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Reduced postischemic macrophage infiltration and interstitial fibrosis in osteopontin knockout mice

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Background. Osteopontin (OPN) is a phosphoprotein that is up-regulated in several experimental models of renal disease, including ischemia/reperfusion injury. OPN has been described as a macrophage chemoattractant, may serve as a survival factor for tubular cells, and is implicated in the development of tubulointerstitial fibrosis. However, the precise role of this protein in renal pathophysiology remains unclear.

Methods. OPN knockout and wild-type mice were subjected to 30 minutes of warm renal ischemia combined with a contralateral nephrectomy, and sacrificed at six different time points, ranging from 12 hours to seven days after reperfusion. Besides functional and morphological parameters of postischemic acute renal failure (ARF), macrophage infiltration, apoptosis and expression of collagen types I and IV were investigated.

Results. Postischemic ARF in OPN knockouts and wild-types showed a similar course and severity, without significant differences in either functional or morphological disease parameters. However, macrophage infiltration was significantly diminished in OPN knockouts after five and seven days, in cortex as well as in the outer stripe of the outer medulla (OSOM). Furthermore, OPN knockout mice showed significantly enhanced apoptosis in the injury phase and significantly less collagen I and IV expression in the regeneration phase of postischemic ARF.

Conclusions. There was no influence of OPN protein on the severity or course of functional impairment or morphological injury in the first seven days after an ischemic insult to the kidney. However, our results demonstrate that OPN favors macrophage recruitment to the postischemic kidney, inhibits apoptosis, and stimulates the development of renal fibrosis after an acute ischemic insult.

Osteopontin (OPN) is a highly acidic phosphoprotein, first isolated from bone, but also expressed in several other tissues, including renal tubular epithelium. OPN expression is implicated in a wide array of pathophysiological processes, including bone remodeling and osteoporosis [1–3], tumor metastasis [4], vascular pathology [5, 6] and wound healing [7].

In the normal kidney, OPN is mainly produced by the distal nephron [8, 9] and secreted into urine [10], where it may serve to prevent urinary stone formation [11–13]. OPN up-regulation has been demonstrated in several experimental models of renal disease [14–18], including ischemia/reperfusion (I/R) injury [19–21]. However, the precise mechanism in which OPN is functioning in renal pathophysiology remains unclear.

Osteopontin has been described a macrophage chemoattractant [22–25] and its up-regulation in renal disease is also implicated in tubulointerstitial fibrosis [15, 23, 26, 27].

Osteopontin can bind to different integrins through its RGD (arginine-glycine-aspartic acid) cell adhesion sequence [28–32], but probably also through non-RGD domains [33, 34]. The cell adhesion and migration properties of OPN [35–37] also may be of importance for its role in acute tubular necrosis.

Osteopontin may act as a 'survival factor' for tubular cells, either through inhibition of inducible nitric oxide synthase (iNOS) induction [38] or through inhibition of apoptosis [39, 40]. Decreased iNOS up-regulation could limit cell injury by diminishing the production of NO and its cytotoxic metabolites such as peroxynitrite. The inhibition of iNOS expression by OPN was confirmed by Noiri et al, who describe enhanced presence of iNOS and NO, along with more severe I/R injury in OPN knockouts at 24 hours after reperfusion [41].

The present study evaluates whether OPN has a functional role in postischemic macrophage infiltration, renal fibrosis and apoptosis. OPN knockout mice were subjected to renal ischemia and reperfusion and followed for up to seven days. Mice lacking OPN protein showed a significant inhibition of macrophage infiltration, increased apoptosis and less tubulointerstitial fibrosis.

Key words: osteopontin, ischemia, macrophage infiltration, fibrosis, apoptosis, phosphoprotein.

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METHODS

Animals and experimental design

OPN knockout mice. A breeding colony was started with four breeder couples of 129JV × black Swiss hybrid strain, hemizygous for a targeted deletion of the osteopontin gene, which were the generous gift of Dr. L. Liaw. Offspring were genotyped with polymerase chain reaction (PCR) performed on tail DNA as described earlier [7]. Briefly, a three-primer PCR reaction was performed to amplify a 600 bp product for the normal allele and a 500 bp product from the mutated allele. On agarose gel electrophoresis OPN knockouts (KO) showed a single band of 500 bp, whereas wild-types (WT) showed a single band at 600 bp.

Animals were kept in a 12-hour light/dark cycle, with free access to standard mouse chow and tap water. All animal procedures were carried out in accordance with the *NIH Guidelines for the Care and Use of Laboratory Animals* No. 85-23 (1985) and approval of the Antwerp University Ethical Committee.

Osteopontin knockouts were used between 90 and 120 days of age, with age- and gender-matched wild-type littermates as controls. The animals were subjected to 30 minutes of warm ischemia of the left kidney, combined with a right nephrectomy. Five animals of each genotype were sacrificed 12 hours and 1, 2, 3, 5 and 7 days after reperfusion.

Ischemia model. A model of warm ischemia and reperfusion injury on a single kidney was adapted for use in mice [42]. This model was designed to inflict a moderate acute renal failure with tubular necrosis and limited animal mortality.

Mice were anesthetized with prewarmed solution (37°C) of Ketamine (Ketalar®; Parke-Davis, Morris Plains, NJ, USA) 80 mg/kg and xylazine (Rompun®; Bayer, Wuppertal, Germany) 16 mg/kg, administered intraperitoneally. Immediately after loss of righting reflex, they were placed on a heated surgical pad (surface temperature 37°C), where they were kept during the whole procedure. An abdominal midline incision was made and the left renal pedicle exposed and clamped for 30 minutes with a Kleinert-Kutz microvessel clamp. The right renal pedicle was exposed and ligated with a silk 4/0 ligature. The excised right kidney was fixed as described below and used as control. Left renal surface temperature was measured immediately before clamp release. The reperfusion was monitored de visu. The abdomen was closed in two layers using 4/0 resorbable sutures. Animals were kept on the heated pad until restoration of the righting reflex.

Blood and urine sampling. Preoperatively and before sacrifice animals were kept in metabolic cages for 24-hour urine sampling. Urine output was measured with a graded pipette and urine samples were frozen at −20°C until analysis. Blood samples were taken from the peri-orbital sinus under ketamine-xylazine anesthesia. Blood was allowed to clot at room temperature and centrifuged at 13,000 rpm for 15 minutes. Serum was stored at −20°C until analysis. After preoperative blood sampling animals were left to recuperate for two days before the ischemia procedure.

Determination of biochemical parameters. Serum creatinine determination was performed using a routine autoanalyzer system (Vitros 750 XRC) at the Biochemistry Laboratory of the Antwerp University Hospital. Urinary creatinine was determined by a colorimetric method (Creatinine Merckotest, Diagnostica Merck, Germany). Proteinuria was determined by the Bradford method. Creatinine clearance (in mL/min/100 g body weight) was calculated according to the standard formula.

Tissue collection. At sacrifice, animals were exsanguinated via the retro-orbital sinus under ketamine-xylazine anesthesia. The left kidney was quickly excised and decapsulated. Sagittal slices of kidney tissue were fixed in formol calcium for 90 minutes and methacarn for four hours and were subsequently processed for paraffin embedding.

Immunohistochemistry. Osteopontin staining was performed with OP199, a polyclonal antibody against rat OPN (gift of Dr. C.M. Giachelli), which has been shown to cross-react with mouse OPN [43]. Staining was performed as described before [19], but sections were counterstained with methyl green.

Immunohistochemical staining for collagen types I and IV was performed with rabbit polyclonal antibodies against mouse collagen types I and IV (Biodesign International, Saco, ME, USA) on methacarn-fixed kidney tissue, as for the OPN staining.

Renal morphology was evaluated on formol calcium-fixed sections stained for proliferating cell nuclear antigen (PCNA) with a mouse monoclonal antibody (Clone PC10; Dako, Glostrup, Denmark) using the ARK-kit (Dako) according to the manufacturer's instructions. Sections were counterstained with periodic acid Schiff (PAS).

For staining of macrophages we used the rat monoclonal antibody F 4/80 (Clone C1: A3-1; Immunosource, Halle-Zoersel, Belgium) on formol calcium-fixed tissue. Antigen retrieval was performed with a five minute incubation in trypsin solution (Sigma Chemical Co., St. Louis, MO, USA). After blocking with normal rabbit serum and blocking endogenous peroxidase activity with 0.03% H_2O_2 in methanol, sections were incubated with primary antibody overnight. After washing, sections were incubated with a biotinylated, mouse adsorbed rabbit anti-rat IgG antibody (Vector Laboratories, Burlingame, CA, USA) and subsequently with avidin and biotinylated horseradish peroxidase (AB-complex; Vector Laboratories). Color development was performed with diaminobenzidine in the presence of 0.03% H_2O_2. Sections were counterstained with methyl green.

Scoring of morphological injury and regeneration. In every tissue section 40 cross sections of proximal tubules (PT) in the outer stripe of the outer medulla (OSOM) were scored, as described in [19]. Scoring was performed blinded to the identity of the sections. Tubules were divided into four morphological categories: normal, sublethally damaged, necrotic or regenerating. PT cross sections showing a continuous epithelium with an intact periodic acid Schiff (PAS) stained-positive brush border were considered normal. PT with damaged brush border or loss of brush border were considered sublethally injured. Tubules with a discontinuous epithelium or cross sections containing necrotic cells were considered to have suffered necrosis or cell loss. Defined as regenerating were those PT cross sections containing large PCNA positive nuclei, combined or not with a flat epithelium with large nuclei and a very narrow cytoplasmic rim, or cross sections showing some restoration of cytoplasmic volume with still large nuclei protruding into the tubular lumen.

Quantification of immunohistochemical data. For OPN (10 fields in cortex and OSOM at magnification ×320), F4/80 (10 fields in cortex and OSOM at magnification ×200) and collagen types I and IV staining (30 fields in cortex and OSOM at magnification ×400), the area % of the immunohistochemical signal was measured using a computerized image analysis system (Kontron 400 2.0).

TUNEL-staining

DNA strand breaks were detected on formol calcium-fixed sections, after incubation with TdT (Boehringer, Indianapolis, IN, USA) and FITC-labeled d-UTP (Fluorogreen; Amersham, Little Chalfont, Buckinghamshire, UK), with a rabbit anti-FITC antibody (Dako) and color development with AEC (amino-ethylcarbazole) in the presence of 0.9% H_2O_2. Sections were lightly counterstained with hematoxylin.

Apoptotic bodies were counted in tubular epithelium as well as in the interstitium of cortex and OSOM. As the TUNEL (terminal deoxynucleotide transferase uridine triphosphate nick-end labeling) technique frequently results in false positive staining when necrosis or cell proliferation are present [44], only TUNEL-positive objects meeting the following morphological criteria were considered apoptotic: small, densely TUNEL positive, rounded objects (apoptotic bodies) or cells with a small, very dense, TUNEL-positive nucleus.

Statistics. Data are presented as mean ± SD or as percentages, unless indicated otherwise. Statistical analysis was performed with *Systat 7.0 for Windows* and *SPSS 10.0* statistical software. For animal and image analysis data non-parametric tests were used: Kruskall-Walllis H test and Mann-Whitney U test, with Bonferroni correction for multiple comparisons. Data from the morphological evaluation were analyzed using Pearson's χ^2-test. Values $P < 0.05$ were considered significant.

RESULTS

Mortality

In both groups, 4 out of 30 animals died prior to planned sacrifice. As mortality was highest in the five and seven day groups (one knockout in the 3 days group also died) and obstruction revealed no peculiarities, animals most likely died of the consequences of acute renal failure. Data from these animals were excluded from further analysis.

Quality controls of the ischemia model

Renal surface temperature. The use of a heated surgical pad could not prevent a limited drop in body temperature, but this was comparable in both genotypes: renal temperature was 33.32 ± 0.76 and 33.40 ± 0.46°C in respectively OPN KO and WT mice (data not shown).

OPN staining. Immunohistochemical staining confirmed the absence of OPN protein in OPN KO mice, in control as well as postischemic kidneys. In WT mice, a spectacular up-regulation of OPN staining after renal I/R was found in renal cortex (Fig. 1A) as well as OSOM (Fig. 1B), which was already highly significant 12 hours after reperfusion, and lasted throughout the seven-day study period. In keeping with our previous findings in the rat [18, 19], OPN up-regulation in distal tubules was early and sustained, with an apical staining pattern, whereas proximal tubules remained largely OPN negative when injured, showing OPN staining in a vesicular, perinuclear pattern when uninjured or regenerating.

Functional results

Creatinine clearance values revealed the occurrence of postischemic acute renal failure, with equal severity and course in OPN knockouts and wild-types: in both genotypes the creatinine clearance decreased toward a minimum after 24 hours: 0.27 ± 0.21 and 0.24 ± 0.21 mL/min/100 g body weight in OPN knockouts and wild-types, respectively. Partial restoration of glomerular filtration occurred over the rest of the study period (Fig. 2A). Serum creatinine values showed an equal increase in both genotypes, with a maximum after 24 hours (data not shown).

Proteinuria was not significantly different between the genotypes either. In both OPN knockouts and wild-types a peak in urinary protein content was reached 24 hours after reperfusion with values of 2.20 ± 1.13 and 2.58 ± 1.37 mg/24 h for OPN KO and WT mice, respectively (Fig. 2B).

Morphological injury and regeneration

Ischemic renal injury mainly affected proximal tubules (PT) of the outer stripe of the outer medulla (OSOM), that is, the S3 segment of the PT. Tubular cells lost their brush border, detached from the tubular basement mem-

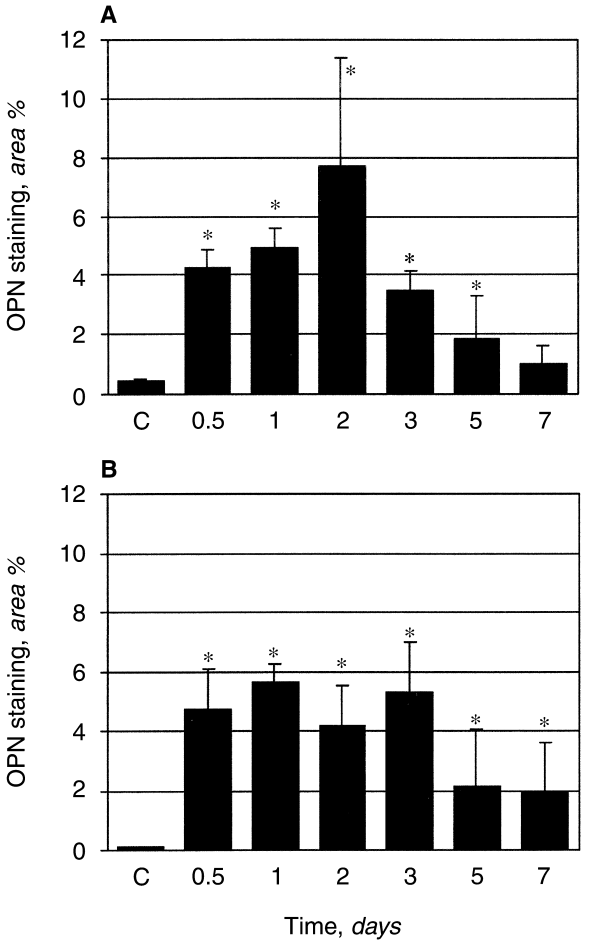


Fig. 1. Osteopontin (OPN) was up-regulated after renal ischemia in wild-type mice in the (*A*) cortex and (*B*) outer stripe of the outer medulla (OSOM). Immunohistochemical staining was performed with polyclonal antibody OP199. The area % stained positive was quantified with a computerized image analysis system. OPN wild-type mice showed a significant up-regulation of OPN protein, which was already present 12 hours after reperfusion and lasted throughout the study period. No OPN staining was found in kidneys of OPN KO mice (data not shown).

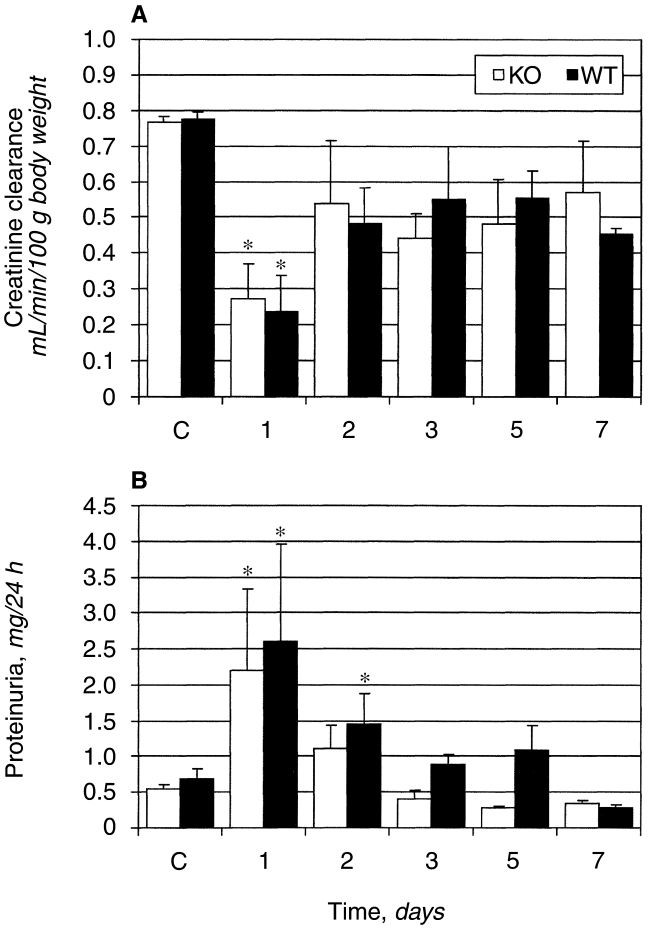


Fig. 2. Osteopontin had no effect on functional parameters of postischemic injury. (*A*) Creatinine clearance, calculated according to the standard formula, showed moderate renal function impairment 24 hours after reperfusion, which was comparable between OPN knockout and wild-type mice. Data are means ± SEM. (*B*) Proteinuria, determined with the Bradford method, showed an approximately fourfold increase 24 hours after reperfusion, reflecting renal injury. However, no significant differences were found between OPN knockouts and wild-types. Data are means ± SEM; $^*P < 0.05$ vs. controls.

brane or became necrotic. Cortical PT, on the other hand, suffered limited, mostly sublethal injury. To assess the severity of morphological injury, OSOM PT cross sections were classified into four categories: normal, sublethally damaged, tubules showing signs of necrosis or cell loss and regenerating tubules (Fig. 3) [19]. Morphological evaluation revealed no statistical differences between both genotypes at any time point (Pearson's χ^2 test). OPN KO and WT mice showed comparable percentages of sublethally damaged and necrotic tubules over the whole study period, reflecting a similar severity of morphological damage in both genotypes. Morphological injury peaked after 24 hours (with 32.1% and 30.0% of the OSOM PT showing suffering necrosis or cell loss in OPN KO and WT mice, respectively) and slowly subsided afterwards. Moreover, the percentages of regenerating tubules (peaking at 29.2% in KO and 30.4% in WT mice after 3 days) were comparable in both groups as well.

Thus, in this study, the absence of OPN protein did not aggravate nor alleviate the functional or morphological consequences of acute postischemic renal injury.

Macrophage infiltration

Staining with an anti-F4/80 antibody revealed prominent macrophage infiltration in both cortex and OSOM of wild-type mice, where from day 3 onwards the number of infiltrating macrophages was significantly increased in comparison with right kidney control values (Fig. 4).

In contrast, OPN knockout mice showed significantly reduced macrophage infiltration after acute renal ischemia. Macrophage infiltration was significantly less in the OSOM of OPN KO mice on days 5 and 7 after reperfusion (Fig. 4B, and photomicrographs in Fig. 7 A–D). Al-

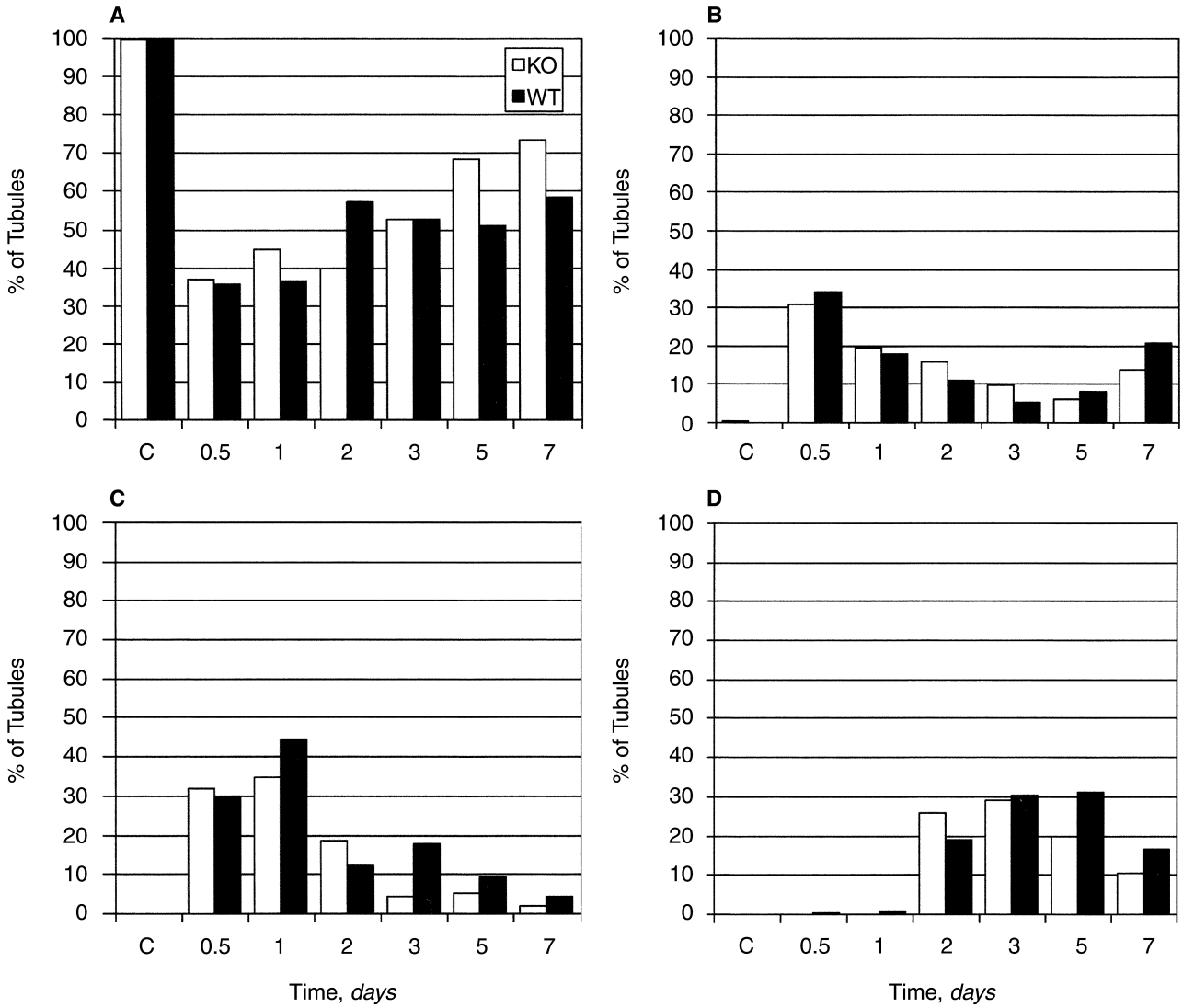


Fig. 3. Acute renal ischemia caused a comparable degree of morphological injury in the OSOM of OPN knockout and wild-type animals. Morphological injury was evaluated on PAS-PCNA staining. Forty proximal tubules were scored as follows: morphologically normal (*A*), sublethally injured (*B*), showing necrosis/cell loss (*C*), or showing signs of regeneration (*D*). The percentage of normal tubules dropped drastically, toward a minimum at 12 hours after reperfusion. Part of the damaged tubules only showed sublethal injury to the brush border (maximal 12 h after reperfusion), whereas others lost their epithelial continuity due to necrosis or detachment of tubular cells (already important after 12 h but only reaching its peak 24 h after reperfusion). After two days regenerating tubules started appearing, which coincided with the gradual increase in the percentage of normal tubules. Over the entire study period, no statistically significant differences (Pearson's χ^2 test) were observed between OPN KO mice and their WT counterparts reflecting a comparable severity of injury as well as a similar speed in regeneration in both genotypes.

though macrophage influx in the cortex was much more limited, the cortex of OPN KO mice also showed significantly less F4/80 staining in comparison with wild-type animals (Fig. 4A). These findings confirm a role for OPN in recruitment of inflammatory macrophages to the interstitium of the postischemic kidney.

Apoptosis

Apoptosis was evaluated in tubular epithelium and interstitium of both cortex and OSOM. Apoptosis was found predominantly early in the injury phase, peaking at 12 hours after reperfusion. In the OSOM, no significant differences in the number of apoptotic cells were found between both genotypes (Fig. 5B), whereas in the cortex (Fig. 5A) OPN knockout mice showed significantly more apoptosis in both tubular epithelium (after 12 hours and after 2 days) and in the interstitium (12 hours after reperfusion).

Collagen I and IV immunostaining

To evaluate the possible influence of OPN on the development of tubulointerstitial fibrosis after an acute ischemic insult to the kidney, we investigated the presence of collagen types I and IV on days 5 and 7 after reperfusion.

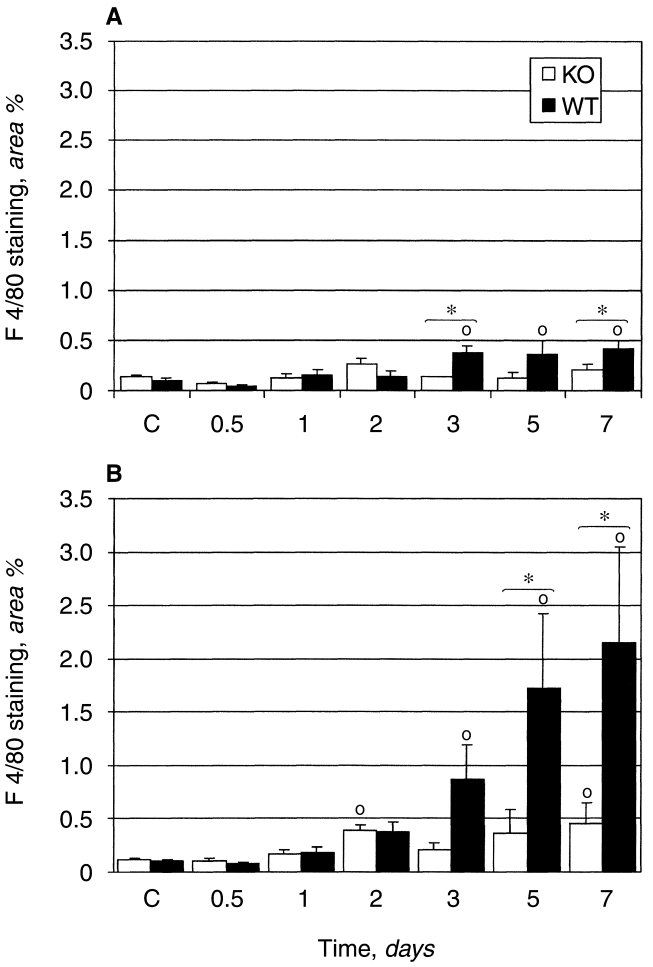


Fig. 4. OPN knockout mice showed significantly less macrophage infiltration in the postischemic kidney. Macrophage infiltration was quantified with a computerized image analysis system on sections stained for the murine monocyte/macrophage marker F 4/80. (*A*) Cortex. Macrophage infiltration was significantly increased in postischemic kidneys from wild-type animals from day 3 onwards, whereas no significant macrophage influx was seen in the renal cortex of OPN KO animals. (*B*) OSOM. In normal mice, ischemic injury caused a prominent infiltration of macrophages in the renal interstitium of the OSOM. In contrast, the OSOM of OPN knockouts showed significantly less macrophage staining from day 3 onwards, indicating a role for OPN in the recruitment of macrophages to the postischemic kidney. $*P < 0.05$ KO vs. WT; $\circ P < 0.05$ vs. control.

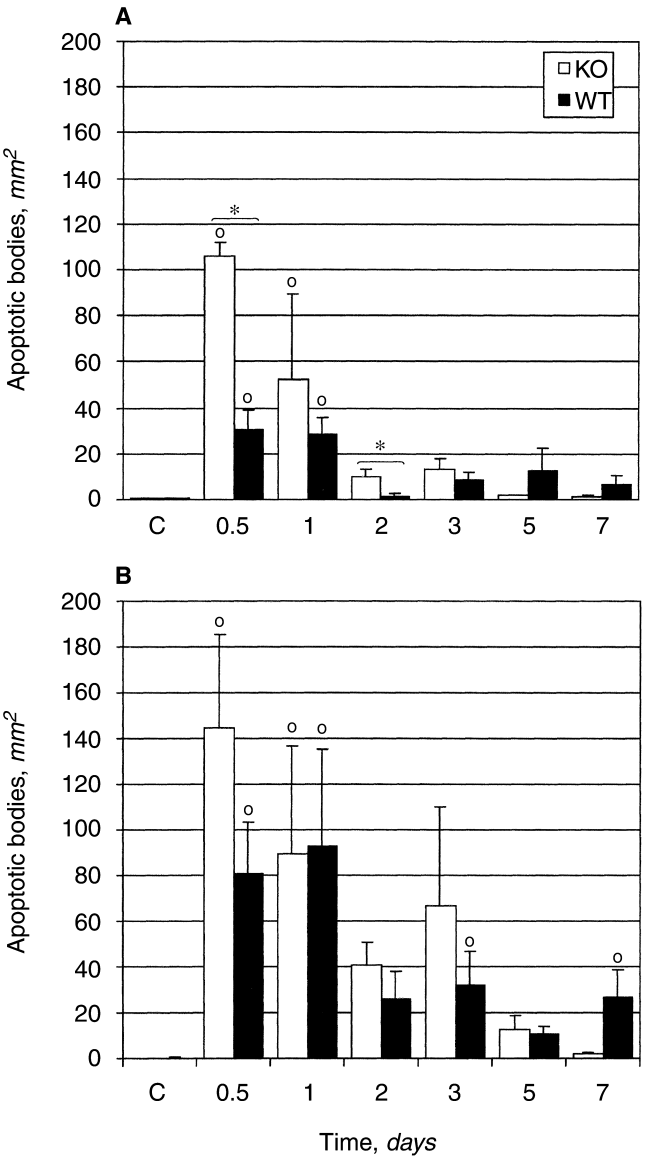


Fig. 5. Less apoptosis was found in the cortex of OPN KO mice during the injury phase of postischemic acute renal failure. (*A*) Cortex. Apoptotic bodies were mainly found very early after ischemia reperfusion, reaching a maximum already 12 hours after reperfusion. OPN knockouts showed significantly more apoptosis after 12 hours and 2 days when compared to wild-types. (*B*) OSOM. No significant differences in apoptosis between OPN KO and WT mice were found in OSOM, although more apoptosis was observed in the OSOM than in the cortex. $*P < 0.05$ KO vs. WT; $\circ P < 0.05$ vs. control.

A prominent up-regulation of collagen I staining was found in OPN WT mice in cortex and even more so in OSOM, which was nearly abrogated in the cortex and significantly diminished in the OSOM of OPN knockouts (Figs. 6A and 7 E–H).

Collagen IV deposition was found to be significantly increased in postischemic kidneys of WT mice ($P = 0.025$ and $P = 0.016$ in cortex and OSOM, respectively). In contrast, OPN KO mice showed complete abrogation of this increase in collagen IV expression after acute ischemic injury ($P = 0.655$ and $P = 0.565$ for postischemic vs. control values in cortex and OSOM, respectively; Fig. 6B).

These results indicate that OPN knockout mice are less prone to develop tubulointerstitial fibrosis after acute renal injury.

DISCUSSION

In this study the role of OPN in acute ischemia/reperfusion injury and subsequent regeneration was investigated. OPN KO mice and WT controls were subjected

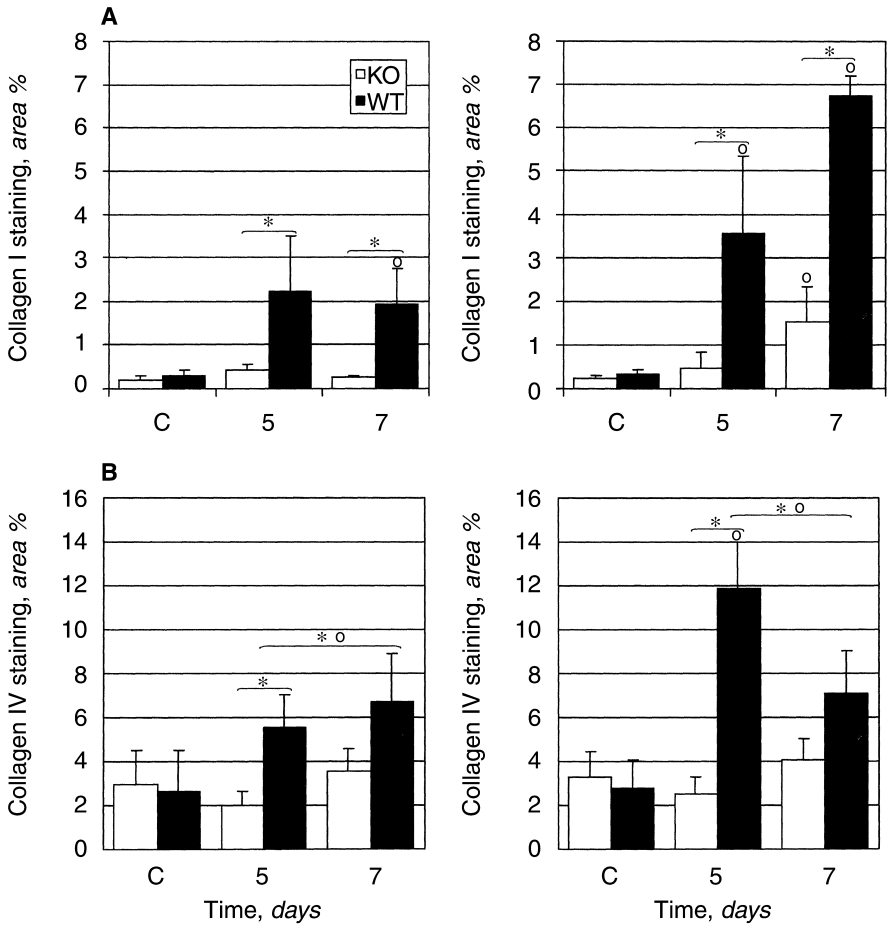


Fig. 6. OPN knockout mice showed less deposition of collagen types I and IV in the postischemic kidney. (*A*) Collagen I staining. Quantification of collagen I deposition was performed with a digital image analysis system on sections of days 5 and 7 after reperfusion stained with a polyclonal antibody against murine collagen type I. OPN wild-type mice already displayed a dramatic increase in collagen I immunostaining in the regeneration phase of postischemic acute renal failure, whereas in OPN knockout mice this collagen up-regulation was nearly completely abrogated in the renal cortex (no significant difference in collagen I staining between postischemic kidneys 5 to 7 days after reperfusion and control right kidneys) and significantly diminished in the OSOM. (*B*) Collagen IV staining. Sections immunohistochemically stained with a polyclonal antibody against murine collagen type IV were analyzed with a computerized image analysis system. In OPN knockout mice the collagen IV staining was not up-regulated 5 to 7 days after ischemic renal injury, whereas OPN wild-type mice showed a significant up-regulation of collagen IV immunostaining in both cortex and OSOM, in comparison to both OPN knockout and control kidneys. These findings indicate a profibrotic role for OPN after acute I/R injury. $^{*}P < 0.05$ KO vs. WT; $^{\circ}P < 0.05$ vs. control.

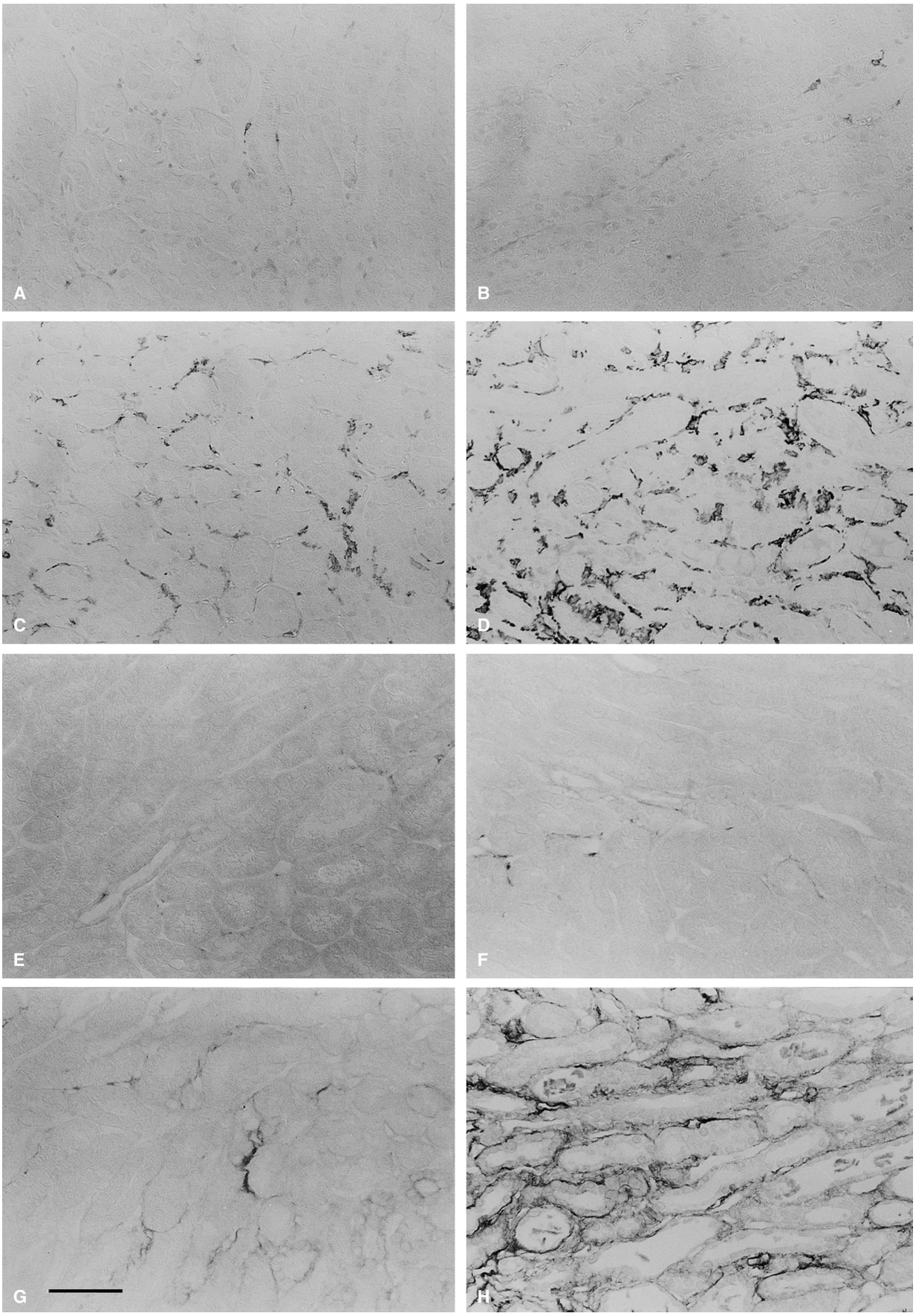
to 30 minutes of warm renal ischemia combined with contralateral nephrectomy, a model designed to create moderate postischemic renal failure with limited animal mortality [42].

In OPN knockout mice the severity and course of postischemic renal injury and subsequent regeneration was completely parallel to that of wild-type controls, functionally and morphologically. These results suggest that OPN protein does not play a functional nor morphological role of importance in the course of postischemic ARF in mice.

These findings are in contrast with the results obtained by Noiri et al [41], who found significantly higher serum creatinine levels and more prominent morphological injury in OPN KO mice than in WT controls 24 hours after a 30-minute bilateral renal ischemia. A different ischemia model, along with a different OPN KO construct [45], against another genetic background (129 × C57Bl/6 F2 strain) possibly may account for this discrepancy. The occurrence of redundancy in our animals is not a likely explanation, in view of the marked differences found in macrophage infiltration and collagen immunostaining.

Through interaction with integrins, which are redistributed over the entire cell surface in injured cells [46], OPN could influence tubular cell attachment possibly limiting cell desquamation and intratubular cast formation. This is supported by experiments showing that rats suffered less severe acute ischemic injury and showed less cast formation after administration of RGD-peptides, which act as OPN-analogs [47]. On the other hand, after acute I/R injury the kidney is massively infiltrated by leukocytes, mainly macrophages and T cells, and several experimental approaches preventing leukocyte infiltration also conferred functional protection against ischemic injury [42, 48, 49]. In this study, immunohistochemical staining for the murine macrophage marker F4/80 revealed significantly limited macrophage infiltration in postischemic kidneys of OPN KO mice. These findings confirm the well documented role of OPN protein in the chemoattraction of macrophages [22–25]. Thus, OPN is one of the factors responsible for the recruitment of a macrophage-rich leukocyte infiltrate into the interstitium of the postischemic kidney

Moreover, OPN knockouts also showed significantly less collagen staining five to seven days after reperfusion,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demonstrating the contribution of OPN to the development of renal fibrosis after an acute insult to the kidney. These findings are unlikely to be solely attributable to altered wound healing, as Liaw et al showed less organized deposition of somewhat smaller collagen fibrils in OPN knockout mice after skin incisional wounding, but the overall wound healing capacity of OPN knockouts was found to be intact and wound tensile strength was unchanged [7].

The mechanism by which OPN contributes to the development of tubulointerstitial fibrosis remains to be elucidated. OPN could either directly stimulate fibrosis, induce profibrotic mediators or exert its fibrogenic effect through chemoattraction of macrophages. A growing body of evidence links the persistence of a macrophage infiltrate with the development of tubulointerstitial fibrosis and the progression toward chronic renal failure [50]: since macrophages have been shown to produce matrix proteins (collagen I among others) [51], protease inhibitors (such as, TIMP-1) [52], and profibrogenic cytokines like transforming growth factor-β (TGF-β) [53].

Our results fit in with those of Ophascharoensuk et al, who found less macrophage infiltration along with diminished collagen expression and increased apoptosis in OPN KO mice in the early phase after unilateral ureteral obstruction [23]. In our experiment, the increased apoptosis in OPN knockouts was only significant early after reperfusion (12 hours) and in the cortex, which may be related to the extent of injury being much more pronounced in the OSOM, preferentially causing necrosis of heavily injured tubular cells, although the relative contributions of apoptosis and necrosis to ischemic acute renal failure are still unclear [54]. By inhibiting apoptosis, OPN could act as a survival factor for tubular cells, possibly through interaction with the $\alpha_v\beta_3$ integrin, which has been shown to mediate cell survival through the nuclear factor-κB (NF-κB) pathway in endothelial cells [40].

Since no functional or morphological differences were observed between OPN knockout mice and their wild-type counterparts, we have an excellent model to study the effect of the macrophage infiltrate on the development of renal fibrosis after an acute insult to the kidney. It is well known that tubulointerstitial fibrosis is the most important parameter determining the long-term outcome of various forms of renal disease [55, 56], and as described earlier, tubulointerstitial inflammation and the persistence of a leukocyte infiltrate in the renal interstitium may lead to fibrosis.

In this study, we demonstrated that OPN knockout mice are less prone to develop renal fibrosis after an acute ischemic insult, indicating a profibrotic role for OPN in renal pathophysiology. This was supported by the presence of fewer macrophages and less collagen deposition in OPN KO mice after 12 weeks of chronic renal failure (Persy et al, *www.TheScientificWorldJournal.com* 2002, pp 177–179; abstract, 3rd ICORP Conference, San Antonio, TX, 9–12 May 2002).

Osteopontin KO and wild-type mice were subjected to ischemia/reperfusion injury in a single kidney model, which mimics the transplant setting. The results of this study can be relevant for the field of renal transplantation, as the enhancing effect of OPN on renal fibrosis indicates a possible role for OPN in the development of chronic allograft failure. Second, OPN may contribute to interstitial inflammation in the graft. Further, in a recently published paper Mazzali et al demonstrated that OPN KO mice were partially protected against cyclosporine nephrotoxicity: mice lacking OPN showed lower mortality, less arteriolopathy, less macrophage infiltration and less interstitial collagen deposition in response to cyclosporine administration [57].

In summary, OPN knockouts showed no significant difference in severity or course of postischemic ARF, either functionally or morphologically, when compared to their wild-type counterparts. However, macrophage infiltration and tubulointerstitial fibrosis were significantly reduced in the absence of OPN protein, confirming the role of OPN in macrophage recruitment and indicating a role in the development of tubulointerstitial fibrosis.

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Fig. 7. Photomicrographs. (*A–D*) Mice lacking OPN showed significantly less macrophage infiltration after acute renal ischemia. F4/80 immunostaining showed that in control mice, the presence of macrophages in kidney interstitium was rare, for knockouts (A) as well as for wild-types (B). After I/R injury, F4/80 staining was much less prominent in OPN knockouts than in wild-types, confirming the role of OPN in macrophage recruitment. (C) The OSOM of OPN KO mice showed only a slight increase in F4/80 staining on day 7 after reperfusion. (D) In contrast, the OSOM of OPN WT mice showed a prominent macrophage infiltration 7 days after the ischemic insult. (*E–H*) Ischemic injury induced significantly less collagen I deposition in kidneys of OPN KO mice. Collagen I immunostaining showed very limited presence of collagen I in in the OSOM of non-ischemic control kidneys, of both KO (E) and WT (F) mice. After acute ischemic injury, there was very little increase in collagen I immunostaining in OPN KO mice, whereas OPN WT mice showed a spectacular up-regulation of collagen I. (G) The OSOM of OPN KO mice showed only a slight increase in collagen I deposition 7 days after reperfusion. (H) In the OSOM of OPN WT mice a strong increase in collagen I deposition was in the renal interstitium was seen 7 days after the ischemic insult (Original magnification ×400. Bar represents 50 μm).

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