Kidney International, Vol. 45 (1994), pp. 515–524

Osteopontin expression in angiotensin II-induced tubulointerstitial nephritis

CECILIA M. GIACHELLI, RAIMUND PICHLER, DONNA LOMBARDI, DAVID T. DENHARDT, CHARLES E. ALPERS, STEPHEN M. SCHWARTZ, and RICHARD J. JOHNSON

Pathology Department, and the Division of Nephrology, Department of Medicine University of Washington, Seattle, Washington, and Nelson Biological Laboratories, Rutgers University, New Jersey, USA

Osteopontin expression in angiotensin II-induced tubulointerstitial nephritis. Osteopontin is an arginine-glycine-aspartate (RGD) containing secreted phosphoprotein recently shown to stimulate a local macrophage influx when injected subcutaneously in mice. We examined the effect of angiotensin II infusion on renal injury and osteopontin expression in the rat kidney by *in situ* hybridization and immunohistochemistry. Preceding pathologic changes in tubular and interstitial cells, a dramatic increase in renal osteopontin protein and mRNA levels was observed primarily in epithelial cells of the distal tubules, collecting ducts and Bowman's capsule. Although both cortex and medulla showed increased osteopontin levels, the effect was most pronounced in the renal cortex which normally showed very little constitutive osteopontin expression. Interestingly, regions of the kidney expressing high osteopontin levels correlated with sites of monocyte/macrophage accumulation. These observations, coupled with recent findings that osteopontin may be a pro-inflammatory protein, suggests that osteopontin over-expression may facilitate monocyte/macrophage accumulation at the sites of renal tubulointerstitial injury.

In many glomerular and extraglomerular renal diseases the risk for progression to end-stage renal failure depends largely on the severity of the injury to the tubulointerstitial compartment [1, 2]. In several diseases associated with tubulointerstitial injury, the degree of interstitial mononuclear cell infiltration correlates both with the degree of renal dysfunction and the risk for progression [reviewed in 3]. Understanding the pathogenesis of tubulointerstitial injury and fibrosis is therefore of paramount interest.

We have studied a model of angiotensin II (Ang II)-mediated hypertension in rats induced by continuous infusion of Ang II by subcutaneous minipump [4]. In this model, a decrement in renal function occurred and was associated with significant phenotypic and proliferative changes in the various renal cell populations. Most notably, focal tubulointerstitial injury with tubular atrophy and dilation, cast formation, and interstitial fibrosis with type IV collagen deposition developed. These changes were associated with tubular and interstitial cell proliferation, local PDGF B-chain mRNA expression, and an interstitial inflammatory infiltrate composed predominantly of monocyte-macrophages.

A possible role for Ang II in mediating tubulointerstitial injury is supported by the observation that tubulointerstitial disease occurs in models of hypertension [5] and in a model of ureteric obstruction associated with elevated renin [6]. Diamond and Anderson have also reported that the tubulointerstitial injury that occurs in an experimental model of nephrotic syndrome is prevented if angiotensin II generation is blocked by angiotensin I converting enzyme inhibitors [7].

In this paper, we explore a potential mechanism by which angiotensin II and/or hypertension may mediate the macrophage influx that occurs in tubulointerstitial disease. Specifically, a dramatic increase in the expression of osteopontin mRNA and protein occurs focally in cortical renal tubules during the course of angiotensin II infusion. The elevated expression of osteopontin occurred early, and was followed first by a monocyte/macrophage influx and subsequently, tubular damage. The observations that the macrophage localized almost exclusively to sites of tubular osteopontin expression is of interest because osteopontin has previously been shown to bind with high affinity to murine macrophages and to induce a macrophage rich cellular infiltrate upon *in vivo* inoculation in mice [8]. This suggests that the tubulointerstitial damage that accompanies Ang II-induced hypertension may result from an osteopontin-mediated monocyte/macrophage accumulation in the interstitium.

Methods

Experimental protocol

The Ang II infusion protocol has been described previously [4]. The kidney tissues obtained from that study (group 1; $N = 12$) in addition to a separate group of similarly treated rats (group 2, $N = 6$) were used in these studies. Briefly, male Sprague-Dawley rats (Zivic Miller, Allison Park, Pennsylvania, USA) weighing 400 to 550 g (~3 month old) received continuous Ang II infusion (200 ng/min) via subcutaneous osmotic minipumps (Alzet model 2002, ALZA Corp., Palo Alto, California, USA) containing Ang II (Sigma, Chemical Co., St. Louis, Missouri, USA) dissolved in Ringer's lactate [4]. Control rats had subcutaneous osmotic minipumps containing Ringer's lactate only. Rats in group 1 underwent renal biopsy by flank

Received for publication May 28, 1993
and in revised form September 2, 1993
Accepted for publication September 2, 1993

incision under ether anesthesia at seven days and were sacrificed at 14 days. Systolic arterial blood pressures were measured by tail cuff plethysmography in conscious, restrained rats as previously described [9]. Blood pressures were normal through day 3; in group 1 angiotensin II-infused rats blood pressures were 156 ± 14 at 7 days and 172 ± 7 mm Hg at 14 days; respective values in control rats were 124 ± 15 and 138 ± 21 mm Hg as previously reported [4]. Rats in group 2 (control and Ang II-infused, $N = 6$) were treated identically as those in group 1 except that renal biopsies were obtained from three rats in each treatment group four days following infusion, and the remaining three were killed at day 7, and dissected kidney cortexes and medullas were collected for fixation, RNA and protein extract preparation. Blood pressures in group 2 at day 6 were 188 ± 16 mm Hg in angiotensin II-infused rats and 135 ± 10 mm Hg in control rats.

Histology and immunohistochemistry

Methyl Carnoy's-fixed tissues were embedded, processed, and 4 μm sections stained with the periodic acid/Schiff reagent (PAS) with hematoxylin counterstain or with Gomori's trichrome. Additional 4 μm sections were immunostained using an indirect avidin-biotin immunoperoxidase method [4] with either a murine monoclonal antibody to rat osteopontin, MPIIIB10 (1) (obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, and the Department of Biological Sciences, University of Iowa, Iowa City, Iowa, USA under contract NO1-HD-6-2915 from the NICHD), an affinity-purified rabbit polyclonal antibody, 2arC, directed to recombinant mouse osteopontin [10], or a specific murine monoclonal antibody, ED-1 (Bioproducts for Science, Indianapolis, Indiana, USA), to monocyte, macrophage, and dendritic cells [11]. Double immunolabeling of the same tissue sections was performed with the 2arC and ED-1 antibodies in which the 2arC was detected by a biotinylated goat anti-rabbit IgG (Vector), and the ED-1 was detected by immunogold with a gold-labeled goat antimouse IgG (Amersham). Negative controls for the immunostaining consisted of replacing each of the primary antibodies with equivalent concentrations of an irrelevant murine monoclonal or rabbit polyclonal antibody.

In situ hybridization

Osteopontin mRNA transcripts were detected in formalin-fixed tissues from control rats and angiotensin II infused rats by *in situ* hybridization with osteopontin cRNA probes generated from the rat smooth muscle osteopontin cDNA, 2B7 [12]. Both sense and antisense ^{35}S-labeled cRNA probes were prepared as previously described [13]. Hybridizations were performed overnight at 55°C using 300,000 cpm/ml cRNA probe [13].

Northern and Western blot analyses

Kidneys were excised from group 2 control and angiotensin II treated rats and dissected into cortex and medulla. Portions of these tissues from each animal were quick frozen in liquid nitrogen and ground into a fine powder under liquid nitrogen. One sample of tissue powder was processed for RNA preparation by the method of Chomczynski and Sacchi [14]. Northern blot analysis was performed as previously described using 2B7 [12], and an oligomer targeted to rat 28S ribosomal RNA obtained as a gift from Dr. Tom Barrett at the University of Washington. Autoradiographic analysis was carried out using a Scanjet IIp (Hewlett-Packard) scanner/densitometer and ImageQuant software (Molecular Dynamics). Cortical and medullary protein extracts were obtained from a second portion by dissolving the frozen powder in Laemmli buffer [15] containing the proteinase inhibitors PMSF (40 mg/ml), bestatin (4 μg/ml), pepstatin (0.7 μg/ml), EDTA-Na_2 (0.37 μg/ml), aprotinin (2 μg/ml), and leupeptin (0.5 μg/ml). Samples were forced through an 18 g needle and boiled for five minutes, centrifuged for five minutes at 10,000 × g, and supernatants containing osteopontin were recovered. Protein was quantitated by the microBCA protein assay (Biorad). Polyacrylamide gel electrophoresis was performed according to the Laemmli procedure [15]. Proteins were transferred to nitrocellulose filters using a biorad electroblot apparatus. Western blots were performed using MPIIIB10 (1) as the primary antibody and alkaline phosphatase-linked secondary antibody. Purified osteopontin was prepared from 12-day-old rat aortic smooth muscle cell culture supernatants using DEAE-sepharose chromatography followed by barium citrate precipitation as described [16].

Results

Angiotensin II infusion induces tubular interstitial nephritis in rat kidney

We previously showed that infusion of Ang II at levels which produce a moderate level of hypertension in rats induced a substantial, focal tubulointerstitial cell injury [4]. Figure 1 illustrates the progression of this renal injury. After seven days of angiotensin II infusion, some focal areas of mildly dilated tubules were observed, but the majority of the kidney cortex showed very little morphological differences when compared to untreated rat kidney (compare Fig. 1 A and C). Notably, an inflammatory infiltrate consisting predominantly of ED1+ monocyte/macrophages as determined by immunostaining of adjacent sections (not shown) was evident at this time. In contrast, after 14 days of Ang II infusion (Fig. 1B), pronounced tubulointerstitial injury was evident with large areas showing intratubular cast formation, tubular atrophy, and widening of the interstitial areas with early fibrosis. At this time, most pathological changes were visualized in the renal tubular epithelium involving primarily distal tubules and collecting ducts, but with some injury to proximal tubule cells. Rare glomeruli showed features of segmental hyalinosis. Injury was often but not invariably occurring in areas around blood vessels, possibly accounting for the focal nature of the response. An interstitial inflammatory cell infiltrate was also evident in areas of structural damage which by immunostaining with the ED-1 antibody was shown to be primarily monocyte-macrophage rich (not shown). Renal vascular injury was also marked with some arterioles displaying hypercellularity and fibrinoid necrosis. These changes are in striking contrast to the normal morphology observed in kidneys infused for 7 or 14 days with vehicle alone (Fig. 1 C and D, respectively).

Angiotensin II-infused kidneys express elevated osteopontin protein

Tissue sections were stained for osteopontin with MPIIIB10(1), a mouse monoclonal antibody to rat osteopontin.

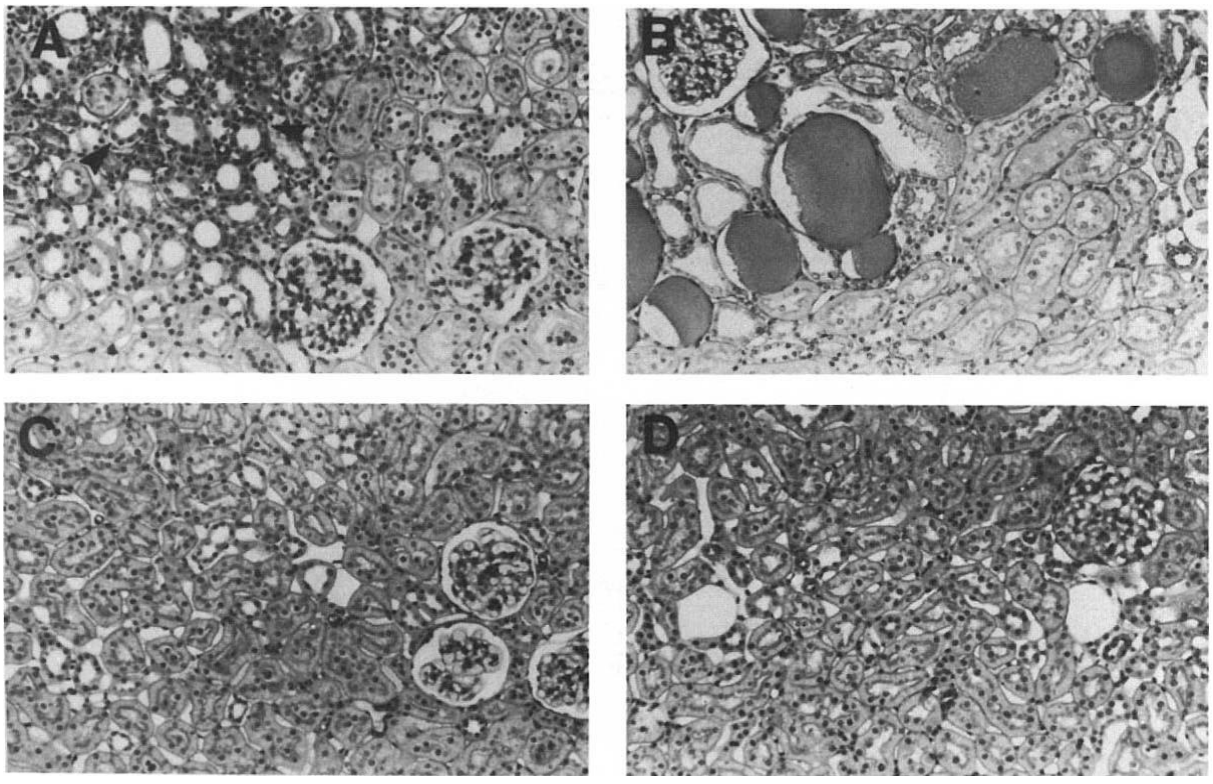


Fig. 1. *Angiotensin II infusion induces focal tubulointerstitial fibrosis.* Seven days after Ang II infusion (**A**) rare areas with mild tubular dilation and infiltration of mononuclear cells were noted in some biopsies (arrows) however, the majority of the kidney cortex showed the normal morphology which was also observed in rats infused with vehicle alone for 7 days (**C**). In contrast, 14 days following angiotensin II infusion (**B**), the cortex displayed dramatic morphological changes including tubular dilatation and atrophy, intratubular cast formation, widening of the interstitial areas, and inflammatory mononuclear cell infiltration. Rats infused with vehicle alone for 14 days showed normal kidney morphology (**D**). Sections were stained with Gamori's trichrome with PAS. Original magnification = 100×.

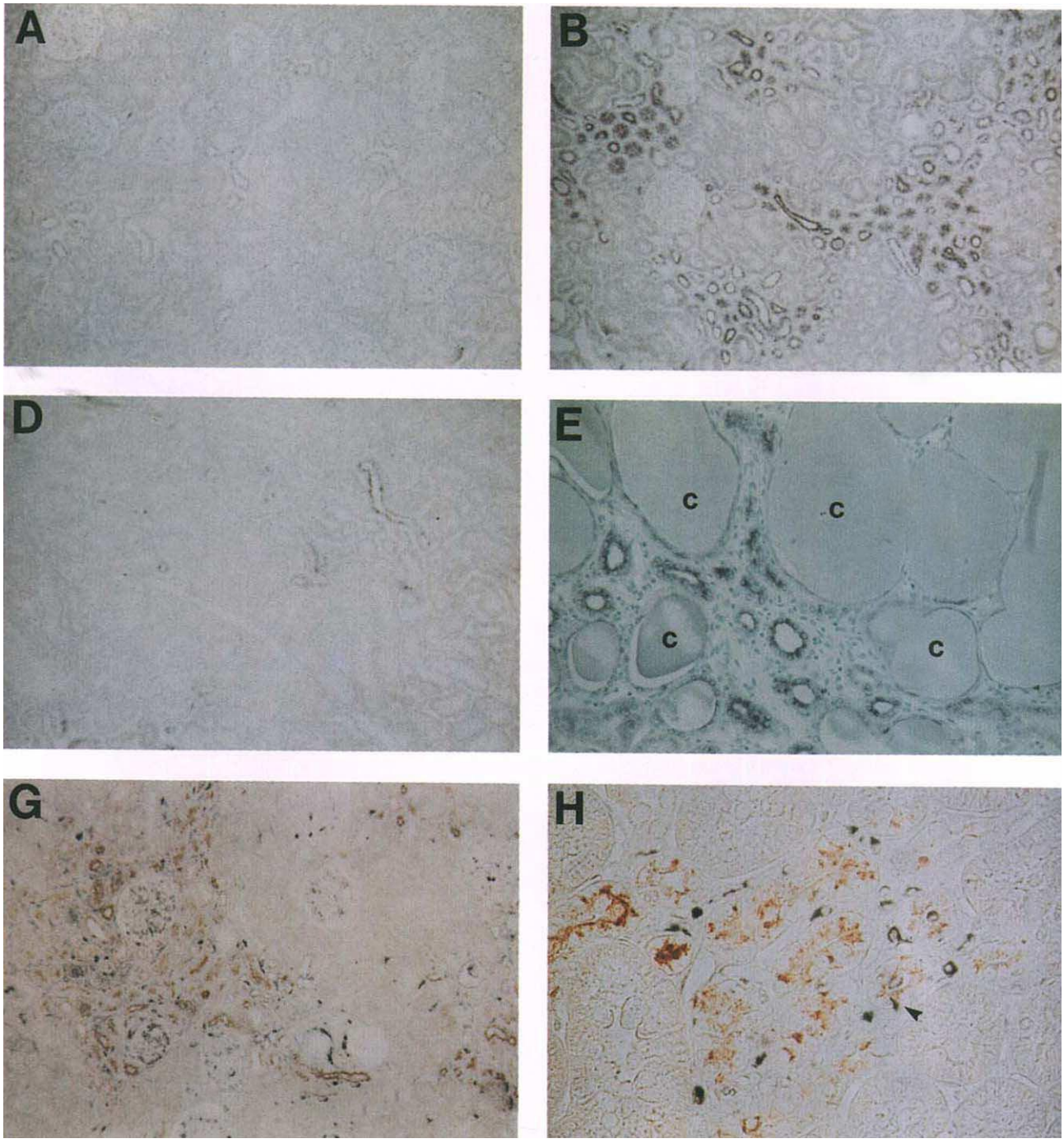
Figure 2A shows MPIIIB10(1) staining of a seven-day vehicle-infused rat renal biopsy. Most cortical structures were negative for osteopontin, although occasional weak staining was observed in some tubular epithelial cells of the distal tubules and parietal epithelial cells of Bowman's capsules and cortices. In the medulla (not shown), a much higher level of staining was observed which was confined to tubular epithelium, collecting ducts and uroepithelial lining cells, in agreement with previous findings [17]. An identical staining pattern was observed in rats infused for 14 days with vehicle alone or in untreated rats, or when immunostaining was performed with the rabbit polyclonal antibody to osteopontin (2arC) (data not shown).

In contrast, seven days after angiotensin II infusion, the level of osteopontin protein was dramatically elevated in tubular epithelium and Bowman's capsule cells (Fig. 2 B and C). The most striking effect was seen in the renal cortical regions of each biopsy which normally expressed very little osteopontin. At this time, very little morphologic evidence of tubular damage was observed by PAS stain as shown in Figure 1A. Thus, elevated osteopontin levels appeared to precede the dramatic structural changes seen in kidney tubules following chronic angiotensin II infusion.

After 14 days of infusion, morphological evidence of tubular damage was pronounced and focal areas of dilated or atrophied tubules with cast formation were numerous. These were invariably surrounded by tubules which were markedly positive for osteopontin (Fig. 2 E). In addition, casts were often lined with cells strongly positive for osteopontin protein. Interestingly, the staining pattern observed in many positive tubular cells both at day 7 and day 14 appeared to be polarized such that most of the signal was detected on the apical side of the cell (Fig. 2 C and F). The significance of this polarized staining is unclear, but might indicate directed secretion of osteopontin into the lumen.

The most dramatic changes in osteopontin staining occurred in the renal cortex which normally expressed very little constitutive osteopontin. No consistent difference in osteopontin protein levels was observed in medullary regions between vehicle- and angiotensin-infused rats (not shown). In both Ang II and vehicle infused rats, osteopontin protein was occasionally seen in the medial layer of renal blood vessels (not shown). No consistent differences were seen in vascular osteopontin staining between these groups.

To validate the specificity of MPIIIB10(1) for rat osteopontin, Western blot analysis was performed. For this experiment, rats from either vehicle- or Ang II-infused groups were sacrificed at seven days. Kidneys were excised from each animal and dissected into cortical and medullary regions. Protein extracts from each were prepared and analyzed by Western blotting. As shown in Figure 3, vehicle-infused renal medullas contained a protein of approximately 66 kD which reacted strongly with



MPIIIB10(1) and had the same mobility as purified smooth muscle cell-derived osteopontin. A second protein at ~50 kD was also weakly detected by this antibody. The identity of this band is unclear, but it might represent a differentially processed form of osteopontin, since osteopontin has been shown to be differentially phosphorylated and proteolytically cleaved in other systems [17]. Rats treated with Ang II for seven days showed similar sized antigenic proteins. As seen in the immunohistochemical analysis, kidney cortices showed even more striking changes in osteopontin protein expression. Very little immunoreactive 66 kD and 50 kD proteins were observed in extracts from vehicle infused renal cortex. Equivalent protein loading was assessed by Coomassie Blue staining of an identically loaded gel (not shown).

Osteopontin mRNA is synthesized by renal epithelial cells, and correlates with osteopontin protein levels

Since osteopontin has been reported to circulate in the blood [18], and has been shown to be expressed by cells of the

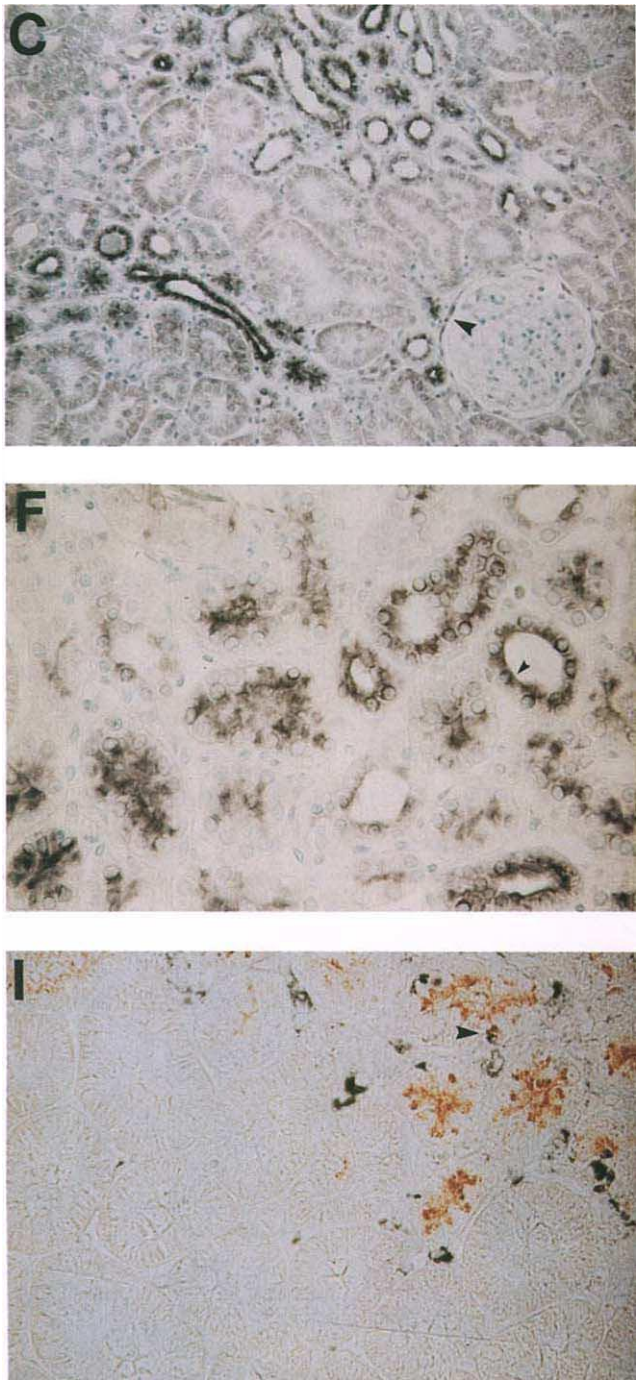


Fig. 2. *Immunolocalization of osteopontin protein and ED-1+ cells in the kidneys of Ang II-infused rats.* MPIIIB10(1) was used to localize osteopontin protein in rat kidneys following infusion with (**A**) vehicle alone for 7 days, (**B**) and (**C**) Ang II for 7 days, (**D**) vehicle alone for 14 days, and (**E**) and (**F**), Ang II for 14 days. Note the focal osteopontin staining pattern, as well as the apical distribution of the protein in many tubules (arrows). (**G**), (**H**), and (**I**), Double immunostaining was performed on kidney biopsies 14 days after Ang II treatment using 2arC (orange) and ED-1 (black) antibodies as described in the **Methods**. Most ED-1+ cells are found in a peritubular arrangement around osteopontin+ tubules, in some cases contacting osteopontin rich regions (arrows). Original magnification for A, B, D, E = 100×, G = 20×, and C, F, H, and I = 200×.

monocytic lineage [19], we used *in situ* hybridization to determine whether the increased osteopontin protein levels were due to increased synthesis of osteopontin by the tubular cells themselves, or whether accumulation at these sites was due to exogenous sources (that is, the infiltrating macrophage, or reabsorption from the tubular fluid). Vehicle infused kidney showed low intensity signal over tubular epithelial and Bowman's capsule cells in the kidney cortex with increased signal over collecting ducts and epithelial lining cells in medullary regions using the antisense osteopontin probe (Fig. 4A). Fourteen days following Ang II infusion, extremely high intensity signal was observed focally in cortical tubular epithelial cells (Fig. 4B), and Bowman's capsules. Identical results were obtained from rats infused for seven days with Ang II (not shown). Counterstaining of *in situ* sections with PAS indicated that the majority of the osteopontin mRNA signal was associated with distal tubules. At no time was specific signal seen in cells of the interstitium, suggesting that the tubular epithelial cells themselves, and not inflammatory cells or interstitial fibroblasts, synthesize the elevated osteopontin seen at this time after injury. Specificity of the *in situ* hybridization was confirmed by probing similar tissue from Ang II-infused or control kidneys with the sense osteopontin cRNA probe in which no silver grains were observed above background (not shown).

Northern blot analysis (Fig. 5) showed that all cortex and medulla samples, regardless of Ang II treatment, contained a single 1.6 kb transcript hybridizing to 2B7. This is the expected size for the rat osteopontin mRNA. In general, osteopontin mRNA levels in untreated rats were higher in rat medulla than in the cortex. In agreement with *in situ* results, Ang II-treated rat cortices had higher levels (an average of 7-fold) of osteopontin mRNA than untreated cortices.

Colocalization of OPN expressing tubular cells with monocyte/macrophage infiltrates

Monocyte/macrophage infiltration is an important component of the tubulointerstitial injury observed in these studies. Utilizing the ED-1 antibody [11], we observed that the monocyte/macrophage infiltrates were present early (readily detected at day 7 of angiotensin infusion; Fig. 2), and were focally distributed in a pattern strikingly similar to that observed for elevated osteopontin protein (Fig. 2 B and E). Thus, we utilized a double staining technique in order to determine whether the osteopontin + and ED-1 + cells were found in the same focal region. For these experiments it was not possible to use the mouse anti-osteopontin monoclonal antibody, since ED-1 is also a mouse monoclonal antibody of the same isotype, and we therefore utilized a rabbit polyclonal anti-mouse osteopontin antibody, 2arC. This antibody has been shown to be specific for recombinant mouse osteopontin [10], and gave results identical to MPIIIB10(1) when used for single immunostaining of biopsy specimens of vehicle- and angiotensin-infused rat kidneys (not shown). The results of double immunostaining using 2arC and ED1 are shown in Figure 2 G, H and I. ED-1 + monocytes/macrophages were found almost exclusively in regions in which tubules were expressing osteopontin. The ED-1 + cells were found most often in a peritubular distribution (Fig. 2 H and I). Occasionally, however, ED-1 + cells were found between

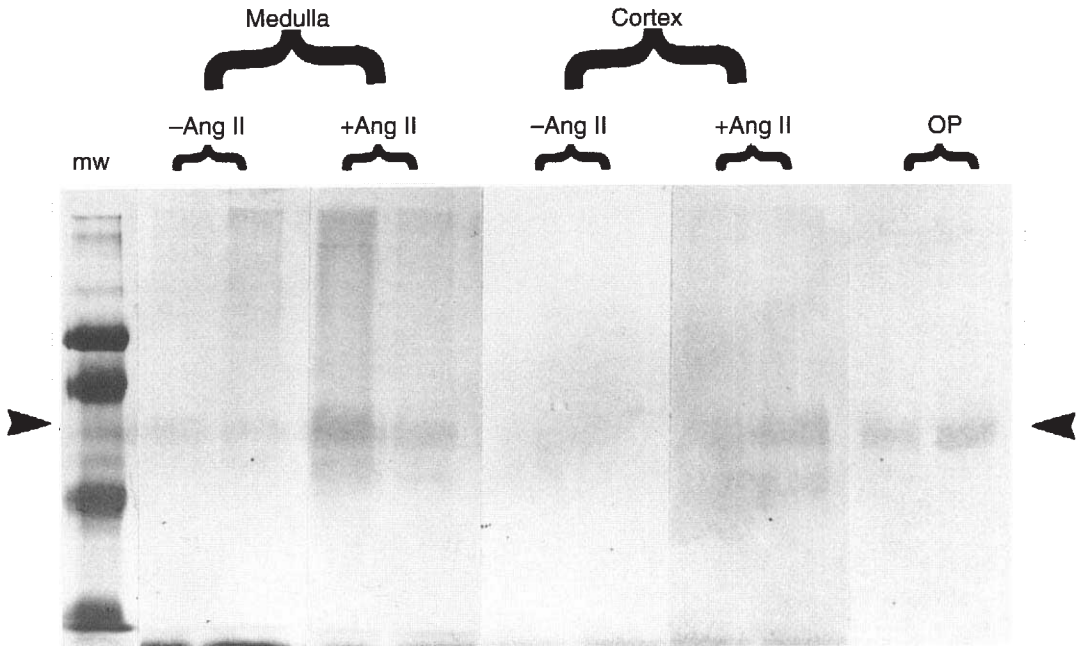


Fig. 3. *Western blot analyses of osteopontin protein in kidney cortices and medullas.* Rats were either infused with vehicle alone (-Ang II) or angiotensin II (+Ang II) for 7 or 14 days and protein extracts were prepared from kidney medullas (med) or cortices (cor) and processed for Western blot analyses using the MPIIIBI0(1) antibody. The results obtained from each of two representative animals is shown in comparison to results obtained with purified osteopontin (OP). The migration of molecular size markers (mw) is also shown. Arrows indicate the position of migration of 66 kD osteopontin.

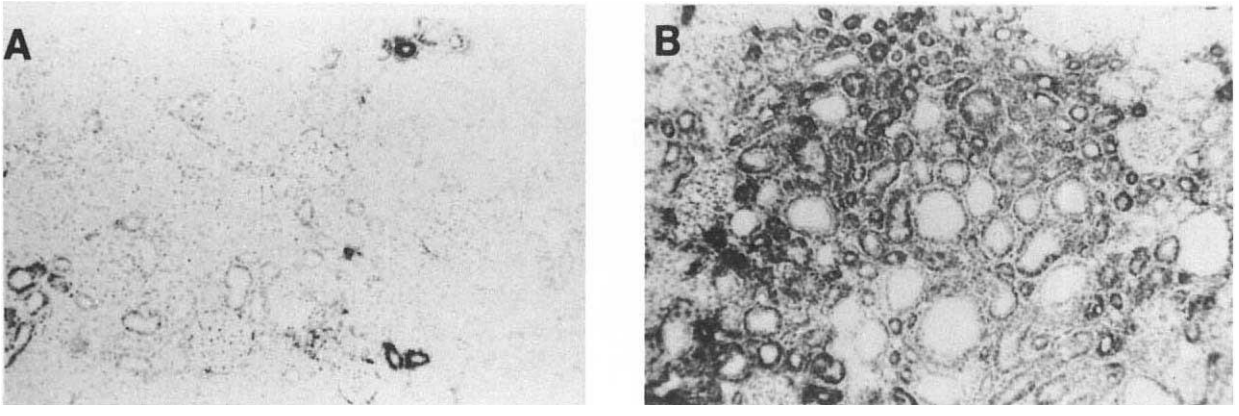


Fig. 4. *In situ hybridization for osteopontin mRNA in Ang II infused rat kidneys.* Rats were infused for 14 days with vehicle alone (**A**) or angiotensin II (**B**), and *in situ* hybridization was performed using a ^{35}S-labeled-2B7 riboprobe. Photomicrographs of the resulting hybridization signals were obtained at 200× magnification on the same field using bright field illumination. Original magnification = 200×.

osteopontin positive tubular epithelial cells or within the tubular lumen (Fig. 2 H, arrows). No staining was observed when normal mouse IgG and normal rabbit IgG were used to replace the primary antibodies in these double staining experiments (not shown).

Elevated osteopontin protein precedes ED1 + monocyte/macrophage accumulation

After four days of Ang II infusion, elevated osteopontin protein was readily detectable in focal tubules throughout the kidney cortex, Fig. 6A). At this time, there was no detectable accumulation of ED-1 + cells around the osteopontin + structures, as shown by staining of an adjacent section with ED1 (Fig. 6B).

Discussion

We have investigated the effect of angiotensin II infusion on osteopontin expression in the rat kidney using RNA hybridization and immunochemical methods. We have shown that osteopontin is constitutively expressed in rat kidney, at high levels in tubular and uroepithelial cells of the medulla, but at a much lower level in distal tubules and Bowman's capsules in the

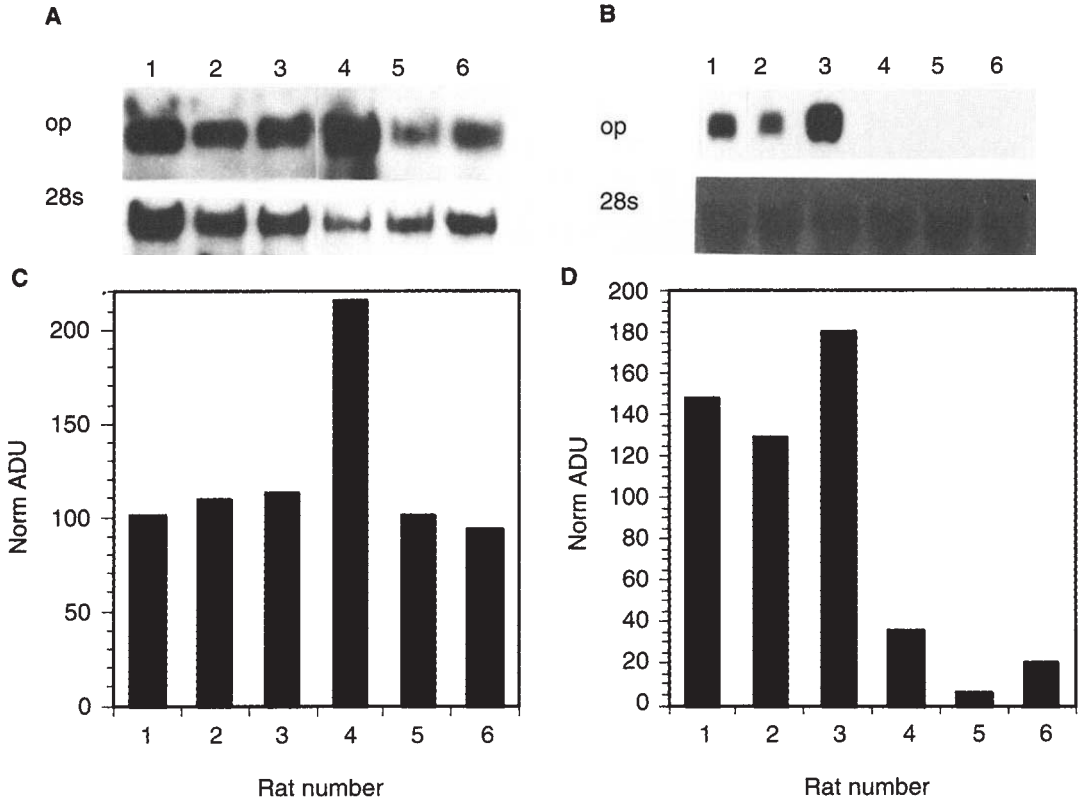


Fig. 5. *Northern blot analysis of osteopontin mRNA levels in kidney cortices and medullas.* Total RNA was extracted from portions of tissue samples prepared as described in Figure 3, and subjected to Northern blot analysis using the 2B7 osteopontin cDNA probe (OP), followed by the 28S ribosomal RNA oligomer probe (28S) for (**A**) or photography of the 28S rRNA band visualized by UV shadowing of the Northern membrane (**B**). The autoradiograms obtained from these analyses on rat medullas (A) and cortices (**B**) are shown. Rats 1, 2, and 3 were treated with Ang II for 7 days while rats 4, 5, and 6 received vehicle alone. Exposure times were 16 hours for OP and 2 hours for 28S. The original autoradiogram showed faint bands for OP mRNA in (B) lanes 4, 5, and 6, but these did not reproduce well. The graph below the autoradiograms shows the results represented as the OP/28S ratio in arbitrary units and were obtained by densitometric analysis of the blots as described in the **Methods**.

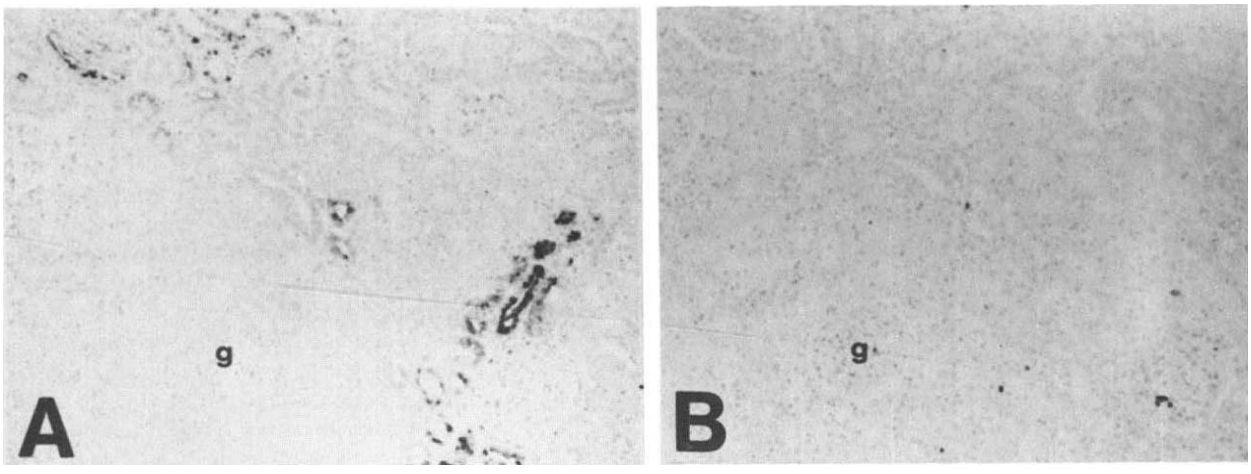


Fig. 6. *Induction of osteopontin protein precedes ED-1+ cell accumulation.* Immunolocalization of osteopontin (**A**) or ED-1+ monocytes/macrophages (**B**) 4 days following Ang II infusion. Sections were counterstained with hemotoxylin. The letter "g" indicates the position of the same glomerulus in the adjacent sections of panel A and B. Original magnification = 100×.

cortex. These data are in good agreement with previous observations of normal rat kidney osteopontin distribution [17] and recent findings in mouse kidney [20]. After 7 or 14 days of Ang II infusion, however, the level of osteopontin expression in the kidney cortex was dramatically elevated. The Ang II treatment protocol used in our study also produced focal, tubulointerstitial injury characterized by a monocyte/macrophage rich inflammatory infiltrate, tubular atrophy and dilation, cast formation, and fibrosis. The striking increase in osteopontin protein and mRNA preceded histological evidence of severe tubular

damage and was focal in nature, suggesting that elevated cortical osteopontin may be an early marker for tubular damage, and might also have a role in the tubulointerstitial disease produced by Ang II. The colocalization of osteopontin+ tubules with ED-1+ inflammatory cells suggests that this role might be to facilitate monocyte/macrophage accumulation at sites of renal damage.

Osteopontin is a highly acidic, secreted glycoprotein, also known as uropontin, eta-1, secreted phosphoprotein I, and bone sialoprotein I [reviewed in 21]. It was originally isolated as a matrix molecule in bone [22], but has since been found to be secreted by many tumor and transformed cells [10, 18] as well as normal cells of the kidney, immune system, placenta and decidua, brain, bone marrow, inner ear, and blood vessels [12, 17, 22–24]. Although the exact function of osteopontin is unknown, clues regarding its role in these various tissues have been obtained from primary sequence analysis and *in vitro* studies. Osteopontin contains two highly acidic domains as well as phosphorylated amino acid residues which have been proposed to be involved in divalent cation binding [21]. Most interestingly, osteopontin contains a canonical arginine-glycine-aspartate (RGD) cell adhesion sequence, and purified osteopontin has been shown to facilitate adhesion of cultured fibroblasts and osteosarcoma cells [25, 26] and most recently, smooth muscle cells (unpublished observation).

It is intriguing to speculate that the elevated osteopontin levels seen in the kidney after Ang II infusion in our experiments might be due to a direct effect of Ang II on cortical epithelial cells. Ang II binding sites have been measured on proximal convoluted tubules, pars recta, medullary collecting tubules and distal tubules [27]. Furthermore, we have found that Ang II, at a concentration of 10 μM was a potent direct inducer of osteopontin mRNA expression and secretion in cultured vascular smooth muscle cells [16, 28]. However, we cannot exclude the possibility that Ang II infusion stimulates osteopontin expression indirectly, perhaps via some systemic effect such as, for example, elevated blood pressure. Alternatively, elevated cortical osteopontin levels might be related to other processes occurring as a consequence of Ang II infusion, such as tubular cell proliferation or inflammation [4]. Both of these possibilities must be considered since osteopontin has recently been shown to be associated with cellular proliferation *in vitro* [28], and a number of inflammatory cytokines such as TGF-β, IL-1α, TNF-α, and LIF have been shown to stimulate osteopontin synthesis in various cell types [29–31].

A possible function for osteopontin in Ang II-induced renal disease was suggested by our finding that ED-1 + monocyte/macrophage cells were found in a peritubular distribution almost exclusively around tubular cells expressing high levels of osteopontin. This observation would be consistent with the hypothesis that Ang II either directly or indirectly stimulates osteopontin synthesis in tubular cells, and it in turn stimulates accumulation of immune cells around responding tubular structures. Our finding that osteopontin protein expression preceded monocyte/macrophage accumulation four days following Ang II-infusion is consistent with this possibility. Thus, it is tempting to speculate that inappropriate expression of osteopontin by kidney tubules might be important in directing an inflammatory response, a step which is thought to be critical for the development of tubulointerstitial injury [32].

How might osteopontin achieve this? Several hypotheses based on the reported properties of osteopontin seem likely, but are by no means the only possibilities. First, osteopontin might be chemotactic for monocytes and macrophages in the damaged kidney. This idea is supported by the recent finding that subcutaneous injection of osteopontin (termed eta-1 in that study), stimulated a macrophage-rich inflammatory infiltrate in the mouse, and that mouse peritoneal macrophages contain a large number of high affinity osteopontin binding sites (10,000/cell) [8]. Although the receptor responsible for osteopontin binding was not identified in that report, osteopontin has been shown to bind to the integrin $\alpha v\beta 3$ [33, 34] which has been previously identified on the macrophage [35]. Secondly, osteopontin might act to stimulate accumulation of monocyte or macrophage to selected tubules by acting as a preferred adhesive support, much like the role postulated for fibronectin at sites of inflammation [36]. Lastly, osteopontin may act to inhibit monocyte or macrophage chemotaxis, thus allowing accumulation of these cells at the appropriate target areas. Whatever the mechanism, if osteopontin is an important proinflammatory agent in tubular disease, we would predict that it would be elevated in other models of tubular injury where inflammation is thought to be an important part of the pathogenesis of the disease. Preliminary experiments in tubulointerstital nephritis associated with three rat models of glomerulonephritis support this hypothesis since elevated osteopontin is found in all cases (unpublished observations).

The observation that osteopontin is constitutively expressed at low, but detectable levels in the normal kidney cortex and at higher levels in the renal medulla would appear to counter the hypothesis that tubules express osteopontin as a chemotactic/adhesive factor for monocytes. However, it is possible that osteopontin in normal kidney tubules remains intracellular and/or is directionally secreted into the urine, and thus unavailable for interaction with interstitial cells. This would be consistent with recent observations by Shiraga et al that osteopontin is present in human urine [37]. These investigators have also proposed that osteopontin may play a role in regulating mineralization in the normal kidney based on the observation that osteopontin (termed uropontin in that report) inhibited calcium oxalate crystal growth *in vitro*. In the tubular injury described here we have observed some necrosis and it is thus conceivable that osteopontin could be released extracellularly into the interstitial space as a result. Alternatively, Ang II might directly effect the compartmentalization of renal osteopontin. In either case, osteopontin might become available for interaction with interstitial cells only in the context of tubular injury.

Chemoattractants derived from renal cells and cell adhesion molecules are thought to play a critical role in tubulointerstitial fibrosis by recruiting inflammatory cells to tubular areas, yet very little is known about the molecule(s) which might mediate this effect. Rovin et al have begun characterization of a lipid factor which may be involved in monocyte/macrophage chemotaxis that occurs following unilateral ureteral obstruction [38]. In a different approach, Heeger et al [39] have identified the presence of murine RANTES (muRANTES) in proximal tubular epithelial cells. MuRANTES is an 8000 D protein member of a new family of small cytokines (Scy superfamily/intercrines) which have a variety of proinflammatory or reparative properties in tissues [40]. Another member of the family,

HuRANTES, the human homologue of MuRANTES, is a selective chemoattractant for CD4+ lymphocytes and monocytes [41], and is responsive to the modulating influence of paracrine factors which have a role in the development of renal parenchymal injury [39]. In addition, leukocyte adhesion molecules are most likely involved in leukocyte attachment and accumulation. The intercellular adhesion molecules (ICAM) 1 and 2, vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1) [reviewed in 42] are primary candidates. ICAM-1 and VCAM-1 have been found to be increased on renovascular endothelium and tubular epithelium in renal allograft rejection [43–46], and both are increased in proximal tubules in experimental and human glomerulonephritis in association with tubulointerstitial infiltrates [47, 48].

In conclusion, our studies are the first to document the activation of osteopontin during Ang II-induced tubulointerstitial injury in the rat. Furthermore, the temporal pattern of expression and co-localization of osteopontin and macrophage in the kidney cortex suggests a role for osteopontin in facilitating accumulation of these cells near the cortical tubules. This hypothesis is strengthened by previously described effects of osteopontin on monocyte-derived cells of the bone and peritoneal cavity [8, 21]. Furthermore, these data, together with our recent finding of elevated levels of osteopontin during rat arterial neointima formation and in human atherosclerotic plaques [16] suggests that osteopontin may have a broader role than previously suspected in disease processes.

Acknowledgments

This work was supported by grants DK-43422 and DK-02142 and NIH grants HL03174 and the O'Brien Kidney Center Grant DK-47659. Dr. Giachelli is also supported by grant #3124R1 from the Council for Tobacco Research. We thank Manuela Almeida and Nancy Bae for their technical assistance, and Tom Barrett (University of Washington, Seattle, Washington) for the gift of the 28S ribosomal oligomer probe.

Reprint requests to Cecilia M. Giachelli, Ph.D., Pathology Department, SJ60, School of Medicine, University of Washington, Seattle, Wa 98195, USA.

References

1. Bohle A, Mackenson-Haen, von Gise H: Significance of tubulointerstitial changes in the renal cortex for the excretory function and concentration ability of the kidney. A morphometric contribution. *Am J Nephrol* 7:421–433, 1987
2. Nath KA: Tubulointerstitial changes as a major determinant in the progression of renal damage. *Am J Kid Dis* 20:1–17, 1992
3. McCluskey RT, Bhan AK: Cell-mediated mechanisms in renal diseases. *Kidney Int* 21(Suppl 11):S6–S12, 1982
4. Johnson RJ, Alpers CE, Yoshimura A, Lombardi D, Pritzl P, Floege J, Schwartz SM: Renal injury from angiotensin II mediated hypertension. *Hypertension* 19:464–474, 1992
5. Wilson C, Byrom FB: Renal changes in malignant hypertension. *Lancet* 1:136–139, 1939
6. Klahr S: New insights into the consequences and mechanisms of renal impairment in obstructive nephropathy. *Am J Kid Dis* 18:689–699, 1991
7. Diamond JR, Anderson S: Irreversible tubulointerstitial damage associated with chronic aminonucleoside nephrosis, amelioration by angiotensin 1 converting enzyme inhibition. *Am J Pathol* 137: 1323–1331, 1990
8. Singh RP, Patarca R, Schwartz J, Singh P, Cantor H: Definition of a specific interaction between the early T lymphocyte activation 1 (Eta-1) protein and murine macrophages in vitro and its effect upon macrophages *in vivo*. *J Exp Med* 171:1931–1942, 1990
9. Daeman MJAP, Lombardi DM, Bosman FT, Schwartz SM: Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. *Circ Res* 68:450–456, 1991
10. Craig AM, Smith JH, Denhardt DT: Osteopontin, a transformation-associated cell adhesion phosphoprotein, is induced by 12-O-tetradecanoylphorbol 13-acetate in mouse epidermis. *J Biol Chem* 264:9682–9689, 1989
11. Dijkstra CD, Dopp EA, Joling P, Kraal F: The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2, and ED3. *Immunology* 54:589–599, 1985
12. Giachelli C, Bae N, Lombardi D, Majesky M, Schwartz S: Molecular cloning and characterization of 2B7, a rat mRNA which distinguishes smooth muscle cell phenotypes in vitro and is identical to osteopontin (secreted phosphoprotein I, 2aR). *Biochem Biophys Res Commun* 177:867–873, 1991
13. Wilcox JN, Smith KM, Williams LT, Schwartz SM, Gordon D: Platelet-derived growth factor mRNA detection in human atherosclerotic plaques by *in situ* hybridization. *J Clin Invest* 82:1134–1143, 1988
14. Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
15. Laemmli UK: Cleavage of structural protein during assembly of the head of bacteriophage T4. *Nature* 277:680–685, 1970
16. Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM: Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. *J Clin Invest* 92:1686–1696, 1993
17. Ullrich O, Mann K, Haase W, Koch-Brandt C: Biosynthesis and secretion of an osteopontin-related 20-kDa polypeptide in the Madin-Darby canine kidney cell line. *J Biol Chem* 266:3518–3525, 1991
18. Senger DR, Perruzzi CA, Papadopoulos A: Elevated expression of secreted phosphoprotein I (osteopontin, 2ar) as a consequence of neoplastic transformation. *Anticancer Res* 9:1291–1299, 1989
19. Miyazaki Y, Setoguchi M, Yoshida S, Higuchi Y, Akizuki S, Yamamoto S: The mouse osteopontin gene: Expression in monocytic lineages and complete nucleotide sequence. *J Biol Chem* 265:14432–14438, 1990
20. Lopez CA, Hoyer JR, Wilson PD, Waterhouse P, Denhardt DT: Heterogeneity of osteopontin expression among nephrons in mouse kidneys and enhanced expression in sclerotic glomeruli. *Lab Invest* 69:355–363, 1993
21. Butler WT: The nature and significance of osteopontin. *Connect Tissue Res* 23:123–136, 1989
22. Heinegard D, Hultenby K, Oldberg A, Reinholt F, Wendel M: Macromolecules in bone matrix. *Connect Tissue Res* 21:3–11, 1989
23. Young MF, Kerr JM, Termine JD, Wewer UM, GeWang M, McBride OW, Fisher LW: cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). *Genomics* 7:491–502, 1990
24. Nomura S, Wills AJ, Edwards DR, Heath JK, Hogan BLM: Developmental expression of 2ar(Osteopontin) and SPARC (Osteonectin) RNA as revealed by in situ hybridization *J Cell Biol* 106:441–450, 1988
25. Oldberg A, Franzen A, Heinegard D: Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. *Proc Natl Acad Sci USA* 83:8819–8823, 1986
26. Somerman MJ, Fisher LW, Foster RA, Sauk JJ: Human bone sialoprotein I and II enhance fibroblast attachment in vitro. *Calcif Tissue Int* 43:50–53, 1988
27. Mugais SK, Kauffman S, Katz AI: Angiotensin II binding sites in individual segments of the rat nephron. *J Clin Invest* 77:315–318, 1986
28. Gadeau A-P, Campan M, Millet D, Candresse T, Desgranges

C: Osteopontin overexpression is associated with arterial smooth muscle cell proliferation in vitro. *Arteriosler Thromb* 13:120–125, 1992
29. NODA M, RODAN GA: Type β transforming growth factor regulates expression of genes encoding bone matrix proteins. *Connect Tissue Res* 21:71–75, 1989
30. JIM CH, MIYAURA C, ISHIMI Y, HONG MH, SATO T, ABE E, SUDA T: Interleukin 1 regulates the expression of osteopontin mRNA by osteoblasts. *Mol Cell Endocrinol* 74:221–228, 1990
31. NODA M, VOGEL RL, HASSON DM, RODAN GA: Leukemia inhibitory factor suppresses proliferation, alkaline phosphatase activity, and type I collagen messenger ribonucleic acid level and enhances osteopontin mRNA level in murine osteopblast-like (MC3T3E1) cells. *Endocrinol* 127:185–190, 1990
32. KUNCIO GS, NEILSON EF, HAVERTY T: Mechanisms of tubulointerstitial fibrosis. *Kidney Int* 39:550–556, 1991
33. OLDBERG A, FRANZEN A, HEINEGARD D, PIERSCHBACHER M, RUOSLAHTI E: Identification of a bone sialoprotein receptor in osteosarcoma cells. *J Biol Chem* 263:19433–19436, 1988
34. MIYAUCHI A, ALVAREZ J, GREENFIELD EM, TETI A, GRANO M, COLUCCI S, ZAMBONIN-ZALLONE A, ROSS FP, TEITELBAUM SL, CHERESH D, HRUSKA KA: Recognition of osteopontin and related peptides by an avβ3 integrin stimulates immediate cell signals in osteoclasts. *J Biol Chem* 166:20369–20374, 1991
35. ATHANASOU NA, QUINN J, HORTON MA, MCGEE JO'D: New sites of cellular vitronectin receptor immunoreactivity detected with osteoclast-reacting monoclonal antibodies 13C2 and 23C6. *Bone Miner* 8:7–22, 1990
36. MOSESSON MW: The role of fibronectin in monocyte/macrophage function. *Prog Clin Biol Res* 154:155–175, 1884
37. SHIRAGA H, MIN W, VANDUSEN WJ, CLAYMAN MD, MINER D, TERRELL CH, SHERBOTIE JR, FOREMAN JW, PRZYSIECKI C, NEILSON EG, HOYER JR: Inhibition of calcium oxalate crystal growth in vitro by uropontin: Another member of the aspartic acid-rich protein superfamily. *Proc Natl Acad Sci USA* 89:426–430, 1992
38. ROVIN BH, HARRIS KPG, MORRISON A, KLAHR S, SCHREINER GF: Renal cortical release of a specific macrophage chemoattractant in response to ureteral obstruction. *Lab Invest* 63:213–220, 1990
39. HEEGER P, WOLF G, MEYERS C, SUN MJ, O'FARRELL SC, KRENSKY AM, NEILSON EG: Isolation and characterization of cDNA from renal tubular epithelium encoding murine Rantes. *Kidney Int* 41:220–225, 1992
40. OPPENHEIM JJ, ZACHARIAE COC, JUKAIDA N, MATSUSHIMA K: Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu Rev Immunol* 9:617–648, 1991
41. JONGSTRA J, SCHALL TJ, DYER BJ, CLAYBERGER C, JORGENSEN J, DAVIS MM, KRENSKY AM: The isolation and sequence of a novel gene from a human functional cell line. *J Exp Med* 165:601–614, 1987
42. MAIN IW, NIKOLIC-PETERSON DJ, ATKINS RC: T cells and macrophages and their role in renal injury. *Semin Nephrol* 12:395–407, 1991
43. FAULL RJ, RUSS GR: Tubular expression of intercellular adhesion molecules-1 during renal allograft rejection. *Transplantation* 48: 226–230, 1989
44. MOOLENAR W, BRUIJN JA, SCHRAMA E, FERRONE S, DAHA MR, ZWINDERMAN AU, HOEDEMAEKER PJ, VAN ES LA, VAN DER WOUDE FJ: T-cell receptors and ECAM-1 expression in renal allografts during rejection. *Transplant Int* 4:140–145, 1991
45. BRISCOE DM, POBER JS, HARMON WE, COTRAN R: Expression of vascular cell adhesion molecule-1 in human renal aloografts. *J Am Soc Nephrol* 3:1180–1185, 1992
46. ALPERS CE, HUDKINS KL, DAVIS CL, MARSH CL, RICHES W, MCCARTY JM, BENJAMIN CK, CARLOS TM, HARLAN JM, LOBB R: Expression of vascular cell adhesion molecule-1 (VCAM-1) in kidney allograft rejection. *Kidney Int* 44:805–816, 1993
47. SERON D, CAMERON JS, HASKARD DO: Expression of VCAM-1 in the normal and diseased kidney. *Nephrol Dial Transplant* 6:917–922, 1991
48. WUTHRICH RP, JEVNIKAR AM, TAKEI F, GLIMCHER LH, KELLEY VE: Intracellular adhesion molecule-1 (ICAM-1) expression is upregulated in autoimmune murine lupus nephritis. *Am J Pathol* 136:441–450, 1990