Measurement of Urinary Clusterin as an Index of Nephrotoxicity (43335)

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Abstract. **Measurements of tissue immunoassayable clusterin, a protein associated with programmed cell death and tissue reorganization, were performed in rats treated with nephrotoxic doses of gentamicin sulfate. Adult Lewis rats were treated with 100 mg/kg/day of gentamicin sulfate for 12 days. Urine, serum, and tissue levels of clusterin protein were measured, as were urinary *N*-acetylβ-glucosaminidase (NAG) and serum creatinine levels. Induction of renal injury by gentamicin was detectable within 4 days by increased levels of urinary *N*-acetylβ-glucosaminidase (from 280 ± 66 (mean ± SD) to 910 ± 210 nmol/mg creatinine), and within 9 days of initiating gentamicin treatment by increased serum creatinine (from 0.5 ± 0.1 to 1.2 ± 0.4 mg/dl). Paralleling these changes, renal, urinary, and serum levels of clusterin increased 10-, 116-, and 3-fold ($P < 0.05$). Treatment with gentamicin sulfate did not increase clusterin levels in the seminal vesicle, ventral prostate, testis, or epididymis. The measurement of urinary or serum clusterin may play a role in the early detection of renal injury.**

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Clusterin is a dimeric glycoprotein with molecular mass of 80–85 kDa (1), originally isolated from the ram, that has been found in immunoreactive forms in multiple species (2). Clusterin protein or mRNA have been independently investigated under a number of different names, including sulfated glycoprotein-2 (SGP-2) (3), testosterone-repressed prostate message-2 (TRPM-2) (4) or protein-2 (TRPP-2) (5), and human serum protein (SP-40,40) (6). It is produced constitutively in many tissues, but is found at the highest protein levels in the epididymis and testis (7). In some tissues, this protein or its mRNA has been associated with programmed cell death (5) and tissue remodeling.

Programmed cell death (apoptosis) is an orderly process of separation of cells, degradation of DNA, and fragmentation and resorption of the cellular tissue (8, 9). In contrast to coagulative necrosis, programmed cell death is thought to result in a minimal immunological response. The process of programmed cell death requires gene expression and protein production (5). This process is well suited to the maintenance of homeostasis in tissues, allowing the resorption or replacement of cells without resulting in significant injury or destruction of gross tissue architecture or affecting adjacent cell function. Interestingly, clusterin found in human seminal plasma has been shown to inhibit complement-mediated hemolysis (6). It is possible that clusterin modulates the local immunological response to programmed cell death by complement inhibition. This action may be important to prevent normal cell injury during tissue remodeling. The cascade of gene expression associated with programmed cell death has been demonstrated previously in the rat kidney after unilateral ureteral obstruction (5, 10). Programmed cell death is thought to represent a ubiquitous remodeling process in most tissues.

Previous studies from other institutions on clusterin production after cell injury have investigated only gene expression or nonquantitative protein level evaluations (11). Clinical application of a marker for cell injury could optimally use fluids, such as blood or urine, or tissue obtained with minimally invasive pro-

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cedures. Our laboratories have the unique ability to quantify clusterin levels in tissues, blood, and urine using a radioimmunoassay developed at The Population Council (7). In this paper, we demonstrate that gentamicin-induced nephrotoxicity increases renal tissue concentrations and urinary excretion of clusterin that can be detected prior to changes in serum creatinine levels.

Materials and Methods

Adult male Lewis rats were treated for 0, 4, 9, or 12 days with intraperitoneal injections of gentamicin sulfate (100 mg/kg/day). For each data point, six rats were treated. All rats were maintained in metabolic cages and complete urinary collections were performed. At the time of sacrifice, renal, liver, prostate, testis, and epididymal samples were snap frozen at −70°C. Blood was obtained at the time of sacrifice by puncturing of the vena cava. The blood was allowed to clot, and sera were collected and stored at −20°C.

Preparation of Organ Cytosols. Tissue samples for clusterin protein determination were blotted dry after removal and then homogenized in 10 mM Tris and 10% glycerol buffer (pH 7.4) at 4°C using a polytron homogenizer. Samples were then centrifuged at 42,000 *g* for 1 hr, and the supernatant was maintained at −20°C until the radioimmunoassay was performed.

Clusterin Radioimmunoassay. The concentrations of clusterin in various tissue extracts were quantified by radioimmunoassay using previously described procedures (7, 12). Each unknown sample was run in duplicate and the concentrations of clusterin in these samples were interpolated against a standard curve calibrated using a pool of crude Sertoli cell-enriched culture medium, and the results were expressed as microliter equivalent (μl eq) of this pool. It was noted that 1 μl of this pool contained 4.31 ng of purified rat clusterin. The minimal detectable dose of the immunoassay was about 0.03 μl eq/assay tube, and 50% displacement was at 3.5 μl eq. The intra- and interassay coefficients of variation were 10% and 16%, respectively.

Urinary, Serum *N*-acetylβ-glucosaminidase, and Creatinine Evaluations. Urinary *N*-acetylβ-glucosaminidase (NAG) was assayed by a modification (13) of the method of Leaback and Walker (14). The urinary NAG activity is expressed as nanomoles of 4-methylumbelliferone liberated from 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide substrate per hour of incubation at 37°C per milligram of urine creatinine. The creatinine in serum and urine was measured by the alkaline picrate (Jaffe) reaction (13).

Results

Renal tissue clusterin levels increased significantly ($P < 0.05$) from 1.6 μg/g in untreated animals to 3.1 μg/g after 4 days of treatment with gentamicin. There was a further increase to 16 μg/g tissue after 12 days in gentamicin-treated animals (Fig. 1). Urinary clusterin protein excretion increased from 80 to 480 μg/mg of creatinine within 4 days of treatment with gentamicin sulfate (Fig. 2); after 12 days, urinary clusterin was further elevated to 9300 μg/mg of creatinine (for all evaluations, $P < 0.05$). The increases in clusterin were associated with a rise in urinary NAG from 280 to 910 within 4 days and peaked after 9 days of gentamicin treatment at 5000 nmol/mg of creatinine. It was still elevated at 3500 nmol of NAG/mg of creatinine after 12 days of treatment (Fig. 2). Serum creatinine increased from 0.5 mg/dl to 1.2 mg/dl at 9 days and to 1.8 mg/dl in rats treated for 12 days with gentamicin (Fig. 3). An increase in serum levels of clusterin protein was significant at 9 days and further increased when evaluated 12 days after the start of gentamicin treatment (Fig. 3). The magnitude of changes in serum clusterin is small relative to those in the kidney and urine.

In view of the fact that clusterin is produced in organs other than the kidney, the levels of this protein

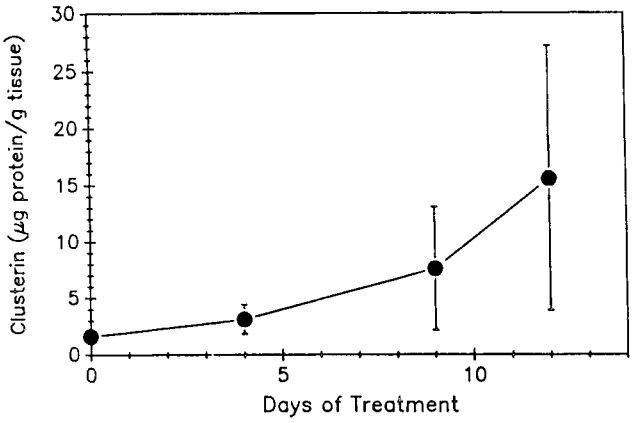

Figure 1. Renal tissue levels of clusterin protein after treatment of rats with 100 mg/kg/day of gentamicin sulfate. All results are presented as mean ± SD, and all error bars indicate 1 SD.

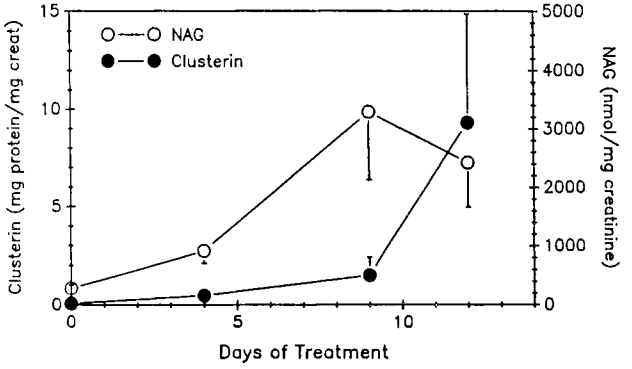

Figure 2. Urinary excretion of clusterin and *N*-acetylβ-glucosaminidase after treatment of rats with 100 mg/kg/day of gentamicin sulfate; mean ± SD.

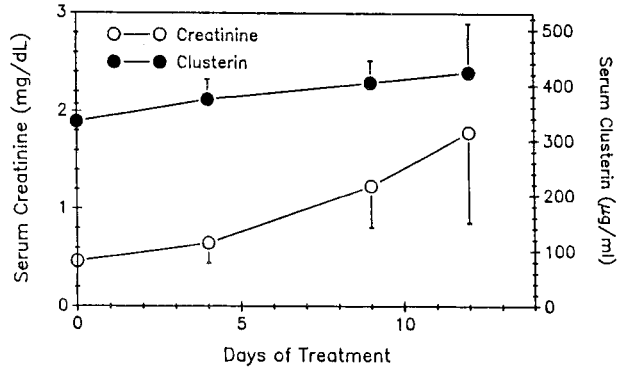

Figure 3. Serum creatinine and clusterin levels in rats treated with gentamicin sulfate (100 mg/kg/day); mean ± SD.

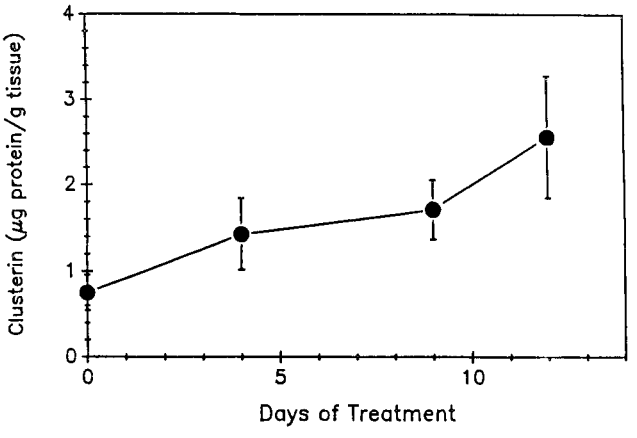

Figure 4. Hepatic tissue levels of clusterin protein in rats treated with gentamicin sulfate (100 mg/kg/day); mean ± SD.

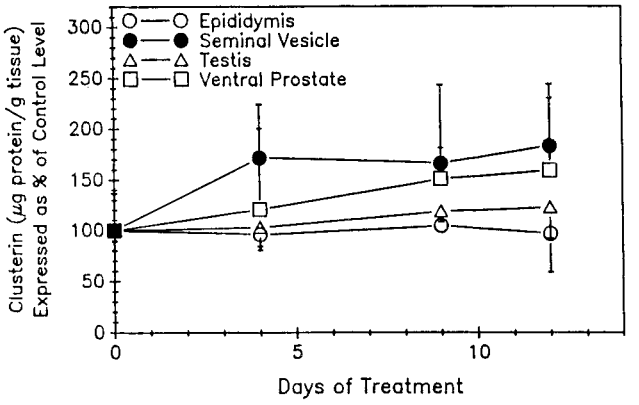

Figure 5. Clusterin protein levels in nonrenal tissues from rats treated with gentamicin sulfate (100 mg/kg/day). All values expressed as a percentage of control clusterin levels; mean ± SD.

were measured in tissues in which either clusterin protein or mRNA had been detected. Clusterin protein levels in the liver also increased from 0.77 to 1.4 μg/g of tissue after 4 days of treatment and further increased over the 12-day course of treatment (Fig. 4). In the male reproductive tract, clusterin levels in untreated animals were: epididymis, 443 μg/g of tissue; testis, 19 μg/g; seminal vesicle, 1.2 μg/g; and ventral prostate, 3.7 μg/g of tissue. Except for the 60% and 70% increases in clusterin concentration in ventral prostrate and seminal vesicles, respectively, other tissues did not change significantly (Fig. 5).

Discussion

Renal injury may be detected by analyzing an increase in serum creatinine, by increased urinary excretion of renal tubular cell enzymes, or by direct measurement of renal function (glomerular filtration rate). The accumulation of creatinine in serum reflects a decrease in glomerular filtration of creatinine, and, therefore, is an indirect measurement of renal function. However, renal tubular injury precedes the detection of increased serum creatinine. Optimally, the detection of renal tubular injury should occur at the time of the onset of cell injury and be differentiated from renal effects of drug administration or other events that cause a renal effect but no renal cellular injury.

Certain renal tubular cell lysosomal enzymes, such as NAG, β-glucuronidase, and β-galactosidase, or brush border enzymes, such as leucine aminopeptidase, may be released from renal tubular cells following tubular damage. Prescott (15) reported that the activity of these enzymes in urine may be a sensitive indicator of proximal tubular injury by toxins. However, the increase in NAG can occur in the absence of other evidence of renal injury (13). Therefore, NAG excretion may not represent actual cell injury, but rather simply a response of the renal tubular cell to the drug. This may be true for the other enzymes as well. Nevertheless, increased NAG excretion can be used as a sensitive indicator of the renal effect of drugs and chemicals (15). β_2-Microglobulin is a serum protein that goes through the glomerulus and is normally reabsorbed by tubular cells. Its increased excretion represents a failure of renal tubular reabsorption, a decrease of renal function. It may not reflect ongoing renal tubular cell injury or cell death.

Clusterin is found in many tissues. An increase in clusterin protein (7) or mRNA (5) has been demonstrated as a response to tissue-specific injury and during tissue remodeling. In the kidney, an increase in clusterin mRNA levels is detected within 10 hr after renal injury induced by ureteral obstruction (5, 10). The finding of increased expression of clusterin gene products after other types of renal injury led us to consider the use of immunoreactive urinary clusterin protein as a marker of drug-induced nephrotoxicity.

Our results indicate that both urinary clusterin and urinary NAG were elevated after only 4 days of gentamicin treatment, the first time point tested. The increase in urinary clusterin paralleled an increase in renal tissue clusterin protein. The increased production of clusterin by renal tissue, rather than merely increased

clusterin clearance, was suggested by the finding of 10- and 100-fold increases of clusterin concentrations in kidney and urine, respectively, versus a 20% increase in serum clusterin over the same time period of gentamicin treatment. The increase in clusterin levels in urine preceded the detection of a change in serum creatinine that occurred only after 9 days of gentamicin treatment. The variable intensity of effect of this dose of gentamicin on glomerular filtration rate in these experiments was reflected in the wide range of serum creatinine levels after 12 days of gentamicin treatment, as seen in Figure 4. Because of the variable effects on renal function, divergent levels of potential markers of renal injury would be expected. Individual animals showed significant variation in nephrotoxic effect of gentamicin sulfate, as reflected in the high standard deviations seen in Figure 3. As would be expected with varied nephrotoxic effects in individual animals, high variations in urinary NAG, urine, and serum clusterin levels, are demonstrated in Figures 1, 2, and 3.

Previous studies in our laboratories have indicated that there is no significant contribution to serum clusterin levels from the male reproductive tract either during organ regression (7) (in the prostate or seminal vesicles after androgen deprivation) or after epididymectomy or unilateral orchiectomy (16).

Taken together, our results indicate that, in the model of gentamicin-induced injury to the rat kidney, measurement of urinary clusterin protein may provide a noninvasive method for the early detection of renal injury. The early detection of renal injury may be useful for the clinician, since significant changes in creatinine clearance may occur before this injury is detected as a change in serum creatinine, the most common clinically used indicator of renal injury. Further studies are needed to learn if elevations in clusterin protein levels are always indicative of renal cell death and not just reversible injury or drug effect.

1. Cheng CY, Chen C-LC, Feng Z-M, Marshall A, Bardin CW. Rat clusterin isolated from primary Sertoli cell-enriched culture medium is sulfated glycoprotein-2 (SGP-2). Biochem Biophys Res Commun **155**:398–404, 1988.
2. Mathur PP, Bardin CW, Voglmayr JK, Cheng CY. Identification of immunoreactive clusterin in multiple species. Mol Androl **2**:127–143, 1990.
3. Collard MW, Griswold MD. Biosynthesis and molecular cloning of sulfated glycoprotein-2 secreted by rat Sertoli cells. Biochemistry **26**:3297–3303, 1987.
4. Bettuzzi S, Hiipakka RA, Gilna P, Liao S. Identification of an androgen-repressed mRNA in rat ventral prostate as coding for sulphated glycoprotein 2 by cDNA cloning and sequence analysis. Biochem J **257**:293–296, 1989.
5. Buttyan R, Olsson CA, Pintar J, Chang C, Bandyk M, Ng P-Y, Sawczuk IS. Induction of the TRPM-2 gene in cells undergoing programmed death. Mol Cell Biol **9**:3473–3481, 1989.
6. O'Bryan MK, Baker HWG, Saunders JR, Kirszbaum L, Walker ID, Hudson P, Liu DY, Glew MD, d'Apice AJF, Murphy BF. Human seminal clusterin (SP-40,40). J Clin Invest **85**:1477–1486, 1990.
7. Grima J, Zwain I, Lockshin RA, Bardin CW, Cheng CY. Diverse secretory patterns of clusterin by epididymis and prostate/seminal vesicles undergoing cell regression after orchiectomy. Endocrinology **126**:2989–2997, 1990.
8. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer **26**:239–257, 1972.
9. Kyprianou N, Isaacs J. Activation of programmed cell death in the rat ventral prostate after castration. Endocrinology **122**:552–562, 1988.
10. Sawczuk IS, Hoke G, Olsson CA, Connor J, Buttyan R. Gene expression in response to acute unilateral ureteral obstruction. Kidney Int **35**:1315–1319, 1989.
11. Bandyk MG, Sawczuk IS, Olsson CA, Katz AE, Buttyan R. Characterization of the products of a gene expressed during androgen-programmed cell death and their potential use as a marker of urogenital injury. J Urol **143**:407–412, 1990.
12. Cheng CY, Marshall A, Grima J, Mathur PP. Tissue distribution and heterogeneity of clusterin in the reproductive tract of rats. Mol Androl **2**:73–84, 1990.
13. Merle LJ, Reidenberg MM, Camacho MT, Jones BR, Drayer DE. Renal injury in patients with rheumatoid arthritis treated with gold. Clin Pharmacol Ther **28**:216–222, 1980.
14. Leaback DH, Walker PB. Studies on glucosaminidase. 4: The fluorimetric assay of N-acetyl-β-glucosaminidase. Biochem J **78**:151–156, 1961.
15. Prescott LF. Assessment of nephrotoxicity. Br J Clin Pharmacol **13**:303–311, 1982.
16. Schlegel PN, Li PS, Aulitzky WK, Saso L, Cheng CY, Goldstein M, Bardin CW. Effect of epididymectomy and orchiectomy on serum clusterin levels. Abstract presented to the New York Section, AUA, Annual Meeting, Budapest, Hungary, October 1–7, 1990.