Lack of In Vivo Function of Osteopontin in Experimental Anti-GBM Nephritis

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Abstract. Osteopontin (Opn) is a potent chemoattractant for mononuclear cells that is upregulated in various inflammatory states of the kidney. Opn is believed to contribute to mononuclear cell infiltration and renal injury. The importance of Opn was examined in vivo in rapidly progressive glomerulonephritis in Opn knockout mice. Glomerulonephritis was induced by intravenous injection of rabbit anti-mouse glomerular basement membrane antiserum in mice that had been presensitized to rabbit IgG. Immunologic responsiveness to rabbit IgG (assessed by cutaneous delayed-type hypersensitivity and antibody titers) showed no significant difference between wild-type and Opn −/− mice. Proteinuria was also similar in both groups. Glomerular crescent formation was not different in Opn +/+ and −/− groups (26 ± 6% versus 29 ± 7%). Tubulointerstitial infiltration was assessed qualitatively and showed no significant difference between the two genotypes.

Osteopontin (Opn) is a soluble secreted phosphoprotein that was first isolated from rat, human, and bovine bone (1). Despite its name, it is not only expressed in bone but also detected in numerous organs and tissues, with a predilection for luminal epithelial surfaces (2–5). The presence of an Arg-Gly-Asp domain has triggered much speculation over its function (6). In experimental anti-glomerular basement membrane (anti-GBM) nephritis, Opn mRNA and protein upregulation have been observed in glomeruli and tubules of rat kidneys (16,17). As in other forms of renal injury, this upregulation of Opn has also been associated with mononuclear cell infiltration and renal injury in this model. The anti-GBM nephritis model therefore has been used to study the effect of blocking antibodies against Opn to determine the role of Opn in vivo. Anti-Opn-treated rats that experienced anti-GBM nephritis displayed a milder pathology, suggesting an important role for Opn in promoting glomerular inflammation (17).

In various pathologic conditions in rodents and humans, Opn is found to be highly upregulated in response to several insults, especially in the kidney (11–15). The presence of inflammatory cells associated with these sites of upregulation suggests that Opn could play a significant role in the development of such conditions.

In experimental anti-glomerular basement membrane (anti-GBM) nephritis, Opn mRNA and protein upregulation have been observed in glomeruli and tubules of rat kidneys (16,17). As in other forms of renal injury, this upregulation of Opn has also been associated with mononuclear cell infiltration and renal injury in this model. The anti-GBM nephritis model therefore has been used to study the effect of blocking antibodies against Opn to determine the role of Opn in vivo. Anti-Opn-treated rats that experienced anti-GBM nephritis displayed a milder pathology, suggesting an important role for Opn in promoting glomerular inflammation (17).

Taking advantage of recently generated Opn-deficient mice (18), we induced accelerated anti-GBM nephritis to compare the severity of renal disease between wild-type and Opn −/− mice. We show that Opn-deficient mice do not have less renal injury compared with their wild-type littermates, suggesting that Opn does not play an important chemoattractant role in this model of renal injury.

Materials and Methods

Animals

Female Opn knockout mice (Opn −/−), originally in a mixed 129 Sv/C57Bl/6 background, were crossed once with C57Bl/6 wild-type Opn +/+ mice. The F1 mice were then set up for intercross to obtain homozygous Opn −/− and +/+ F2 littermates. All animals were kept...
under pathogen-free conditions in sterilized filter-top cages, and all animal manipulations were carried out in a sterile laminar flow hood.

**DNA Extraction and Purification**

At 4 wk of age, mice were anesthetized with methoxyflurane and marked with numbered earmarks (Hauptner Instrumente GmbH, Dietlikon, Switzerland). One-centimeter tail segments were cut and then further processed using the DNeasy™ tissue kit rodent tail protocol (Qiagen, Valencia, CA). DNA concentration was assessed by OD₂₆₀ measurements.

**PCR Analysis of DNA for Genotype Determination**

To determine the genotype of the mice, we used two different PCR reactions with the following primers: reaction 1, 5’-AAT AGT CGA AGT GGA CCT GG-3’ (Neo 219 up) and 5’-GAT CAA GCC ATA GCC CTT CA-3’ (Opn 538 down); reaction 2, 5’-TCC AAT GAA AGC CAT GAC C-3’ (Opn Ex 6) and 5’-GAA GAG TGA GTG AAT CTG C-3’ (Opn Unt 6).

Reaction 1 was used to determine the correct insertion of the neomycin cassette and yielded a product of 346 bp. In reaction 2, we used primers that flank the neomycin cassette insertion site, yielding products of 331 bp in the wild type and 1451 bp with the disrupted allele. For the PCR, 500 ng template DNA was used. Cycling parameters were chosen as follows: 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. The reaction mix contained 1.5 mM MgCl₂, 200 µM dNTP, 1 mM of primers, 2 U of Taq polymerase, and 1× reaction buffer in a total volume of 40 µl. All reagents were from Promega (Madison, WI).

**Immunologic Response to Rabbit IgG**

Cutaneous delayed-type hypersensitivity (DTH) and antibody response to rabbit IgG were examined in Opn+/+ mice to measure their immunologic competence. Six Opn+/+ and six Opn−/− mice were immunized with 0.2 mg of rabbit IgG for the induction of anti-GBM nephritis. After 10 d, 20 µl of either rabbit IgG or bovine serum, both solutions at 1 mg/ml, were subcutaneously injected in the footpads of the mice. The right foot was chosen for the injection of rabbit IgG and the left for bovine serum. After 24 and 48 h, both feet were measured using a Mitutoyo dial thickness gauge, model 7301 (Mitutoyo Corp., Kawasaki-Shi, Japan). Anti-rabbit IgG antibodies titers were measured by enzyme-linked immunosorbent assay as follows: microtiter plates were coated with rabbit IgG at 1 µg/ml in phosphate-buffered saline, washed, and blocked with bovine serum albumin (20 mg/ml in phosphate-buffered saline). Sample sera were incubated overnight at dilutions of 1:2000 for IgG₁ and 1:5000 for IgG₂. A goat anti-mouse IgG coupled to alkaline phosphatase was used as secondary antibody, and the disodium salt of p-nitrophenyl phosphate was used as substrate. OD₆₅₀ was measured after 20 min of incubation at room temperature.

**Induction of Anti-GBM Nephritis**

The anti-GBM serum was obtained after immunization of rabbits with mouse GBM in complete Freund’s adjuvants as described (19). Wild-type and Opn knockout mice were immunized between 8 and 12 wk of age with 0.2 mg of rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.2 ml of complete Freund’s adjuvants. Glomerulonephritis was induced after 6 d by injection of 0.25 ml of rabbit anti-mouse IgG in the tail vein. Mice were analyzed 8 d (n = 9 for each genotype) or 5 wk (n = 5 for each genotype) after injection. In addition, six untreated mice of each genotype were used as controls. Proteinuria was assessed using dipsticks (Albustix, Bayer Diagnostics, Basingstoke, United Kingdom), and results were classified on an arbitrary scale from 0 (no proteinuria) to 4 (10 to 20 mg/ml).

**Kidney Preparation for Histologic Staining**

Mice were killed under methoxyflurane anesthesia. The left kidney was excised for periodic acid-Schiff (PAS) staining. At least 100 glomerular cross sections (GCS) were analyzed for each animal, and the number of crescents was registered as percentage of GCS counted. Assessment of formation of thrombi within the glomerular capillaries was based on at least 100 GCS. Scores ranged from 0 (no thrombosed capillaries) to 3 (more than one half of the capillaries thrombosed). The single scores were added and then divided by the number of GCS counted, giving the average per GCS. Tubulointerstitial cellularity was assessed qualitatively, indicating the degree of infiltration as mild, moderate, or strong. All histologic evaluations were performed without knowledge of the mouse genotype.

**Immunofluorescent Staining**

The right kidney was snap-frozen for immunolabeling. The following rat anti-mouse antibodies were used: anti-CD3 (clone KT3), anti-MHC class II (1-A₁b,d), clone M5/114.15.2, and anti-macrophage scavenger receptor (clone 2F8). Cy3-labeled goat anti-rat IgG was used as second antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Six-micrometer sections were fixed for 10 min in acetone at 4°C and rinsed in Tris-buffered saline (TBS). Primary antibodies diluted in TBS were allowed to bind during 3 h at room temperature. Sections were then washed twice for 15 min in TBS and afterward incubated for 1 h with the second antibody at room temperature. After washing, the sections were mounted in Immu-mount (Shandon, Pittsburgh, PA). Positively stained cells on at least 100 glomerular cross sections were counted on each slide.

**RNA Extraction and Northern Blot Analysis**

Total RNA was extracted from one half kidney, using the RNeasy™ protocol (Qiagen). RNA concentration was assessed by OD₂₆₀ measurement. Aliquots of 20 µg of RNA were loaded onto 1.5% agarose gels and then transferred on Hy Bond-XL blotting membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Blots were cross-linked with ultraviolet light; afterward, methylene blue staining of ribosomal RNA was performed. The Opn cDNA was generated by reverse transcription-PCR as described (20). The regulated upon activation, normal T cell expressed and secreted (RANTES) and monocyte chemoattractant protein-1 (MCP-1) cDNA probes were generated by restriction of plasmids (kindly donated by Dr. Gunter Wolf, University of Hamburg, Germany). Blotting membranes were prehybridized for 4 h at 65°C and then hybridized overnight with probes previously labeled by random priming with ³²P-dCTP. After washing, blots were exposed to Kodak X-Omat AR films (Kodak, Rochester, NY). Autoradiographs were digitized with a CCD camera, and files were analyzed densitometrically using the Scion Image software (Scion Corp., Frederick, MA).

**In Situ Hybridization**

Riboprobes labeled with digoxigenin-11-UTP were synthesized in vitro using the transcription kit SP6/T7 (Roche Molecular Biochemicals, Rotkreuz, Switzerland) from full-length mouse cDNA. RNA probes were degraded by alkaline hydrolysis to fragments of approximately 200 bp in length. Paraffin-embedded sections were hybridized overnight at 68°C, and hybridized probes were detected using alkaline...
phosphatase-coupled sheep antidiogoxigenin antibody and 5-bromo-4-chloro-3-indolyl phosphate as a substrate (20).

Statistical Analyses

Data are expressed as mean ± SEM. Data analysis was performed by ANOVA using the In Stat program (GraphPad Software, San Diego, CA). Tubulointerstitial infiltration was analyzed by χ² contingency analysis.

Results

Opn Knockout Mice Show Normal Cellular and Humoral Immune Response to Rabbit IgG

Opn +/+ and −/− mice had similar cutaneous DTH responses (Figure 1A). The production of specific anti-rabbit IgG antibodies was also determined, as depicted in Figure 1B. IgG2a (associated with a T helper cell type 1 response) did not show a difference between the Opn +/+ and −/− mice. IgG1 (associated with a T helper cell type 2 response) also showed no relevant difference between Opn +/+ and −/− mice (P > 0.05).

Anti-GBM Nephritis Is Similar in Wild-Type and Opn −/− Mice

After anti-GBM disease induction, proteinuria increased markedly (Figure 2). There was no difference between Opn +/+ and −/− mice in the pattern of development or severity of the proteinuria. The disease severity was assessed by PAS staining (Figure 3). Sections from Opn +/+ and Opn knockout mice presented the same histologic picture, showing glomerular damage with crescent and thrombi formation (Figure 3, C and D). The percentage of glomeruli with crescent formation was 26 ± 6% for Opn +/+ mice and 29 ± 7% for the knockout mice (n = 9 for each group; Figure 4A). The number of thrombotized capillaries on a scale from 0 to 3 also showed no relevant difference between genotypes: Opn +/+ mice scored an average of 1.3 ± 0.3 and Opn −/− mice scored 1.4 ± 0.3 (Figure 4B). Tubulointerstitial infiltration was assessed qualitatively and also revealed no difference between the two genotypes (mild to moderate infiltration for both groups, P > 0.05).

In a long-term experiment, five Opn +/+ and five Opn −/− mice were killed 5 wk after disease induction. Evaluation of PAS-stained paraffin-embedded sections showed persistent renal disease. Crescent formation per 100 GCS was 28 ± 6 in Opn +/+ and 19 ± 8 in Opn −/− mice (P > 0.05). Tubulointerstitial infiltration also showed no significant difference between Opn +/+ and −/− mice (P > 0.05).

Opn Knockout Mice Present the Same Type of Infiltration as Wild-Type Littermates

Immunofluorescent staining of kidneys with anti-GBM disease revealed similar glomerular and tubulointerstitial infiltration in both genotypes (Figure 5). There was a 5.0 ± 1.3-fold (Opn +/+ ) and 5.9 ± 1.3-fold (Opn −/− ) increase of CD3-positive T cells in glomeruli compared with normal control mice (Figure 6A) and a 3.0 ± 0.3-fold (Opn +/+ ) and 3.6 ± 0.4-fold (Opn −/− ) increase of MHC class II-positive cells (macrophages and dendritic cells; Figure 6B). When sections were stained for CD4 (helper T cells), CD8 (cytotoxic T cells), and scavenger receptor (macrophages), there was also no relevant difference in the quantity of these infiltrating cells between Opn +/+ and Opn knockout mice (data not shown).
Opn and Chemokine Expression in Opn Wild-Type and −/− Mice

After induction of anti-GBM disease, Opn mRNA detected by Northern blot analysis increased 5.1 times in Opn +/+ mice. In −/− mice, no Opn signal could be detected in controls and in animals with anti-GBM disease, as expected (Figure 7). Because the expression of Opn has not been studied in mice with anti-GBM nephritis, we also assessed the distribution of
Opn by \textit{in situ} hybridization (Figure 8). There was a typical overexpression in proximal tubular cells in Opn \textit{+/+} mice, as described previously in other models of nephritis in mice and rats (11,20–22). Opn-positive cells could be found in Bowman’s capsule (Figure 8) and occasionally in cellular crescents (not shown). The glomerular tuft remained negative. In knockout mice, no hybridization signal could be detected, as expected (Figure 8C).

The upregulation of additional chemokines was assessed by Northern blot analysis. RANTES and MCP-1, known macrophage and T cell chemoattractants, were investigated for possible overexpression to compensate for Opn (23). In both genotypes, the upregulation of RANTES and MCP-1 was prominent in the kidneys with anti-GBM nephritis (Figure 9). Again, there was no significant difference between wild-type and knockout mice. Overall, the level of chemokine upregulation correlated with the severity of the disease in individual mice (data not shown).

**Discussion**

Our data demonstrate that there is a marked elevation of Opn in the tubulointerstitial compartment in wild-type mice after...
anti-GBM nephritis induction, whereas mice with both Opn alleles disrupted by homologous recombination are unable to form functional forms of the protein. Despite the complete absence of Opn, knockout mice developed rapidly progressive anti-GBM nephritis. The course of the disease was identical in wild-type littermates with respect to proteinuria, histopathologic severity, and upregulation of chemokines. As the DTH and the humoral immune responses were also not different in Opn \(-/-\) mice compared with Opn \(+/+\) littermates, these data suggest that Opn does not play a major pathogenic role in the development of anti-GBM nephritis in mice.

Previous reports have shown that Opn can play a significant role in the recruitment of leukocytes to sites of inflammation. In vitro studies have shown that several integrins bind Opn with a strong avidity and that Opn acts as a chemoattractant for mononuclear cells (7,8,24). In vivo studies have also revealed that Opn acts as a chemoattractant for macrophages and that this property could be counteracted by neutralizing antibodies (9). It has therefore been speculated that Opn might have pathogenic potential in inflammatory diseases in vivo.

In animal models of kidney disease, Opn mRNA and protein levels are increased (13–15,20,25–29). The upregulation of Opn in the kidney occurs mostly in proximal tubular epithelial cells and in cells of Bowman’s capsule (12), as well as in the MRL-Fas\textsuperscript{lo} mouse with murine lupus nephritis (20). In all of these studies, a close

Figure 7. (A) Northern blot analysis of RNA shows a marked increase in Opn mRNA expression in Opn \(+/+\) mice after induction of anti-GBM nephritis. Knockout mice show no signal in treated and control groups, as expected. (B) Methylene blue staining of Northern blot reveals equal amounts of loaded total RNA.

Figure 8. (A) In situ hybridization shows focal expression of Opn mRNA in proximal and distal tubulus in the cortex as well as in the medulla in Opn \(+/+\) mice with anti-GBM nephritis. (B) Crescents and glomerular tufts remain deprived of hybridization signal. Additional signals are found in cells lining the parietal epithelium of Bowman’s capsule of some glomeruli (arrow). (C) No signal is detected in mice carrying the disrupted allele \((-/-\)).
correlation has been found between the degree of tubulointerstitial macrophage infiltration and the level of enhanced Opn expression. However, most of these studies were purely associative in nature and could not definitively prove the chemotactic potential of Opn in vivo.

It is important to note that there is no Opn expression in glomeruli of mice with anti-GBM nephritis, as well as in most other experimental models of immune renal injury. However, some Opn expression was described within the glomerulus in rats with anti-GBM nephritis, suggesting that Opn could have chemoattractive properties in glomeruli as well (16,17). In our study, we could not detect Opn expression in glomeruli in mice, and the possibility of a species difference therefore must be considered.

The study by Yu et al. (17) showed that treatment of rats with anti-GBM nephritis using an anti-Opn antibody resulted in a significant reduction of proteinuria and glomerular injury. The beneficial effect of the anti-Opn antibody in that study, however, may not have been solely due to blocking of glomerular Opn. It is known that in the anti-GBM nephritis, injury is mainly a manifestation of a cell-mediated immune response similar to that observed in cutaneous DTH (30,31). In the previously mentioned study, anti-Opn treatment of rats reduced the skin swelling in the DTH response to rabbit Ig. These findings were attributed to reduced macrophage influx (17) and suggested, therefore, that the antibody also inhibited cellular immune responses not only in the kidney. This is in striking contrast to our study, in which we could not detect a difference in the DTH or in the humoral immune response to rabbit IgG.

A diminished responsiveness to DTH can be caused by several factors and in general cannot be attributed to a single protein because of the complexity and redundancy of the immunologic network. A model to investigate the individual role of proteins or chemokines in the pathogenesis of anti-GBM nephritis must therefore be neutral with regard to the general DTH response to be convincing. It is known that in anti-GBM models in which the DTH response is reduced, the disease generally is less pronounced and milder in severity (19). In our work, despite the complete absence of Opn in the knockout mice, there was no difference in the measured DTH between Opn +/+ and Opn −/− mice. It is therefore unlikely that Opn plays a dominant role in the ability to respond to the DTH challenge. The reduction in DTH response induced by anti-Opn treatment must be further investigated and cannot rely only on Opn neutralization. Nonspecific actions of the antibody OP199, used by Yu et al. (17,24) to neutralize Opn could lead to binding of other epitopes, possibly on T cells. This possibility is not completely excluded because this antibody was tested only for cross reactions against vitronectin and fibronectin.

Infiltration of macrophages is essential and plays a key role in the development of anti-GBM nephritis (32–34). We found that macrophage infiltration in glomeruli and in the interstitium was not less pronounced in Opn knockout mice, suggesting
that compensatory mechanisms could be in place in Opn \(-/-\) mice. Other chemokines were investigated to determine whether known macrophage chemotactants were upregulated more markedly in Opn \(-/-\) than in \(+/+\) mice. We found that the expression of MCP-1 and RANTES mRNA was not different in knockout and wild-type littermates. Our data therefore cannot support an active compensation mechanism by these two chemokines but do not exclude that other chemotactant pathways could be in place. The absence of Opn expression in the glomerular tuft and the lack of compensatory mechanism, as far as investigated in our work, raise questions about the overall relevance of Opn in anti-GBM nephritis in mice and possibly in other inflammatory renal diseases.

Recently, it was shown that Opn could play a protective role in renal injury via its antagonizing effect on inducible nitric oxide synthase (iNOS) and by protecting from apoptosis. Noiri et al. (35) showed that Opn knockout mice have a reduced tolerance to renal ischemia. The outcome after 30 min of clamping of the renal artery was worse in Opn \(-/-\) mice, and a more pronounced upregulation of renal iNOS was detected in Opn \(-/-\) mice. This is in agreement with several in vitro studies that have shown that Opn is an inhibitor of iNOS (36,37). It is known that the inhibition of iNOS leads to reduced macrophage cytotoxicity (38,39). In Opn \(-/-\) mice with anti-GBM nephritis, the lack of Opn could unleash the full potential of NO in activating macrophages, thereby compensating for the absence of Opn. However, this mechanism is unlikely to operate in vivo in anti-GBM nephritis, as it has been shown that iNOS deficiency does not influence the course of anti-GBM nephritis (40). Protection by Opn from apoptosis in kidney tubular and interstitial cells has also been recently shown (41). This finding could also explain the lack of protection against inflammation by the absence of the chemotactant Opn in the knockout mice.

In summary, our study demonstrates that Opn does not play a relevant role in the pathogenesis of anti-GBM nephritis in mice. The infiltration of inflammatory cells and the severity of the disease despite the complete absence of Opn clearly show that this molecule is not responsible for the mononuclear cell infiltration in anti-GBM nephritis. A putative protective role of Opn was not investigated in these experiments, leaving this question open for future studies.

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