial cells and macrophages. Thereby, its levels may reflect more directly the inflammatory status of the vasculature [6, 7]. Recently, several clinical investigations have demonstrated that elevated plasma PTX3 levels are associated with cardiovascular [8, 9] and chronic kidney diseases (CKD) [10, 11]. Furthermore, plasma PTX3 levels are inversely associated with body mass index (BMI) suggesting that PTX3 may play a role in obesity and metabolic syndrome [12, 13]. Interestingly, with the approach of genome-wide scan and linkage analysis, chromosome 3q is found to be linked with diabetes and DN in many ethnic groups [14–16].

The gene encoding for PTX3 protein is located in chromosome 3q25.32 and resides in the linkage region. Yilmaz et al. have shown that PTX3 is positively associated with proteinuria in Turkish subjects with T2DM hypertensive patients, while renin angiotensin system blockade lowers plasma PTX3 levels in the patients [17, 18]. However, there are gender and racial differences of plasma PTX3 levels [7, 19], by which the association of PTX3 with kidney dysfunction may be influenced.

In the present study, we examined plasma PTX3 levels in a Malay cohort, including NGT subjects, T2DM patients with and without DN. We also analyzed plasma PTX3 levels according to BMI. The aim of our study was to investigate the association of plasma PTX3 levels with T2DM and DN in this Malay population. Data from our study are also useful for better understanding the different effects of PTX3 in DN and CKD.

2. Patients and Methods

2.1. Patients and Controls. Malaysia is a country with multicultures and multiethnic populations. We collected the samples of subjects with NGT and patients with T2DM from the collaborating centers all over Malaysia. The ethnic distribution of our study subjects was 67.6% Malay, 15.3% Indian, 14.8% Chinese, and 2.3% Indigenous Sabahans and Sarawakians. To avoid the error caused by ethnic stratification, Indian, Chinese, Indigenous Sabahans, and Sarawakians were excluded in the present study. Finally, 103 (50 males/53 females) Malay individuals with NGT (controls) and 193 (99/94) Malay patients with T2DM (cases) were included into the analyses. Diagnoses of T2DM were done based on the World Health Organization (WHO) criteria [20]. The diagnoses of DN were based on urine albumin-to-creatinine ratio (ACR) suggested by ADA [21]. The patients with T2DM and normoalbuminuria (ACR < 3.5 mg/mmol) were considered as controls for DN, while the patients with macroalbuminuria (ACR ≥ 35 mg/mmol) and ESRD who needed dialysis were included as the cases for DN. Except for the patients with ESRD, all other subjects were required to give urine under fasting conditions early in the morning. Clinical characteristics of all Malay subjects with NGT and T2DM with and without DN are summarized in Table 1.

All subjects answered a set of questionnaires and underwent clinical and physical examinations. Informed consent was obtained from all subjects, and the study was approved by the local ethical committees. Data and materials transfer agreement from the Institute for Medical Research, Malaysia to Karolinska Institutet, Sweden was signed prior to the study.

2.2. Clinical Characterization. Body weight and height were measured using a calibrated digital scale (Seca, Birmingham, UK). The WHO/International Association for the Study of Obesity (IASO)/International Obesity Task Force (IOTF) has proposed BMI cut-off values of 23.0–24.9 kg/m^2 for classification of overweight and of ≥25.0 kg/m^2 for obesity for adult Asians [22]. Based on the Malaysian clinical practice guidelines, subjects with BMI value ≥23.0 kg/m^2 are considered as overweight [23]. Systolic and diastolic blood pressures were measured using a digital sphygmomanometer (Omron Healthcare, Inc., Lake Forest, USA) after 5 minutes resting. Creatinine in serum and urine were measured using Randox Assayed Multisera (Randox Laboratories Ltd., Crumlin, UK). Urine or serum was mixed with sodium hydroxide in biochemistry analyzer (Selectra E). Creatinine in alkaline solution reacted with picric acid to form a coloured complex. The amount of the complex formed was measured at wavelength of 490 to 510 nm. The reading of complex measured is directly proportional to the creatinine concentration. Estimate of glomerular filtration rate (GFR) in the dialysis patient was calculated by the mean of renal urea and creatinine clearance from a 24-hour urine correction [24].

2.3. Plasma PTX3 Measurement. A total of 25 mL venous blood samples were collected from each subject early in the morning after an overnight fasting and then stored at −80°C. Plasma PTX3 concentrations were determined using a commercial enzyme-linked immunosorbent assay kit (Quantakine DPTX 30; R&D Systems Inc., Minneapolis, USA). Experiments with the PTX3 assay were carried out according to the manufacturer's instructions. Briefly, 20 μL of standard and plasma samples was assayed duplicate in the microtiter plate wells coated with a specific PTX3 monoclonal antibody followed by incubation at room temperature for 2 hours. The wells were then washed four times with a buffered surfactant solution. Anti-PTX3 polyclonal antibody conjugated to alkaline phosphatase was added to each well and incubated for two hours at room temperature. After washing step, 200 μL of substrate solution was added to each well followed by incubation for 30 minutes at room temperature. The solution of 2 N sulfuric acid was added to each well to stop the reaction. Absorbance was measured at 450 nm with corrections set at 540 nm using microplate reader. The values of plasma PTX3 levels were extrapolated from a curve drawn using a standard PTX3.

2.4. Statistical Analyses. All data were expressed as mean (95% CI) for normally distributed variables and as geometric means (95% CI) for nonnormally distributed variables. The Kolmogorov-Smirnov test was initially used to test the data for normality. Normal probability plots were created and parameter distributions were transformed to the common logarithm for obtaining a normal distribution before performing statistical analysis. The one-way analysis of variance (ANOVA) was used for comparisons involving more than two groups or independent t-test for comparison between two groups. Pearson and Spearman analyses were conducted to determine correlations with continuous and noncontinuous variables, respectively. Statistical significance was defined as the P value of below 0.05. All analyses were performed using PASW Statistic Base 18 (SPSSInc, Chicago, USA).

Table 1: Clinical and laboratory characteristics of Malay subjects with normal glucose tolerance, and type 2 diabetes patients with and without diabetic nephropathy.

Gender	Male				Female			
Group (N)	NGT (50)	T2DM − DN (40)	T2DM + DN (59)	P values[a/b]	NGT (53)	T2DM − DN (51)	T2DM + DN (43)	P values[a/b]
Age (years)	58 (56–60)	55 (53–58)	55 (52–58)		56 (54–59)	53 (50–56)	56 (53–59)	
Diabetic duration (years)	—	14 (12–17)	14 (12–17)		—	12 (9–15)	12 (9–15)	
BMI (kg/m^2)	24.9 (23.6–26.2)	27.7 (26.4–29.1)	27.6 (26.0–29.1)	0.010/NS	27.2 (25.9–28.5)	29.1 (27.2–30.7)	27.2 (25.1–29.4)	
SBP (mm Hg)	138 (132–144)	137 (129–144)	159 (147–170)	NS/0.001	137 (132–143)	144 (137–150)	149 (137–160)	
DBP (mm Hg)	83 (79–85)	82 (79–85)	83 (79–88)		84 (80–87)	87 (83–91)	79 (73–85)	NS/0.047
FPG (mmol/L)	5.6 (5.0–6.1)	7.3 (5.3–9.4)			5.0 (4.74–5.6)	7.7 (5.6–9.9)		
HbA1c (mmol/mol)	31 (29–33)	43 (33–52)	31 (21–41)		31 (30–33)	38 (30–44)	37 (26–46)	
eGFR (mL/min/1.73 m^2)	102 (88–117)	93 (81–105)	50 (42–58)	NS/<0.001	103 (92–113)	89 (79–98)	50 (36–64)	NS/<0.001
Creatinine (μmol/L)*	75.9 (69.2–83.2)	81.3 (67.6–93.3)	158.5 (134.9–195.0)	NS/<0.001	57.5 (53.7–63.1)	63.1 (60.3–75.9)	141.3 (112.2–117.8)	NS/<0.001
ACR (mg/mmol)*	0.89 (0.66–1.13)	1.03 (0.63–1.44)	179.0 (133.8–224.3)	0.001/<0.001	0.79 (0.64–0.95)	2.01 (0.25–3.76)	304.3 (149.4–459.3)	0.001/<0.001

Data are expressed as mean (95% CI) for normally distributed variables and as geometric means (95% CI) for nonnormally distributed variables*; NGT: normal glucose tolerance; T2DM: type 2 diabetes; BMI: body mass index; SBP and DBP: systolic and diastolic blood pressures; FPG: Fasting plasma glucose; HbA1c: glycosylated hemoglobin; eGFR: estimated glomerular filtration rate; ACR: albumin creatinine ratio; PTX3: pentraxin 3; P values were from tests of NGT versus T2DM − DN[a] and T2DM − DN versus T2DM + DN[b].

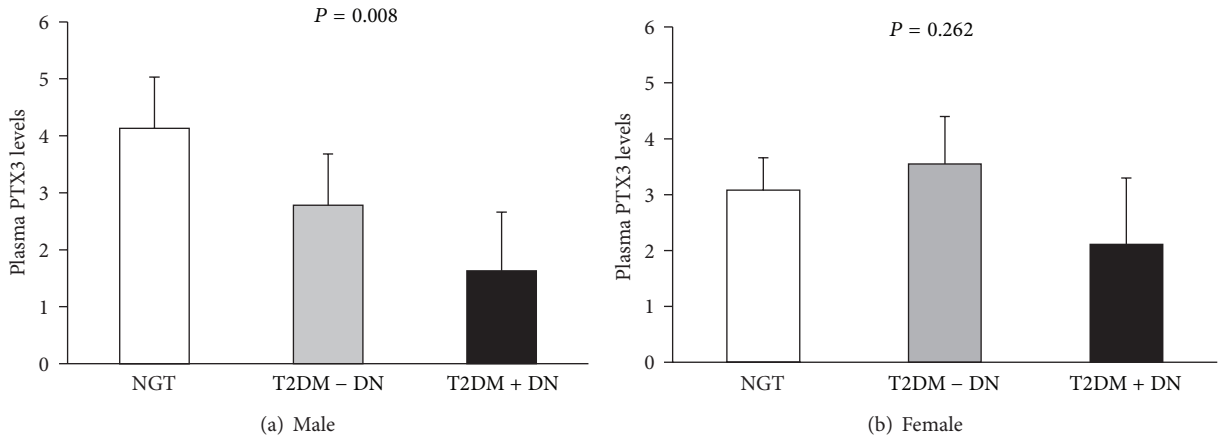

Figure 1: Plasma PTX3 levels in Malay subjects with normal glucose tolerance and type 2 diabetes patients with or without diabetic nephropathy. Data presented as means with 95% CI; *P* values were from ANOVA tests; NGT: normal glucose tolerance; T2DM: type 2 diabetes; DN: diabetic nephropathy.

3. Results

3.1. Gender Differences of Plasma PTX3 Levels in Malay Subjects. In both males and females, there was no difference in age, waist circumference, and HbA1c between T2DM patients and NGT subjects. Although plasma PTX3 levels in males and females differed significantly, PTX3 had no relationship with age in males ($P = 0.647$) or females ($P = 0.626$). Thus, all subsequent analyses of plasma PTX3 levels were done separately in males and females. Our analyses indicated that plasma PTX3 levels in T2DM patients with and without DN were lower as compared with NGT subjects in males (2.62 versus 3.98 ng/mL; $P = 0.021$) but not in females (3.24 versus 3.09 ng/mL; $P = 0.748$).

3.2. Association of Plasma PTX3 Levels with Type 2 Diabetes and Diabetic Nephropathy. We further analyzed the association of PTX3 with T2DM and DN. Figure 1(a) showed that plasma PTX3 levels were consistently decreased from NGT to T2DM without DN and to the patients with DN in males (3.98, 2.78, and 1.63 ng/mL; $P = 0.008$ ANOVA test). Among males, the patients with DN had lower PTX3 levels compared to T2DM without DN (1.63 versus 3.08 ng/mL; $P = 0.013$). In females, however, there was no statistically significant difference of the mean values of plasma PTX3 levels among NGT and T2DM with and without DN (3.09, 3.55, and 2.11 ng/mL; $P = 0.262$, ANOVA test) (Figure 1(b)).

3.3. Correlations of Plasma PTX3 Levels with BMI. There was a negative correlation between plasma PTX3 levels and BMI in male subjects with NGT ($r = -0.390$; $P = 0.012$) (Figure 2) but not in females ($P = 0.330$). The correlation between PTX3 and BMI was not found in all male and female T2DM patients with and without DN. In Malaysia, the adults with BMI value ≥23.0 kg/m^2 are considered to be overweight [23]. To further understand whether the association between PTX3 and DN in T2DM was influenced by BMI, we performed the comparative analyses in the patients with overweight

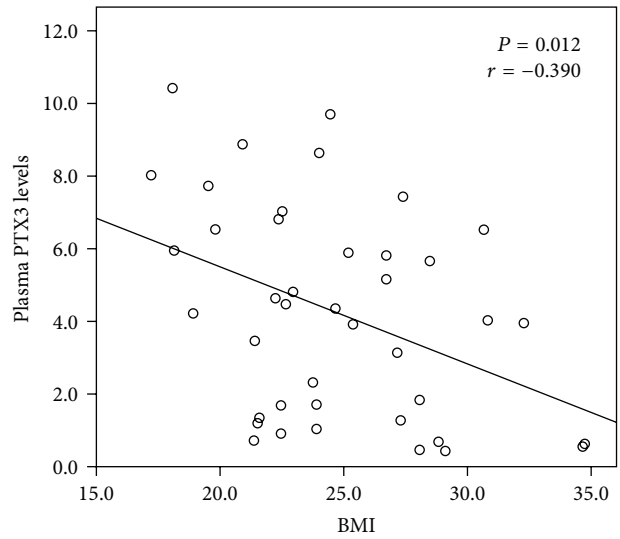

Figure 2: Univariate correlation between plasma PTX3 levels and BMI in Malay men with normal glucose tolerance.

(BMI ≥ 23 kg/m^2) and lean patients (BMI < 23 kg/m^2), respectively. In males with overweight, we found that plasma PTX3 levels were gradually decreased from subjects with NGT to T2DM patients without DN and to the patients with DN (3.68, 2.60 and 1.42 ng/mL; $P = 0.044$, ANOVA test) (Figure 3). In lean males and also in all females, plasma PTX3 levels of NGT and T2DM with and without DN were varied but not with any statistical significance.

4. Discussion

In the present study, we analyzed plasma PTX3 levels in Malay subjects with NGT and T2DM with and without DN. We report a gender difference of plasma PTX3 levels in this population. Lower levels of PTX3 were found to be associated with T2DM and DN in males but not in females.

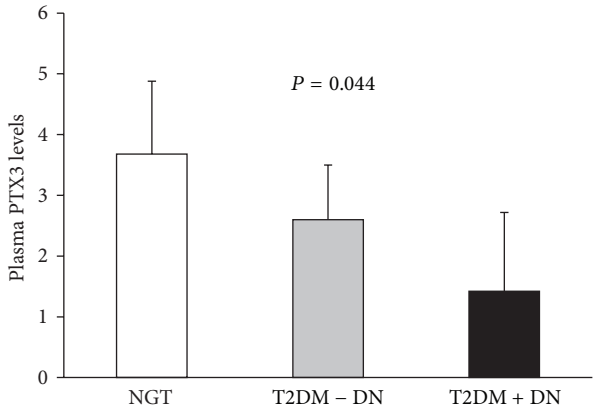

Figure 3: Plasma PTX3 levels in overweight Malay men with normal glucose tolerance and type 2 diabetes patients with or without diabetic nephropathy. Data presented as means with 95% CI; *P* values were from ANOVA tests; NGT: normal glucose tolerance; T2DM: type 2 diabetes; DN: diabetic nephropathy.

Furthermore, PTX3 was found to be inversely associated with BMI in males with NGT. The correlation was not observed both in males and females with T2DM and DN.

Gender is an important factor for the development of T2DM and DN. Males have a higher prevalence of T2DM and DN in many populations including Malaysians [1, 3–5]. Epidemiologic reports have demonstrated that DN is 30% more frequent in males than in females [25]. Genetic studies have also showed that DNA polymorphisms in the genes of sex-determining region Y-box 2, angiotensin II type 1, and type 2 receptors are associated with DN with gender-specific effects [26–28]. Previously, Yamasaki et al. observed that plasma PTX3 levels between males and females in a healthy Japanese population are different [7]. In the present study, we demonstrate that plasma PTX3 levels gradually decreased from NGT to T2DM without DN to T2DM with DN particularly among males but not in females. Furthermore, an inverse correlation between PTX3 and BMI was found in male subjects with NGT. This correlation was not seen in all females and males with T2DM and DN. Taking together, data from previous and present studies implicate that PTX3 most likely has gender-specific effects in T2DM and DN, which should be taken into our consideration in further investigations.

Several studies have reported that increased PTX3 levels are associated with impaired renal function in CKD [10, 11]. The similar association of PTX3 with DN is seen in Turkish patients with T2DM [17]. In the present study, however, we demonstrate that decreased PTX3 levels are associated with DN in Malay men with T2DM. First, the controversy may be caused by the studies in different ethnic populations. Dubin et al. have demonstrated that there are racial differences of PTX3 in term of association with kidney dysfunction [19]. Second, the ages of Turkish T2DM subjects with DN (at 42 years old) [17] are younger and their duration of diabetes are shorter compared to Malay T2DM patients with DN (at the age of 55 years old) in the present study. Furthermore, clinical observations have indicated that two main causes of CKD are diabetes and high blood pressure, which are responsible for up to two-thirds of the cases. The progresses and mechanisms of DN and CKD may be different [29], while PTX3 may have different effects in these two diseases.

We have shown that plasma PTX3 levels were inversely correlated with BMI in males with NGT, which is consistent with previous reports [7, 12, 13]. In the present study, there is a limitation with lack of lean subjects because all patients with T2DM and female subjects with NGT had mean values of BMI at least 27.2 kg/m^2. Recently, a study has demonstrated that PTX3 is expressed in adipose tissue, and its tissue specific expression reflects endothelial dysfunction [30]. Another study has reported that PTX3 is positively correlated with adiponectin [12]. Although we did not analyze plasma levels of adiponectin in this Malay cohort, the accumulated documents have shown that adiponectin is inversely proportional to obesity and T2DM in different populations including Malaysians. Plasma/serum adiponectin levels in the patients with T2DM and obese subjects are decreased compared to that in healthy control subjects [31, 32]. Therefore, we hypothesize that PTX3, as similar to adiponectin, may have protective effects in increased body weight. Further investigation is needed to fully understand the cellular mechanism of PTX3 reduction in T2DM and DN.

In conclusion, the present study provides the first evidence that decreased plasma PTX3 levels are associated with T2DM and DN in Malay men and also suggests that PTX3 may have different effects in DN and CKD.

Abbreviations

ACR: Urine albumin-to-creatinine ratio
eGFR: Estimate of glomerular filtration rate
DN: Diabetic nephropathy
NGT: Normal glucose tolerance
T2DM: Type 2 diabetes
PTX3: Pentraxin 3.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contributions

Peter Stenvinkel, Harvest F. Gu, Kerstin Brismar, and Norhashimah Abu Seman proposed and designed the study; Norhashimah Abu Seman, Anna Witasp, and Björn Anderstam collected experimental data; Wan Nazaimoon Wan Mohamud and Norhashimah Abu Seman collected clinical data; Norhashimah Abu Seman and Harvest F. Gu analyzed the data; and Harvest F. Gu, Norhashimah Abu Seman and Peter Stenvinkel wrote the paper. All of the authors contributed to data interpretation, discussion, and revision of the paper.

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Research Article

Levels of Inflammatory Cytokines in Type 2 Diabetes Patients with Different Urinary Albumin Excretion Rates and Their Correlation with Clinical Variables

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Although the pathogenetic mechanism of DN has not been elucidated, an inflammatory mechanism has been suggested as a potential contributor. This study was designed to explore the relationship between low-grade inflammation and renal microangiopathy in T2DM. A total of 261 diabetic subjects were divided into three groups according to UAE: a normal albuminuria group, a microalbuminuria group, and a macroalbuminuria group. A control group was also chosen. Levels of hs-CRP, TNF-α, uMCP-1, SAA, SCr, BUN, serum lipid, blood pressure, and HbA1c were measured in all subjects. Compared with the normal controls, levels of hs-CRP, TNF-α, uMCP-1, and SAA in T2DM patients were significantly higher. They were also elevated in the normal albuminuria group, $P < 0.05$. Compared with the normal albuminuria group, levels of these inflammatory cytokines were significantly higher in the microalbuminuria and macroalbuminuria group, $P < 0.01$. The macroalbuminuria group also showed higher levels than the microalbuminuria group, $P < 0.01$. Also they were positively correlated with UAE, SBP, DBP, LDL-C, and TC. We noted no significance correlated with course, TG, or HDL-C. Only TNF-α; was positively correlated with HbA1c. This study revealed the importance of these inflammatory cytokines in DN pathogenesis. Further studies are needed to fully establish the potential of these cytokines as additional biomarkers for the development of DN.

1. Introduction

Diabetic nephropathy (DN) has been widely recognized as a major complication associated with type 2 diabetes and is a leading cause of end-stage renal disease. It is characterized functionally by proteinuria and albuminuria and pathologically by glomerular hypertrophy, mesangial expansion, and tubulointerstitial fibrosis [1]. In recent years, our knowledge of the pathophysiological processes that lead to DN has notably improved on a genetic and molecular level. Thus, the classic view of metabolic and hemodynamic alterations as the main causes of renal injury in diabetes has been transformed significantly, with clear evidence indicating that these traditional factors are only a partial view of a much more complex picture. One of the most important changes is related to the participation of immune-mediated inflammatory processes in the pathophysiology of diabetes mellitus and its complications [2, 3]. Whether inflammation plays a role in the pathogenesis of DN and understanding what the underlying mechanisms constitute, these are questions which have yet to be answered [4, 5]. Therefore, it is very important to find new pathogenic pathways that may provide opportunities for early diagnosis and for targets of novel treatments.

C-reactive protein (CRP) is a normal plasma protein that belongs to the pentraxin family, an evolutionary conserved group of proteins involved in acute immunological responses. Levels can rise 100–1000-fold within 24–72 h in a cytokine-mediated response to most forms of tissue injury, infection, or inflammation [6]. In terms of DN, several studies have

examined its relationship with inflammation, leading to conflicting results [7–9]. Some data suggests that CRP may be implicated as a risk factor in DN.

TNF-α is a pleiotropic cytokine that plays an essential role in mediating inflammatory processes. It is cytotoxic to glomerular, mesangial, and epithelial cells and may induce direct renal damage. Several studies found that diabetic patients with nephropathy have higher serum and urinary concentrations of TNF-α than nondiabetic subjects or diabetic patients without renal involvement [10, 11].

Local tissue infiltration of monocytes and macrophages is a characteristic of DN. Recent studies have demonstrated that monocyte chemoattractant protein-1 (MCP-1) is a chemotactic cytokine with a high degree of specificity for monocytes and which may be involved in the infiltration of monocytes and macrophages and plays an important role in the progression of DN [12, 13]. Thus, measuring levels of MCP-1 is of important clinical significance in the diagnosis and intervention of early DN.

SAA is an acute phase protein synthesized in the liver and secreted into the blood with a 1000-fold elevation following inflammation. Transforming growth factor-β (TGF-β) is a cytokine that stimulates the synthesis of acute phase proteins and has been found to be overexpressed in the glomeruli of patients with DN. It is also involved in the induction of extracellular matrix production [14].

As there has been no study done to examine the relationship between inflammatory cytokines and the parameters of UAE, HbA1c, lipids, and blood pressure, the purpose of this study was to detect the levels of hs-CRP, TNF-α, and SAA in serum, as well as the levels of uMCP-1 at different stages of DN, while also describing the relationship between these markers and the various parameters.

2. Subjects and Methods

2.1. Study Subjects. The study was performed on 261 hospitalized patients with T2DM, having an average age of 54.1 years ± 14.2 years. These patients were recruited from the Department of Endocrinology in The First Affiliated Hospital of China Medical University, from September 2011 to November 2012. There were 136 male patients and 125 females. Type 2 diabetes was diagnosed based on the World Health Organization criteria. Patients with cardiac and hepatic diseases, another kidney disease, and infectious diseases were excluded. Patients with a history of diabetic ketoacidosis or hypoglycemic coma during the 3 months preceeding the study were also excluded. None of the patients had an elevated serum creatinine nor used angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs). Also, none of the patients used thiazolidinediones or statins.

Patients were classified into three groups according to urine albumin excretion (UAE) as follows: D1 = normoalbuminuric (patients with urinary albumin levels of <30 mg/g · creatinine (Cr) (112 patients)); D2 = microalbuminuric (patients with microalbuminuria of 30–299 mg/g · Cr (93 patients)); D3 = macroalbuminuria (patients with macroalbuminuria of ≥300 mg/g · Cr (56 patients)). In control group (C), 86 healthy volunteers were also recruited with an average age of 55.2 years ± 12.4 years (45 males, 41 females). The parameters of sex, age, body mass index (BMI), and creatinine were comparable between the study and control groups. General status of the patients and healthy volunteers is shown in Table 1. Informed consents were obtained from the patients before the study began.

2.2. Methods. Blood samples were taken before breakfast in the morning (between 8 AM and 11 AM), after an 8 to 12 hour overnight fast. Samples were collected in sterile tubes, centrifuged at 3000 rpm for 15 minutes at 4°C, and then stored at −70°C until assayed. The urine samples were centrifuged at 2000 rpm/min for ten minutes, and a 2 mL supernatant was removed and stored at −70°C. TNF-α, SAA, and uMCP-1 were measured by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA). Levels of hs-CRP were measured by immunoturbidimetry. TG, LDL-C, HDL-C, SCr, BUN, and HbA1c were measured in all subjects. Levels of uMCP-1 were expressed as values corrected by the urinary creatinine concentration (milligrams of creatinine/deciliter) to exclude the influence of different urine sample concentrations.

2.3. Statistical Analysis. Normally distributed values were analyzed by analysis of variance (ANOVA). Post hoc comparisons of group pairs were performed by Scheffe's multiple comparison test after ANOVA had established significant differences among the groups. Tests of normality between the groups were performed with a Shapiro-Wilk test. Pearson's correlational analysis was used to analyze the levels of inflammation cytokines and various factors. Multiple linear regression analysis and principal component analysis were also used to assess association between lnUAE as a dependent variable and SBP, DBP, lipid,course, HbA1c, uMCP-1, TNF-α, hs-CRP, and SAA as independent variables. Analysis of variance and dependability were processed with IBM SPSS, version 12.0. Values were expressed as mean ± SD. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. Clinical Characteristics of Participants. There were no significant differences between diabetes patients and the control group regarding age, BMI, SCr, BUN, TG, and HDL-C; however, the diabetic patients had higher values of SBP, DBP, TC, LDL-C, and UAE ($P < 0.05$). Among the three diabetes groups there were no significant differences regarding course, SBP, DBP, or TC. However, the levels of HbA1c, LDL-C, and UAE in group D3 were much higher than those in groups D1 and D2 ($P < 0.05$), especially for UAE which was higher in group D2 than in group D1 ($P < 0.01$) (Table 1).

3.2. Levels of hs-CRP, TNF-α, uMCP-1, and SAA. Levels of hs-CRP, TNF-α, uMCP-1, and SAA in groups D1, D2, and D3 were much higher than those in the control group ($P < 0.05$). Among the three diabetes groups, these levels increased

Table 1: Comparison of the general status and study data between diabetes groups and control group.

	C	D1	D2	D3
Number	86	112	93	56
Age (years)	55.2 ± 12.4	54.9 ± 13.1	53.7 ± 12.5	62.6 ± 11.4
BMI (Kg/m^2)	26.5 ± 3.1	26.3 ± 3.7	26.2 ± 2.9	26.8 ± 3.7
Course (year)		8.9 ± 1.3	9.9 ± 1.7	11.8 ± 2.4
SBP (mmHg)	111.3 ± 10.3	124.0 ± 14.3^{b}	130.9 ± 16.9^{b}	135.5 ± 17.3^{b}
DBP (mmHg)	71.4 ± 8.3	77.8 ± 6.5^{b}	82.7 ± 7.8^{b}	86.1 ± 14.5^{b}
SCr (μmol/L)	63.62 ± 8.79	64.96 ± 15.27	69.02 ± 20.32	78.49 ± 24.12
BUN (mmol/L)	4.09 ± 1.01	6.12 ± 1.31	6.41 ± 1.37	6.76 ± 1.70
HbA1c (%)		7.80 ± 3.28	8.89 ± 2.59	9.97 ± 2.91^{c}
TG (mmol/L)	1.69 ± 1.01	1.71 ± 0.97	1.99 ± 1.03	2.13 ± 1.16
TC (mmol/L)	4.33 ± 0.77	5.07 ± 0.83^{b}	5.36 ± 1.35^{b}	6.40 ± 1.76^{b}
LDL-C (mmol/L)	1.92 ± 0.33	3.02 ± 0.67^{b}	2.80 ± 1.23^{a}	3.97 ± 1.31^{bcf}
HDL-C (mmol/L)	1.15 ± 0.12	1.17 ± 0.22	1.13 ± 0.31	1.17 ± 0.30
Hs-CRP (mmol/L)	1.03 ± 0.94	2.41 ± 1.07^{a}	3.95 ± 1.18^{bd}	4.51 ± 1.89^{bdf}
TNF-α (mg/mL)	1.01 ± 0.45	1.99 ± 0.56^{a}	2.73 ± 0.72^{bd}	4.10 ± 0.95^{bdf}
UMCP-1/Ucr (ng/mg)	4.51 ± 2.29	24.70 ± 5.37^{a}	70.59 ± 18.93^{bd}	122.85 ± 63.76^{bdf}
SAA (ug/L)	163.90 ± 37.13	318.31 ± 34.35^{a}	490.13 ± 37.24^{bd}	665.04 ± 64.13^{bdf}
Ln (UAE/Ucr)*	2.19 ± 0.60	2.37 ± 0.86^{a}	4.08 ± 0.58^{bd}	7.34 ± 0.90^{bdf}

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; BUN: blood urea nitrogen; SCr: serum creatinine; HbA1c: glycohemoglobin A1c; TG: triglyceride; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; hs-CRP: high sensitivity C-reactive protein; TNF-α: tumor necrosis factor alpha; uMCP-1: urinary monocyte chemoattractant protein-1; SAA: serum amyloid-A; UAE/Ucr: urine albumin excretion/urinary creatinine.
[a]$P < 0.05$, [b]$P < 0.01$ diabetic patients versus control; [c]$P < 0.05$, [d]$P < 0.01$ D2 and D3 versus D1; [e]$P < 0.05$, [f]$P < 0.01$ D3 versus D2.
*Since the figures of UAE/Ucr were not normally distributed, they were transitioned with ln here (similarly hereinafter).

Table 2: Correlation analysis of inflammation cytokines and various factors.

	hs-CRP		TNF-α		uMCP-1		SAA	
	r	P	r	P	r	P	r	P
Age	0.135	0.244	0.194	0.093	0.249*	0.030	0.212	0.065
BMI	0.111	0.340	−0.096	0.409	−0.065	0.575	−0.069	0.555
Course	0.205	0.158	0.141	0.334	0.169	0.247	0.195	0.179
SBP	0.431**	<0.001	0.522**	<0.001	0.427**	<0.001	0.615**	<0.001
DBP	0.413**	<0.001	0.497**	<0.001	0.279*	0.015	0.507**	<0.001
TG	0.178	0.125	0.121	0.296	0.184	0.111	0.112	0.336
HDL-C	−0.120	0.301	−0.154	0.184	−0.175	0.130	−0.120	0.300
LDL-C	0.507**	<0.001	0.431**	<0.001	0.322**	0.005	0.559**	<0.001
TC	0.510**	<0.001	0.383**	<0.001	0.333**	0.003	0.527**	<0.001
HbA1c	0.235	0.104	0.303*	0.034	0.249	0.085	0.198	0.173
Ln (UAE/Ucr)	0.675**	<0.001	0.813**	<0.001	0.798**	<0.001	0.824**	<0.001

**Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).

consistently with UAE. Levels of hs-CRP, TNF-α, uMCP-1, and SAA in group D3 were significantly higher than those in groups D1 and D2 ($P < 0.01$), and the levels in group D2 were elevated compared to those in group D1 ($P < 0.01$) (Table 1).

3.3. Correlation Analysis of Inflammation Cytokines and Various Factors. Levels of hs-CRP, TNF-α, uMCP-1, and SAA were positively correlated with UAE ($r = 0.675$, $P < 0.001$; $r = 0.813$, $P < 0.001$; $r = 0.798$, $P < 0.001$; $r = 0.824$, $P < 0.001$, resp.), SBP ($r = 0.431$, $P < 0.001$; $r = 0.522$, $P < 0.001$; $r = 0.427$, $P < 0.001$; $r = 0.615$, $P < 0.001$), DBP ($r = 0.413$, $P < 0.001$; $r = 0.497$, $P < 0.001$; $r = 0.279$, $P < 0.05$; $r = 0.507$, $P < 0.001$), LDL-C ($r = 0.507$, $P < 0.001$; $r = 0.431$, $P < 0.001$; $r = 0.322$, $P < 0.01$; $r = 0.559$, $P < 0.001$), and TC ($r = 0.510$, $P < 0.001$; $r = 0.383$, $P < 0.001$; $r = 0.333$, $P < 0.01$; $r = 0.527$, $P < 0.001$), in the T2DM patients. However, there was no significance correlated with course, TG, or HDL-C. It was found that only TNF-α was positively correlated with HbA1c ($r = 0.303$, $P < 0.05$) (Table 2).

Table 3: Predictors of proteinuria in DN by multiple linear regression[a,b].

	Unstandardized coefficients		t	P
	B	Std error		
Constant	−0.357	0.083	−4.304	<0.001
Age	−0.115	0.088	−1.306	0.199
BMI	−0.004	0.081	−0.055	0.956
Course	0.080	0.084	0.948	0.349
HbA1c	0.196	0.085	2.288	0.027
Principal component for SBP and DBP[c]	0.112	0.087	1.298	0.202
Principal component 1 for TG, TC, HDL-C, and LDL-C[d]	0.052	0.077	0.679	0.501
Principal component 2 for TG, TC, HDL-C, and LDL-C[d]	−0.070	0.078	−0.894	0.376
Principal component for hs-CRP, TNF-α, uMCP-1, and SAA[e]	1.184	0.009	13.103	<0.001

[a]Dependent variable: ln (UAE/Ucr).

[b]Each variable was standardized by using Z scores before being entered into the regression model.

[c]Since the values of SBP and DBP were correlated, their unique principal component was substituted for them in the model and the principal component = 0.928 ∗ SBP + 0.928 ∗ DBP. In the formula, each variable was no longer the original variable, but standardized variable and the coefficients before the standardized variables represented the correlation coefficients of principal component and the corresponding original variables. So this formula showed that SBP and DBP were highly correlated and the extracted component could nearly represent the variables of SBP and DBP.

[d]Since the values of TC, TG, HDL-C, and LDL-C were correlated, their two principal components were substituted for them in the model and the principal component 1 = 0.289 ∗ TG + 0.223 ∗ HDL-C + 0.892 ∗ LDL-C + 0.919 ∗ TC, the principal component 2 = 0.770 ∗ TG − 0.793 ∗ HDL-C − 0.090 ∗ LDL-C + 0.128 ∗ TC. In the formulas, each variable was no longer the original variable but standardized variable and the coefficients before the standardized variables represented the correlation coefficients of principal component and the corresponding original variables. So formula 1 showed that LDL-C and TC were highly correlated and component 1 could represent the variables of LDL-C and TC, while formula 2 showed that TG and HDL-C were highly correlated and component 2 could represent the variables of TG and HDL-C.

[e]Since the values of hs-CRP, TNF-α, uMCP-1, and SAA were correlated, their unique principal component was substituted for them in the model and the principal component = 0.841 ∗ hs-CRP + 0.928 ∗ TNF-α + 0.883 ∗ uMCP-1 + 0.944 ∗ SAA. In the formula, each variable was no longer the original variable, but standardized variable and the coefficients before the standardized variables represented the correlation coefficients of principal component and the corresponding original variables. Since only one principal component was extracted among the four inflammatory factors and the correlation coefficients were all close to 1, it showed that the four inflammatory factors were highly correlated and the component could almost contain all the information of the four variables.

3.4. Regression Analysis and Principal Component Analysis of Inflammation Cytokines and DN. To support the results, linear regression analysis and principal component analysis were performed (Table 3). Using lnUAE as a dependent variable and SBP, DBP, lipids, course, HbA1c, uMCP-1, TNF-α, hs-CRP, and SAA as independent variables, it was shown that only constant, HbA1c, principal component for hs-CRP, TNF-α, uMCP-1, and SAA had statistical significance ($t = -4.304$, $P < 0.001$; $t = 2.288$, $P < 0.05$; $t = 13.103$, $P < 0.001$, resp.).

4. Discussion

In recent years, several clinical and animal studies have indicated that inflammatory cytokines play an important role in the development and progression of DN [15, 16]. Based on these findings, the present work was designed to investigate the importance of hs-CRP, TNF-α, uMCP-1, and SAA in the pathogenesis of DN and thus their use as inflammatory markers for DN development in T2DM.

From our data we found a strong and graded association between CRP and UAE in patients with type 2 diabetes. Plasma concentrations of CRP were significantly higher in subjects with T2DM compared to those without T2DM. Besides other studies [17, 18], the results of our work strongly suggest that inflammation seems to play an important and independent role in early microalbuminuria. Taking into account that inflammation and microalbuminuria are both associated with DN, our results are encouraging for the early recognition and treatment of UAE.

There are several mechanisms through which CRP may promote DN. First, enhanced renal inflammation may be a mechanism by which CRP promotes diabetic kidney injury. It is well known that nuclear transcription factor-kappa B (NF-κB) is active in many aspects of immune and inflammation responses in human cells. It has been shown that the NF-κB signaling CRP pathway is activated in DN and that CRP is capable of inducing the production of proinflammatory cytokines such as IL-1β and TNF-α in cultured monocytes or endothelial cells via an NF-κB-dependent mechanism [19–21]. More importantly, CRP itself was induced by high glucose, which, in turn promoted high glucose mediated renal inflammation. This finding suggests that CRP may function as an inflammatory mediator or cofactor of high glucose levels to promote diabetic renal inflammation. This is consistent with previous reports [19, 22].

The present study revealed that serum TNF-α levels were significantly increased in diabetic groups when compared with healthy control subjects, which was confirmed by the significant positive correlation between glucose and TNF-α. On the other hand, it was observed that there was a significant elevation in serum TNF-α values in diabetic microalbuminuric and macroalbuminuric groups in comparison with the diabetic normoalbuminuric group. This was in agreement

with the significant positive correlation between TNF-α and UAE.

TNF-α is known to stimulate prostaglandin production by mesangial cells and may be responsible for the alteration of glomerular microcirculation. This cytokine also induces endothelial procoagulant activity and increases endothelial permeability [23]. Research in animal models as well as other smaller clinical studies has reported interesting data about the importance of TNF-α in the setting of DN [9]. Our results highlighted the possible role of TNF-α in the development and progression of renal injury in diabetic patients. We also found that the effect of TNF-α on albuminuria did not depend on blood pressure, course, or HbA1c. This suggested that TNF-α was an independent factor on DN.

In this study, the level of uMCP-1 was clearly increased in DN. It also appeared earlier than urine microalbumin. It was triggered by increased urinary protein excretion. MCP-1 is a C-C chemokine that exhibits its most potent chemotactic activity toward monocytes. MCP-1 signaling through C-C chemokine receptor type 2 (CCR2) on human mesangial cells has been shown to induce fibronectin mRNA and protein synthesis by a mechanism involving TGF-β1 production and activation of NF-κB in these cells, inducing a fibrotic response [24].

All of these data suggested that MCP-1 played an important part in the progression and development of DN. In the current study, there was a significant positive correlation between MCP-1 and UAE, which suggested that albuminuria and MCP-1 would be the important risk factors of DN. This was in agreement with the results observed by other authors [25, 26].

SAA is a sensitive acute phase protein which was found to be significantly increased and positively correlated with UAE in Japanese patients with type 2 DN [27]. Dalla Vestra et al. [28] have shown that the levels of SAA and CRP in patients with DN increased in the macroalbuminuric stage and that SAA was positively and significantly correlated with UAE. In this study, we found that levels of SAA in T2DM were elevated compared to those in the controls. They increased consistently with UAE and showed a correlation with UAE in the Pearson correlation analyses.

As for DN, the most significant risk factors are hyperglycaemia and hypertension. In order to support our results, the linear regression analysis and principal component analysis were performed. We used lnUAE as the dependent variable and blood pressure, lipids, HbA1c, and inflammatory cytokines as the independent variables. We concluded that hs-CRP, TNF-α, uMCP-1, and SAA had statistical significance with UAE independent of the conventional risk factors. Our results further confirmed the inflammation theory of DN. Chronic inflammation causes kidney damage in diabetic patients by a variety of ways. Inflammatory factors such as hs-CRP, TNF-α, uMCP-1, and SAA constitute a complex cytokine network through which autocrine or paracrine behaviors affect the kidneys due to the expanded effects of these inflammation cascades.

Studies in DN are fraught with difficulty, given the recognized associations with hypertension and dyslipidemia, both of which are known to influence microcirculation. As expected, in our study an increased prevalence of hypertension occurred in the diabetic groups compared with the control group. Moreover levels of hs-CRP, TNF-α, uMCP-1, and SAA were positively correlated with elevated SBP and DBP. This is in agreement with the recent reports made by Morii et al. [25] and may be explained, because hypertension itself is an inflammation reaction. Diabetic patients usually also have lipid disorders. There is research which has shown that TGs and LDL-C are higher and HDL-C is lower in DN groups compared with control groups and non-DN groups. In our study, we found TC and LDL-C were higher in DN groups compared with a control group, especially in the macroalbuminuric group for LDL-C, while TG and HDL-C had no significant difference in these groups.

We also investigated the correlation between inflammation markers and lipidemia and found that the levels of hs-CRP, TNF-α, SAA, and uMCP-1 were positively correlated with TC and LDL-C and had no statistical significance with TG and HDL-C. The reason may be because LDL is easily changed to oxidized low-density lipoprotein (OX-LDL) in DN [29]. Both of them, especially the latter may stimulate the expression of inflammation factors through mesangial cells. We did not find a correlation between inflammation markers and course or HbA1c, but it was present for TNF-α. Perhaps the reason was that the patients we selected had no significant difference in course or HbA1c among the three diabetes groups. In conclusion the present study suggested that in type 2 diabetic patients, increased hs-CRP, TNF-α, uMCP-1, and SAA were associated with DN pathogenesis. Thus, they may be considered as inflammation markers independent of the conventional risk factors that can be used to estimate the progression of DN. As this was a cross-sectional study, it should be confirmed by further longitudinal research. Also in future, new therapeutic potentials such as anti-CRP, anti-TNF-α, anti-MCP-1, or anti-SAA agents could be considered as targets to reduce the risk of renal complications in T2DM.

Conflict of Interests

The authors declare that there is no conflict of interests.

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