Hypoxia-Inducible Factor-1 α Is Involved in the Attenuation of Experimentally Induced Rat Glomerulonephritis

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Key Words

Glomerulonephritis · Habu snake venom · Angiotensin II · Hypoxia-inducible factor-1 α

Abstract

Background/Aim: Among various kidney disease models, there are few rat glomerulonephritis (GN) models that develop in a short time, and with mainly glomerular lesions. Hypoxia-inducible factor (HIF)-1 α is a transcriptional factor that induces genes supporting cell survival, but the involvement of HIF-1 α in attenuating the progression of GN remains to be elucidated. We developed a new model of rat GN by coadministration of angiotensin II (All) with Habu snake venom (HV) and investigated whether HIF-1 α is involved in renal protection. **Methods:** Male Wistar rats were unilaterally nephrectomized on day 1, and divided into 4 groups on day 0; N group (no treatment), HV group, A group (All), and H+A group (HV and All). To preinduce HIF-1 α , cobalt chloride (CoCl₂) was injected twice before injections of HV and All in 11 rats. **Results:** GN was detected only in the H+A group; observed first on day 2 and aggravated thereafter. HIF-1 α was expressed in the glomeruli and renal tubules in the A and H+A groups. In the H+A group, GN was remark-

ably reduced by CoCl₂ pretreatment (44.9 to 12.2%, $p < 0.01$). **Conclusion:** Both HV and All were critical for the development of GN, and HIF-1 α remarkably attenuated the progression of GN.

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Introduction

Many animal studies have been performed in attempts to overcome the poor prognosis of chronic renal failure due to diabetic nephropathy and glomerulonephritis (GN) [1–5]. Although factors involved in the pathogenesis of GN have been intensively investigated, the development of a proper animal GN model with high reproducibility and simplicity as well as a model without time-consuming process is required. Experimental rat models of GN are classified into several groups in terms of the pathophysiological mechanisms of renal diseases. Anti-glomerular basement membrane nephritis was developed with depositions of immune complex using anti-glomerular basement membrane antibody [3, 6], tubulointerstitial injury was caused by cyclosporine A [4] and injury of renal tubules by ischemia [5]. However, there are few rat GN models with mainly pathological features in the glo-

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Fig. 1. Study Protocol. All rats are unilaterally nephrectomized on day 1 and divided into 4 groups on day 0. N group: no injection of reagents. HV group: injection of 3.5 mg/kg of Habu snake venom (HV). A group: continuous administration of 100 ng/min of angiotensin II (AII). H + A group: administration of HV and AII.

meruli that are developed in a short time [7]. Angiotensin II (AII) is known to increase blood pressure through vascular contraction, and to be profoundly involved in cardiovascular hypertrophy and the contraction of intrarenal arteries. AII is also directly involved in the progression of glomerulosclerosis via the effect of hyperfiltration with or without hypertension [8, 9]. Many studies have revealed important factors involved in the pathogenesis of GN or factors aggravating GN, but evaluating further factors that suppress the occurrence of GN is also crucial. To investigate the features of renal protection, we focused on hypoxia-inducible factor (HIF)-1 α . HIF-1 α , a transcriptional factor with formation of a heterodimer with HIF-1 β [10], is post-transcriptionally regulated and its protein level is elevated by hypoxia through inhibition of ubiquitin-mediated degradation. HIF-1 α is known to be a survival factor responsible for inducing lines of genes supporting cell survival such as glucose metabolism (glucose transporters and glycolytic enzymes), vasomotor regulation (heme oxygenase-1 and endothelin-1), angiogenic growth (vascular endothelial growth factor), and anemia

control (erythropoietin and transferrin) [11–13]. Recent studies have demonstrated that non-hypoxic stimuli like AII can also activate HIF-1 α [14, 15], but the role of HIF-1 α induction in attenuating the progression of GN remains to be elucidated. Accordingly, we developed a new rat GN model by coadministration of AII with Habu snake venom (HV) and investigated whether preinduction of HIF-1 α leads to renal protection.

Materials and Methods

Development of Rat GN Model

All experiments were approved by the institutional review board for the care of animal subjects and were performed in accordance with guidelines of Kochi Medical School. Nine-week-old male Wistar rats (180–220 g) were purchased from Japan SLC (Shizuoka, Japan). Rats were unilaterally nephrectomized on day 1. On day 0, the rats were divided into 4 groups. In the first group, no treatment was performed with any reagents or surgical procedure (N group, $n = 6$). In the second group, rats were injected with 3.5 mg/kg of HV (Sigma-Aldrich Co., Steinheim, Germany) through the femoral vein (HV group, $n = 11$). In the third group, rats were continuously adminis-

tered with AII (100 ng/min; Peptide Institute Inc., Osaka, Japan) using Alzet osmotic pumps (DURECT Co., Cupertino, Calif., USA) (A group, $n = 11$). In the fourth group, rats were administered with both HV and AII (H+A group, $n = 22$). Rats were sacrificed on day 1, 2, 3 or 4, and kidneys excised for histochemical analysis (fig. 1).

Measurement of Systolic Blood Pressure

Systolic blood pressure (SBP) was measured by the tail-cuff method with an electro-sphygmomanometer (BP-98A; Softron Co., Tokyo, Japan). SBP was measured in conscious rats every day from day 1 to 2. The SBP value for each rat was calculated as the average of 3 separate measurements at each session. SBP measurement was performed between 9 and 12 a.m. by a single blinded investigator.

Measurements of Serum Urea Nitrogen and Creatinine

Before the sacrifice, blood samples were obtained via an axillary vein for determination of serum urea nitrogen (UN) and creatinine (Cr) levels. Serum UN and Cr levels were determined enzymatically with automation-analysis equipment (Hitachi 7350; Hitachi Co., Ibaragi, Japan) in our laboratory center.

Histological Analysis

To evaluate the progression of GN in our animal model, histological analyses were performed using the periodic acid-Schiff (PAS) and periodic acid-methenamine silver (PAM) reagents. After the specimens were paraffin embedded, 4- μ m-sectioned samples were stained with PAS and PAM reagents and counterstained with hematoxylin. For quantitative analysis, the ratio of damaged glomeruli to all glomeruli in the sectioned sample was calculated and the percentage of GN in the section was evaluated. Moreover, semiquantitative analysis was performed to evaluate more precisely the morphological changes of our GN model according to the protocol in previous studies [16, 17]. A minimum of 20 glomeruli (ranging from 20 to 60 glomeruli) in each specimen were examined and the severity of the mesangiolysis lesion was graded from 0 to 4+ according to the percentage of glomerular involvement; a 1+ lesion represented an involvement of 25% of the glomerulus while a 4+ lesion indicated that 100% of the glomerulus was involved. Thus, the mesangiolysis score (MES) was then obtained by multiplying the degree of damage (0 to 4+) by the percentage of the glomeruli with the lesion. Tubular injuries including tubular necrosis or occlusion of collecting ducts by cast material were graded as mild (1+), moderate (2+), or severe (3+).

Western Blot Analysis

Nuclear protein from whole kidney was prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology Inc., Rockford, Ill., USA). Nuclear protein was electrophoresed using 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, Mass., USA). A monoclonal IgG HIF-1 α antibody α 67 (Novus Biological, Littleton, Colo., USA) was used; a horseradish peroxidase-conjugated antibody (Promega Co., Madison, Wisc., USA) was used as a secondary antibody. The ECL Western blotting systems (Amersham Bioscience, Uppsala, Sweden) was used for detection.

Immunohistochemical Analysis

Paraffin sections including the samples were dewaxed in xylene and rehydrated in a series of ethanol, and then washed in distilled water before staining procedures. According to the instruction pro-

vided by the manufacturer, HIF-1 α was identified with rabbit polyclonal anti-HIF-1 α antibody H-206 (Santa Cruz Biotechnology, Calif., USA) utilizing the catalyzed signal amplification system (Dako, Hamburg, Germany) based on the streptavidin-biotin-peroxidase reaction. Antigen retrieval was performed for 5 min in a preheated Dako target retrieval solution using a microwave. Incubation procedures were performed in a humidified chamber. Following the incubation, specimens were washed 3 times in TBST buffer. The specificity of staining was confirmed by substitution of the primary antibody for a normal rabbit IgG and additionally by an immunohistochemical reaction without a primary antibody but with the secondary antibody alone.

An Experiment Using Cobalt Chloride as a Pretreatment

Rats were twice subcutaneously administered 30 mg/kg of cobalt chloride (CoCl_2) at a 12-hour interval (CoCl_2 group) ($n = 11$), followed by unilateral nephrectomy. Then, the rats were administered with HV and AII. As a comparison, rats were injected with 0.9% NaCl solution instead of CoCl_2 , followed by the same protocol as the CoCl_2 group ($n = 11$). After CoCl_2 administration, however, before injection of HV and AII, a kidney was excised as a sample to examine expression level of HIF-1 α (CoCl_2 Pre). Likewise, 2 days after administration of HV and AII, a kidney was also excised (CoCl_2 Day 2). To compare the expression level of HIF-1 α by CoCl_2 before GN and the severity of pathology of GN, we investigated whether preinduction of HIF-1 α is involved in renal protection.

Statistical Analysis

Data are reported as mean $\pm$ SEM. A paired t test was used for paired samples and Student's t test was used to compare the 2 groups. One-way layout analysis of variance or repeated measures of analysis of variance were used to compare multiple groups. If the p value was significant, Scheffé's multiple comparison was performed. A p value < 0.05 was considered significant.

Results

AII Combined with HV Developed GN

Morphological studies using PAS and PAM staining revealed that there are no glomerular or tubular injuries in N group (fig. 2a), HV group (fig. 2b), A group (fig. 2c), however, GN was detected only in the H+A group (fig. 2e). Although renal tubular casts were observed, glomerular changes were scarcely observed on day 1 after AII and HV administration (fig. 2d, 3). GN was initially detected on day 2 (fig. 2e, f, 3), followed by further aggravation during the time course (data not shown). Renal tubular injury including tubular necrosis was not remarkable, and extensive cellular infiltration was not found in the interstitial regions (fig. 3). On the other hand, characteristic focal and segmental mesangiolysis, explained as capillary aneurysmal ballooning, was observed with dilatation of glomerulus (fig. 2e, f). The rate of occurrence of GN on day 2 was $44.9 \pm 2.6\%$, and the MES score of the H+A

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group was 199 ± 15 (fig. 3). On the other hand, in the HV group, less than 2% had morphologic changes of mesangiolysis during 4 days, and the MES score was 10 ± 5 (fig. 2b, 3). Moreover, in the A group, there were no morphologic changes during the time course (fig. 2c).

Changes in Serum UN and Cr

Serum UN and Cr were 18.4 ± 0.7 and 0.31 ± 0.01 mg/dl, respectively, on day 2 in the N group. In the H+A group, serum UN and Cr levels increased to 41.5 ± 4.0 and 0.57 ± 0.05 mg/dl, respectively, on day 2; significantly higher than those in the N group (fig. 4a, b). In contrast, serum UN and Cr levels in the H+A group on day 1 (24.0 ± 1.8 and 0.42 ± 0.02 mg/dl, respectively) were similar to the level of the N group. There were no significant differences in serum UN and Cr level among the HV, A and N groups.

SBP Response

SBP values of each group are shown in figure 4c. There were no significant differences in SBP after nephrectomy among the 4 groups. Administration of AII caused a significant increase of SBP on day 1 (186 ± 4 mm Hg) and persisted to day 2 (192 ± 1 mm Hg). SBP in the H+A group on day 2 (183 ± 3 mm Hg) was comparable to that in the A group. Administration of HV had no influence on SBP during the 2 days.

Expression Level of HIF-1 α Protein

Western blot analysis revealed that the expression level of HIF-1 α protein increased in the H+A and A groups (fig. 5a), compatible with the results of immunohistochemical analysis. Expressions of HIF-1 α protein were observed in the A and H+A groups, but protein expres-

Fig. 2. Glomerulonephritis is developed with the combination of HV and AII, and HIF-1 α is induced in the intact glomeruli. There are no glomerular or tubular injuries in N group (a), HV group (b), A group (c) and H+A group on day 1 (d). Damaged glomeruli, characterized by extensive mesangiolysis, are observed in H+A group on day 2. PAS staining. Magnification, $\times 100$ (e). Focal and segmental mesangiolysis with large capillary aneurysmal ballooning are observed in the H+A group on day 2. PAM staining. Magnification, $\times 400$ (f). The number of GN was significantly less in pretreatment with CoCl_2 than without. PAS staining. Magnification, $\times 100$ (g). Immunoreactive HIF-1 α -positive signals are not detected in the N group (h). Nuclear HIF-1 α signals are observed in a glomerulus and tubules in the A group. Magnification, $\times 200$ (i). A glomerulus in the H+A group on day 2 possesses intact cells with HIF-1 α -positive signals; in contrast, other parts have few HIF-1 α signals due to mesangiolysis. Magnification, $\times 200$ (j).

Fig. 3. Semiquantitative analysis of morphologic changes in our glomerulonephritis model. The main lesion in the H+A group is initially detected on day 2 as mesangiolysis in glomeruli; however, there are no tubular lesions of necrosis except for tubular casts; in contrast, there are no morphological changes in the N and A groups. MES = Mesangiolysis score.

sion was not detected in the N and HV groups. These data suggest that HIF-1 α was induced mainly by AII, and, at least in part, was related to the pathogenesis of GN or to the defense mechanism against the progression of GN.

Induction of HIF-1 α in Glomeruli and Renal Tubules

Immunohistochemical study demonstrated positive nuclear staining of HIF-1 α in glomeruli, renal tubules (fig. 2i, j), collecting ducts and epithelium of the papilla (data not shown) in the A and H+A groups. In contrast, no positive nuclear signals were detected in the N (fig. 2h) and HV (data not shown) groups. HIF-1 α -positive cells were mainly detected in mesangial cells in glomeruli (fig. 2i, j). As demonstrated, especially in the H+A group (fig. 2j), HIF-1 α was expressed in the intact part of the glomerulus, but not in the injured part of the same glomerulus. Furthermore, nuclear HIF-1 α -positive signals were observed in smooth muscle cells in peripheral renal arteries (data not shown).

CoCl_2 Pretreatment Inhibits the Progression of GN

To further investigate whether HIF-1 α is involved in the development of nephropathy or in the antiprogessive action, we pretreated rats with CoCl_2 . As demonstrated in figure 5b, pretreatment with CoCl_2 increased HIF-1 α expression before administration of HV and AII (Pre-1),

Fig. 4. Serum UN, Cr and SBP are increased with the combination of HV and AII. The serum UN (**a**) and Cr (**b**) levels in the H+A group on day 2 are significantly higher than other groups. SBP increases significantly with administration of AII (A and H+A groups) (**c**).

suggesting that HIF-1 α was induced by CoCl₂ before development of GN. Even on day 2, the expression level of HIF-1 α was increased in the CoCl₂ group (CoCl₂ Day 2-1). In the CoCl₂ group, focal mesangiolysis with glomeruli enlargement was still observed, but the number of GN was much less than in those without CoCl₂ pretreatment (fig. 2g).

Thus, 7 of 11 rats (63.6%) with CoCl₂ pretreatment were rescued from GN alone, while the other 4 (36.4%) were not; showing a comparable severity level of GN with the non-CoCl₂ group. As demonstrated in figure 5b, unlike Pre-1, Pre-2 did not induce HIF-1 α with CoCl₂ and showed no CoCl₂ suppression of GN. The ratio between rats rescued or not rescued from GN was comparable with that between preinduction and noninduction of HIF-1 α

by CoCl₂, as demonstrated in figure 5c. In the CoCl₂ group, the rate of GN from each rat decreased to $12.2 \pm 2.1\%$, which was in great contrast to $44.9 \pm 2.6\%$ in the non-CoCl₂ group. Furthermore, serum UN and Cr levels on day 2 were significantly lower in the CoCl₂ than in the non-CoCl₂ group ($p < 0.05$) (fig. 6a, b), despite comparable SBP values between the 2 groups (fig. 6c).

Discussion

In this study, we developed a new model of GN induced by both HV and AII. This model has several distinct characteristics. First, GN developed rapidly, and was detected on the second day after administration of

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Fig. 5. The protein level of HIF-1 α is increased by administration of HV and AII, and pretreatment of CoCl₂ increases HIF-1 α expression before development of GN. HIF-1 α is not detected in the N and HV groups (Day 2). However, HIF-1 α is detected in A (Day 2) and H+A (Days 1 and 2) groups (**a**). The CoCl₂ group, in accord with the level of HIF-1 α induction, was divided into 2 groups. HIF-1 α is greatly induced before the development of GN (CoCl₂ group Pre-1), and is followed by a high level (CoCl₂ group Day 2-1); in contrast, it is not

efficiently induced (CoCl₂ group Pre-2), and also is scarcely detected on day 2 (CoCl₂ group Day 2-2) (**b**). The rate of preinduction of HIF-1 α by CoCl₂ is comparable with that of the inhibition of GN by CoCl₂ (**c**).

Fig. 6. Pretreatment with CoCl₂ attenuates GN. Serum UN (**a**) and Cr (**b**) levels in the CoCl₂ group on day 2 are significantly decreased compared to those in the non-CoCl₂ group. There is no significant difference in SBP between the CoCl₂ and non-CoCl₂ groups (**c**).

HV and AII. Many models of GN have been reported including 5/6 nephrectomized and Thy-1.1 nephritis models [18, 19]. However, these models take a long time to develop nephropathy. In contrast, our protocol induced GN in 2 days, suggesting that one of the advantages our model has over others is in terms of the time course. Further, pathological findings were restricted to glomerular regions without remarkable tubular or interstitial lesions. Since our GN model developed within 2 days, it also has advantages for disclosing the specifically critical time point of the development of GN. Furthermore, the development rate of GN was almost 100%, indicating the high reproducibility of our model. This basis of the rat model was initially developed by Barnes et al. [20] who reported that the progression of AII-induced renal injury was accelerated by pre-existing injury induced by HV; our model, which now optimizes the reproducibility of GN, is a modification of theirs.

Habu-induced nephropathy was reported to develop within 1 day by a dose of 2.0–4.0 mg/kg HV (in our model 3.5 mg/kg) and the main pathological change was ‘mesangiolysis’ [21, 22]. However, for reasons we have not as yet ascertained, in our study no rats showed Habu-nephropathy-specific pathological findings during the first week in the HV group. On the other hand, AII is one of the major factors responsible for the pathogenesis of GN, because it remarkably increases glomerular pressure causing hyperfiltration, production of extracellular matrix and expression of lines of genes involving GN [23–25]. Further, since AII has some ischemic effects on the kidneys, there is the possibility that an AII-induced ischemic effect causes the GN depicted in our model. However, as demonstrated in this study, glomerular injury was predominantly observed, and was not associated with renal tubular lesions, i.e. tubular necrosis suggesting renal ischemia. Therefore, in accordance with the pathological characteristic of this GN, AII-induced renal ischemia may not be responsible for its development in our model. Additionally, in this study, SBP increased in the A and H+A groups, but GN was not induced in the A group. Therefore, GN in our model was induced not by HV or AII alone, but by the combination of HV and AII, independent of any increase in systemic blood pressure.

HIF-1 α is a master transcriptional factor, transactivating the expression of many genes important for cell survival under hypoxic conditions [11–13, 26]. These genes are responsible for glycolysis, angiogenesis, proliferation and iron metabolism, all of which are induced by hypoxic stress; thus, the induction of HIF-1 α is a marker of hypoxia. HIF-1 α is regulated at the post-translational level by

the proteasome system through ubiquitination with von Hippel-Lindau (VHL) protein [27, 28]. As previously reported, this regulation of HIF-1 α protein level is dependent on the concentration of oxygen. Hypoxia induces enhancement of HIF-1 α protein stability leading to the elevation of the protein level due to inhibition of degradation by VHL. Therefore, hypoxia induces adaptation in cells including induction of HIF-1 α ; the hypoxic pathway. On the other hand, a line of evidence recently accumulated suggests that HIF-1 α is also regulated independently of oxygen concentration through the nonhypoxic pathway [14, 15]. AII is reported to regulate HIF-1 α both at transcriptional and post-translational levels in vascular smooth muscle cells cultured under normoxic condition through the AII type 1 receptor [14, 15]. Moreover, HIF-1 α is also post-translationally regulated in several cell lines in the presence of tumor necrosis factor- α or nitric oxide independent of oxygen contents [29, 30].

As demonstrated in this study, immunoreactivity of HIF-1 α was not detected in the N group (no treatment group), but HIF-1 α was detected in the nuclei of glomerular, tubular and epithelial cells of the papilla by administration of AII alone or AII and HV together. This is the first evidence showing that HIF-1 α was detected in the kidney by AII, independent of systemic hypoxic stress. As indicated here, HIF-1 α was found to be expressed only in intact, not damaged glomeruli. Even within a glomerulus, only the intact part of glomerular cells expressed HIF-1 α . Considering the fact that induction of HIF-1 α is one of the defense mechanisms for cell survival [31–33], our data indicate that induction of HIF-1 α is a marker of glomeruli survival; indeed, it could be a marker of renal protection.

To further investigate whether HIF-1 α is involved in the progression or protection of GN, preinduction of HIF-1 α was performed with CoCl₂ before administration of HV and AII. Surprisingly, the induction of HIF-1 α by CoCl₂ pretreatment attenuated the progression of GN; the level of GN was reduced from 44.9 to 12.2% and the incidence of GN was reduced from 100 to 36.4%. Furthermore, as indicated, the preinduction of HIF-1 α actually affects the inhibition of GN, because the rate of HIF-1 α induction was parallel with that of the attenuation of GN. Therefore, our data suggest that HIF-1 α is involved, at least in part, in the defense mechanism against the progression of GN, and hence could be a marker for renal protection.

AII is reported to induce HIF-1 α [14, 15] and plays a partial role in the renal protective effect; however, the other effects of AII, such as increasing glomerular pressure and modulating gene expression involving in the renal

failure, may overcome any protective effect of AII-induced HIF-1 α , and so as a result it may lead to the progression of GN.

In conclusion, we developed a highly reproducible GN model by combining HV and AII. Preinduction of HIF-1 α remarkably attenuated the progression of GN, indicating that HIF-1 α was involved in the defense mechanism of the kidney.

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