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# Osteopontin expression in human crescentic glomerulonephritis

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**Osteopontin expression in human crescentic glomerulonephritis.**

*Background.* Osteopontin is a molecule with diverse biological functions, including cell adhesion, migration, and signaling. The expression of osteopontin has been demonstrated in a number of models of renal injury in association with accumulations of monocyte/macrophages, including recent reports of osteopontin expression in glomerular crescents in a rat model of anti-glomerular basement membrane glomerulonephritis.

*Methods.* Glomerular expression of osteopontin in biopsies of human crescentic glomerulonephritis ($N = 25$), IgA nephropathy with crescents ($N = 2$), and diffuse proliferative lupus glomerulonephropathy with crescents ($N = 1$) was studied by immunohistochemistry, *in situ* hybridization, and combined immunohistochemistry/*in situ* hybridization. Additionally, antibodies to cell-specific phenotypic markers were used to identify cellular components of the glomerular crescent, which express osteopontin protein and mRNA.

*Results.* All of the crescents present in the biopsies studied contained a significant number of cells that expressed osteopontin protein and mRNA, demonstrated by immunohistochemistry and *in situ* hybridization, respectively. Using replicate tissue sections and combined immunohistochemistry/*in situ* hybridization, we showed that the majority of the strongly osteopontin-positive cells are monocyte/macrophages. In addition to the very strong and cell-associated localization, a weaker and more diffuse pattern of osteopontin protein and mRNA expression could be seen in a number of crescents. None of the osteopontin mRNA-expressing cells could be identified as parietal epithelial cells, CD3-positive T cells, or α-smooth muscle actin-positive myofibroblasts. Interstitial monocyte/macrophages did not express osteopontin, except when located in a periglomerular inflammatory infiltrate.

*Conclusions.* Macrophages present in the human glomerular crescent express osteopontin protein and mRNA at a high level. This expression supports a role for osteopontin in the formation and progression of the crescentic lesion via chemotactic and signaling properties of the molecule.

**Key words:** renal injury, anti-glomerular basement membrane glomerulonephritis, monocytes, macrophages, inflammation, crescentic lesion.

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Osteopontin is a secreted phosphoprotein that has a number of diverse biological functions, including cell adhesion, migration, and signaling. Originally isolated from bone, it has been shown to be expressed in a number of different tissues, including kidney, lung, liver, bladder, pancreas, and breast [1, 2]. Its expression has also been demonstrated in vascular smooth muscle cells and macrophages *in vitro* and *in vivo* [3–6]. Osteopontin has been shown to be chemotactic for vascular smooth muscle cells *in vitro* [7] and monocyte/macrophages *in vivo* [8, 9] and has been described in a number of models of injury in association with accumulations of macrophages in tissue [10–15]. In some settings, osteopontin may act as a cell survival factor for renal cells [13, 16].

These chemotactic and trophic features of osteopontin, in addition to its up-regulation in the tubulointerstitium in a variety of rodent models of renal injury [11, 12, 14, 15, 17–19], suggest that osteopontin could be an important mediator of the glomerular injury that occurs in crescentic glomerulonephritis. It is generally accepted that both monocyte/macrophages and glomerular epithelial cells are major components of the cellular crescent. Osteopontin, if present in or near crescents, could contribute to the recruitment of monocytes/macrophages in this process. This hypothesis is considerably strengthened by recent studies in the rat model of anti-glomerular basement membrane (GBM) glomerulonephritis; these studies have demonstrated the expression of osteopontin in crescentic glomeruli [20]. In this model, osteopontin appeared to be expressed predominantly by visceral and parietal epithelial cells, and this expression preceded macrophage influx [20]. Most infiltrating monocytes/macrophages had no demonstrable osteopontin expression in this model. In further studies in the same rat model, blocking osteopontin by treating the animals with neutralizing antibody to this molecule resulted in the amelioration of disease [21], indicating the importance of osteopontin for the evolution of the disease process.

We sought to extend these observations in rodents to

**Table 1.** Antibodies directed against cell-specific markers

| Antigen | Target cell | Host | Conditions | Source and clone or code | Reference |
|---|---|---|---|---|---|
| Actin | Myofibroblasts, smooth muscle cells | Mouse | No pretreatment | Dako 1A4 | [28] |
| Cytokeratins | Tubular and parietal epithelial cells | Mouse | No pretreatment | Dako AE1/AE3 | [38, 58] |
| CD3 | T cells | Rabbit | Antigen retrieval | Dako A 452 | [33, 59] |
| CD68 | Macrophages, monocytes | Mouse | Antigen retrieval | Dako PGM-1 | [26] |
| VCAM (CD106) | Parietal epithelial cells | Goat | Antigen retrieval | R & D BBA-19 | [34, 35] |
| HAM56 | Macrophages, monocytes, endothelium | Mouse | Standard ABC | Dako HAM56 | [27] |
| P27 | Visceral epithelial cells | Goat | Standard ABC | Santa Cruz sc-528G | [31] |
| WT-1 | Visceral epithelial cells | Rabbit | Standard ABC; antigen retrieval | Santa Cruz sc-192 | [32] |

corresponding human disease. We did this by examining the expression of osteopontin mRNA and protein in human renal biopsies demonstrating crescentic glomerulonephritis. In glomeruli involved by crescent formation, we demonstrate that osteopontin is expressed by cells localized both within areas of the urinary space involved by crescents and within the glomerular tufts. Further characterization of the cells expressing osteopontin mRNA demonstrates that a majority of these cells are monocyte/macrophages. These studies provide clear evidence that osteopontin is present and may participate in the events of crescentic glomerulonephritis but that important differences in patterns of osteopontin expression may exist between human disease and rodent models. These mechanistic differences could limit the applicability of therapeutic approaches targeting osteopontin derived from animal models of crescentic glomerulonephritis to the treatment of analogous human disease.

## METHODS

### Tissue

Core needle biopsies were obtained from the University of Washington Medical Center. Biopsies that had been diagnosed as crescentic glomerulonephritis ($N = 19$), IgA nephropathy with crescents ($N = 2$), and diffuse proliferative lupus glomerulonephritis with crescents ($N = 1$) were studied. As controls, biopsies of diabetic nephropathy ($N = 2$), membranous glomerulonephritis ($N = 2$), IgA nephropathy without crescents ($N = 5$), and protocol transplant biopsies ($N = 6$) were used. Additional control tissue consisted of macroscopically normal appearing cortex taken from kidneys resected for localized neoplasms ($N = 4$). All of the surgical tissue specimens were fixed in 10% neutral buffered formalin. In addition, six cases of crescentic glomerulonephritis fixed in methyl Carnoys solution (60% methanol, 30% chloroform, 10% acetic acid) were utilized. All fixed tissues were processed and embedded in paraffin according to standard protocols.

### Antibodies

*Osteopontin.* LF7 is a rabbit polyclonal antibody directed against the intact osteopontin protein molecule isolated from bone (Table 1) [22]. Its specific recognition of osteopontin has been characterized by Western blotting, and its ability to detect osteopontin by immunocytochemistry in fixed tissue sections has been previously demonstrated [5, 23, 24]. Further demonstration of the specificity of this antibody comes from previous complementary studies that localize expression of osteopontin mRNA to sites of peptide expression in tissue sections [25].

*Monocytes/macrophages.* PGM1 (Dako, Carpenteria, CA, USA) is a well-characterized murine monoclonal antibody directed against the CD68 epitope present on human monocytes and macrophages. Its specificity has been demonstrated by Western blotting, and it has been shown to be reactive in formalin-fixed, paraffin-embedded tissue following antigen retrieval [26].

HAM56 (Dako) is a murine monoclonal IgM that is reactive with human monocytes and macrophages and also demonstrates cross-reactivity with endothelial cells [27].

*α-Smooth muscle actin.* 1A4 (Dako) is a murine monoclonal antibody specific for α-smooth muscle actin [28]. It has been extensively characterized by Western blotting and has been previously shown to identify smooth muscle actin in methyl Carnoys and formalin-fixed tissue using immunohistochemical procedures [29, 30].

*Podocytes.* P27 kip1 (p27; Santa Cruz Biotechnology, Santa Cruz, CA, USA) is an affinity-purified goat polyclonal antibody raised against peptides corresponding to amino acids 181 to 298 at the carboxy terminus of human p27. It has been characterized by Western blotting and immunoprecipitation and has been shown to immunolocalize to the nuclei of human podocytes [31].

WT-1 (C-19) is a rabbit polyclonal antibody that reacts with the Wilms Tumor antigen. It has been characterized by Western blotting and immunoprecipitation. WT-1 has been shown to react with both fetal and adult visceral epithelial cells [32].

T cells used in this study were CD3 (Dako), an affinity-purified rabbit polyclonal raised against synthetic human CD3 peptide. This antibody reacts with the T-lymphocyte–associated CD3 antigen and has been characterized by comparison of cell and tissue immunostaining patterns with other established anti-CD3 antibodies [33].

*Parietal epithelial cells.* Polyclonal goat anti-vascular cell adhesion molecule (VCAM; BBA-19; R&D, Minne-

apolis, MN, USA) has been characterized by Western blotting. VCAM has been shown to be widely expressed on human parietal epithelial cells [34, 35].

*AE1/AE3.* AE1/AE3 (Dako) is a mixture of two mouse monoclonal IgG1 fractions that is a specific cocktail pan reactive with human cytokeratins. It reacts with normal epithelium in a variety of tissues and has been characterized by Western blotting [36, 37]. Cytokeratin expression has been demonstrated in parietal epithelial cells within glomerular crescents [38].

### Immunohistochemistry

Both formalin- and methyl Carnoys-fixed, paraffin-embedded tissue sections were deparaffinized, rehydrated, and then incubated in 3% hydrogen peroxide to block endogenous peroxidases. For antibodies requiring antigen retrieval (Table 1), the tissue sections were immersed in Antigen Retrieval Solution (Vector Laboratories, Burlingame, CA, USA) that had been preheated to 95°C and then heated for 30 minutes in a household vegetable steamer. Following steam heating, the slides were allowed to cool for 20 minutes at room temperature and were washed in phosphate-buffered saline (PBS). The sections were then incubated sequentially with 10% normal serum (only used for polyclonal antibodies to block nonspecific binding), primary antibody, biotinylated secondary antibody (Vector Laboratories), and the avidin-biotin-horseradish peroxidase (HRP) complex (ABC; Vector). The immunohistochemical signal was then visualized with 3,3′-diaminobenzidine (Sigma, St. Louis, MO, USA) with nickel chloride enhancement to give a black-brown reaction product. After methyl green counterstaining, the slides were dehydrated and coverslipped. For all samples, negative controls for the immunohistochemistry included substitution of the primary with antibody an irrelevant IgG from the same species (Dako).

### *In situ* hybridization

Human osteopontin cDNA in plasmid pBluescript SK(−) (plasmid OP-10) was obtained from Dr. Larry Fisher (National Institutes of Health) [39]. It contains a 1493 bp fragment of the human osteopontin gene, which includes the entire protein-encoding sequence of the human osteopontin Ia gene. The plasmid was linearized with Xba 1 and Xho 1 and then transcribed into both antisense and sense (negative control) riboprobes using reagents from Promega (Madison, WI, USA), except $^{35}$S UTP, which was obtained from NEN (Boston, MA, USA). The details of this procedure have been previously published [40]. Needle biopsy kidney tissue, which had been fixed in 10% formalin and embedded in paraffin, was deparaffinized following standard protocol. The sections were washed with 0.5 × standard saline citrate (SSC; 1 × SSC = 150 mmol/L NaCl, 15 mmol/L Na citrate, pH 7.0) and digested with 10 μg/mL proteinase K (Sigma) in Tris buffer (500 mmol/L NaCl, 10 mmol/L Tris, pH 8.0) for 30 minutes at 37°C. Several 0.5 × SSC washes were followed by prehybridization for two hours in 50 μL of prehybridization buffer (50% formamide, 0.3 mol/L NaCl, 20 mmol/L Tris, pH 8.0, 5 mmol/L ethylenediaminetetraacetic acid, 1 × Denhardt's solution, 10% dextran sulfate, 10 mmol/L dithiothreitol, 50 μg/ml yeast tRNA) at 50°C. The hybridizations were started by adding 500,000 cpm of $^{35}$S-labeled riboprobe in 25 μL of prehybridization buffer. The hybridization was allowed to proceed overnight at 50°C. After hybridization, sections were washed with 0.5 × SSC, treated with RNase A (20 μg/mL, 30 minutes at room temperature), washed in 2 × SSC (2 × 2 min), followed by three high stringency washes in 0.1 × SSC/0.1% Tween 20 (Sigma) at 55°C and several 2 × SSC washes, dehydrated, and air dried.

The slides hybridized with the $^{35}$S-labeled probe were dipped in NTB2 nuclear emulsion (Kodak, Rochester, NY, USA) and exposed in the dark at 4°C for three to 10 days. After developing, the sections were counterstained with hematoxylin and eosin and were dehydrated, mounted, and viewed. Positive cellular labeling was defined as five or more silver grains concentrated over a single cell.

Negative controls included hybridization performed on replicate tissue sections using the sense riboprobe.

### Combined immunohistochemistry and *in situ* hybridization

Slides were first hybridized and washed as discussed earlier in this article. Following the *in situ* hybridization stringency washes, the slides were incubated with primary antibody (PGM1 HAM56, AE1/AE3, or 1A4) for one hour at room temperature. After washing, the slides were incubated with HRP-conjugated secondary antibody, and then DAB without nickel chloride enhancement was used to produce a brown reaction product. Following immunostaining, the slides were washed, dehydrated, and then dipped in NTB-2 emulsion.

## RESULTS

A total of 22 biopsies containing crescentic glomeruli were studied by immunocytochemistry and *in situ* hybridization. This included 19 cases of segmentally necrotizing and crescentic glomerulonephritis of nonimmune complex type, two cases of IgA nephropathy with crescents, and one case of diffuse proliferative lupus glomerulonephritis. An additional six cases of crescentic glomerulonephritis, which had been fixed in methyl Carnoys solution, were studied by immunohistochemistry. There was a total of 110 crescentic glomeruli present, which were primarily cellular ($N$ = 43) and fibrocellular ($N$ = 67) in composition (Table 2). Crescentic glomeruli were sorted based on their morphologic appearance in a rou-

**Table 2.** Number of osteopontin mRNA expression cells aND CD68 positive monocyte/macrophages by glomerular lesion type. The numbers shown represent the average number of positive cells per glomerulus (total number of positive cells/number of glomeruli) seen in single label *in situ* hybridization and immunohistochemistry slides

| | | Cellular crescent | | | Fibrocellular crescent | | | Sclerotic glomeruli | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Case | Diagnosis | *N* | Opn ISH+ | CD68+ | *N* | Opn ISH+ | CD68+ | *N* | Opn ISH+ | CD68+ |
| 1 | cGN | 3 | 8.7 | 13.3 | 0 | NA | NA | 3 | 0 | 0 |
| 2 | cGN | 2 | 3 | 3.5 | 1 | 10 | NP | 2 | 0 | 0.5 |
| 3 | cGN | 1 | 3 | 3 | 0 | NA | NA | 11 | 0.8 | 0 |
| 4 | cGN | 2 | 10 | —[a] | 0 | NA | —[a] | 0 | NA | —[a] |
| 5 | cGN | 0 | NA | NA | 3 | 4 | —[a] | 0 | NA | NA |
| 6 | cGN | 1 | 12 | —[a] | 4 | 10.5 | —[a] | 0 | NA | NA |
| 7 | cGN | 6 | 11 | 3.5 | 4 | 3.75 | 3 | 3 | 0 | 0 |
| 8 | cGN | 1 | 9 | 6 | 5 | 9.2 | 10.6 | 0 | NA | NA |
| 9 | cGN | 3 | 9 | 6 | 3 | 10.3 | 2 | 0 | NA | NA |
| 10 | cGN | 0 | NA | NA | 3 | 5 | —[a] | 0 | NA | NA |
| 11 | cGN | 1 | 5.5 | ND | 2 | 3 | ND | 3 | 0 | ND |
| 12 | cGN | 0 | NA | NA | 2 | 7 | ND | 2 | 0 | NA |
| 13 | cGN | 2 | 7 | ND | 1 | 8 | ND | 0 | NA | NA |
| 14 | cGN | 3 | 3 | 6.3 | 10 | 9.3 | 7.5 | 0 | NA | NA |
| 15 | cGN | 1 | 4 | ND | 2 | 3.5 | ND | 0 | NA | NA |
| 16 | cGN | 0 | NA | NA | 2 | 6 | ND | 0 | NA | NA |
| 17 | cGN/ANCA | 4 | 14.5 | 10 | 6 | 11 | 12.8 | 0 | NA | NA |
| 18 | cGN/ANCA | 0 | NA | NA | 2 | 7 | ND | 0 | NA | NA |
| 19 | cGN/lupus | 1 | 6 | 13 | 1 | 29 | 4 | 2 | 0 | 0 |
| 20 | IgA, cresc. | 0 | NA | NA | 1 | 2 | ND | 0 | NA | ND |
| 21 | IgA, cresc. | 1 | 2 | ND | 1 | 4 | ND | 0 | NA | NA |
| 22 | DPLGN | 1 | 4 | ND | 2 | 6.5 | ND | 0 | NA | NA |

Abbreviations used: ISH, *in situ* hybridization; cGN, crescentic glomerulonephritis; NA, not applicable; ND, not done; NP, not present; ANCA, anti-nuclear cell antigen positive; IgA, cresc., IgA nephropathy with crescents; DPLGN, diffuse proliferative lupus glomerulonephritis.

[a] Immunohistochemistry done, results too weak to count positive cells

tine hematoxylin and eosin stain; cellular crescents appeared to be composed of organized layers of cells, whereas fibrocellular crescents were composed of a mixture of cells and extracellular matrix.

### Glomerular osteopontin expression

All of the tissues, both methyl Carnoys and formalin fixed, were utilized for immunohistochemistry. Additionally, all of the formalin-fixed specimens were used for *in situ* hybridization to demonstrate osteopontin mRNA expression. A majority of the crescentic glomeruli were shown to express osteopontin protein by immunocytochemistry. This expression was usually localized to individual cells within the crescent, as well as to cells within the glomerular tuft (Fig. 1A). Rarely, a strong, more diffuse extracellular pattern of expression could be seen distributed over the crescentic lesion (Fig. 2A). The most common pattern of expression seen is shown in Figure 3A, with individual cells expressing a high level of osteopontin protein, as well as moderate expression spread throughout the crescent. In the noncrescentic control tissues, there was no osteopontin protein localized to glomeruli (data not shown).

As can be seen in Table 2, all of the crescentic glomeruli contained cells that expressed osteopontin mRNA. In many cases, there were distinct osteopontin mRNA-positive cells within the residual glomerular tuft as well as a weak, more diffuse hybridization signal spread over the crescent (Figs. 1B, 2B, and 3 C, D). Localization of osteopontin protein by immunocytochemistry in replicate sections demonstrated a similar pattern of expression (Fig. 1A, 2A, and 3A) with both weak to moderate expression in the crescent and strong expression by individual cells. Using combined immunohistochemistry and *in situ* hybridization, osteopontin protein and mRNA were localized to the same cells present in the crescentic glomeruli (data not shown). Morphologically normal appearing glomeruli within the same biopsies generally did not contain osteopontin expressing cells, although an occasional circulating cell could be seen within the glomerular tuft or closely opposed to Bowman's capsule, which did express osteopontin mRNA (Fig. 4 A, C, D) or protein (Fig. 5 A, B). Occasional osteopontin mRNA expressing cells could also be seen in periglomerular inflammatory infiltrates (Fig. 4 C, D). Tubular expression of osteopontin mRNA and protein was widespread (Figs. 1–6) and was expressed by both proximal and distal tubules. Glomeruli of the nondiseased adult kidney, diseased kidneys without crescent formation, and kidneys undergoing transplant rejection did not contain any osteopontin-positive cells demonstrated by either *in situ* hybridization or immunocytochemistry, although tubular expression remained widespread.

### Phenotype of osteopontin-expressing cells

Cellular crescents are known to contain several different cell types, including glomerular epithelial cells, mac-

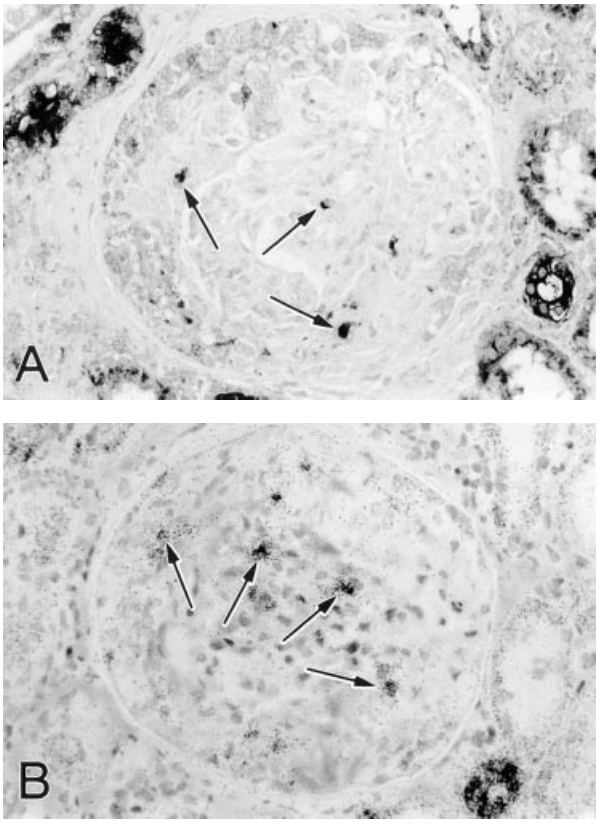



**Fig. 1. Osteopontin expression in a cellular crescent.** (*A*) Immunohistochemistry with antiosteopontin antibody, LF7, demonstrating individual strongly positive cells within the crescent and glomerular tuft (arrows), as well as a more diffuse, weak staining within the crescent. There is widespread tubular expression of osteopontin protein. (*B*) *In situ* hybridization using osteopontin antisense riboprobe in a replicate tissue section demonstrates osteopontin mRNA expression localized to individual cells within the crescent and the glomerular tuft (arrows). Strong hybridization signal can also be seen in a neighboring tubule.

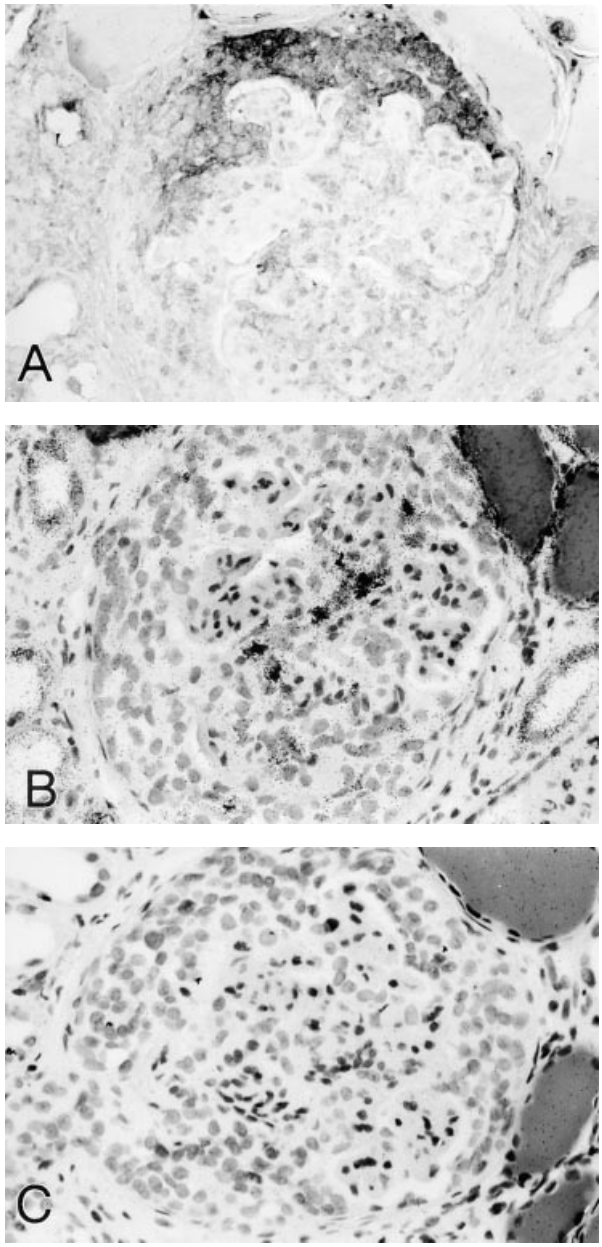



**Fig. 2. Osteopontin expression in a cellular crescent.** (*A*) Immunohistochemistry demonstrates osteopontin protein localized in a strong, diffuse pattern within the crescent. (*B*) *In situ* hybridization in a replicate tissue section demonstrates osteopontin mRNA expression localized primarily to cells within the glomerular tuft. (*C*) Negative control *in situ* hybridization using the sense osteopontin riboprobe in a replicate tissue section, demonstrating the specificity of the hybridization procedure.

rophages, lymphocytes. As the crescent progresses to a more fibrous state, fibroblasts or myofibroblasts may infiltrate from the interstitium. Because osteopontin mRNA signal localized to cells within both the crescent and the glomerular tuft, we sought to identify these cells.

To determine the cell type present within the crescentic glomeruli that expressed osteopontin, tissue sections were immunostained with antibodies directed against osteopontin, α-smooth muscle actin, monocytes/macrophages (CD68 and Ham56), T cells (CD3), vascular cell adhesion molecule (VCAM; CD106), p27 and Wilms tumor antigen (WT-1), and also hybridized with the osteopontin riboprobe. All of the crescentic glomeruli contained a significant number of CD68-positive macrophages (2-30 CD68+ macrophages/crescentic glomerulus, median = 8.9; Table 2). The expression of CD3-positive T cells was less frequent (0 to 3 per crescentic glomerulus). Many crescents also contained cytokeratin-expressing cells (Fig. 6 E, F) and VCAM-expressing cells (data not shown), indicating that a major component of these crescents was parietal epithelial cells, which normally express these markers. In some of the fibrocellular crescents, α-smooth muscle actin-expressing myofibroblasts could also be identified (data not shown). The expression of WT-1 and p27, markers of mature visceral epithelial cells, was largely absent in the crescents. As can be seen in Figures 3 and 4, the cell-specific pattern of osteopontin expression appeared to match that of the macrophage marker

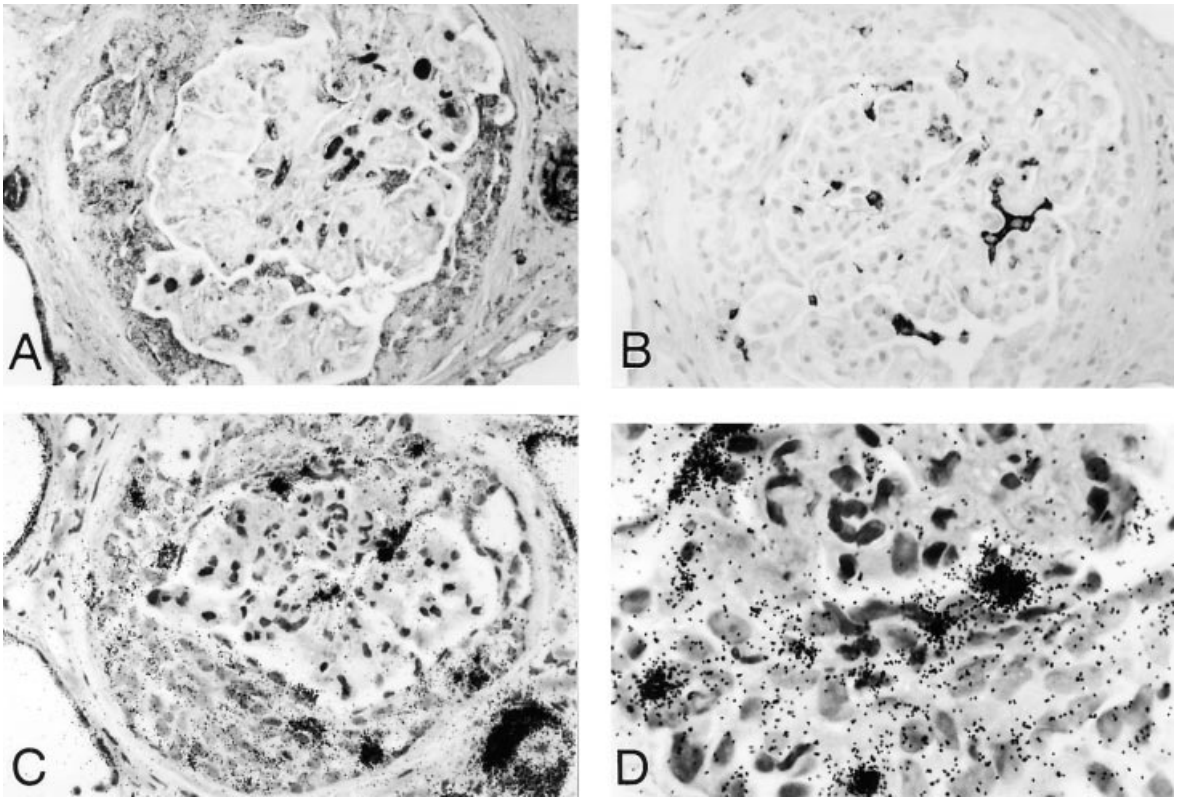



**Fig. 3. Osteopontin expression and macrophage localization in a cellular crescent.** (*A*) Immunohistochemistry demonstrates osteopontin protein strongly expressed by individual cells within the glomerular tuft and moderate, diffuse expression within the crescent. (*B*) In a replicate section, CD68-positive macrophages are localized both within the glomerular tuft and the crescent. (*C* and *D*) Low- and high-power views of osteopontin *in situ* hybridization in a replicate section demonstrates strong mRNA expression by individual cells within the glomerular tuft and the crescent, as well as weak hybridization signal spread over the crescent.

CD68 closely. Because of the complexity of composition of the cellular and fibrocellular crescentic lesions, it was impossible to positively identify the cells that express osteopontin using serially sectioned slides.

Therefore, to more accurately identify the cells within the crescent, which express osteopontin, combined immunohistochemistry and *in situ* hybridization were performed on a subset of the biopsies. As can be seen in Figure 6 A–D, the majority of cells that demonstrated strong hybridization signal with the osteopontin riboprobe were monocytes/macrophages, as demonstrated by their colabeling with both the Ham56 and CD68 antibodies. Interestingly, monocytes and macrophages present in the tubulointerstitium (Fig. 6 G, H) did not appear to be positive for osteopontin mRNA expression. Although not every cell that showed positive osteopontin hybridization signal could be definitively identified as a macrophage, none of the osteopontin mRNA-positive cells were colabeled with AE1/AE3 (anticytokeratin; Fig. 5 E, F), anti–α-smooth muscle actin (data not shown), or anti-CD3 (data not shown), that might indicate cells of epithelial, myofibroblast, or lymphocyte origin, respectively.

## DISCUSSION

In this study, we retrospectively examined 25 cases of crescentic glomerulonephritis, 2 cases of IgA nephropathy with crescents, and 1 case of diffuse proliferative lupus glomerulonephritis with crescents for the presence of osteopontin. In all of the cases studied, there was significant expression of osteopontin protein and mRNA within the crescentic and segmentally sclerotic glomeruli, as well as rare expression within histologically normal-appearing glomeruli. In noncrescentic control biopsies, no osteopontin expression could be identified in glomeruli. This finding is in agreement with previously published results [25], in which we found osteopontin expressed only in tubular epithelium of mature adult kidney.

Osteopontin protein and mRNA expression demonstrated a similar pattern of expression, with both weak to moderate expression spread throughout the crescent and very strong expression by individual cells. Rarely, immunohistochemistry demonstrated strong osteopontin protein expression throughout the crescent, as shown in Figure 2. In this case, however, osteopontin mRNA expression was limited to individual cells present in the glomerular tuft, suggesting that the osteopontin protein

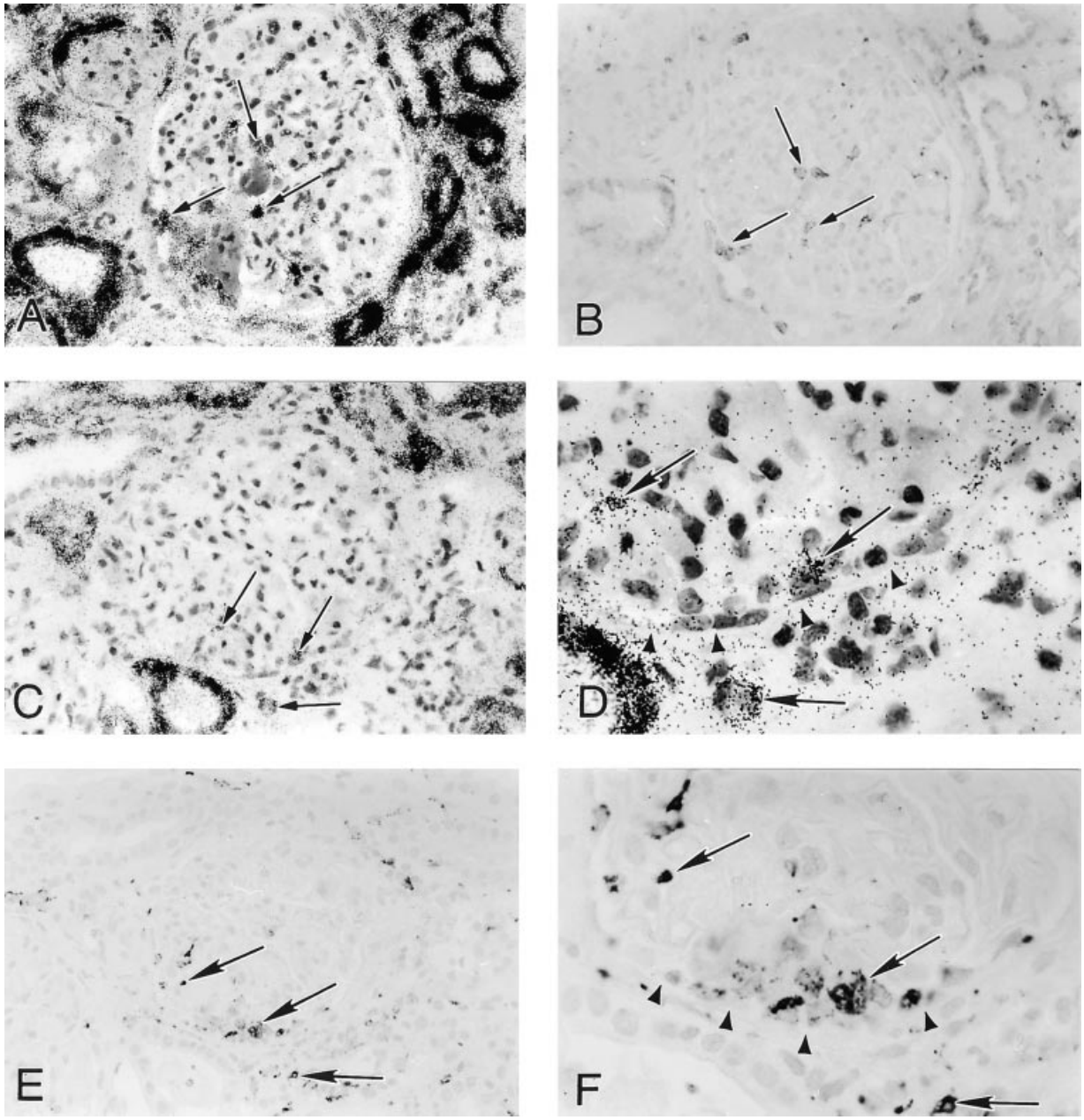



**Fig. 4. Osteopontin mRNA expression in normal-appearing glomeruli within crescentic glomerulonephritis (GN) biopsies coincides with macrophage localization.** (*A*) *In situ* hybridization demonstrates osteopontin mRNA-expressing cells localized to the glomerular tuft and closely opposed to Bowman's capsule (arrows) within in a glomerulus uninvolved by an established crescent. (*B*) Immunohistochemistry in a replicate tissue section demonstrates CD68-positive macrophages localized in a similar pattern to that shown in A (arrows). (*C* and *D*) Low- and high-power view of *in situ* hybridization for osteopontin. Strong hybridization signal can be seen in tubular epithelial cells (T) surrounding a normal appearing glomerulus within a crescentic GN biopsy. Osteopontin mRNA-expressing cells (arrows) can be seen closely opposed to Bowman's capsule (defined by arrowheads), in a periglomerular inflammatory infiltrate, and rarely within the glomerular tuft. (*E* and *F*) Low- and high-power view of replicate tissue to that shown in C and D, which has been immunostained with anti-CD68 antibody. CD68-positive macrophages (arrows) can be seen in the urinary space, closely opposed to Bowman's capsule and in a periglomerular infiltrate.

was secreted by these cells and may have been bound to cells or extracellular matrix present in the crescent. The generally weak hybridization signal seen spread diffusely in the crescents by *in situ* hybridization with the osteopontin riboprobe is difficult to interpret. It may be background due to the very strong hybridization signal localized to individual cells present in the crescent, or it could represent a very low level of osteopontin mRNA expression in a number of cells present in the crescents.

The majority of the osteopontin-expressing cells present within the glomeruli in the crescentic biopsies are monocyte/macrophages, as identified by immunostaining

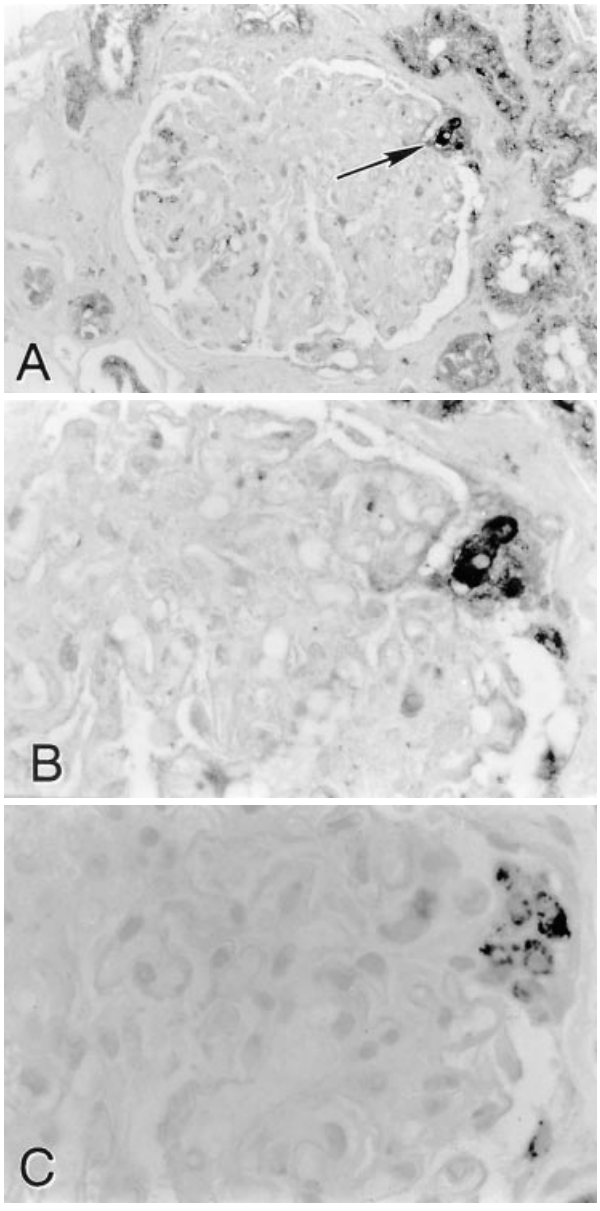



**Fig. 5. Osteopontin protein expression in normal-appearing glomerulus within a crescentic GN biopsy.** (*A* and *B*) Low- and high-power view of immunohistochemistry with LF7 antibody demonstrates osteopontin protein expression in a normal-appearing glomerulus from a biopsy of crescentic GN. Osteopontin-positive cells can be seen in a small cluster within the urinary space, closely opposed to Bowman's capsule (arrow) and in surrounding tubular epithelium. (*C*) High-power view of a replicate tissue section immunostained with anti-CD68 antibody. The same cluster of cells seen in A and B is shown to be CD68-positive macrophages.

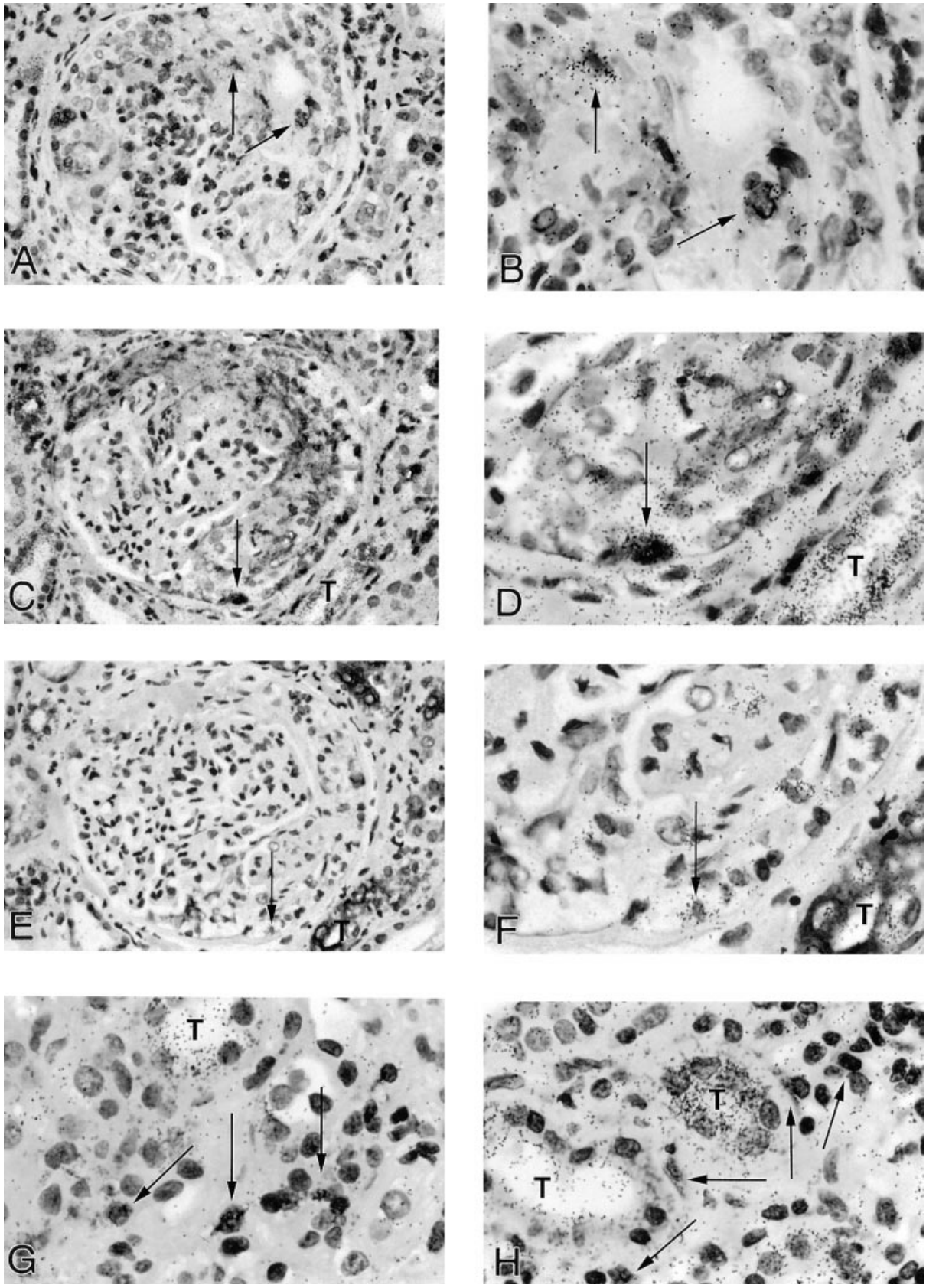
of replicate tissue sections and by combined *in situ* hybridization and immunohistochemistry. None of the osteopontin mRNA-positive cells could be identified as T cells, cytokeratin expressing epithelial cells or α-smooth muscle actin positive myofibroblasts. However, because of sensitivity limitations associated with the combined immunohistochemistry and *in situ* hybridization technique, we cannot exclude the possibility that osteopontin mRNA is expressed, perhaps at a lower level, by other cell types present in the crescent.

These results are in contrast to those reported recently in a rodent model of anti-GBM crescentic glomerulonephritis [20, 21]. In this rat model, osteopontin expression appeared to localize predominantly to intrinsic renal epithelial cells and preceded the influx of macrophages.

Although the biopsies of human crescentic glomerulonephritis (GN) undoubtedly represent a more chronic stage of disease than that examined in the rodent model, within each biopsy, glomeruli could be seen that represented different stages of crescent formation. Even in very small, cellular crescents composed of two to three layers of cells, CD68-positive monocyte/macrophages were present, and it was these cells in which osteopontin expression could be detected. Additionally, in a number of cases with features of crescentic glomerulonephritis, histologically normal-appearing glomeruli demonstrated rare osteopontin-producing cells closely opposed to Bowman's capsule, which were shown, upon double labeling and in replicate tissue sections, to be CD68-positive macrophages. These may represent the very earliest stage of crescent formation. There was no significant difference in the average number of osteopontin-positive cells between cellular and fibrocellular crescents (Table 2). The total number of osteopontin mRNA expressing cells appeared rather to vary from one individual to another and not between different crescents within the same individual. Globally sclerotic glomeruli, which were generally devoid of cells, did not demonstrate significant osteopontin mRNA or protein.

Crescentic glomerulonephritis is an inflammatory glomerular disease that is mediated by the immune system and demonstrates many of the pathologic features of delayed-type hypersensitivity (DTH) [41, 42]. Over the past several years, it has been shown in experimental

**Fig. 6. Combined immunohistochemistry and *in situ* hybridization.** (*A* and *B*) Low- and high-power view of a crescentic glomerulus immunostained with anti-CD68 antibody (DAB appears as a green-brown color) and hybridized with osteopontin antisense riboprobe. There are several CD68-positive monocyte/macrophages that also demonstrate osteopontin mRNA expression (silver grains) localized within the crescent (arrows). (*C* and *D*) Low- and high-power view of same glomerulus shown in A and B, immunostained with HAM56 antibody and hybridized with osteopontin antisense riboprobe. A positive osteopontin hybridization signal can be seen in a monocyte/macrophage closely opposed to Bowman's capsule (arrow). An osteopontin positive tubule (T) can be seen in lower right corner. (*E* and *F*) Low- and high-power view of same glomerulus shown in A–D, immunostained with anticytokeratin antibody and hybridized with osteopontin antisense riboprobe. The arrow indicates the same osteopontin mRNA-positive cell shown in C and D. None of the cytokeratin-positive cells (brown) within the crescent hybridized with the osteopontin riboprobe. The tubular epithelium (T) shown in the lower right of the photograph demonstrates colocalization of cytokeratin protein and osteopontin mRNA. (*G* and *H*) High-power view of tubulointerstitium from the same biopsy shown in A–F. The sections were immunostained with HAM56 (G) or CD68 (H) antibody and hybridized with the osteopontin antisense riboprobe. Osteopontin-positive tubules (T) can be seen, as well as monocyte/macrophages (arrows), which do not express osteopontin mRNA.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models of crescentic GN that CD4+ T cells are essential for the development of crescents. This has been demonstrated both in depletion studies using monoclonal antibodies [43] and in mice genetically deficient in CD4+ T cells [44]. The glomerular accumulation of DTH mediators, namely CD4+ T cells and macrophages, has been well documented in human crescentic GN [45–48]. It is unknown which factors may induce osteopontin expression in macrophages within crescentic lesions. Monocytic cell lines *in vitro* have been shown to up-regulate osteopontin expression in response to various cytokines, including interleukin-1 [49] and tumor necrosis factor-α [50]. This study is the first to report that the macrophages that participate in the human glomerular crescentic lesion appear to represent some subclass of activated macrophage, which is phenotypically distinct from the interstitial macrophages present in the same tissue that do not express detectable levels of osteopontin.

Osteopontin is a multifunctional molecule and has been associated with cell adhesion, signaling, and migration [reviewed in 2, 51]. Up-regulated osteopontin expression in tubular epithelium in association with interstitial macrophage infiltration has been demonstrated in various rodent models of experimental nephritis [11, 12, 14, 52] and in mature human kidney [25]. Expression of osteopontin by parietal epithelial cells has also been reported in sclerotic glomeruli of aging mice [53]. Recently, several studies have shown *de novo* expression of osteopontin in experimental models of crescentic GN [20], and follow-up studies demonstrated that blocking osteopontin with neutralizing antibodies ameliorated the disease [21]. Osteopontin expression by macrophages has been reported in cardiovascular lesions and wound healing [5, 6, 24, 54] and *in vitro* [49, 50, 55]. Additionally, osteopontin has been shown to be a potent chemotactic molecule for macrophages both *in vivo* [8] and for macrophages and smooth muscle cells *in vitro* [7, 56]. Thus, our finding that macrophages within the glomerular crescent express osteopontin would support a role for osteopontin in an autocrine feedback loop, which could potentiate the disease process by promoting the further accumulation of monocytes and macrophages and perhaps the migration of myofibroblasts into the crescent.

Recently, binding of endothelial cells to osteopontin via the $\alpha_v\beta_3$ integrin was shown to rapidly induce nuclear factor-κB (NF-κB) activity and protect the endothelial cells from apoptosis [16]. NF-κB belongs to a family of transcription factors that are involved in the up-regulation of chemotactic proteins and adhesion molecules, cell proliferation, matrix protein crosslinking, and myofibroblast differentiation [reviewed in 57]. In addition to mediating directly macrophage and myofibroblast chemotaxis, the osteopontin-positive macrophages present may be interacting with intrinsic renal cells via the NF-κB signaling pathway to contribute to the pathogenesis of the crescentic lesion further.

In this study, we have examined a series of human crescentic GN biopsies for the expression of osteopontin. In all of the cases studied, there was significant expression of osteopontin within the crescentic glomeruli. None of the control tissues demonstrated osteopontin expression within glomeruli. Using combined *in situ* hybridization and immunocytochemistry, we show that it is primarily macrophages and not intrinsic renal cells that are producing high levels of osteopontin within the crescentic glomeruli. Because of its properties as a chemotactic agent and modulator of the NF-κB signaling pathway, osteopontin may be a potential target of intervention in human crescentic GN.

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