The Role of Osteopontin in the Development of Albuminuria

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Osteopontin (Opn; or secreted phosphoprotein 1 [Spp1]) is a pleiotropic cytokine that is broadly expressed and upregulated during inflammation, cancer, and various other conditions.1–3 Secreted Opn can bind to αVβ3 integrin (vitronectin receptor) and can induce phosphoinositide-3-kinase/Akt-dependent NF-κB activation and urokinase plasminogen activator (uPA) secretion in cancer cells.1–3 Opn can also induce NF-κB activation through both IKK- and extracellular signal–regulated kinase–mediated pathways, which stimulates uPA-dependent matrix metalloproteinase 9 (MMP-9) activation. All of these could contribute to increased motility of cancer cells, invasion, tumor growth and metastasis.1–3 In addition, recent studies by Wei et al.4 showed that αVβ3 integrin (one of the Opn receptors) is a critical regulator of proteinuria.

Gene expression studies showed that higher Opn levels strongly correlate with more severe diabetic albuminuria and glomerulosclerosis in various diabetic nephropathy models.5 Gene profiling experiments performed on acute and chronic glomerulonephritis6,7 and transplant renal disease8 also found Opn mRNA to be highly increased and that it was one of the top genes that predicted renal damage.5,9 Similarly, gene array studies performed on isolated podocytes identified Opn as the gene with the highest increase in mRNA expression after sheer stress10; however, the reports are conflicting as to whether Opn plays a role in murine autoimmune glomerulonephritis.6,11

Here we show that Opn is regulated in mouse models of type 1 diabetic nephropathy, LPS-, and puromycin aminonucleotide (PAN)-induced proteinuria and also in children with nephrotic syndrome. We found that Opn deletion (Spp1−/− mice) confers a protection against the development of albuminuria. We propose that podocytes are an important target of Opn, because we found that Opn treatment led to IkBα phosphorylation, upregulation of MMP-2 and MMP-9, and increased podocyte motility.

Because Opn is a secreted protein, we examined Opn levels in urine samples obtained from children with nephrotic syndrome. Thirty-six urine samples were obtained (at the time of the kidney biopsy), including 12 from control subjects without any evidence of renal disease and 24 patients with nephrotic syndrome. Patient characteristics are shown in Fig...
The indication for the renal biopsy was nephrotic syndrome with atypical presentation (age, presence of hypertension) or resistance to standard dosage of steroid treatment. FSGS was the most common cause of nephrotic syndrome in our cohort (58 to 66%), with a similar frequency between steroid-sensitive nephrotic syndrome (SSNS) and steroid-resistant nephrotic syndrome (SRNS). Urinary Opn levels were significantly increased in children with nephrotic syndrome to 824 ± 272 ng/mg creatinine compared with 135 ± 16 ng/mg creatinine in control subjects (P = 0.029). Urine Opn levels did not correlate with gender, proteinuria, renal histology, or GFR. Opn levels correlated with clinical response to steroids. Urine Opn levels (drawn after steroid treatment) were highly increased in children with SSNS (1611 ± 476 ng/mg creatinine), whereas they were not significantly different from control subjects in children who did not respond to the course of steroids (180 ± 61 ng/mg creatinine) (Figure 1B). Renal Opn expression was increased in kidney biopsies of patients with SSNS, indicating that the kidney is the likely source of increased Opn levels (Figure 1C).

These observations suggest that urinary Opn levels might be a useful biomarker of steroid sensitivity in children with nephrotic syndrome. SRNS of children is associated with a bad prognosis, because this disease is often resistant to other secondary forms of treatment and leads to ESRD.12 The renal histology is often not a reliable predictor of treatment response. A biomarker that predicts outcome would be useful to prevent treatment with steroids that are associated with significant risk and adverse effect. Our study suggested that urine Opn levels might be a useful marker. Our study used a cross-sectional study design, and urine was collected from children after they were treated with prednisone; therefore, additional larger and prospective studies are needed to determine whether Opn can prospectively predict steroid sensitivity.

In addition to patients, we examined the expression and regulation of Opn in animal models of albuminuria. Twenty-four hours after LPS injection, 6-wk-old male C57/B6J mice developed steroid-sensitive and transient proteinuria (Figure 2A). This rapid and abundant albuminuria was not associated with significant glomerular changes under light microscopy (Supplemental Figure 2B).
1); however, quantitative real-time-PCR analysis performed on whole-kidney lysates showed a significant increase in Opn mRNA level (Figure 2B).

Single (intraperitoneal) injection of PAN to Sprague-Dawley rats caused severe nephrotic syndrome (Figure 2E) as early as 4 d after the injection. We found increased Opn mRNA levels in PAN-treated rats. Although it did not reach statistical significance (Figure 2F) in whole-kidney homogenate, the increase was significant and much greater (approximately 70-fold) in glomerular extracts (Figure 2G).

The Opn mRNA level was also significantly increased in kidneys of 16-wk-old male Akita mice (Figure 2D), which is a model of diabetic nephropathy. This model developed significantly increased albuminuria and mesangial expansion as well (Figure 2C and Supplemental Figure 1).

Immunostaining studies showed increased podocyte-specific Opn immunostaining in the glomeruli of LPS-treated mice and in 16-wk-old male Akita mice compared with control mice (Figure 2, H through P). The specificity of the antibody was tested on kidney sections obtained from Opn knockout mice (Figure 2, J and M).

Our next question was whether Opn plays a role in the development of albuminuria. To answer this question, we took advantage of the Opn knockout mice. Opn null (B6.Cg-Spp1tmBlh/J or Spp1−/−) mice are viable without any renal abnormalities.15 Six-week-old male Opn knockout mice were administered an injection of 20 μg of LPS (intraperitoneally). Wild-type mice developed significant albuminuria 24 h after the injection; however, albuminuria did not appear in Opn knockout mice (Table 1).

We also tested whether Opn plays a role in the development of diabetic albuminuria and mesangial expansion. We generated combined mutant mice that both were Opn null and had the Ins2Akita mutation (both strains were in C57/B6J background). Body weight and blood glucose levels were not different in male wild-type Ins2Akita and in the Opn null Ins2Akita groups (Tables 1 and 2). Wild-type (L) or Opn knockout (M) 16-wk-old male Akita mice. (N and O) Kidneys obtained from LPS-treated mice were stained with anti-Opn antibody (green; N) and with anti-synaptopodin antibody (red; O). (P) Overlay of synaptopodin and Opn staining.
type Ins2\textsuperscript{Akita} mice had increased albuminuria at 16 wk age.\textsuperscript{14,15} Albuminuria was significantly lower in the Spp1\textsuperscript{−/−} Ins2\textsuperscript{Akita} mice compared with nondiabetic controls. Mesangial expansion, a hallmark of diabetic glomerulopathy, was also significantly decreased in Ins2\textsuperscript{Akita} mice with Opn deletion (Supplemental Figure 2). In summary, Opn knockout mice showed protection from LPS- and diabetes-induced albuminuria.

Further cell culture experiments examining the role and regulation of Opn in cultured podocytes showed that LPS and hyperglycemia induced upregulation of Opn mRNA in podocytes in vitro. This response is reminiscent of the observed in vivo increase of Opn expression in podocytes in diabetic or LPS-treated mice (Supplemental Figure 3). Activation of the protein kinase C and the extracellular signal–regulated kinase pathways likely via the release of intracellular reactive oxygen species seems to play an important role in regulating Opn mRNA levels in cultured podocytes (Supplemental Figure 4).

We evaluated the effect of exogenous recombinant Opn in vitro on cultured murine podocytes. After Opn treatment, we observed an increase in IκBα phosphorylation, peaking at approximately 40 min (Figure 3A and Supplemental Figure 5) and a decrease in total IκBα levels. Preincubation of the cells with Bay11-7082 and pyrrolidine dithiocarbamate (PDTC) inhibited IκBα phosphorylation, indicating NF-κB activation. Similarly, Opn treatment increased the Bay11- and PDTC-sensitive NF-κB p50 DNA binding (Figure 3B). These observations suggest that Opn treatment leads to the activation of the NF-κB pathway in cultured podocytes.

Increased Opn expression in cancer cells regulates basement membrane turnover via the activation of NF-κB, subsequently increasing expression of key extracellular matrix enzymes, uPA, MMP-2 and -9. Recent observations indicated that MMP is involved in glomerular disease development.\textsuperscript{16} We found a time-dependent increase in MMP-2 and -9 and uPA mRNA levels after Opn treatment (Figure 3C). To test whether the increased MMP transcript levels are the consequence of the NF-κB activation, we incubated cells with inhibitors of the NF-κB pathway, including Bay11-7082 and PDTC. We found that whereas MMP-2 transcription was sensitive to the inhibitors of the NF-κB pathway, MMP-9 was not sensitive to Bay11-7082 or PDTC (Figure 3D). We performed wound-healing assays to examine the functional effect of Opn on podocytes, because podocyte motility has been strongly linked to the development of proteinuria.\textsuperscript{14,17} We found that Opn-treated podocytes migrated faster than control untreated cells, and they covered the wounded area faster than control cells (Figure 3E). The NF-κB pathway seems to be an important regulator of podocyte migration, because inhibitors of this pathway (Bay11-7082, PDTC) blocked Opn-induced podocyte migration (Figure 3, E and F). In summary, we found that Opn-treated podocytes have increased motility and increased expression of matrix-degrading enzymes. This is likely to be the consequence of Opn-induced enhanced NF-κB signaling.

What could the relationship between Opn levels and SSNS be? Although we attempted to study different animal models of albuminuria, we note that the PAN- and LPS-induced albuminuria models are sensitive to steroid treatment,\textsuperscript{18} indicating a correlation with our human data. The differential expression of Opn in SSNS could also suggest that the pathomechanism of SSNS might be different from SRNS, with Opn and downstream NF-κB perhaps being involved in SSNS. Glucocorticoids are known to interfere with the NF-κB pathway, via inhibiting the DNA binding of NF-κB p65 subunit.\textsuperscript{19} This opens the possibility that glucocorticoids might be effective in SSNS via interfering with the NF-κB pathway (downstream of Opn) in

### Table 1. Phenotype analysis of LPS-treated wild-type (C57/B6J) Opn knockout (Spp1\textsuperscript{−/−}) mice\textsuperscript{a}

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (wk)</th>
<th>Body Weight (g)</th>
<th>Albumin/Creatinine (μg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57/B6</td>
<td>9</td>
<td>6</td>
<td>20.9 ± 2.0</td>
<td>71.6 ± 23.0</td>
</tr>
<tr>
<td>C57/B6 LPS</td>
<td>10</td>
<td>6</td>
<td>20.4 ± 1.0</td>
<td>292.4 ± 37.0\textsuperscript{b}</td>
</tr>
<tr>
<td>Spp1\textsuperscript{−/−}</td>
<td>8</td>
<td>6</td>
<td>19.9 ± 1.0</td>
<td>63.4 ± 23.0</td>
</tr>
<tr>
<td>Spp1\textsuperscript{−/−} LPS</td>
<td>10</td>
<td>6</td>
<td>21.8 ± 1.0</td>
<td>91.8 ± 35.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Body weight and albuminuria were measured at the time animals were killed, 24 h after LPS injection. Albuminuria was measured by murine albumin-specific ELISA kit and creatinine with colorimetric reaction and expressed as μg albumin/mg creatinine.

\textsuperscript{b}Statistically significant difference, P < 0.05 versus C57/B6 control groups.

### Table 2. Phenotype analysis of wild-type, Ins2\textsuperscript{Akita}, and Spp1\textsuperscript{−/−} Ins2\textsuperscript{Akita} mice\textsuperscript{a}

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (wk)</th>
<th>Body Weight (g)</th>
<th>Blood Glucose (mg/dl)</th>
<th>Albumin/Creatinine (μg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57/B6</td>
<td>10</td>
<td>16</td>
<td>31.0 ± 1.0</td>
<td>204.0 ± 14.0</td>
<td>9.0 ± 4.0</td>
</tr>
<tr>
<td>Akita</td>
<td>10</td>
<td>16</td>
<td>24.0 ± 2.0</td>
<td>559.0 ± 40.0\textsuperscript{b}</td>
<td>438.0 ± 98.0\textsuperscript{b,c}</td>
</tr>
<tr>
<td>Spp1\textsuperscript{−/−}</td>
<td>8</td>
<td>16</td>
<td>31.0 ± 1.0</td>
<td>246.0 ± 20.0</td>
<td>17.0 ± 6.0</td>
</tr>
<tr>
<td>Spp1\textsuperscript{−/−} Akita</td>
<td>9</td>
<td>16</td>
<td>26.0 ± 2.5</td>
<td>575.0 ± 25.0\textsuperscript{b}</td>
<td>82.0 ± 8.0\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Body weight, blood glucose, and albuminuria were measured at the time animals were killed, at 16 wk of age. Albuminuria was measured by murine albumin-specific ELISA kit and creatinine with colorimetric reaction and expressed as μg albumin/mg creatinine.

\textsuperscript{b}Statistically significant difference, P < 0.05 versus C57/B6 control groups.

\textsuperscript{c}Statistically significant difference, P < 0.05 Akita versus Spp1\textsuperscript{−/−} Akita mice.
podocytes. The presence of glucocorticoid receptors have been demonstrated in cultured podocytes, a finding consistent with our hypothesis. We also found that dexamethasone inhibits Opn-induced podocyte motility (data not shown).

Finally, our results offer the possibility of using Opn inhibitors as a novel therapeutic target of albuminuria. Peptides that target RGD molecules such as Opn have already been developed by various pharmaceutical laboratories as a potential therapy for cancer and metastasis.

**CONCISE METHODS**

**Animals**

Mice with homozygous deletion of Opn (B6.Cg-Spp1^tmBlh/J, stock no. 004936), Ins2Akita mice on C57/B6J background, and wild-type C57/B6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Genotyping PCR was performed on genomic DNA according to published protocol (http://www.jax.org).

Puromycin (150 mg/kg body wt; Sigma-Aldrich Co., St. Louis, MO) was injected intraperitoneally into male Sprague-Dawley rats (Charles River Co., Wilmington, MA) that weighed 100 g (n = 6). Rats developed severe proteinuria on day 4, and they were killed on day 8 after the injection. Glomeruli were isolated by differential sieving. All procedures were approved by the Albert Einstein College of Medicine Animal Care and Use Committee.

**Phenotypic Analysis**

Blood glucose was measured by OneTouch Ultra2 Glucometer (Johnson & Johnson, New Brunswick, NJ). Albuminuria was determined using a mouse albumin-specific ELISA kit (Exocell Laboratories, Philadelphia, PA), and urine creatinine was determined using Creatinine Companion (Exocell), following the manufacturer’s protocol.

**Immunohistochemistry**

Immunohistochemistry was performed either on OCT-embedded frozen or formalin-fixed paraffin-embedded kidney sections as...
described previously (15) using anti-Opn antibody obtained from University Hybridoma Bank (Iowa), and some was also a gift from Dr. David D. Dendhardt (University of New Jersey); antisynaptopodin antibody was obtained from Sigma-Aldrich.

QRT-PCR
QRT-PCR results were normalized to ubiquitin C and HPGRT gene expression using the ΔΔCT value method. Supplemental Table 1 contains all primer sequences and further details.

Cell Wound-Healing Assay
Cell wound-healing assay was performed as described previously.²³

Western Blotting
Western blotting was performed as detailed in the supplemental information. The following primary antibodies were used in this study: Total-IκBα was purchased from Cell Signaling (Danvers, MA), and phospho-IκBα (Ser32) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Patient Characteristics
Pediatric patients with nephrotic syndrome were recruited at the Montefiore Medical Center (Bronx, NY). The institutional review board for clinical trials involving human subjects approved the study protocol. Fresh midstream urine samples were collected at the clinic or at the time of the kidney biopsy. Control kidney biopsy samples were collected from living transplant allografts right after removal from the donor (time 0).

NF-κB p50 Nuclear Binding
NFκB p50 nuclear binding assay was performed using TransFactor Colorimetric kit (Clontech BD, Mountain View, CA) according to the manufacturer’s protocol.

Human Osteopontin Quantikine ELISA Kit
Human Osteopontin Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) was used for quantification of urine Opn levels. The assay was performed according to the manufacturer’s protocol.

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DISCLOSURES
None.

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Supplemental information for this article is available online at http://www.jasn.org/.