Kidney International, Vol. 60 (2001), pp. 635–640

Osteopontin expression in human cyclosporine toxicity

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Background. Osteopontin is a secreted phosphoprotein that has a number of diverse biological functions, including cell signaling, mediation of cell adhesion, migration, and chemoattraction of monocytes/macrophages. Up-regulation of osteopontin expression by proximal tubular epithelium has been demonstrated in both human and rodent models of renal injury in association with macrophage influx.

Methods. We studied the expression of osteopontin protein and mRNA in renal donor biopsies ($N = 7$) and renal transplant biopsies with cyclosporine A toxicity ($N = 23$) by immunohistochemistry and in situ hybridization. Serial tissue sections were immunostained with a monocyte/macrophage marker, CD68, to demonstrate the pattern of macrophage infiltration.

Results. Strong osteopontin expression was observed in the majority of pretransplant donor biopsies in the absence of any macrophage infiltration. In the biopsies with cyclosporine toxicity, osteopontin expression was widespread and demonstrated moderate immunohistochemical signal intensity that did not correlate with the number of interstitial macrophages present.

Conclusions. Strong osteopontin protein and mRNA expression by tubular epithelium was observed in pretransplant donor biopsies and in biopsies with cyclosporine toxicity without an inflammatory cell infiltration. Therefore, osteopontin expression alone is insufficient to serve as the principal mediator of intrarenal monocyte/macrophage influx in the transplant setting.

The use of immunosuppressive medications, including cyclosporine, has revolutionized solid organ transplantation. Survival rates for renal grafts have steadily improved over the past decade [1]. However, soon after its use was established, the adverse effects of chronic cyclosporine use became apparent [2, 3]. Renal cyclosporine toxicity is a lesion that can be characterized by afferent arteriolopathy, but that generally lacks additional specific histologic features or prominent interstitial infiltration by inflammatory cells.

Osteopontin is a secreted phosphoprotein that has a number of diverse biological functions involving cell adhesion, migration, and signaling [4–7]. Originally isolated from bone, osteopontin expression has been demonstrated in a number of different tissues, including kidney, lung, liver, bladder, pancreas, and breast [8]. Osteopontin is both expressed by and chemotactic for vascular smooth muscle cells and monocyte/macrophages in vitro and in vivo [5, 9–16]. In rodent and human kidney, osteopontin is constitutively expressed by distal tubular epithelium [8, 17–19]. Up-regulation of osteopontin expression by proximal tubular epithelial cells has been described in a number of rodent models of renal injury in association with monocyte/macrophage infiltrates [15, 20–25] and recently in a rat model of ischemia/reperfusion [26]. These studies have suggested that osteopontin is likely to be important in mediating mononuclear leukocyte accumulation and localization in renal injuries.

We previously demonstrated a correlation between up-regulated osteopontin expression in proximal tubular epithelium and the number of CD68-positive macrophages present in the tubulointerstitium of the mature human kidney [17]. In addition to being chemotactic for macrophages, osteopontin has been shown to be expressed by macrophages in some settings, including crescentic glomerulonephritis [11, 27]. These chemotactic and trophic features of osteopontin, in addition to its demonstrated up-regulation in the tubulointerstitium in a variety of rodent models of renal injury, suggest that osteopontin might be an important mediator of the macrophage influx. In this study, we examined donor biopsies and transplant biopsies with cyclosporine toxicity, both of which demonstrate tubular damage but no macrophage influx.

METHODS

Tissue

Core needle biopsies were obtained from the University of Washington Medical Center (Seattle, WA, USA). The morphological diagnosis of cyclosporine toxicity was confirmed by the clinical responses of patients to therapeutic interventions (that is, lowering the cyclosporine dose without increasing the dosage of other immunosup-

Key words: transplantation, macrophages, renal injury, renal biopsy, immunosuppression, nephrotoxicity.

Received for publication December 7, 2000
and in revised form February 16, 2001
Accepted for publication March 3, 2001

Table 1. Donor biopsies

Case	% OP	OP score	CD68/HPF
1	26.1	0	3.4
2	49.9	1	9.5
3	8.8	1	5.9
4	91.9	2	6.8
5	66.7	2	10.3
6	80.7	3	15.2
7	94.0	3	8.1
Average	59.7	1.7	8.5

Abbreviations are: OP, osteopontin; HPF, high-power field.

pressive agents). The study included biopsies of donor kidneys obtained immediately prior to transplantation ($N = 7$) and biopsies with cyclosporine toxicity ($N = 23$). Most of the biopsies included a portion of tissue fixed in 10% neutral-buffered formalin and another portion fixed in methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% acetic acid). A subset of the cases with cyclosporine toxicity was available fixed only in 10% neutral-buffered formalin ($N = 11$). All fixed tissues were processed and embedded in paraffin according to standard protocols.

Antibodies

LF7 is a rabbit polyclonal antibody directed against the intact osteopontin (bone sialoprotein I) protein molecule isolated from bone [28].

PGM1 (Dako, Carpenteria, CA, USA) is a well-characterized murine monoclonal antibody directed against the CD68 epitope present on human monocytes and macrophages [29]. The specificity and use of these antibodies has been previously described [17, 27].

Immunohistochemistry

Immunohistochemistry was performed using a standard avidin-biotin peroxidase protocol as previously described [17, 27]. Double-label immunohistochemistry was also performed on the methyl Carnoy's fixed sections. First, slides were immunostained using LF7 and 3,3′-diaminobenzidine to visualize osteopontin with a brown reaction product. Following a phosphate-buffered saline (PBS) rinse and further block with 3% hydrogen peroxide, the slides were incubated sequentially with anti-CD68 overnight at 4°C, horseradish peroxide-conjugated anti-mouse antibody (Dako) and then developed with the Vector VIP substrate kit (Vector, Burlingame, CA, USA) to give a purple color reaction.

In situ hybridization

In situ hybridization was performed using a riboprobe transcribed using human osteopontin cDNA in plasmid pBluescript SK(−) (plasmid OP-10), which was obtained from Dr. Larry Fisher (National Institutes of Health, Bethesda, MD, USA) [30], as previously described [17, 27].

Scoring

Ten random cortical areas were scored by counting the total number of tubular cross-sections in each high-power field (HPF) and the number of tubular cross-sections that demonstrated positive osteopontin expression. One HPF is equivalent to an area of 0.173 mm^2 as determined using a stage micrometer (Olympus, Tokyo, Japan). These data are shown as the percentage of the total tubular cross-sections that were positive for osteopontin. Additionally, the intensity of staining in proximal tubular segments was graded semiquantitatively, with a scale of 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining. CD68-positive monocyte/macrophages were counted in ten equivalent high-power cortical fields and were expressed as the average number of CD68-positive cells present per HPF.

Statistical analysis was performed using the InStat® Program, Version 3.0 for Windows (Intuitive Software for Science, San Diego CA, USA). The nonparametric Spearman rank correlation test was used to determine the correlation between osteopontin score and the number of CD68-positive cells present in the tubulointerstitium. Additionally, the nonparametric Kruskal–Wallis test was used to compare the means of osteopontin scores and the percentage of osteopontin-positive tubules.

RESULTS

All renal biopsies

All renal biopsies in this study ($N = 30$) were scored for both the percentage of osteopontin positive tubules and osteopontin immunostaining intensity, as well as the number of interstitial macrophages. Distal tubules served as an internal control for the osteopontin immunostaining, as they constitutively express osteopontin protein and did so uniformly in this study. In all of the cases studied, the distribution pattern of osteopontin mRNA expression demonstrated by in situ hybridization correlated with the immunostaining pattern observed.

Donor renal biopsies

The donor biopsies used in this study generally had no specific pathologic abnormality with no prominent mononuclear inflammatory cell infiltrate or tubulitis. Two of the biopsies showed mild arteriosclerosis, and one of the biopsies had focal glomerulosclerosis. Osteopontin expression by proximal tubules in the donor control tissues varied widely, from no or little detectable expression ($N = 3$) to widespread strong expression ($N = 4$; Table 1 and Fig. 1). The number of CD68-positive monocyte/macrophages also varied considerably from one biopsy to another, from 3.4 to more than 15 cells per HPF (mean 8.4). The number of donor biopsies available for study was too low to calculate a correlation. As shown in

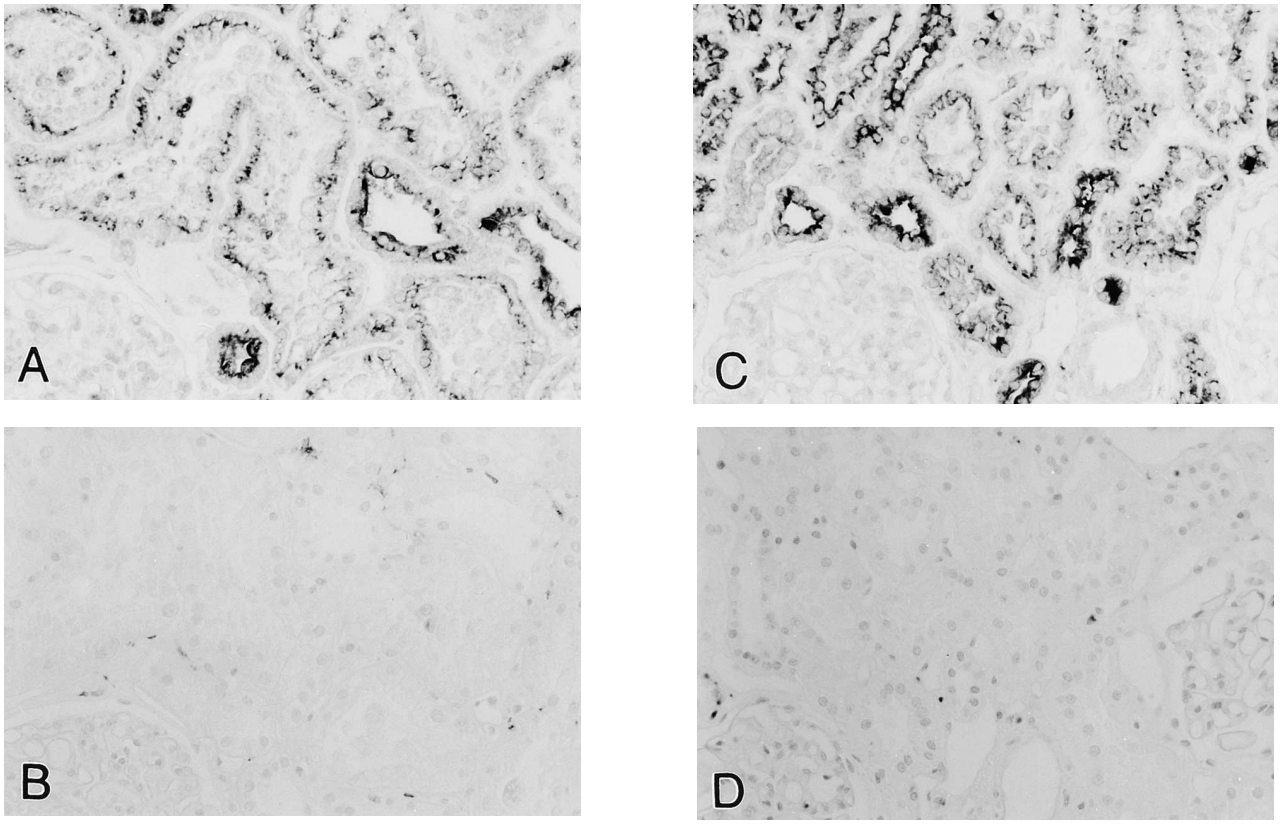

Fig. 1. Osteopontin expression in pretransplant donor biopsies. Replicate tissue sections from two pretransplant donor biopsies immunostained with anti-osteopontin antibody (*A* and *C*) and anti-CD68 antibody (*B* and *D*). Biopsies demonstrate widespread, strong immunohistochemical staining for osteopontin by all tubular epithelium with a small amount of monocyte/macrophage accumulation.

Table 2. Cyclosporing toxicity biopsies

Case	% OP	OP score	CD68/HPF
<5 CD68/HPF			
1	79	1	0.9
2	53.2	1	1.7
3	66	2	2
4	91	2	3
5	57	2	3.5
6	55.6	1	4.5
7	34.8	1	4.6
Average	62.4	1.4	2.9
5–15 CD68/HPF			
8	52.5	1	5.5
9	88.6	2	5.6
10	87	2	5.7
11	56	2	6.9
12	73.4	1	7.1
13	61	1	7.4
14	71	2	7.7
15	97	2	10.7
16	84.5	2	13.3
17	90	2	13.3
18	63	1	13.8
19	90	3	14.9
Average	76.2	1.8	9.3
>15 CD68/HPF			
20	61.6	1	16.3
21	95	1	18.4
22	81.9	2	19.8
23	73	3	46
Average	77.9	1.8	25.1

Biopsies are divided into three groups based on the number of macrophages present per high-power field (HPF). OP is osteopontin.

Figure 1, strong osteopontin protein expression by proximal tubular epithelium was often present in the absence of a prominent macrophage accumulation. This pattern was also seen in the double-label immunohistochemistry (data not shown).

Cyclosporine toxicity

The biopsies with cyclosporine toxicity generally had a small, focal interstitial mononuclear inflammatory cell infiltrate and variable degrees of tubular atrophy and occasionally demonstrated a pattern of striped interstitial fibrosis. All of the biopsies had some degree of arteriolar hyalinosis, predominantly subendothelial.

In the majority of biopsies with cyclosporine toxicity that we examined, osteopontin protein and mRNA expression by proximal tubules was widespread and exhibited low-to-moderate signal intensity (Table 2 and Fig. 2). In contrast to the widespread osteopontin expression, the number of macrophages present in the tubulointerstitium was generally low and was independent of the intensity of osteopontin expression by proximal tubular epithelium, as no significant correlation could be discerned ($r = 0.18$, $P = 0.3920$). A weak but significant correlation was found between the overall percentage of osteopontin-positive tubules and the number of macrophages ($r = 0.45$, $P = 0.0292$). As shown in Table 2, when the biopsies were separated into groups based on the number of interstitial macrophages present (less than 5 per HPF, 5 to

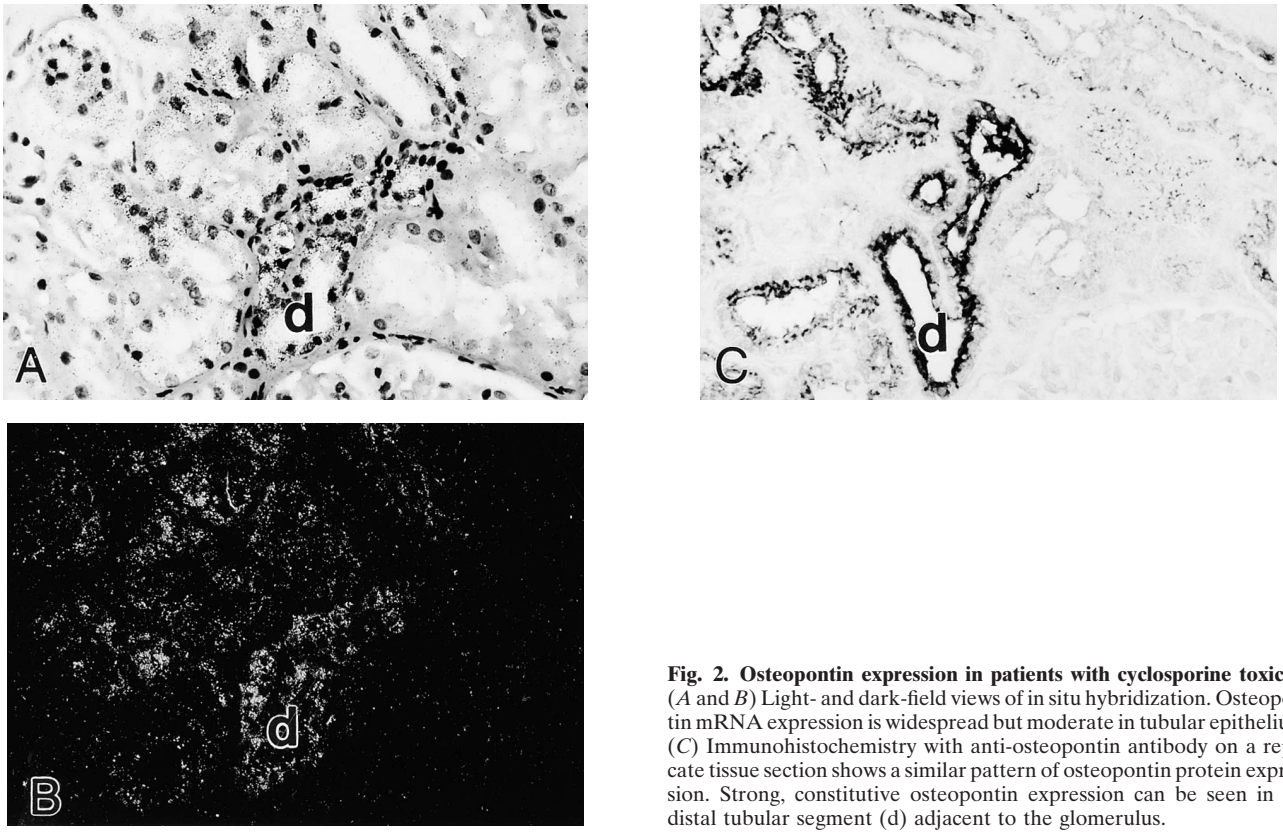

Fig. 2. Osteopontin expression in patients with cyclosporine toxicity. (*A* and *B*) Light- and dark-field views of in situ hybridization. Osteopontin mRNA expression is widespread but moderate in tubular epithelium. (*C*) Immunohistochemistry with anti-osteopontin antibody on a replicate tissue section shows a similar pattern of osteopontin protein expression. Strong, constitutive osteopontin expression can be seen in the distal tubular segment (d) adjacent to the glomerulus.

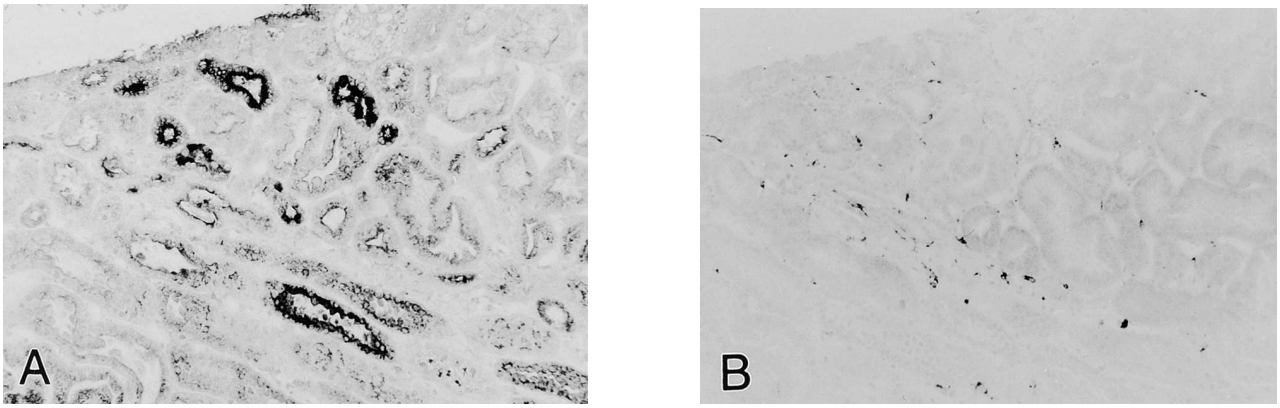

Fig. 3. Focal, strong osteopontin expression in patients with cyclosporine toxicity. Immunohistochemistry on a biopsy with cyclosporine toxicity demonstrates focal up-regulation of osteopontin protein expression by tubular epithelium (*A*) in association with a focal, modest macrophage influx (*B*).

15 per HPF, and greater than 15 per HPF), there was no statistically significant difference in the observed osteopontin expression. Focal, strong expression of osteopontin with corresponding focal accumulations of CD68-positive macrophages was apparent in several of the cyclosporine toxicity biopsies studied (Fig. 3).

DISCUSSION

Osteopontin is a secreted phosphoprotein that has been shown to be chemotactic for macrophages, both in vivo and in vitro [15, 16]. In addition, osteopontin expression by tubular epithelium has been found to be up-regulated in a number of models of renal injury [20–23, 31]. We have previously shown that osteopontin expression by proximal tubular epithelium in histologically normal human kidney tissue is very low to absent and is increased in association with interstitial macrophage infiltration [17]. Studies utilizing osteopontin knockout mice, however, have had conflicting results depending on the injury model studied. In one study, osteopontin

knockout mice were shown to have reduced macrophage influx in a model of obstructive nephropathy [21]. However, Bonvini et al found no difference between osteopontin knockout mice and littermate controls in a model of anti-glomerular basement membrane nephritis [32].

In this study, we examined the expression of osteopontin protein and mRNA in both pretransplant donor biopsies and biopsies with cyclosporine toxicity. These are both conditions with tubular injury but are usually not associated with prominent macrophage influx. In the majority of donor biopsies, there was strong expression of osteopontin protein and mRNA by proximal tubular epithelium, as demonstrated by the intensity of immunohistochemistry and in situ hybridization signal, as well as an increase in the total number of positive tubular segments when compared with normal human kidneys [17]. This increase in osteopontin expression was not associated with macrophage accumulation.

Ischemia is known to induce osteopontin expression in renal proximal tubular epithelium [26, 33]. In a rat model of renal ischemia, it has been shown that distal tubules rapidly increase their osteopontin expression, with a maximal expression at 24-hours postischemia, while proximal tubules show a delayed response, with maximal expression after five to seven days [26]. Noiri et al found that the osteopontin knockout mouse demonstrated a reduced tolerance to renal ischemia and postulated that osteopontin may serve as a protective molecule in this setting [34]. Although we have no data available on the cold ischemia time of the renal grafts included in this study and, therefore, cannot correlate ischemia to the level of osteopontin expression observed, an induction via cold ischemia appears to be likely. This up-regulation would be consistent with a response to ischemia and may be protective for the tubular epithelial cells.

Renal cyclosporine toxicity is a lesion that can be characterized by afferent arteriolopathy, but that generally lacks additional distinctive histologic features or prominent interstitial infiltration by inflammatory cells. In vivo, cyclosporine acts as a vasoconstrictive agent and has been demonstrated in animal models to increase tubular osteopontin expression [23, 35, 36]. In this study, we also found an increased number of osteopontin-positive tubules with widespread, moderate expression of osteopontin protein and mRNA in biopsies with the diagnosis of cyclosporine toxicity. The staining intensity did not correlate with the number of interstitial macrophages present in the biopsies. Occasionally, the biopsies with cyclosporine toxicity demonstrated a pattern of focal strong osteopontin expression in association with very focal macrophage accumulations. This may reflect localized ischemia induced by the vasoconstrictive effects of cyclosporine.

In summary, osteopontin expression was up-regulated in proximal tubules in renal allografts prior to transplantation after a period of cold ischemia and in patients with cyclosporine toxicity. This up-regulation was manifested both by the increased numbers of osteopontin-positive proximal tubules and an increase in the immunohistochemical and in situ hybridization signal for osteopontin peptide and mRNA within positive tubules. Constitutive expression of osteopontin by distal tubules was unaffected by these various conditions. Monocyte influx was not associated with up-regulated osteopontin expression in these settings. In aggregate, our findings indicate that osteopontin expression is a ubiquitous and nonspecific marker of tubular injury and, in some settings, is insufficient to produce macrophage infiltration.

ACKNOWLEDGMENTS

This work was supported by an O'Brien Kidney Research Center of Excellence grant (NIH grant DK47659) and NSF grant EEC9529161.

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