Advanced glycation end-product induces fractalkine gene upregulation in normal rat glomeruli

Yuichi Kikuchi, Toshihiko Imakiire, Toshitake Hyodo, Taketoshi Kushiya, Keishi Higashi, Naomi Hyodo, Shigenobu Suzuki and Soichiro Miura

Second Department of Internal Medicine, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama, 359-8513 Japan

Abstract

Background. We previously reported that fractalkine was upregulated in streptozotocin-induced diabetic kidneys. Fractalkine in diabetic kidneys was detected on glomerular capillaries and the mesangium. This upregulation was suppressed by treatment with angiotensin-converting enzyme inhibitor (ACE-I) or aminoiguanidine. We examined what factors induce fractalkine upregulation in normal rat glomeruli.

Methods. Glomeruli were collected from the kidneys of normal Sprague–Dawley rats by a microdissection method. Ten glomeruli were incubated in a solution with glucose, mannitol, angiotensin II, tumour necrosis factor (TNF)-α and advanced glycation end-product (AGE)–bovine serum albumin (BSA) for 1, 2 and 4 h. Fractalkine mRNA expression in glomeruli was examined by reverse transcription–polymerase chain reaction.

Results. Fractalkine mRNA levels in the 30 mM glucose solution significantly increased (121%) compared with those in the control or 30 mM mannitol solution at 1 h. Fractalkine mRNA levels in the 15 mM glucose solution showed no significant differences at 1 or 2 h, but significantly increased (106%) after 4 h incubation. Fractalkine mRNA levels in 10⁻⁶–10⁻⁸ M angiotensin II solution showed no significant differences. Fractalkine mRNA levels in 5 or 10 ng/ml TNF-α solution significantly increased compared with those in the control in a time- and dose-dependent manner (by 94 to 253%). Fractalkine mRNA levels in the 50–200 μg/ml AGE–BSA solution also increased compared with those in BSA solution in a time- and dose-dependent manner (by 119 to 261%). By pre-incubation with MG132, a nuclear factor-κB inhibitor, fractalkine upregulation by AGE–BSA or 30 mM glucose was completely suppressed.

Conclusions. High glucose levels, AGE formation and cytokine activation in diabetes may induce fractalkine upregulation in the kidneys and lead to progression of diabetic nephropathy.

Keywords: angiotensin II; diabetic nephropathy; glucose; nuclear factor-κB; tumour necrosis factor-α

Introduction

Macrophage infiltration in the kidneys plays a crucial role in the progression of diabetic nephropathy [1]. In diabetic patients, the number of macrophages significantly increased in the moderate stage of glomerulosclerosis compared with the mild stage [2]. Patients with type 2 diabetic nephropathy with many activated macrophages in the kidneys had a poor renal prognosis [3]. Upregulation of chemokines is one of the most important events linked to macrophage infiltration. In streptozotocin (STZ)-induced diabetic rats or non-insulin-dependent diabetic models, the expression of monocyte chemoattractant protein-1 (MCP-1) in the kidneys was elevated [4,5]. It was also proven that urinary levels of MCP-1 and interleukin-8 in patients with diabetic nephropathy increased compared with healthy controls [6].

Fractalkine is the fourth chemokine type (CX3C motif), having three amino acid residues between the first and second cysteines [7]. Soluble fractalkine has chemoattractant activity for T cells and monocytes, whereas cell surface-bound fractalkine, which is induced on activated endothelial cells, promotes strong adhesion of those leukocytes via its receptor, CX3CR1. Ito et al. showed that the expression of fractalkine in the glomeruli in prolonged models of mesangial proliferative glomerulonephritis induced by anti-Thy 1
AGE induces fractalkine upregulation in glomeruli

antibody 1-22-3 increased compared with those in reversible models [8]. This suggested that a high blood flow rate and glomerular hypertension were important factors for fractalkine expression because they were suppressed by treatment with an angiotensin II receptor antagonist. Chen et al. reported that cytokines, such as tumour necrosis factor (TNF)-z and interleukin (IL)-1B, and growth factors induced fractalkine upregulation in mesangial cells, and that this upregulation was mediated by nuclear factor (NF)-kB activation [9,10].

We previously reported that fractalkine expression increased in STZ-induced diabetic kidneys [11]. Fractalkine expression in diabetic kidneys was detected along with glomerular and peri-tubular capillaries at 4 weeks, and also partially detected in mesangial lesions at 8 weeks. This upregulation was suppressed by treatment with angiotensin-converting enzyme inhibitor (ACE-I) or aminoguanidine. Although these results suggested that fractalkine upregulation in diabetic glomeruli might be induced by glomerular hypertension, angiotensin II and advanced glycation end-product (AGE), the precise mechanisms are still unknown. In this study, we examined what diabetes-related substances induced fractalkine upregulation in normal rat glomeruli, which were collected by the microdissection method. We especially focused on whether AGE increased fractalkine expression in glomeruli, because both ACE-I and aminoguanidine suppressed the formation of AGE [12,13]. Moreover, modulation of the induced changes with the NF-kB inhibitor was also analysed, based on the previous findings that fractalkine upregulation in mesangial or proximal tubular cells was mediated by nuclear factor (NF)-kB activation [9,14].

Materials and methods

Materials

AGE-bovine serum albumin (BSA) was purchased from BioVision (Mountain View, CA), recombinant rat TNF-z from R&D systems (Minneapolis, MN) and MG132, an NF-kB inhibitor, was from Calbiochem (Cambridge, MA). Angiotensin II, BSA and STZ were obtained from Sigma (St Louis, MO). The vanadyl ribonucleoside complex (VRC) was obtained from New England BioLabs (Beverly, MA).

Preparation of glomeruli

A total of 60 pathogen-free male Sprague–Dawley rats, weighing 100–120 g, were used. Rats were given free access to tap water and standard rat chow. Animal care followed the guidelines of the National Defense Medical College for the care and use of laboratory animals in research. In eight rats, STZ (65 mg/kg) was injected through the tail vein to induce diabetes. The blood glucose level was assayed through the tail vein 2 days after the STZ injection, and rats with blood glucose levels higher than 300 mg/dl were used. After anaesthesia by intraperitoneal injection with sodium pentobarbital (50 mg/kg), the left kidney was perfused with solutions A and B. Glomeruli were rapidly microdissected in a dish filled with solution A with VRC at 4°C. Eighty to 100 glomeruli were obtained per rat. Glomeruli were transferred into a new dish, which was filled with solution A, to wash out VRC. After washing, 10 glomeruli were transferred into each Eppendorf tube with a different incubation solution. The composition of solution A was as follows (mmol/l): 130 NaCl, 5 KCl, 1 NaH2PO4, 1 MgSO4, 1 Ca lactate, 2 Na acetate, 5.5 glucose and 10 HEPES; the pH was adjusted to 7.4 with NaOH. Solution B was prepared by adding 1 mg/ml of BSA and collagenase (type 1) to solution A [15].

Incubation study

Every 10 microdissected glomeruli was transferred into an Eppendorf tube filled with solution A with or without 15 or 30 mM glucose, 30 mM mannitol, 10–10–6–10–8 M angiotensin II, 5 or 10 ng/ml TNF-a, 50–200 μg/ml AGE–BSA or BSA. The samples were incubated for 1, 2 and 4 h at 37°C. Pre-incubation with MG132 was performed for 30 min at 37°C and then 200 μg/ml AGE–BSA, BSA or 30 mM glucose was added. Each set of the identical incubation study was repeated at least seven times. Each time, fresh glomeruli were prepared from another rat.

Reverse transcription coupled with competitive polymerase chain reaction

After incubation, each sample was centrifuged at 15 000 r.p.m. for 5 min, and the supernatant was discarded. In each sample, 8 μl of 2% Triton X-100 and 10 μl of reverse transcription mixture containing a random primer, RNase inhibitor and dithiothreitol were added. The total volume of the sample was 20 μl. Samples were incubated at 42°C for 50 min for reverse transcription. Reverse transcription was stopped by heating the sample at 70°C for 15 min. Samples were stored at 4°C until the polymerase chain reaction (PCR) was performed.

A 2 μl aliquot of cDNA samples was used to assess the β-actin (a housekeeping gene) mRNA levels. The concentration of primer was 0.25 μM, and the total PCR mixture was adjusted to 50 μl. The annealing temperature was 55.0°C, and the PCR cycle was 34 cycles. The PCR product was 285 bp in length. The other cDNA samples were used to examine the fractalkine mRNA levels. A specific primer for fractalkine was designed on different exons. The fractalkine sense primer was defined by bases 98–117 (5'-CCCTCGGCATGACGAAATGCA-3'), and the antisense primer by bases 781–800 (5'-AGGCCCTGGAGATTTCCTCTG-3') (GenBank AF030358). The cDNA PCR amplification product was predicted to be 703 bp in length. The competitor was synthesized by a competitive DNA construction kit (TAKARA BIO INC., Shiga, Japan). The size of the competitor was 540 bp. The primer for fractalkine was used at a concentration of 0.5 μM, and the total PCR mixture was adjusted to 100 μl. The annealing temperature was 55.0°C, and the PCR cycle was 34 cycles. After PCR, the PCR product was ethanol precipitated.
After centrifugation at 15,000 r.p.m. for 30 min at 4°C, the supernatant was discarded and the DNA pellet was resuspended with 7.5 μl of distilled water and 1.5 μl of 6× loading buffer. Samples were electrophoresed in a 1.5% agarose gel in TAE. The PCR products were visualized by ethidium bromide staining. The mRNA levels were normalized to the levels of β-actin mRNA, and the fractalkine mRNA levels were quantified by comparison with that of the competitor on ethidium bromide staining using a densitometer (ATTO, Tokyo, Japan).

Statistical analysis

Results are expressed as the mean±SEM. Statistical analyses were performed using analysis of variance (ANOVA) followed by Fisher’s multiple comparisons. A P-value of <0.05 was considered significant.

Results

Effect of glucose or mannitol on fractalkine mRNA expression in glomeruli

Eight normal Sprague–Dawley rats were used (n = 8). Microdissected glomeruli were incubated with 15 or 30 mM glucose for 1, 2 and 4 h at 37°C. In solution A (control solution), 5.5 mM glucose was included. The fractalkine mRNA levels in glomeruli incubated with 30 mM glucose significantly increased (by 121%) compared with those in controls at 1 h, and returned to control levels after 4 h incubation (Figure 1A). The fractalkine mRNA levels in glomeruli incubated with 15 mM glucose showed no significant differences at 1 or 2 h, but significantly increased (by 106%) after 4 h incubation (Figure 1A). The fractalkine mRNA levels in glomeruli incubated with 30 mM mannitol solution did not show significant differences at 1 h. The fractalkine mRNA levels in glomeruli incubated with 30 mM glucose significantly increased compared with those in equivalent concentrations of mannitol (Figure 1B).

Effect of angiotensin II on fractalkine mRNA expression in glomeruli

Fractalkine expression in STZ-induced diabetic glomeruli was suppressed by the treatment with ACE-I [11]. Therefore, we examined the effect of angiotensin II on fractalkine mRNA expression in glomeruli. Microdissected glomeruli were incubated with 10⁻⁶, 10⁻⁷ and 10⁻⁸ M angiotensin II (n = 8). The fractalkine mRNA levels in glomeruli incubated with any concentration of angiotensin II showed no significant differences from 1 to 4 h incubation in this experimental protocol (Figure 2). Because the sensitivity for angiotensin II in diabetic glomeruli may be different from that in normal glomeruli, we also examined the effect of angiotensin II on fractalkine expression in STZ-induced diabetic glomeruli. Eight diabetic rats at 2 weeks after STZ injection were used. The fractalkine mRNA levels in diabetic glomeruli significantly increased compared with those in age-matched controls (by 53%, P < 0.005). The effect of 10⁻⁶–10⁻⁸ M angiotensin II on fractalkine expression in diabetic glomeruli showed similar results to that in normal glomeruli in which no significant alteration was observed (data not shown).

Effect of TNF-α on fractalkine mRNA expression in glomeruli

Microdissected glomeruli were incubated with 5 or 10 ng/ml TNF-α for 1, 2 and 4 h (n = 7). The fractalkine mRNA levels in glomeruli incubated with 5 ng/ml
TNF-α significantly increased (by 94%) compared with those in controls at 1 h. After 4 h incubation, the fractalkine mRNA levels increased further by 145% in a time-dependent manner. The fractalkine mRNA levels in glomeruli incubated with 10 ng/ml TNF-α significantly increased compared with those in controls for 1–4 h. This upregulation was also time dependent. Moreover, the fractalkine mRNA levels incubated with 10 ng/ml TNF-α increased compared with those in 5 ng/ml TNF-α at all points, although there were no significant differences (Figure 3).

**Effect of AGE–BSA on fractalkine mRNA expression in glomeruli**

Microdissected glomeruli were incubated with 50, 100 or 200 µg/ml AGE–BSA for 1 h (n = 8). As a control, incubation with 200 µg/ml BSA was performed. Fluorescence of AGE was confirmed by fluorescence spectrophotometry, and glycated BSA showed a 7000% increase in fluorescence compared with control BSA. The fractalkine mRNA levels in glomeruli incubated with 50 or 100 µg/ml AGE–BSA significantly increased (by 119 or 132%, respectively) compared with those in controls. There were no significant differences between 50 and 100 µg/ml AGE–BSA. The fractalkine mRNA levels in glomeruli incubated with 200 µg/ml AGE–BSA significantly increased (by 232%) compared with those in controls, even when compared with those in 50 or 100 µg/ml AGE–BSA (Figure 4A).

Glomeruli were incubated with 100 µg/ml AGE–BSA for 1, 2 and 4 h (n = 7). The fractalkine mRNA levels in glomeruli significantly increased compared with those in controls at any time, and showed time dependency (by 132 to 261%) (Figure 4B).

**Effect of the NF-κB inhibitor on fractalkine mRNA upregulation by AGE or glucose**

Activation of NF-κB is reported to play a crucial role in fractalkine upregulation in glomeruli [9]. Therefore, we examined whether the NF-κB inhibitor MG132 suppressed fractalkine upregulation in glomeruli by AGE–BSA or 30 mM glucose. Glomeruli were incubated with solutions with or without 10 µM MG132 for 30 min and then with 200 µg/ml AGE–BSA (n = 7) or 30 mM glucose (n = 7) for 1 h. The fractalkine mRNA levels in glomeruli incubated with AGE–BSA without MG132 significantly increased (by 121%) compared with those in controls. Pre-incubation with MG132 completely suppressed the fractalkine upregulation by AGE–BSA in glomeruli (Figure 5A). The fractalkine mRNA levels in glomeruli incubated with 30 mM glucose without MG132 significantly increased (by 64%). This upregulation was also completely suppressed by pre-incubation with MG132 (Figure 5B).

**Discussion**

In this study, we demonstrated that high ambient glucose, TNF-α and AGE induced fractalkine upregulation in normal rat glomeruli. High glucose is a starting point in the pathogenesis of diabetic nephropathy. Incubation with 30 mM glucose for 1 h significantly increased fractalkine mRNA levels in glomeruli. Even when incubated with 15 mM glucose, fractalkine mRNA expression was upregulated. On the other hand, incubation with 30 mM mannitol for 1 h did not affect the fractalkine mRNA expression in glomeruli. These data suggest that high ambient glucose, but not hyperosmolality, might directly induce increased fractalkine gene expression in glomeruli. However, the
fractalkine mRNA levels in glomeruli incubated with 30 mM glucose returned to control levels after 4 h incubation. The reason why the fractalkine mRNA levels in glomeruli incubated with 15 mM glucose were higher than those with 30 mM glucose at 4 h was unknown. These findings suggest that glucose, even in moderate concentration, may stimulate fractalkine upregulation in glomeruli, although the upregulation by glucose was temporary.

Angiotensin II induces not only contraction of glomerular efferent arterioles, resulting in glomerular hypertension, but also accelerated production of transforming growth factor (TGF)-β and accumulation of collagen and extracellular matrix in the mesangium [16]. Activation of the renin–angiotensin system is an important mechanism in the progression of diabetic nephropathy. Therefore, the use of ACE-I and angiotensin II receptor blockade at the early stage of human diabetic nephropathy is recommended. In STZ-induced diabetic rats, ACE-I suppressed fractalkine upregulation in glomeruli [11]. Therefore, we hypothesized that angiotensin II might increase fractalkine mRNA expression in glomeruli. However, 10⁻⁶–10⁻⁵ M angiotensin II did not affect fractalkine mRNA expression in glomeruli in this experimental protocol. Although the sensitivity for angiotensin II in the diabetic kidney may be different from that in normal kidneys, angiotensin II did not show any significant differences in fractalkine expression in STZ-induced diabetic glomeruli. In our previous study, the suppression of fractalkine upregulation in diabetic glomeruli by ACE-I might be due to another mechanism.

That cytokines such as TNF-α and IL-1β upregulated fractalkine expression in mesangial cells in a time- and dose-dependent manner has been reported by Chen et al. [10]. Diabetic states, including
high glucose condition and oxidative stress, induce the activation of various types of cytokines. Urinary and renal interstitial TNF-α levels in STZ-induced diabetic kidneys increased prior to the rise in albuminuria [17]. TNF-α, as well as TGF-β, may be one of the important cytokines in the onset and progression of diabetic nephropathy. Therefore, we examined whether TNF-α induced fractalkine mRNA upregulation in fresh microdissected glomeruli, but not cell culture, in our method. Although the fractalkine mRNA levels in glomeruli incubated with 5 or 10 ng/ml TNF-α at 1 h showed the same degree as those in 30 mM glucose, the upregulation by TNF-α further increased in a time-dependent manner. This result was consistent with the report by Chen et al. [9].

Production and accumulation of AGE induces a variety of diabetic complications, including nephropathy. AGE stimulates synthesis of fibronecin and type IV collagen in mesangial cells, induces upregulation of cell adhesion molecules and mediates monocyte migration. The accumulation of AGE in glomeruli may alter the turnover, elasticity and ionic charge of the basement membrane. Therefore, suppression of AGE formation is also an important treatment in diabetic nephropathy [18]. Aminoguanidine inhibits the formation of AGE. Our previous study showed that aminoguanidine suppressed the fractalkine upregulation in STZ-induced diabetic glomeruli [11]. This finding suggested that AGE plays a crucial role in fractalkine upregulation in glomeruli. As expected, the fractalkine mRNA levels in glomeruli incubated with AGE–BSA significantly increased compared with those in controls in a time- and dose-dependent manner. The increased levels were of a similar degree to those in TNF-α. Of interest, ACE-I not only inhibited upregulation of cytokines and growth factors [19], but also attenuated AGE formation and accumulation of AGE in diabetic glomeruli [12]. Although treatments with ACE-I or aminoguanidine in diabetic rats for 4 weeks showed no significant difference in fractalkine expression, the expression was significantly suppressed by these treatments for 8 weeks. This result suggests that ACE-I and aminoguanidine suppress fractalkine upregulation by the inhibition of AGE formation, because production and accumulation of AGE in vitro require several weeks under a high glucose condition. AGE was reported to promote expression and secretion of TNF-α in human endothelial cells [20]. The possibility that increased expression of TNF-α by AGE induced fractalkine upregulation in glomeruli could not be completely ruled out. However, the fractalkine mRNA levels in glomeruli incubated with AGE–BSA at 1 h were of a similar degree to those in 10 ng/ml TNF-α. This finding suggests that AGE itself may stimulate fractalkine expression in glomeruli. This is the first report that AGE may modulate fractalkine mRNA expression in glomeruli.

Both TNF-α-stimulated rat mesangial cells and human albumin-stimulated human proximal tubular cells showed increased fractalkine expression, and the upregulation was suppressed by NF-κB inhibitors [9,10,14]. MG132 inhibits degradation of IkB, nuclear translocation of p65/NF-κB and p65/NF-κB binding activation, and cannot inhibit the c-Jun/AP-1 pathway [10]. Pre-incubation with MG132 completely suppressed AGE- or 30 mM glucose-induced fractalkine upregulation in normal rat glomeruli. The fractalkine upregulation by AGE or glucose in glomeruli might be also mediated through the NF-κB signalling pathway.

In conclusion, the high glucose levels, AGE formation and cytokine activation in diabetes may induce fractalkine upregulation in the kidneys and aggravate diabetic nephropathy.

Conflict of interest statement. None declared.

References


