VHL modification is involved in progression of experimentally-induced rat glomerulonephritis

Running title: Pre-induction of VHL accelerates glomerulonephritis.

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Abstract

Background/Aims: We previously demonstrated that angiotensin II (AII) combined with Habu snake venom (HV) induces glomerulonephritis (GN) in rats, the lesion of which is restricted to the glomeruli, on day 2 following administration of both reagents. However, the mechanisms by which those cause GN are unclear. We also indicated a role for hypoxia-inducible factor (HIF)-1α in attenuating the progression of GN. However, a role of VHL in GN and mechanisms by which HV regulates the pathogenesis of GN remains unclear.

*Methods and Results: Im*munohistochemical analysis revealed that VHL is weakly expressed in the renal tubules alone; however, HV caused elevation of VHL expression in the injured glomeruli including endothelial cells and partially podocytes. Western blot analysis revealed that VHL expression was increased in HV-treated kidney, compared with AII-treated or normal kidney. An in vitro study also showed HV-induced elevation of VHL expression. To investigate whether VHL pre-induction causes GN aggravation, we utilized thrombin, an inducer of VHL. Thrombin alone did not cause renal injuries; however, thrombin pre-treatment accelerated the development of GN even on day 1 following administration.

Conclusion: We suggest that VHL pre-induction by thrombin aggravates GN, and that the increase in VHL expression due to HV might be involved in accelerating onset of GN.

Key words
glomerulonephritis, VHL, renal protection

Introduction

Our previous study demonstrated that our protocol using angiotensin II (AII) and Habu snake venom (HV) efficiently induced glomerulonephritis (GN) with high reproducibility [1]. Our GN model was developed based on the protocol first reported by Barnes JL et al. [2] with a modification. This group has investigated several pathological features of GN, but they have not focused on the dysregulation of self-defense mechanisms affecting the pathogenesis of the GN. Our protocol for inducing GN has several advantages compared to others; first, it is a very easy method; second, higher reproducibility; third, rapid induction of GN in two days, fourth, the lesion is restricted to glomeruli. GN is induced by AII and HV and is consistently observed on the second day (day 2) after administration, but not at day 0 or day 1. Therefore, this model can easily and reliably induce GN in a rapid time course.

The previous study characterized the time course of GN and identified a protective effect of hypoxia-inducible factor (HIF)-1α on the progression of GN. It has been reported that the level of HIF-1α protein is tightly regulated by von Hippel Lindau (VHL) protein, which forms a complex with HIF-1α during normoxic conditions but not in hypoxia [3,4]. The role of HIF-1α was fully investigated in the previous study; however, the role of VHL in the progression of GN induced by our protocol remains unclear. Furthermore, the mechanisms by which GN is induced by a combination of AII and HV, in a rapid time course, also remain to be investigated. AII is known to play a crucial role in the progression of GN [5,6]. AII increases glomerular pressure by preferentially constricting extraglomerular arterioles, and activates proliferation of mesangial cells as well as extracellular matrix production. On the other hand, HV is a potent inducer of tissue necrosis and hemorrhage, and it is also reported to induce glomerular injury, i.e., mesangiolysis [7-9]. Even on the basis of these findings, as HV alone actually did not produce the typical lesions of GN during the first several days following administration, the pathogenesis of GN induced by our protocol was unclear.

Therefore, in the present study, we focused on the role of VHL in the progression of GN, evaluated VHL expression and localization in GN, examined whether VHL expression is induced by several reagents, including HV, and finally whether such an inducer of VHL could affect the time course of GN.

Materials and Methods

Development of Rat GN Model

According to our previous study, GN was induced by AII and HV [1]. Briefly nine week-old male Wistar rats (180-220g) from Japan SLC (Shizuoka, Japan) were unilaterally nephrectomized on day -1 (n=39). On day 0, rats were intravenously injected with 3.5 mg/kg of HV (Sigma-Aldrich Co., Steinheim, Germany), and then AII was continuously administered (100ng/min, Peptide Institute, Inc., Osaka, Japan) by Alzet osmotic pumps (DURECT Co., Cupertino, CA) (n=15). For comparison, AII (n=9) or HV (n=9) alone was administered after nephrectomy. For preinduction of VHL, one unit of thrombin was administered intraperitoneally on day -1 (n=15), during the nephrectomy, followed by the same protocol [10]. Rats were sacrificed on day 1 (n=15) and 2 (n=6), and the kidneys were excised for further analysis.

Measurement of Serum Urea Nitrogen

Before sacrifice, blood samples were obtained to measure serum urea nitrogen (UN) levels with automated analysis (Hitachi 7350, Hitachi Co., Ibaragi, Japan) in our center.

Histological Analysis

To evaluate the progression of GN in our animal model, histological analyses were performed. Four-micrometer sectioned samples were stained with periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. Further samples were also stained with periodic acid-methenamine silver (PAM) reagent. 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, on the basis of the method described by Raij et al. [11], we evaluated the morphological changes in GN using the mesangiolysis score (MES), which was obtained by multiplying the degree of damage (0 to 4+) by the percentage of glomeruli with a lesion.

Western Blot Analysis

Protein from whole kidney was prepared using T-PER Extraction Reagents (Pierce Biotechnology, Inc. Rockford, USA). Western blot analysis was performed according to the methods described in our previous study [12]. Kidney extract was mixed with sample buffer and separated by electrophoresis on 15% SDS-PAGE gels, then transferred to a membrane (Immobilon-P, Millipore Corp., Bedford, Mass., USA). A monoclonal anti-VHL antibody Ig32 (1:500, BD Biosciences, San Jose, CA, USA) or polyclonal anti-VEGF antibody, sc-152 (1:250, Santa Cruz Biotechnology, Inc., Santa

Cruz, CA, USA) was used in conjunction with a horseradish peroxidase-conjugated secondary antibody (Promega Co., Madison, WI, USA). The ECL Western blotting system (Amersham Bioscience, Piscataway, NJ, USA) was used for detection. For in vitro studies, experiments were performed 3-5 times, each time in duplicate.

Immunohistochemical Analysis

An immunohistochemical study was performed on paraffin sections from HV alone or HV+AII-treated rats. VHL was identified with a monoclonal anti-VHL antibody (1:100, BD Biosciences), and synaptopodin with a monoclonal anti-synaptopodin antibody (1:10, Progen Biotechnik GmbH, Heidelberg, Germany), according to the manufacturer's instructions, utilizing the catalyzed signal amplification (CSA) system with Dako Target Retrieval Solution (Dako, Hamburg, Germany), based on the streptavidin-biotin-peroxidase reaction. Antigen retrieval was performed for 5 min in a preheated Dako Target Retrieval Solution (pH 6.0) using a microwave, followed by further steps including inhibition of intrinsic peroxidase, blocking and reaction with a primary antibody.

In vitro study

HEK 293 cells derived from human embryonic kidney cells were incubated in DMEM supplemented with 10% FBS with antibiotics to obtain 100% confluency. After serum starvation, cells were treated with 1 μg/ml HV, and samples were prepared for Western blot analysis in order to examine VHL protein levels. The optimal concentration of HV was determined by the cell survival (MTT) assay, because the concentration, more than 10 μg/ml HV, was identified to be toxic to the cells. Cells were also incubated with thrombin (1 U), which is recently reported to be an inducer of VHL [10].

Statistical Analysis

Data are reported as mean ± 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, Scheffe's multiple comparison was performed. A $p < 0.05$ was considered significant.

Results

GN developed on day 2 following administration of AII and HV.

As demonstrated in Figure 1 with PAS and PAM staining, GN induced by our protocol, using AII and HV, was observed two days after the administration (day 2); however, on day 1 GN was not clearly identified. The pathological features of GN were characterized as mesangiolysis and glomerular hypertrophy without remarkable renal tubular injury on day 2. Typical focal and segmental mesangiolysis was observed on day 2 with large capillary aneurysmal ballooning, as clearly demonstrated by PAM staining (Figure 1).

VHL protein expression was enhanced by HV in renal tubules and glomerulus.

To investigate the role of VHL in the progression of GN, an immunohistochemical study was performed using an anti-VHL antibody. In the control kidney, immunoreactivity of VHL was sparsely detected in the renal tubules, with very weak signal intensity. The negative control study using a secondary antibody alone did not show any specific signals throughout the kidney (data not shown). Other than the tubules, VHL protein expression was not detected in the glomeruli from the control kidney (Figure 2a, 2b). Furthermore, only AII administration did not induce any changes in the VHL staining pattern, compared with the control (data not shown). However, 24 hour of HV administration greatly increased VHL expression in the kidney. Increased VHL immunoreactivity was further detected throughout the entire kidney including not only renal tubules but also glomeruli. The VHL signals were remarkably enhanced in the renal tubules, compared with that in the control kidney. Moreover, HV-treated kidney showed an increase in VHL expression in glomerular cells, including endothelial cells and partially podocytes (Figure 2b). This VHL expression pattern induced by HV was comparable with that by HV+AII group even on day 2 (Figure 2c). In HV+AII group, VHL expression was observed in glomerular endothelial cells and partially detected in podocytes, which were identified by a staining pattern with an antibody against synaptopodin. Therefore, these results suggest that VHL induction by HV is involved in glomerular injury.

In accordance with these immunohistochemical results, Western blot analysis showed that the HV-treated kidney expressed more VHL protein than the control (Figure 2d). In contrast, AII alone did not induce VHL expression, suggesting that HV is involved in modification of VHL expression. Compared with the results of VHL protein expression in HEK293 cells (Figure 3), those from in vivo whole kidneys suggested that one band

corresponds to VHL and the other is non-specific (Figure 3). Taken together, it was indicated that HV, which is required for GN induction by our protocol however AII is not enough to do so, is responsible for VHL expression in the progression of GN.

HV increases VHL protein expression in HEK293 cells.

To further examine at the cellular level whether HV is involved in increasing VHL expression, HEK293 cells were treated with HV after serum deprivation. VHL protein expression was increased by HV in a time-dependent manner with a peat at 4 hours (Figure 3). Compared to the results of VHL expression from whole kidneys (in vivo), this in vitro study revealed that the lower band in vivo corresponded to VHL in vitro. The concentration of HV was critical for inducing VHL protein, because a higher concentration was toxic to the cells, as evaluated by the cell survival assay, we used HV at a concentration of 1 μg/ml. Therefore, it is suggested that HV plays a role in increasing VHL protein expression even in vitro. In contrast, VEGF expression in HEK293 was reciprocally attenuated by a prolonged treatment with HV, as demonstrated in Figure 3.

Thrombin induces VHL protein expression in HEK293 cells.

Thus far, it was demonstrated that HV induces VHL expression in glomerulus, which might be involved in GN development. In contrast, as our previous study revealed, HV did not increase expression of HIF-1α protein, which plays a protective role in renal injury. Consequently HV modulated the ratio between VHL protein and HIF-1α protein levels. On the basis of this result, it is further hypothesized that a VHL inducer may accelerate and aggravate GN. Therefore, HEK293 cells were treated with 1 U thrombin, already reported as a VHL inducer in a specific cell line [10]. As shown in Figure 4, thrombin slowly increased VHL expression in HEK293 cells with sustained elevation even following 24 h; in contrast, HIF-1α was not induced by thrombin (data not shown). Therefore, it is suggested that thrombin and HV are both VHL inducers in HEK293 with different time courses.

Thrombin pretreatment accelerates the development of GN.

To investigate whether preceding VHL induction can affect GN development, 1U thrombin was administered during the nephrectomy. As shown in Figure 5a thrombin accelerated the GN development. Morphological changes, including mesangiolysis and glomerular hypertrophy with less interstitial lesions including tubular injuries, were observed on day 1 in GN with thrombin, one day faster than GN without thrombin

pretreatment. In accordance with the effect of thrombin on the pathological lesions in GN, serum UN was also increased on day 1 in the pretreated group (thrombin (+)), compared with the non-pretreated group (thrombin (-)) (34.6±2.5 mg/dl vs. 26.4±0.4 mg/dl, n=15, $p<0.05$) (Figure 5b). Furthermore, MES and the occurrence rate of GN in the pretreated group was more than in the non-pretreated group: 30.3±4.9 vs. 7.1±1.2, respectively, for MES (n=15, $p<0.05$); 11.1±1.8% vs. 3.9±1.1%, respectively for the GN occurrence rate (n=15, $p<0.01$). These results suggest that preinduction of VHL by thrombin accelerates renal injury and aggravates GN progression.

Discussion

Following our recently published study using a specific GN model [1], which was developed by AII with HV, we further investigated the role of VHL in the progression of GN. The present study disclosed novel insights into the role of VHL. First, VHL was expressed in intact renal tubules with low signal intensity; however, the protein expression level was increased dramatically by HV as well as HV+AII, along with further increased VHL expression in the glomeruli, probably endothelial cells and partially podocytes. Second, VHL protein expression was also induced by HV in vitro. These results suggest that HV alone is involved in VHL induction, and with a combination of AII, HV is responsible for the pathogenesis of the GN, because AII alone is not enough in our protocol to induce GN. Third, thrombin enhanced VHL expression in vitro. Fourth, VHL pre-induction by thrombin accelerated GN development. Therefore, it is suggested that VHL plays a role in development of GN.

So far, several studies dealing with HIF-1α have been performed, indicating that pre-induction of HIF-1α before pathological insults activates a self-defense mechanism and suppresses further aggravation of the organ or cellular injury [13-16]. Our previous study also concluded that HIF-1α pre-induction by chemical hypoxia remarkably attenuated the GN progression [1]. In contrast, there are no representative studies in the nephrology field that extensively investigate the role of VHL in renal injuries induced by ischemia and/or reperfusion and GN. Since there are no pure reagents available as VHL antagonists or agonists, it is quite difficult to investigate the direct effects of VHL. Therefore, we recognize that such a fact leads to the limitation of the present study.

However, it is known that VHL is a crucial protein, malfunction of which is responsible for the pathogenesis of VHL disease characterized by renal cancer with hypervascularity. Abnormal VHL protein can neither bind to HIF-1α, nor degrade it through the proteasome, resulting in the accumulation of HIF-1α and transactivation of its downstream angiogenic genes including VEGF. However, the precise mechanisms by which VHL protein expression and function are regulated, are unclear. Recently, thrombin has been first reported to induce VHL in a rapid fashion in a specific renal cancer cell [10]. In the present study we investigated the role of VHL in the pathogenesis of our GN model, therefore, we used thrombin. Thrombin is a serine protease involved in cancer progression, reorganization of the cytoskeleton and RhoA activation [10]. Furthermore, in the previous papers [17,18], thrombin was reported to induce HIF-1α especially in smooth muscle cells. In our study, thrombin increased VHL protein expression, but did not induce the HIF-1α protein expression, both in vivo and in vitro for unknown reasons. This might be due to the different cell types and

experimental conditions used in the study. We also recognize that thrombin possesses pathophysiologically multiple effects involved in cellular injuries and its effect of VHL induction is one of them. Thus, other possible effects than VHL induction could not be excluded from the GN aggravation in the case of thrombin.

According to our study, thrombin pre-treatment accelerated GN development. Our GN protocol usually induces the typical glomerular injury on day 2 after administration of HV+AII, but on day 1 no injured glomeruli were observed. In contrast, with thrombin pre-treatment, typical glomerular injury was clearly and more often seen even on day 1. Thrombin is reported to be involved in fibrin formation and blunting of the fibrinolysis system. In addition, it transduces its signal through protease-activated receptor (PAR)-1, resulting in proliferation of fibroblasts and glomerular epithelial cells [19,20]. Then, as a supportive finding, PAR-1 knockout mice showed less severe renal failure in an anti-GBM glomerulonephritis model [21], suggesting the importance of thrombin as GN inducer, especially involved in crescentic glomerulonephritis. However, thrombin alone did not induce any renal injuries resembling our GN within our experimental time course. Therefore, it is suggested that thrombin aggravates the GN development mediated by possibilities including VHL induction. Surprisingly, HV was also revealed to induce VHL in the kidney, not only in the renal tubular epithelial cells, but also in glomeruli including podocytes and Bowman's capsular epithelia. Consequently, the thrombin pre-treatment may result in more VHL induction with HV than HV alone.

Our rat GN model is considered to show a distinct type of glomerular injury mainly derived from endothelial cell injury, i.e. mesangiolysis, which is seen in thrombotic microangiopathy [22]. Recent studies including pathogenesis of mesangiolysis indicates that endothelial cells and podocytes are closely regulated with mutual crosstalks through a VEGF signal, which is a well-known cell survival signal [23-27]. According to those studies, they suggest that down-regulation of VEGF in podocytes as well as endothelial cells increases a risk of renal injuries [23]. In addition, fine tuned VEGF production in endothelial cells or podocytes has a pivotal role in the cell survival, and through the survival signal mesangial cells are further regulated [24]. As revealed in the present study, HV induced glomerular VHL expression in vivo, especially in endothelial cells, on the other hand, HV reciprocaly decreased VEGF expression in vitro. Taken together, it is speculated that HV-induced injuries mainly in endothelial cells or podocytes decrease the survival signal and cause further development of renal injury. Reminding that VEGF is a major target gene transactivated by HIF-1α, and VHL plays an important role in regulating the HIF-1α protein level, induction of VHL appears be a negative modulator of the survival signal between endothelial cells and podocytes.

According to our study, VHL was further expressed in glomeruli in response to HV, but not to AII. In accordance with this, our GN protocol produced glomeruli-restricted lesions without remarkable renal interstitial injuries, suggesting that HV is involved in the pathogenesis of the glomerular injury. However, we have already recognized that glomeruli with VHL induction by HV caused the injuries, but renal tubules with VHL expression were intact even in the GN. The issue would be investigated by a future study, but differences of cell types in the kidney might be involved in that.

Another recent study has shown that HV accelerates the permeability of endothelial cells through VEGF type 1 receptor [28] and activates plasminogen activator activity [29], contributing to extensive stress on glomeruli, as glomeruli are rich in capillaries. Therefore, in addition to our speculated mechanisms including VHL induction, HV might increase extravasation through glomerular capillary endothelial cells and aggravate the regional response in glomeruli with reduced cell survival signals. These various effects of HV including increased VHL induction might contribute to the GN progression.

As a study limitation, our present study demonstrates only the correlation between VHL induction and GN development due to the lack of the pure antagonists of VHL available at present. A further study would be needed to investigate whether the VHL induction is exactly an aggravating factor or not.

In conclusion, induction of VHL is involved in hampering the cell protective mechanisms; and pre-treatment with thrombin further aggravates GN development. VHL plays a role in regulating the progression of GN.

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Figure legends

Figure 1

Angiotensin II (AII) combined with Habu snake venom (HV) induces glomerulonephritis (GN) 2 days, but not 1 day, after the administration.

On day 2, glomerular injury was specifically observed including focal and segmental mesangiolysis, and glomerular hypertrophy. However, tubular injury and cellular infiltration in the interstitium were scarcely detected on day 2. In contrast, on day 1 the typical glomerular injury was not detected. PAS (left panels) and PAM (right panels) staining. Representative data were shown from each group. Magnification, x200.

Figure 2

Immunohistochemical study of VHL in the kidney .

HV (3.5 mg/kg) alone was intravenously administered through the femoral vein of a Wistar rat, and 24 hours later VHL expression in the whole kidney was evaluated by immunohistochemical study, in comparison with a non-treated rat. In the control a weak signal of VHL expression was sparsely observed in the renal tubules, whereas the glomerulus did not express VHL (a). In contrast, the HV-treated kidney more strongly expressed VHL throughout the renal tubules; furthermore, the injured glomerulus also showed immunoreactivity for VHL. The pattern of VHL immunoreactivity in the glomerulus was comparable with that of endothelial cells and partially podocytes (b). The HV+AII-induced VHL expression pattern in glomeruli (day 2) was almost the same as HV alone (b, c). The comparison of staining patterns in VHL and synaptopodin suggested that VHL was expressed by endothelial cells and podocytes. Magnification x100 (a) and x200 (b, c). Counterstaining was performed in both (a), (b) and (c). The representative immunohistochemical data were shown from each group (n=6 in each). Western blot analysis using the HV, AII or HV+AII-treated kidney demonstrated that VHL expression was increased by HV. Lower bands corresponded to VHL, and upper bands were non-specific ones. Representative data were shown (n=6) (d). Upper band, VHL; lower band, nonspecific (It was differentiated by comparing Western blot data from rats with those from humans).

Figure 3

HV increases VHL expression.

HEK 293 cells were treated with HV 1μg/ml after serum starvation. VHL was induced by HV in a time-dependent manner. In contrast, VEGF expression was decreased by the

HV treatment. Representative data were shown from 5 independently performed experiments

Figure 4

Thrombin induces VHL protein expression in HEK293 cells.

Thrombin, which has been reported to induce VHL in some specific cell line [10], was evaluated to possess the potency to increase VHL protein expression. Twenty four hours later VHL expression was induced by thrombin (1U). Representative data were shown from 3 independent experiments

Figure 5

Thrombin pretreatment accelerates the time course of GN development.

On day 1, GN was not morphologically detected; however, thrombin (1U) treatment 24 hours before the nephrectomy accelerated the development of glomerular injury, which was seen even on day 1 (n=15). Thrombin-treated kidney showed typical mesangiolysis and glomerular hypertrophy without remarkable renal tubular injury (n=15) Magnification x100) (a). In accordance with the morphological changes, the serum UN concentration was increased in thrombin-treated rats (thrombin (+)), compared with non-treated rats (thrombin (-)) (b) (n=15, $P<0.05$). Furthermore, both the mesangiolysis score (MES) and occurrence rate of GN, in thrombin-treated rats (thrombin (+)), were remarkably increased, compared with the non-treated rats (thrombin (-)) (c) (n=15, MES $p<0.05$; GN occurrence $p<0.01$).

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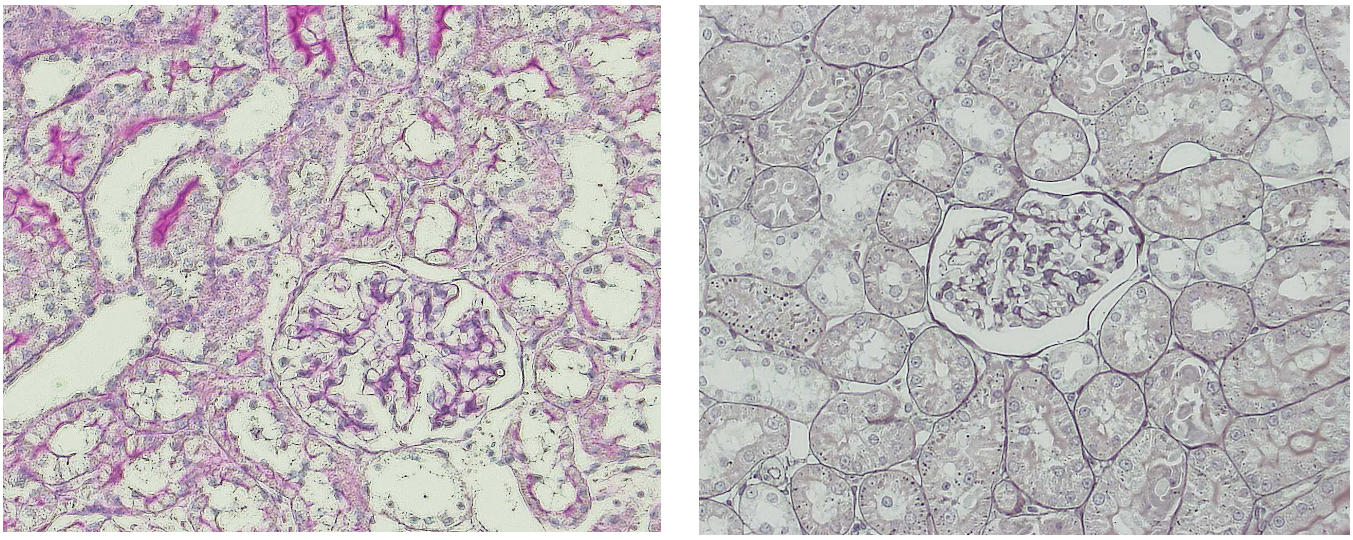
Figure 1

day 0



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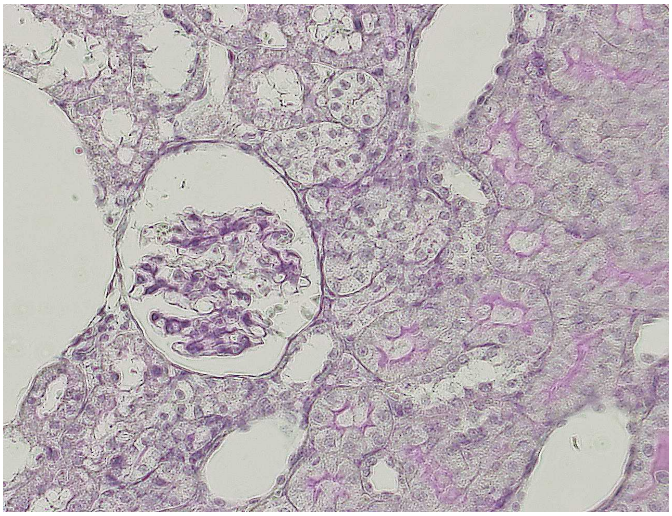

day 2

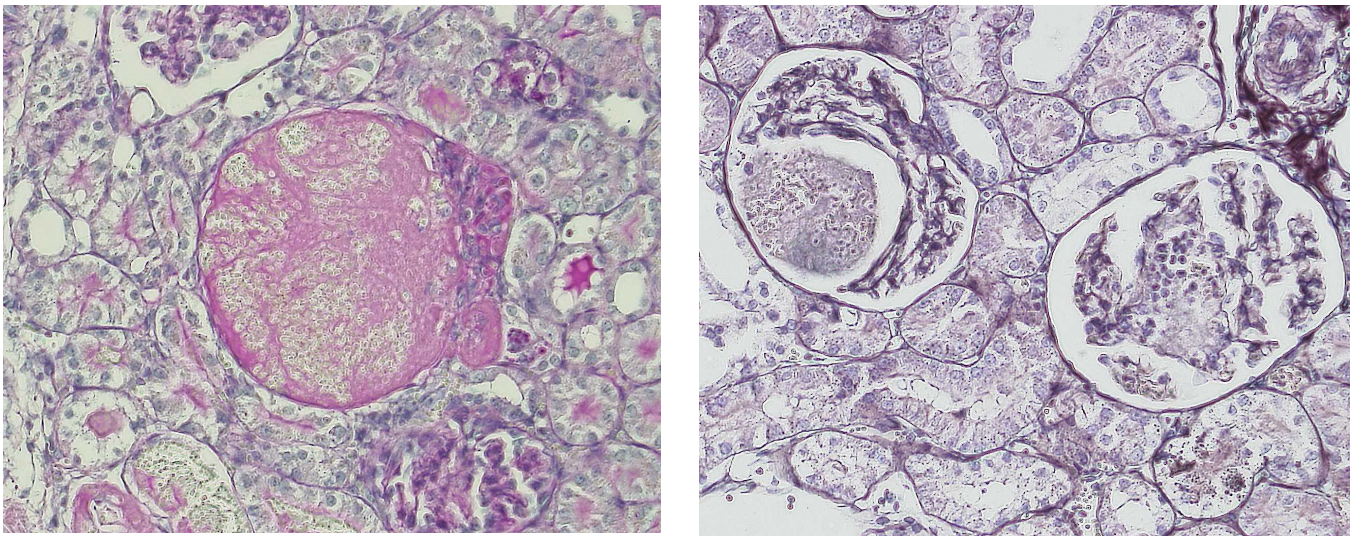

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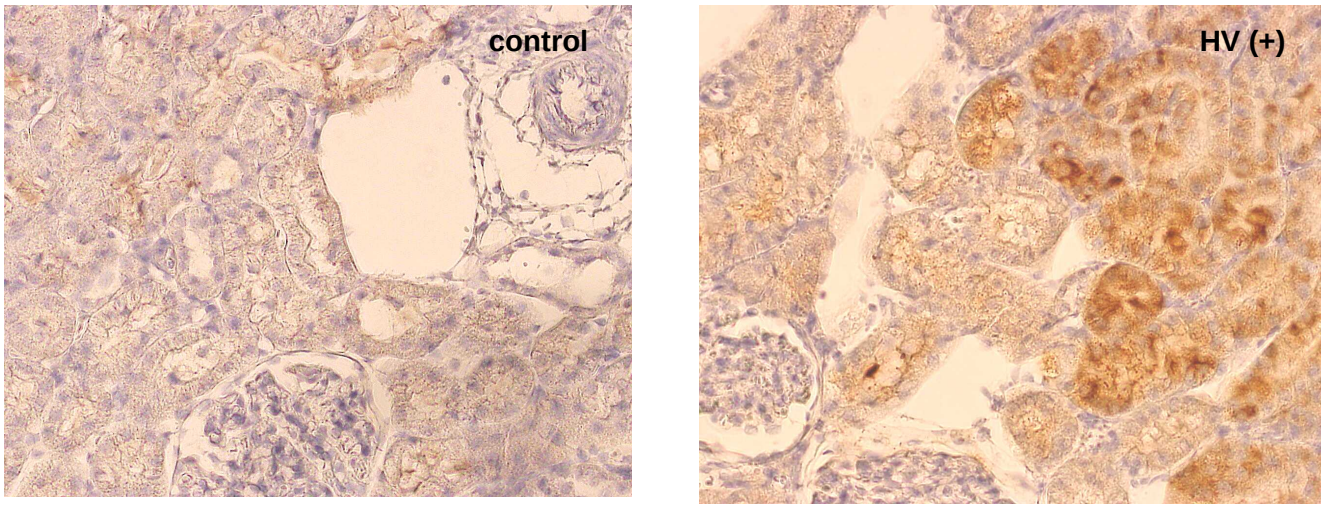
Figure 2

a



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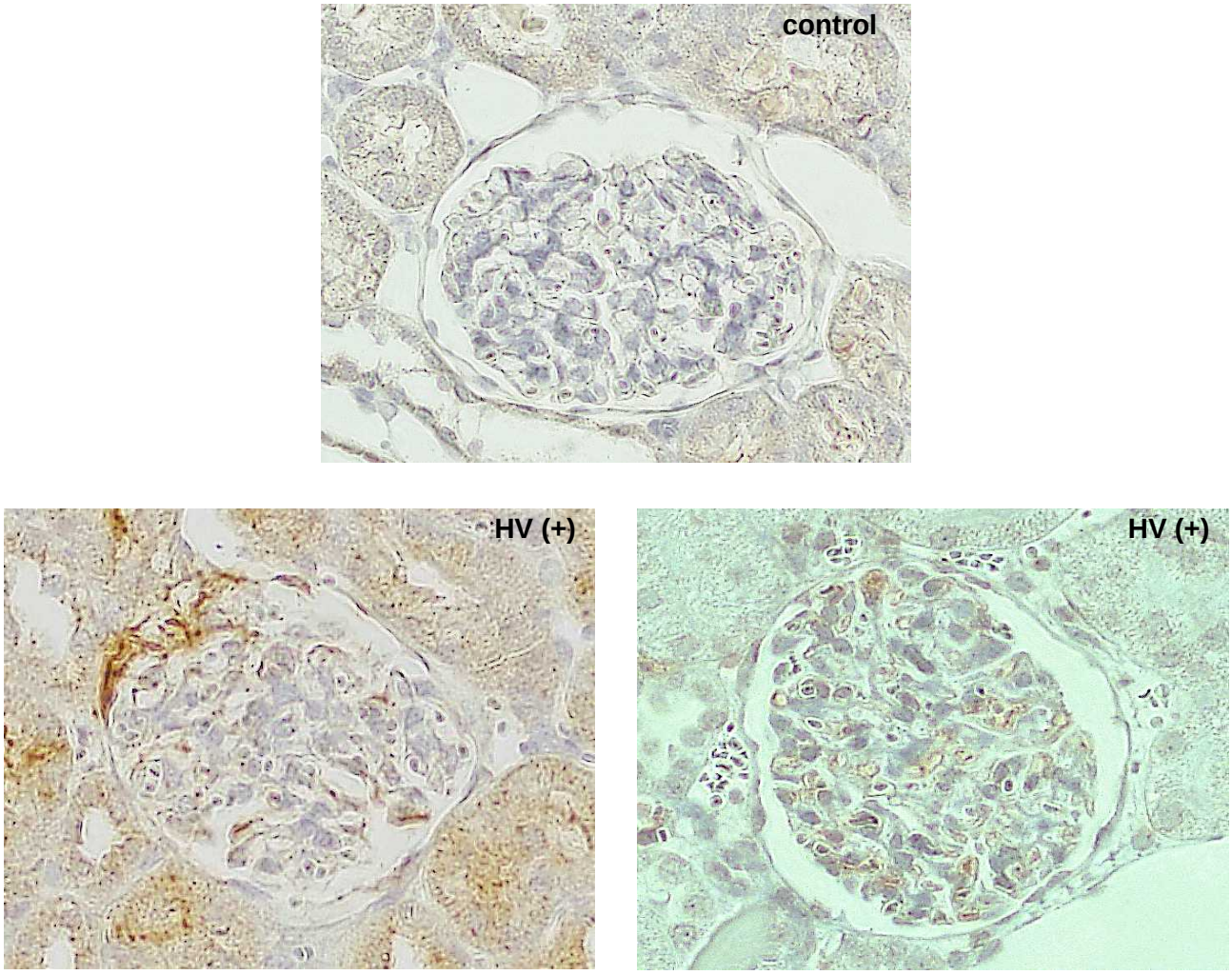

Kudo Y, et al.
Figure 2

c

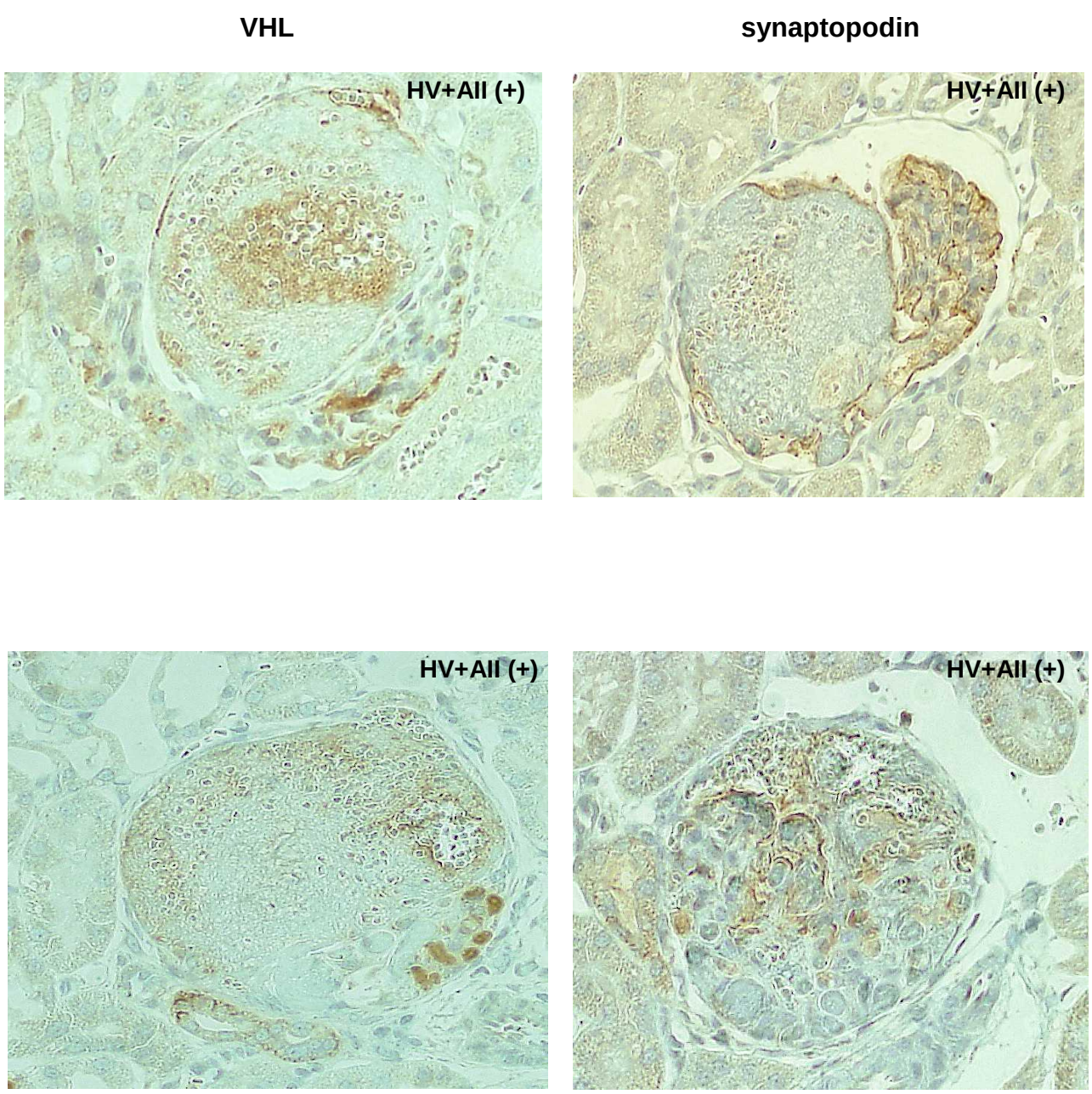

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Figure 2

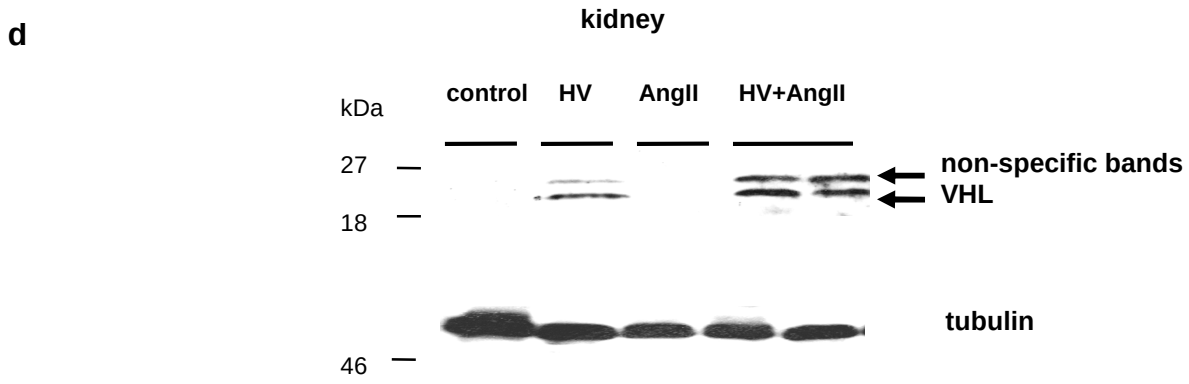

Figure 3

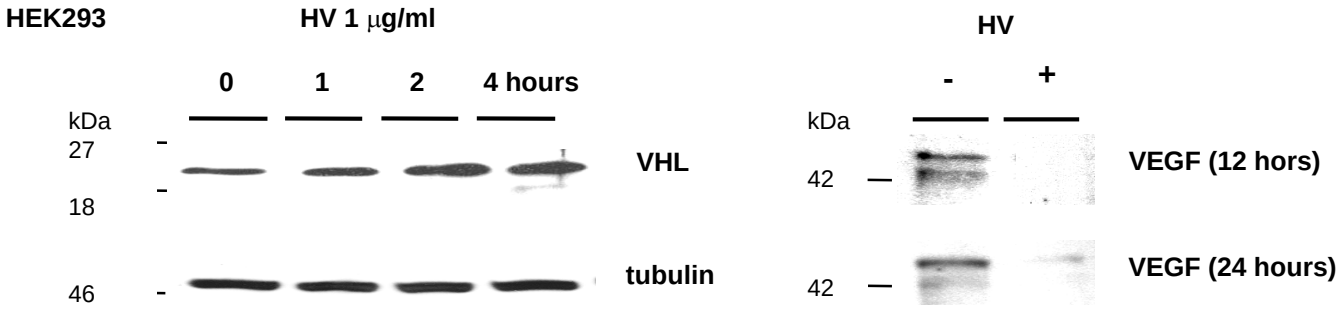

Figure 4

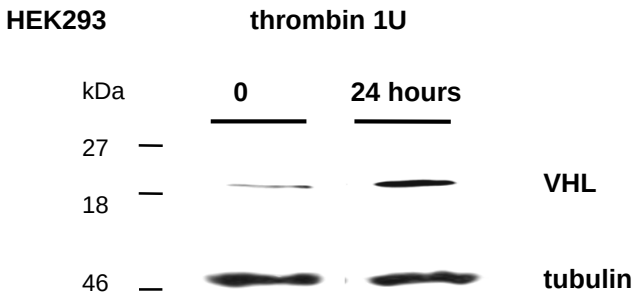

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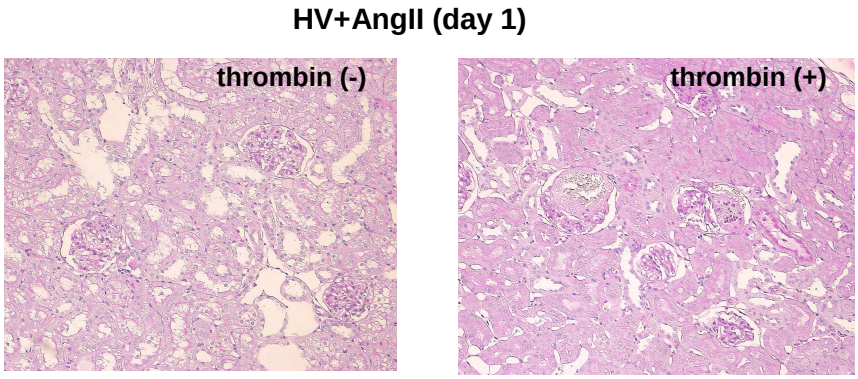
Figure 5

a



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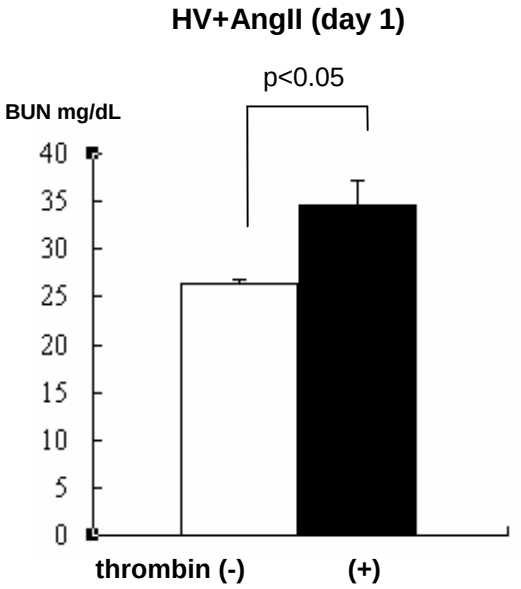

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