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Decreases in Podocin, CD2-Associated Protein (CD2AP) and Tensin2 May Be Involved in Albuminuria during Septic Acute Renal Failure

Takashi KATO1,2), Yoko MIZUNO-HORIKAWA3) and Shinya MIZUNO1,4)*

1)Division of Molecular Regenerative Medicine, Department of Biochemistry and Molecular Biology, Osaka University Graduate School of Medicine, 2–2–B7 Yamadaoka, Suita, Osaka 565–0871, 2)Research Division for Regenerative Drug Discovery, Center for Advanced Science and Innovation, Osaka University, 2–1 Yamadaoka, Suita, Osaka 565–0871, 3)Protein Research Institute, Osaka University, 1–2 Yamadaoka, Suita, Osaka 565–0871 and 4)Division of Virology, Department of Microbiology and Immunology, Osaka University Graduate School of Medicine, 2–2–B7 Yamadaoka, Suita, Osaka 565–0871, Japan

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ABSTRACT. Podocytes have a peculiar structure constituting slit diaphragm (SD) and foot process (FP), and play essential roles in the glomerular filtration barrier. There is now ample evidence that SD- and FP-associated molecules, such as podocin and CD2-associated protein (CD2AP), are down-regulated during albuminuria of chronic kidney disease. However, it is still unclear whether these molecules are altered during acute renal failure (ARF) with albuminuria. Using lipopolysaccharide (LPS)-treated mice as a model of septic ARF, we provide evidence that the expression of SD- and FP-associated molecules becomes faint, along with albuminuria. In the LPS-treated mice, urinary albumin levels gradually increased, associated with the elevation of blood urea nitrogen levels, indicating the successful induction of albuminuria during septic ARF. In this pathological process, glomerular podocin expression became faint, especially at 36 hr post-LPS challenge (i.e., a peak of albuminuria). Likewise, LPS treatment led to a significant decrease in CD2AP, an anchorage between podocin and F-actin. With regard to this, tensin2 is a novel molecule that stabilizes F-actin extension. Interestingly, glomerular tensin2 expression levels were also decreased during the albuminuric phase, associated with losses of glomerular F-actin and synaptopodin under septic states. As a result, there were some lesions of podocytic FP effacement, as shown by electron microscopy. Based on these data, we emphasize the importance of concomitant decreases in podocin, CD2AP and tensin2 during septic ARF-associated albuminuria.

KEY WORDS: albuminuria, CD2AP, podocin, sepsis, tensin2.

Plasma ultra-filtration occurs through the glomerular filtration barrier, which consists of highly specialized visceral epithelial cells called podocytes, fenestrated capillary endothelium, and an intervening glomerular basement membrane (GBM). Among them, podocytes serve as a molecular sieve that selectively restricts the filtration of different molecules on the basis of their size and charge [14]. Podocytes have octopus-like cells comprising a cell body and cytoplasmic extensions called major processes, which divide into actin-rich foot process (FP) interdigitating over each capillary loop and counteracting the distensive forces [9, 12]. Each foot process is attached to its neighbor along the length by an intercellular adherent-type junction, the slit diaphragm (SD), which is localized just above the GBM.

There is a closed correlation between the degree of proteinuria and the prognosis of chronic kidney disease (CKD) [36]. Therefore, it is important to elucidate the mechanisms whereby proteinuria occurs and develops at genetic and molecular levels. Recent genetic studies identified podocin, encoded by NPHS2, as a mutant gene that causes autosomal recessive steroid-resistant nephritic syndrome [3, 29]. Likewise, CD2-associated protein (CD2AP) mutations are associated with sporadic nephrotic syndrome and focal and segmental glomerulosclerosis [11, 32]. These genomic studies lead to the determination of a molecular topology for SD, required for maintaining the homeostasis of podocytes-podocytes [21]. Notably, the decreases of SD-related molecules (such as podocin and CD2AP) are observed in many types of CKD, regardless of the primary etiology [1, 19].

In contrast to CKD, little is known about the molecular basis of albuminuria during acute renal failure (ARF), including sepsis. Sepsis-mediated renal injury is a typical phenotype of ARF that is associated with albuminuria [2, 33]. During the sepsis, glomerular thrombosis becomes evident via up-regulations of inflammatory cytokines, and then local permeability and hypoxia enhance renal damage, associated with a loss in renal blood flow [26, 30]. Even under low glomerular filtration, albuminuria is often noted in septic patients [2, 33] and in rodents [31]. Thus, we hypothesize that albuminuria may occur during sepsis via the possible down-regulation of SD-related proteins, such as podocin and CD2AP, but it is still unclear whether these molecules are involved in albuminuria during septic ARF.

To test this hypothesis, we used lipopolysaccharide (LPS)-treated mice as a model of septic ARF, because LPS-treated mice manifest albuminuria, as reported [31]. Using this mouse model, we provided evidence that expressions of podocin and CD2AP by podocytes becomes faint, concomitantly with a peak of urinary albumin excretion. The "syn-
chronized” decreases of cytoskeletal components, such as synaptopodin, F-actin and tensin2, are also discussed here, all of which could be responsible for the onset of albuminuria during sepsis.

MATERIALS AND METHODS

Animal models: Eight-week-old female mice (C57BL/6J strain) were purchased from SLC (Hamamatsu, Japan). LPS (E. coli O111:B4) was obtained from Sigma (St. Louis, MO., U.S.A.). Sepsis was induced via a single injection of LPS at a dose of 8 mg/kg (i.p.), based on a recent report [15]. Mice were sacrificed at 0, 12, 24, 36 and 48 hr post-LPS challenge. All the experiments were performed, according to the regulations on animal experiments at Osaka University, with an approval by Animal Research Committee of Osaka University Graduate School of Medicine.

Determination of urinary albumin levels by SDS-PAGE: The samples were collected as spot urine at each time-point to be examined. The albuminuria was evaluated using SDS-PAGE, where its concentration (mg/ml) was determined, based on the band size of bovine serum albumin under densitometry [16].

Blood chemistry: At the scheduled autopsy, blood was collected from the submaxillary artery, and plasma was obtained at 0, 12, 24, 36 and 48 hr post-LPS challenge (n=6 per each time point). To evaluate the renal dysfunction, blood urea nitrogen (BUN) levels were measured by a urease indophenol method with a kit (urea nitrogen-B test, Wako, Osaka, Japan) [25].

Immunohistochemistry: Right kidneys were fixed in 10% formalin and 70% ethanol for 24 hr, and embedded in paraffin. The kidneys were cut into 4-µm sections and were subjected to the histological processes. To investigate the level of a slit diaphragm protein, the sections were identified using an anti-podocin goat IgG (sc-22298, Santa Cruz, Santa Cruz, CA, U.S.A.). To identify FP proteins, anti-CD2AP rabbit IgG (sc-9137, Santa Cruz), anti-synaptopodin mouse IgG (65194, Progene, Heidelberg, Germany), or anti-tensin2 rabbit IgG (Transgenic, Kobe, Japan) was applied onto renal sections, as the primary antibodies. For antigen retrieval, the sections were treated with citrate buffer (pH 6.0). The sections were incubated at 4°C for overnight, washed with phosphate-buffered saline followed by detection of fluorescence with Alexa546- or Alexa488-labeled antibodies (Invitrogen, Carlsbad, CA, U.S.A.).

Morphometry: We quantified the glomerular expressions of podocin, CD2AP and tensin2 according to a staining score [16]. Briefly, >20 glomeruli were randomly chosen, and expression levels in each glomerulus were scored based on the fluorescence extent, as followed: 0; none, 1; faint, 2; mild, 3; moderate, and 4; strong. The extent of molecular expressions was expressed as the individual index (i.e., total score/glomerular number examined) [16].

Immunoprecipitation and Western blotting: Kidney tissue homogenates were prepared from mice in lysis buffer, as reported [16, 25]. The renal extraction was incubated overnight at 4°C with goat anti-podocin antibody. The complexes were precipitated with protein A/G sepharose. These immunoprecipitates were washed three times and dissolved in sample buffer. The samples were separated by SDS-PAGE and electroblotted onto PVDF membranes. The membranes were blotted with anti-podocin goat IgG and held overnight at 4°C, then subsequently labeled with peroxidase-conjugated antibody against goat IgG (Santa Cruz). The resultant signals were detected using ECL-plus-chemiluminescence (Amersham, Little Chalfont, U.K.).

Statistical analysis: Data were expressed as mean ± SD. A Student’s t-test, ANOVA analysis or Mann Whitney U-test was used to compare the group means. P-values <0.05 were considered significant.

RESULTS

Induction of albuminuria and ARF in LPS-treated mice: The changes in BUN and urinary albuminuria were checked after LPS treatment. A single injection of LPS (8 mg/kg, i.p.) resulted in the apparent increases in BUN levels (Table 1), indicating the onset and progression of septic ARF, as reported [15]. As expected, albumin levels gradually increased in the urine samples, with a peak at 36 hr post-LPS challenge (Table 1). These clinico-biochemical findings indicated the successful induction of septic ARF and albuminuria in our mouse model.

Decrease in podocin expression in the renal glomeruli of septic mice: Although albuminuria became evident 24–48 hr post-LPS challenge, there were no significant differences in the number of podocytes between saline- and LPS-treated groups (not shown). Thus, we focused on the degree of podocin, an SD-associated molecule [9]. Immune fluorescence analysis revealed that podocin was extensively noted along GBM (i.e., linear pattern) in the saline group (Fig. 1A, left). On the other hand, LPS treatment apparently reduced the glomerular podocin expression, with a loss of linearly positive signals (i.e., granular pattern) (Fig. 1A, right). Consistently, there was a difference in the podocin staining score between both groups (36 hr: saline, 3.76 ± 0.14 vs. LPS, 2.10 ± 0.04, P<0.01).

Immune blot analysis of podocin strengthened this histological finding: a podocin-positive band, with a molecular size of 45-kD, was detected by immune precipitation and blotting before LPS challenge. The molecular size of the podocin-positive band was not altered by saline, but itsTable 1. Renal dysfunction and albuminuria after LPS injection

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>BUN (mg/ml)</th>
<th>uAlb (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20.9 ± 1.88</td>
<td>9.2 ± 11.4</td>
</tr>
<tr>
<td>12</td>
<td>63.3 ± 11.3</td>
<td>57.1 ± 43.4</td>
</tr>
<tr>
<td>24</td>
<td>116.9 ± 19.5</td>
<td>494.7 ± 139.6</td>
</tr>
<tr>
<td>36</td>
<td>132.3 ± 32.9</td>
<td>981.4 ± 186.4</td>
</tr>
<tr>
<td>48</td>
<td>63.9 ± 18.7</td>
<td>546.1 ± 226.8</td>
</tr>
</tbody>
</table>

uAlb: urinary albumin. BUN: blood urea nitrogen. Data are expressed as a mean ± SD (n=6).
LOSSES OF PODOCIN AND TENSIN2 DURING SEPSIS

expression level was reduced to half of the basal control (Fig. 1B). These histological and biochemical data demonstrated a decrease in podocin during septic ARF.

Reduced expression of CD2AP leading to actin-associated molecules: CD2AP is essential for the stabilization of SD structure as a common intra-cellular bridge among nephrin, podocin and F-actin [21]. In the saline-injected control group, CD2AP was detected in the extra-capillary areas of glomeruli (i.e., podocyte area) (Fig. 2A). By contrast, CD2AP-positive signals became faint at 36 hr after LPS treatment (Fig. 2E). A significant difference in the CD2AP staining score was seen between both groups (36 hr: saline; 3.67 ± 0.17 vs. LPS; 2.25 ± 0.63, P<0.05).

The change in F-actin was examined, since it is connected to podocin via CD2AP [9, 21]. Consistent with the decreases in podocin and CD2AP, glomerular expression of F-actin in the LPS-treated group (36 hr) was also reduced compared to that of the control (Fig. 2B and 2F). The change in synaptopodin, a key player for actin dynamism of podocytes [9], was then examined. As a result, LPS reduced the expression of synaptopodin (Fig. 2C and 2G), concomitantly with the losses of CD2AP and F-actin.

We next focused on tensin2, a novel molecule that is required for stable expression of synaptopodin during congenital nephrotic syndrome in mice (ICGN strain) [17]. Tensin2 was detected in the extra-capillary areas (i.e., podocyte-localized area) of the saline-injected control mice (36 hr) (Fig. 2D). By contrast, LPS treatment resulted in a significant loss of tensin2 at 36 hr post-LPS challenge (Fig. 2H). Indeed, there was a significant difference in the tensin2 staining score between the control and septic groups (36 hr: saline; 2.50 ± 0.29 vs. LPS; 1.69 ± 0.29, P<0.05). This is, to our knowledge, the first report to show a decrease in tensin2 expression during acquired kidney diseases.

Detection of FP effacement by electron microscopy: Since losses of SD- and FP-associated molecules were associated with the albuminuria, we hypothesized that these molecular changes would lead to a change in the ultra-structure of podocytes. To test our hypothesis, the morphology of FP was evaluated by an electron microscopy. In the saline-injected control mice, podocytic FP located on GBM seemed normal (Fig. 3A and 3B). By contrast, there were effaced findings, with an FP-FP fusion, especially 36 hr post-LPS challenge (i.e., albuminuric stage) (Fig. 3C and 3D). Overall, losses of SD- and FP-associated molecules (such as podocin, CD2AP and tensin2) were considered to elicit albuminuria through the induction of FP effacement.

DISCUSSION

Although urinary albumin excretion is an important hallmark of septic ARF [2, 26, 33], the molecular basis of albuminuria is not fully understood. In this regard, we reported that the loss of nephrin, another key molecule of SD [9], becomes evident during sepsis through a loss in nuclear localization of WT1, a key transcriptional factor of nephrin

![Fig. 1](image1.png) Podocin reduction in the glomeruli of LPS-challenged mice. (A) Typical finding of podocin expression in saline-treated (left) and LPS-treated (right) groups (× 380). (B) Western blotting for the renal podocin expression at 0 (Pre) and 36 hr post-LPS challenge. For abbreviations see text.

![Fig. 2](image2.png) Immunofluorescence images of podocyte-associated proteins at 36 hr after injection of saline (A-D), or LPS (E-H) (× 380). (A, E) CD2AP, (B, F) F-actin, (C, G) Synaptopodin (SynaPD) and (D, H) tensin2. Red signal: Alexa-546 for CD2AP, F-actin and tensin2. Green signal: Alexa 488 for SynaPD.
to predict proteinuria. Actually, podocin loss is sometimes a marker between nephrin and CD2AP [21]. Furthermore, a loss of key anchorage that strengthens FP-FP contact via a bridge [16]. In this study, we focused on podocin, because it is a target in the LPS-induced decrease in podocin (i.e., paracrine secretion of TNF-α, knockdown of podocin mRNA by siRNA leads to the expanded results of podocyte foot process in the saline group (B) and LPS-treated group (D). Arrows: foot process effacement of podocytes.

In this study, we focused on podocin, because it is a key anchorage that strengthens FP-FP contact via a bridge between nephrin and CD2AP [21]. Furthermore, a loss of podocin, rather than nephrin, seems to be a “strict” marker to predict proteinuria. Actually, podocin loss is sometimes independent of nephrin expression in vivo and in vitro: in some types or stages of CKD, podocin loss does not occur during albuminuria, even if nephrin is decreased [10, 22]. In vitro, knockdown of podocin mRNA by siRNA leads to the concomitant loss of nephrin [7], but inversely, nephrin mRNA knockdown does not elicit podocin down-regulation [8]. Thus, it is still unclear whether podocin expression is altered, or not, during sepsis, even if nephrin is decreased [16], and this background promoted the examination of other SD-associated molecules. As a result, podocin loss became evident, along with the peak of albuminuria, as did nephrin loss [16].

It is important to discuss the molecular mechanism of podocin loss as seen at 36 hr post-LPS. Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), are able to down-regulate podocin transcription in a culture of immortalized podocytes [13]. It is well known that LPS injection rapidly increases TNF-α levels in the blood of rodents (i.e., innate immune system leading to cytokine storm) [15, 30]. We also detected a dramatic increase in blood TNF-α levels (i.e., >1,000-fold increase) within 2 hr post-LPS challenge (not shown). Thus, secretion of TNF-α from macrophages by LPS in distant organs may lead to podocin down-regulation in the glomeruli via an endocrine pathway. In addition, glomerular mesangial cell-derived cytokines, such as transforming growth factor-β, can suppress podocin production in a culture of podocytes [20]. Thus, such an intra-glomerular local system may also participate in the LPS-induced decrease in podocin (i.e., paracrine pathway), and we are now investigating the roles of these cytokines in the regulation of podocin.

Our next attention was paid to the change in CD2AP, an adaptor molecule that provides a bridge between podocin and F-actin [9, 21]. Indeed, CD2AP directly interacts with podocin, or with F-actin to sustain barrier function and morphology of podocyte SD [9, 21]. Of note, mice with CD2AP haplo-insufficiency developed glomerular changes at 9 months of age and had increased susceptibility to glomerular injury by nephritogenic antibodies [18]. In addition, human patients with focal segmental glomerulosclerosis possessed a mutation that was predicted to ablate expression of one CD2AP allele (i.e., 50%-reduction) [18]. Thus, we predict that the half-reduction of CD2AP by LPS may be sufficient, in addition to the decreases of other SD-associated molecules, to elicit albuminuria in this septic model. Since CD2AP is required for stabilization of podocin via inhibition of its degradation [35], the loss of CD2AP may, in part, explain the additional mechanism of podocin loss.

The possible mechanisms for explaining the CD2AP loss during septic ARF should be discussed. A promoter assay revealed that Sp1/Sp3-binding sites of CD2AP-promoter regions is required for up-regulation of CD2AP [34], while inflammatory cytokines, such as TNF-α, suppresses various genes via inhibiting Sp1/Sp3-DNA binding [6]. Thus, cytokine-mediated inactivation of Sp1/Sp3-binding promoter sites may cause the loss of CD2AP during septic ARF, and future studies would shed light on this speculation. The loss of CD2AP was concomitant with that of podocin in our model. With regard to this, production of these molecules is controlled by the same transcriptional factor, Lmx1b [23]. On the other hand, nephrin is up-regulated by another transcriptional factor, WT-1 [16]. Thus, our model is useful for elucidating the transcriptional mechanisms of podocyte-specific gene profiles.

Finally, we focused on a novel protein in nephritic glomeruli. Tensin2 is a member of tensin family, a focal-adhesion molecule that stabilizes F-actin by binding to β-integrin [4], as did α-actinin4 (ACTN4) [21]. Of interest, a loss of tensin2 acts as a switch to trigger cell migration [27]. Tensin2 localizes mainly to podocytes in the kidney [5, 17], and notably, tensin2 homozygous mutation (i.e., tensin2-deficiency) in the mice (i.e., ICGN strain with an ICR background) leads to albuminuria within a few weeks after birth [5, 17, 24]. By contrast, there was no significant albuminuria in the congenic strain carrying the tensin2 mutation on the C57BL/6J genetic background [28], which raises a controversial issue about the role of tensin2 during albuminuria. Using a model of an “acquired” kidney disease (i.e., septic ARF), we found the involvement of decreased tensin2 in septic albuminuria. Tensin2 is required for stability of synaptopodin [17]. In turn, synaptopodin is important for podocytes to sustain FP extension (i.e., podocyte outgrowth) [9, 21, 31]. Thus, we predict that the loss of synaptopodin via down-regulating tensin2, an anchorage between GBM and FP, could enhance the FP effacement and subsequent albuminuria in our septic model.
In summary, it is likely that septic albuminuria occurs through the following cascades: (i) inflammatory cytokines, such as TNF-α, are secreted from LPS-primed macrophages, and they suppress podocin and CD2AP expression; (ii) in addition, tensin2 is down-regulated at a focal adhesion closed to GBM, and then F-actin and synaptopodin are decreased; and (iii) under such a loss of skeletal components, FP effacement becomes evident, resulting in the onset of albuminuria during septic ARF. The present study provides an in vivo model to elucidate the roles and regulatory mechanisms of SD- and FP-associated molecules under sepsis-mediated renal injury.

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