Enhanced glomerular Toll-like receptor 4 expression and signaling in patients with type 2 diabetic nephropathy and microalbuminuria

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Toll-like receptor 4 (TLR4), a component of the innate immune system, is recognized to promote tubulointerstitial inflammation in overt diabetic nephropathy (DN). However, there is no information on immune activation in resident renal cells at an early stage of human DN. In order to investigate this, we studied TLR4 gene and protein expression and TLR4 downward signaling in kidney biopsies of 12 patients with type 2 diabetes and microalbuminuria, and compared them with 11 patients with overt DN, 10 with minimal change disease (MCD), and control kidneys from 13 patients undergoing surgery for a small renal mass. Both in microalbuminuria and in overt DN, TLR4 mRNA and protein were overexpressed 4- to 10-fold in glomeruli and tubules compared with the control kidney and in MCD. In addition, NF-κB signaling was about fourfold higher in the glomeruli. TNF-α, IL6, CCR2, CCL5, and CCR5 mRNAs were markedly (about three- to fivefold) upregulated in microdissected glomeruli. While IL6, CCL2 and CCR5-mRNA, and CD68 were overexpressed in the tubulointerstitial compartment in clinical DN, they were not expressed in microalbuminuria. In a 6-year follow-up of microalbuminuric patients, glomerular TLR4 gene expression was associated with the subsequent loss of kidney function. Thus, innate immunity is activated in the glomeruli of patients with diabetic microalbuminuria. Enhanced TLR4 signaling may contribute to the progression occurring after the incipient, microalbuminuric form of nephropathy evolves to overt disease.

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Although diabetic nephropathy (DN) has consistently been considered a nonimmune disease, an emerging hypothesis is that innate immunity has a role in its development and progression.[1–3] Toll-like receptors (TLRs) are a family of receptors in the innate immune system that mediate signal transduction pathways through the activation of transcription factors that regulate the expression of proinflammatory cytokines and chemokines.[4] These *trans*-membrane proteins are the primary intermediates of the interaction between pathogens and cells.[4,5] However, in addition to microbial products, TLRs can also be activated by endogenous signals of tissue injury, including debris from apoptotic and necrotic cells, oligosaccharides, heat-shock proteins, and nucleic acid fragments.[5,6] TLRs appear to be positioned at a critical site in the pathogenesis of a variety of inflammatory conditions, such as ischemia–reperfusion injury,[7] atherogenesis,[8] and immune-mediated diseases.[9] TLRs are expressed on both leukocyte subsets and nonimmune cells, including native kidney cells.[5,6] Upon stimulation, TLR2 and TLR4 activate the nuclear factor κB (NF-κB) pathway and trigger the production of proinflammatory cytokines and chemokines, and the upregulation of cell surface molecules.[4] TLR4 is upregulated *in vitro* by the presence of high glucose concentration in monocytes,[10] and increased TLR2 and TLR4 expression has been observed in monocytes from patients with type 2 diabetes.[11] Of note, kidney macrophage accumulation has been observed to be associated with albuminuria and renal fibrosis in animal models of type 2 DN,[12] suggesting that kidney infiltration of circulating activated monocytes can cause tissue damage. As a matter of fact, overexpression of TLR4 in the tubulointerstitium, which directly correlates with macrophage infiltration, has recently been observed at a clinical stage of human DN.[13]

The majority of the constitutive TLR2 and TLR4 expression in the kidney is found in tubular epithelial cells, in glomerular endothelial cells, and in podocytes.[5] Behind the view of a monocyte/macrophage-driven inflammatory pathway in the tubulointerstitial compartment of DN, there is mounting evidence that even TLRs expressed by glomerular and kidney tubule cells can recognize endogenous signals and

express molecules that are part of the costimulatory pathway.[14–16] The *in vitro* TLR activation in kidney cells in response to hyperglycemia or to endogenous ligands, which are upregulated in diabetes, raises the possibility that the same mechanism is responsible for initiating cytokine production at an early stage of DN. However, the involvement of TLRs and the cytokine response have been so far studied in patients with clinical diabetic kidney disease and evidence of inflammation has been mainly restricted to the kidney tubulointerstitial compartment.[13] No data on TLR regulation in microalbuminuria are available. Microalbuminuria reflects potentially reversible abnormalities, initiated by dysfunction of the glomerular barrier, which are associated with the glomerular filtration rate (GFR).[17,18] Therefore, the study of molecular events taking place in microalbuminuria may reveal mechanisms occurring at an early stage of disease.

In this study, we tested the hypothesis that a TLR4-driven inflammatory response is enhanced in the kidney of type 2 diabetic patients with microalbuminuria. We tested this postulate with different selected measures. First we studied TLR4 gene and protein expression in kidney biopsies of microalbuminuric diabetic patients and compared the results with those obtained in patients with overt nephropathy. As a second step, to identify specific transcriptional pathways that underlie the pathogenesis of disease, we studied the expression profiles of selected TLR-4 downward genes from the glomeruli and tubulointerstitium in the same patients. As a third step, we examined TLR4 and cytokine distribution and regulation in the early and late phases of DN. Our data demonstrate the activation of innate immunity in the glomeruli of subjects with type 2 diabetes and early nephropathy, and suggest that enhanced TLR4 signaling contributes to the progression that occurs after the incipient, microalbuminuric form of nephropathy evolves to overt, macroalbuminuric nephropathy.

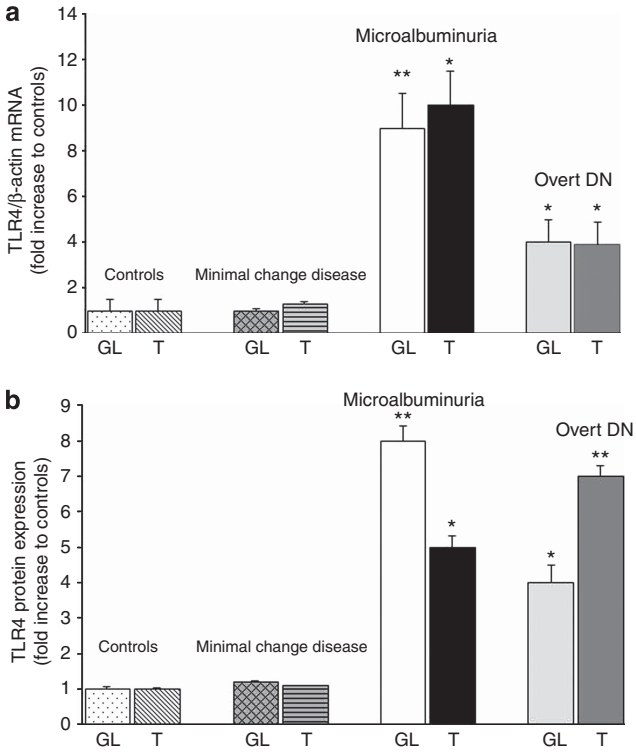

Figure 1 | Expression of (a) Toll-like receptor 4 (TLR4) mRNA and (b) protein in microdissected glomeruli (GL) and tubulointerstitium (T) of patients with microalbuminuria ($n = 12$), overt diabetic nephropathy (DN; $n = 11$), and minimal change disease (MCD; $n = 10$), and in normal control kidney ($n = 13$). TLR4 mRNA expression level was determined by real-time PCR. The expression of TLR4 protein was evaluated by immunohistochemical and image analyses. Values are expressed as fold increase ± s.e.m. to the normal kidney. In comparison with normal and disease controls, (**a**) TLR4 mRNA was markedly overexpressed (~8- to 10-fold increase) in the GL and T of patients with microalbuminuria and, to a lesser extent (~4-fold increase), in patients with overt DN. In microalbuminuria, (**b**) TLR4 protein levels in the GL and T were also upregulated (~8- and 5-fold increase, respectively). TLR4 protein expression remained high (~4- and 7-fold increase in the glomerular and tubulointerstitial compartment, respectively) in overt DN. *$P < 0.05$, **$P < 0.01$ vs. controls and MCD.

RESULTS

TLR4 expression is upregulated both in the glomeruli and in the tubulointerstitium of patients with microalbuminuria

Figure 1a shows TLR4 mRNA levels in the glomerular and tubulointerstitial compartments of microdissected biopsies of normal controls, disease controls (minimal change disease), and diabetic patients. In comparison with normal and disease controls, TLR4 mRNA was markedly overexpressed (~8- to 10-fold increase) in the glomeruli and tubulointerstitium of patients with microalbuminuria and, to a lesser extent (~4-fold increase), in the kidney of patients with overt DN. Mean TLR4 protein levels are shown in Figure 1b and immunohistochemical images in Figure 2. Similar to normal controls, in minimal change disease biopsies TLR4 protein signal was absent or minimal (Figures 1b and 2a and b). TLR4 protein levels in the glomeruli and tubulointerstitium from microalbuminuric and overt DN patients were significantly increased (approximately four- to eightfold vs. normal and disease controls; Figure 1b). In microalbuminuria, the TLR4 signal was detected in glomerular cells, including podocytes (Figure 2c and d), and in tubules (Figure 2e). In patients with overt nephropathy TLR4 was also expressed in glomerular cells (Figure 2f) and along the proximal tubule brush border and tubule cell cytoplasma (Figure 2g and h).

Glomerular NF-κB signaling is upregulated in microalbuminuria

NF-κB is a well-recognized downward TLR4 transcription factor. On the basis of the key role of NF-κB in controlling the expression of genes involved in inflammation, apoptosis, and survival, we examined the expression of NF-κB p65 (phosphorylated p65 subunit; p-p65; Figures 3 and 4) and phosphorylated inhibitor of nuclear factor κB-α (Figure 5) in the kidney of microalbuminuric patients. Normal and diseased control kidneys showed p-p65-positive cells in a very