Induction of Clusterin in Tubules of Nephrotic Rats

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Abstract. Clusterin is a glycoprotein induced after renal tubular cell injury. The purpose of this study was to examine the expression of clusterin in a disease model characterized early in its course by predominant glomerular injury. Male Wistar rats (weighing 251 ± 16 g) were treated with puromycin aminonucleoside (PAN; 15 mg/100 g body wt, subcutaneously; $n = 7$) or vehicle (control; $n = 8$). The kidneys were harvested 6 d after treatment, when rats were nephrotic. Clusterin mRNA was markedly induced in the kidneys of nephrotic rats (8.5-fold *versus* control). Immunohistochemistry studies demonstrated clusterin primarily in tubules in the cortex and medulla. Many of the tubules staining for clusterin were dilated but had no other differentiating morphologic features. Increased numbers of proliferating tubular cells were seen at 6 d, but there was no correlation between these cells and clusterin staining. In contrast to the extent and pattern of clusterin staining, vimentin was seen in only sporadic, dilated tubules, in addition to its expected glomerular localization. An increase in clusterin mRNA was not seen 1, 2, or 4 d after PAN injection. In conclusion, tubular epithelial cell induction of clusterin occurs in the kidneys of nephrotic rats. The appearance of clusterin precedes the development of tubulointerstitial disease and may be a response to the proteinuria. (J Am Soc Nephrol 9: 33–37, 1998)

Clusterin is a heterodimeric glycoprotein induced at times of tissue injury and remodeling (1–4). Numerous functions have been proposed for clusterin, including apoptosis, lipid transport, maintenance of cell interactions, complement defense, and initiation of apoptosis. In the kidney, increased expression of clusterin is seen in both normal-appearing and damaged tubules after a variety of acute tubular injuries (5–11). Glomerular expression of clusterin has been observed in immune complex glomerulonephritis usually in association with the membrane attack complex of complement. The purpose of this study was to examine the expression of clusterin in a non-complement-mediated model of glomerular injury, puromycin aminonucleoside nephrosis (PAN) (12).

Materials and Methods

Puromycin Aminonucleoside Nephrosis

All animal experimentation was conducted in accord with National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Wistar rats were treated with a single intraperitoneal dose of PAN (15 mg/100 g body wt) (Sigma Chemical Co., St. Louis, MO) ($n = 7$) or vehicle (control) ($n = 8$). The kidneys were harvested 6 d after treatment. To determine the time course for changes in clusterin mRNA and urinary protein excretion, a second group of rats was treated as described above except that kidneys were harvested 1, 2, 4, or 6 d after PAN ($n = 2$ to 3 each group).

Before sacrifice, rats were placed in metabolic cages for collection of a 24-h urine sample for measurement of protein and creatinine excretion. At the time of sacrifice, blood was collected for measurement of serum creatinine. The kidneys were harvested, with one snap-frozen in liquid nitrogen for RNA extraction and the other fixed in 10% formaldehyde for immunohistochemistry.

RNA Extraction and Northern Blot Hybridization

Renal RNA was extracted by the method of Chomczynski and Sacchi (13). Clusterin mRNA was quantified by Northern hybridization as described previously (14). The cDNA probe was rat TRPM-2 (clusterin; gift of Martin Tenniswood, W. Alton Jones Cell Science Center, Lake Placid, NY) (15). The probe was labeled by the method of random oligomer-primer labeling (Stratagene) with ^{32}P-dCTP (6000 Ci/mmol; NEN Dupont, Wilmington, DE). Autoradiographs were quantified by computer-assisted videodensitometry as described previously (16).

Immunohistochemistry

For detection of clusterin, a sheep anti-rat polyclonal antibody was used in a dilution of 1:800 (gift of Brendan Murphy, St. Vincent's Hospital, Victoria, Australia) (17). For detection of proliferating cell nuclear antigen (PCNA), a prediluted mouse anti-PCNA monoclonal antibody was used (clone PC10; Zymed Laboratories, San Francisco, CA). Vimentin was detected by a prediluted mouse anti-vimentin monoclonal antibody (Zymed). The specimens were stained using an immunoperoxidase system as described previously (Vector Laboratories, Burlington, CA) (18). Briefly, after 1 h of incubation at 4°C with the primary antibody, the sections were incubated with a biotinylated secondary antibody followed by an avidin:biotinylated horseradish peroxidase complex (Vector Laboratories). The reaction was demon-

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Table 1. General parameters of rats 6 d after PAN or vehicle[a]

Group	Body Weight (g)	Serum Protein (g/dl)	Urine Protein (mg/24 h)	Creatinine Clearance (ml/min)
PAN ($n = 7$)	260 ± 15	3.5 ± 0.2[b]	466 ± 48[b]	0.4 ± 0.1[b]
Control ($n = 8$)	255 ± 12	5.9 ± 0.2	18 ± 4	1.3 ± 0.2

[a] Values are mean ± SEM. PAN, puromycin aminonucleoside.
[b] $P < 0.05$ *versus* control group.

strated with 0.05% 3-3′ diaminobenzidine tetrahydrochloride in 0.01% hydrogen peroxide (Vector Laboratories), and the sections were counterstained with Mayer's hematoxylin (Sigma). For clusterin, positive controls were represented by sections from kidney known to contain clusterin (7); negative controls consisted of sections in which normal rabbit serum was substituted for primary antisera.

Statistical Analyses

Statistical significance was defined as $P < 0.05$, and the results are presented as mean ± SEM. The significance of the differences was analyzed by unpaired *t* test or ANOVA, followed by Student–Newman–Keuls multiple comparison's test.

Results

Proteinuria, hypoproteinemia, and decreased GFR were seen 6 d after PAN injection (Table 1). Clusterin mRNA was markedly induced in the kidneys of PAN-treated rats at this time (control: 310 ± 104 optical density units *versus* PAN: 2528 ± 297; $P < 0.01$) (Figure 1). An increase in clusterin mRNA was not seen 1, 2, or 4 d after PAN injection, but was present on day 6 (Figure 2A). Induction of clusterin followed a similar time course as that for the development of proteinuria (Figure 2B).

Histologic examination of the kidneys 6 d after PAN injection demonstrated minimal light microscopic evidence for tubulointerstitial disease or infiltrates (data not shown). Occasional dilated tubules and tubular casts were observed (Figure 3). Clusterin was present primarily in tubules in the cortex and medulla (Figure 3A). Many of the tubules that stained for clusterin were dilated but had no other differentiating morphologic features. When one cell in a tubule stained for clusterin, the other cells in that tubule were also positive. No clusterin staining was seen in glomeruli or in the interstitium.

An increase in tubular cells with nuclear staining for PCNA was seen in the PAN-treated rats compared with control rats 6 d after injection (54 ± 4 *versus* 2 ± 0.4 positive cells per 40× field; $P < 0.001$) (Figure 3B). No correlation was observed between cells staining for clusterin and those positive for PCNA (Figure 3, A and B). Vimentin staining was confined predominantly to glomeruli in both control and vehicle-treated rats, with tubular staining rarely seen in some dilated tubules (Figure 3C).

Discussion

The major finding of this study was the induction of clusterin in renal tubules, but not glomeruli, 6 d after administration of PAN. The induction of clusterin occurred when the rats were nephrotic, but before light microscopic evidence for tubular damage. The only change present in some of the tubules expressing clusterin was mild dilation.

Clusterin is induced in renal tubules after ischemic and nephrotoxic injuries (5–11,19). The time course for its appearance varies with the model studied, but appears to coincide with increased serum creatinine and morphologic evidence of injury. In some tubules, diffuse cytoplasmic staining is seen, whereas in others clusterin is present only at the apical surface. Clusterin is present in tubular casts, and some sloughed tubular cells and urinary levels correlate with the degree of nephrotoxicity (7, 9). Clusterin is also present in other renal diseases,

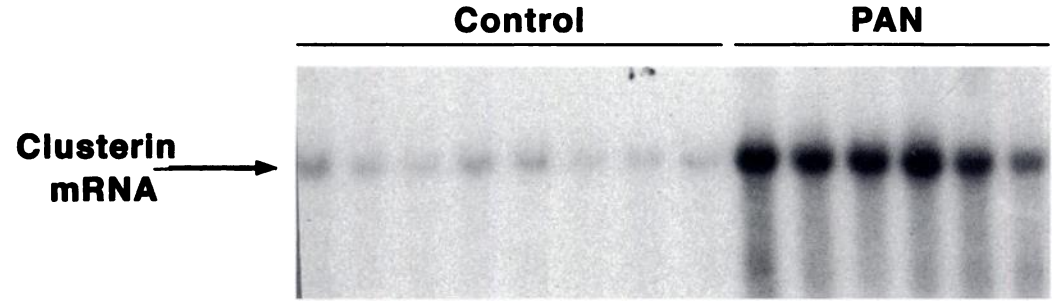

Figure 1. Clusterin mRNA. Northern blot of total kidney RNA harvested 6 d after injection of puromycin aminonucleoside (PAN) or vehicle (Control). The blot has been hybridized with a clusterin cDNA probe. Each lane represents a separate rat. A marked increase of clusterin mRNA was seen in the PAN rats.

including experimental and human cystic disease and in association with immune complexes in glomerulonephritis (20–24).

Despite tubular induction of clusterin 6 d after PAN, we did not see evidence for significant tubular injury. Tubulointerstitial injury eventually develops in this model. Eddy and Michael demonstrated, by careful labeling studies, increased numbers of infiltrating cells as early as 5 d after administration of PAN (25). We did not find evidence for interstitial inflammation in our study, although labeling studies would be needed to be absolutely certain. Early evidence for tubular epithelial cell injury includes increased numbers of proliferating tubular cells and decreased expression of Ia antigen (25,26). The latter finding was first seen on day 5 and was worse in areas of active cellular infiltration. We have confirmed the finding of increased numbers of proliferating cells by PCNA immunohistochemistry, which identifies cells in late G_1 and early S phases of the cell cycle (10). Vimentin, a marker of mesenchymal cells, was present in the glomerular mesangium, as expected. Induction of vimentin occurs in tubular epithelial cells after injury and has been used to identify cells that have dedifferentiated as part of the injury response (10,26,27). The absence of significant tubular staining for vimentin in our study supports the histologic findings of minimal tubulointerstitial injury at this early stage.

In this model, the extent of tubulointerstitial disease correlates with proteinuria and elevations in serum creatinine. These tubulointerstitial changes resolve, as does the nephrotic syndrome. After several months, renal abnormalities recur and are characterized by proteinuria, glomerular sclerosis, and tubulointerstitial scarring (28, 29).

The tubular site for clusterin expression is intriguing. The presence of clusterin may signify the existence of tubular injury, but at a stage when morphology still appears normal. If this is the case, then clusterin may represent an early marker of that injury. The expression of clusterin could denote dedifferentiation of tubular cells as part of their injury response, although the absence of tubular staining for vimentin suggests a more specific function (27).

Clusterin can bind to the membrane attack complex of complement and prevent its insertion into cell membranes (30). Thus, a complement inhibitory role for clusterin is possible in this model, because the complement system may play a role in the pathogenesis of the tubular injury (26).

Induction of clusterin by tubular epithelial cells may be a direct response to the proteinuria, because the development of proteinuria in this study correlated with the appearance of clusterin. Clusterin contains several amphipathic domains that may be involved in lipid or protein binding, or both. For example, clusterin can bind a number of lipids, as well as complement component C9, amyloid-β protein, and IgG (30–33). Thus, clusterin within tubular cells may act as an intracellular scavenger, helping the cell rid itself of potentially toxic proteins that have been reabsorbed. Alternatively, secreted clusterin may bind proteins or lipids in the tubular lumen, protect cell membranes from such compounds, and facilitate their urinary excretion.

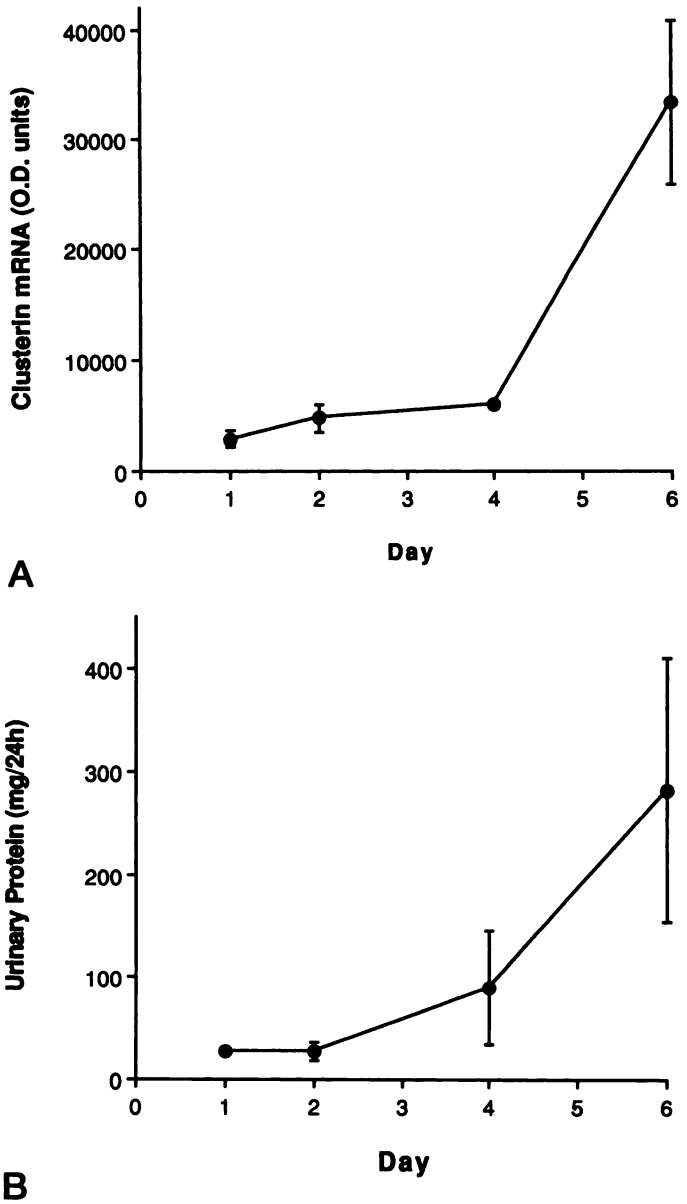

Figure 2. (A) Time course for clusterin mRNA. Renal clusterin mRNA was measured 1, 2, 4, and 6 d after PAN injection. An increase in clusterin mRNA was seen between days 4 and 6. (B) Urinary protein excretion. An increase in 24-h urinary protein excretion was seen after PAN injection. The time course for changes in clusterin mRNA was similar to that for urinary protein excretion. $n = 2$ for days 1, 2, and 4, and $n = 3$ for day 6.

In conclusion, tubular epithelial cell induction of clusterin occurs in the kidneys of nephrotic rats. The appearance of clusterin precedes the development of tubulointerstitial disease and may be a direct response to proteinuria.

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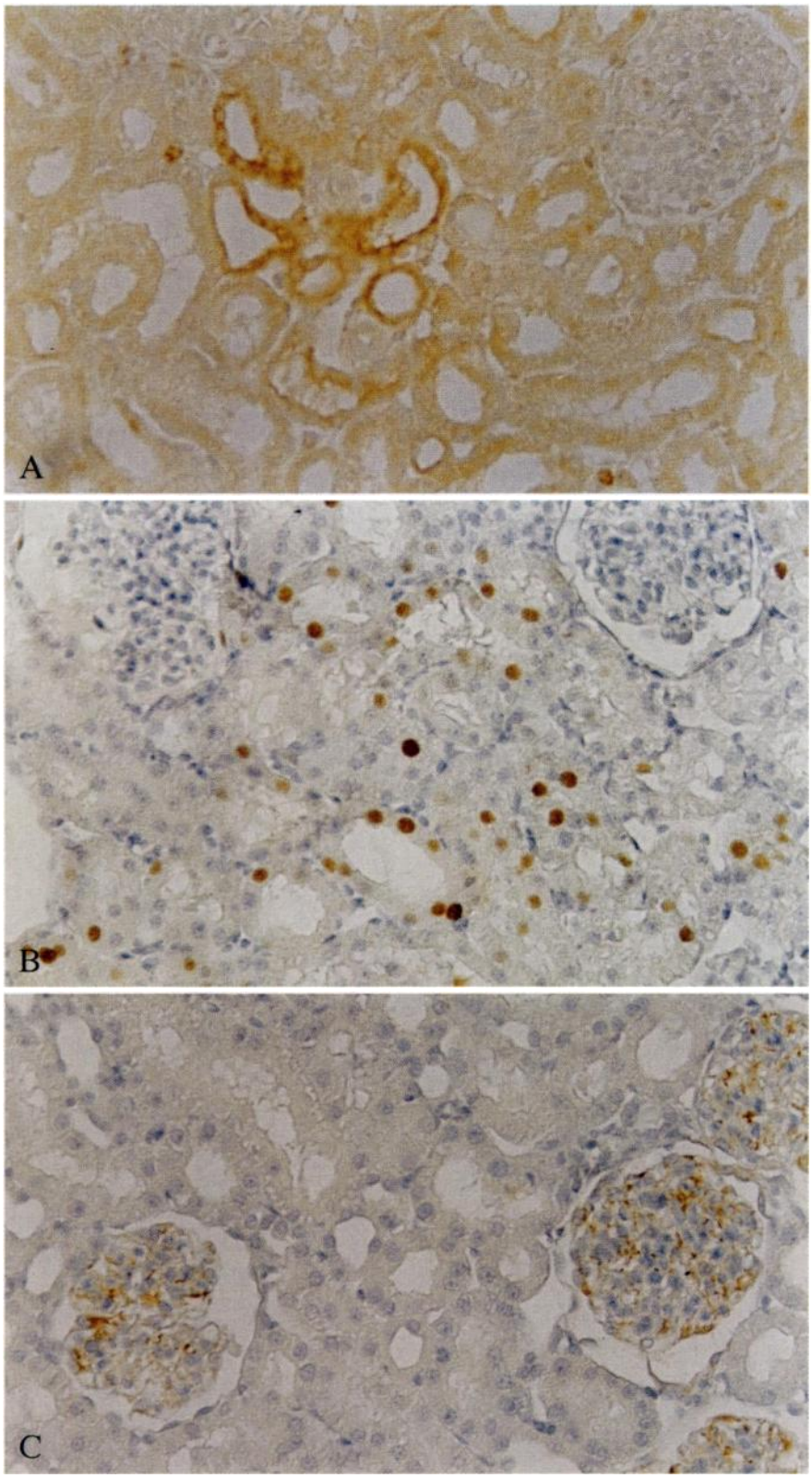

Figure 3. Immunohistochemistry. Sections from a rat kidney 6 d after injection of PAN. (A) Clusterin. Minimal tubulointerstitial disease is present. Clusterin staining, indicated by the brown reaction product, is present in some tubules. The glomeruli did not stain for clusterin. (B) proliferating cell nuclear antigen (PCNA). Brownish-red staining of PCNA-positive nuclei is seen in many tubules. No correlation was seen between tubules staining for clusterin and those containing PCNA-positive cells. (C) Vimentin. Vimentin staining was predominantly confined to glomeruli, a pattern different from that seen for clusterin.

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