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REGULAR ARTICLE

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Urinary clusterin levels in the rat correlate with the severity of tubular damage and may help to differentiate between glomerular and tubular injuries

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Abstract Clusterin is a secreted glycoprotein that is synthesized after several types of tubular injury. We therefore wondered whether the urinary excretion of clusterin could serve as a parameter to determine the severity of tubular damage. Using an affinity-purified rabbit antiserum raised against recombinant clusterin, we established an enzyme-linked immunosorbent assay to measure the urinary excretion of clusterin after bilateral renal ischemia, in the (*cy*/+) rat model of autosomal-dominant polycystic kidney disease and in the FHH rat model of focal segmental glomerulosclerosis. After bilateral renal ischemia, the urinary excretion of clusterin paralleled the excretion of total protein and albumin and correlated with the extent of tubular damage. Male (*cy*/+) rats, but not female (*cy*/+) rats, excreted more clusterin than age-matched (+/+) rats, a finding consistent with the more rapid course of the disease in males. FHH rats presented with pronounced proteinuria and albuminuria but did not excrete increased levels of clusterin. Urinary clusterin levels could therefore serve as a valuable marker for the severity of tubular damage. Furthermore, clusterin may also help to differentiate between tubular and glomerular forms of proteinuria.

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Introduction

Clusterin is a multi-faceted glycoprotein that has been isolated on various occasions and is therefore known by several names, e.g., sulfated glycoprotein-2, glycoprotein III, testosterone-repressed prostate message-2, glycoprotein of 80 kDa, cytolysis inhibitor, and apolipoprotein J (Rosenberg et al. 1993; Rosenberg and Silkensen 1995). Rat (Collard and Griswold 1987) and human (Jenne and Tschopp 1989; Kirszbaum et al. 1989) clusterin is comprised of 426 and 427 amino acids, respectively, and has an apparent molecular mass of 70–80 kDa because of extensive post-translational modifications (Collard and Griswold 1987; Murphy et al. 1988). It is composed of one α-subunit and one β-subunit (Collard and Griswold 1987; Murphy et al. 1988), which arise by cleavage of the precursor peptide (Collard and Griswold 1987) and are joined by a five-disulfide-bond (Kirszbaum et al. 1992). Since clusterin is synthesized in many tissues and is found in various physiologic fluids such as plasma, semen, and cerebrospinal fluid, it has been proposed to serve a variety of functions, including the regulation of complement activity, the protection of cells from stress, the transport of lipids, and sperm maturation. Furthermore, its production is also increased in the early stages of normal organ development and after tissue injury (Rosenberg et al. 1993; Rosenberg and Silkensen 1995).

In the kidney, clusterin is transiently synthesized after the induction of the metanephrogenic mesenchyme. As tubular maturation progresses, clusterin synthesis subsides and nephron segment-specific proteins are produced (French et al. 1993; Harding et al. 1991). In addition to its production during renal development, a reappearance of clusterin has also been observed in various kidney diseases. Clusterin is induced after ureteral

obstruction (Buttyan et al. 1989; Pearse et al. 1992), during ischemia-reperfusion injury (Rosenberg and Paller 1991; Witzgall et al. 1994), and in the peri-infarct region after subtotal nephrectomy (Correa-Rotter et al. 1992). The synthesis of clusterin has also been reported in animal models of hereditary polycystic kidney disease (PKD; Cowley and Rupp 1995; Harding et al. 1991; Obermüller et al. 1997), and its presence in the injured tubular epithelium and in cyst wall epithelia therefore possibly represents the de-differentiated state of these cells. In the post-ischemic kidney, clusterin-positive debris has been seen in the lumen of proximal tubules (Witzgall et al. 1994). These observations suggest that measurement of urinary clusterin exretion may be a useful clinical marker of renal tubular injury.

The purpose of this study has been to develop an enzyme-linked immunosorbent assay (ELISA) to measure urinary clusterin levels in various types of renal injuries and to evaluate the usefulness of urinary clusterin concentrations as a clinical marker. We have therefore measured urinary clusterin levels in a rat model of bilateral renal ischemia (Kränzlin et al. 1996) and in the (*cy*/+) rat model of autosomal-dominant PKD (ADPKD; Cowley et al. 1993; Obermüller et al. 1997; Schäfer et al. 1994), which represent two diseases characterized by tubular injury, and in fawn-hooded hypertensive (FHH) rats as a glomerular injury model (Kriz et al. 1998).

Materials and methods

Animals

All animals were allowed free access to standard rat chow (containing 19% protein) and tap water. Experiments were conducted in accordance with the German Animal Protection Law and were approved by the local government (Regierungspräsidium Karlsruhe, Germany).

Bilateral renal ischemia

Ten Sprague-Dawley rats (3-month-old males) weighing 470 to 550 g were subjected to renal ischemia (n=6) or to a sham operation (n=4) as previously described (Kränzlin et al. 1996). In this model, the serum creatinine values peak on day 1 (~1.1 mg/dl post-ischemia vs ~0.3 mg/dl in sham-operated rats), and the serum urea values peak on day 2 (~140 mg/dl post-ischemia vs ~40 mg/dl in sham-operated rats). Briefly, the animals were anesthetized by an intramuscular injection of ketamine (75 mg/kg b.w.) and xylazine (6 mg/kg b.w.). During the whole procedure, they were placed on a thermostatically controlled heating pad. After an abdominal incision was made, the renal arteries were freed from the surrounding tissue and occluded with a microaneurysm clamp immediately after the injection of 100 IU of heparin into the tail vein. During this time, the open abdomen was covered with moist gauze to minimize fluid loss. The clamps were removed 45 min later, and reperfusion of the kidneys was confirmed by visual inspection. The incision was then sutured, and buprenorphin (0.02 mg/kg b.w.) was administered as an analgesic. Plasma samples were drawn from the retrobulbar plexus 2 days before the operation and on days 3, 4, and 8 after the operation. Urinary samples were collected by housing the rats in metabolic cages from 4 pm to 8 am 2 days before the operation and on days 1, 2, 3, 4, 8, and 15 after the operation.

*The (*cy*/+) rat model for ADPKD*

A total of 43 male and female (*cy*/+) and (+/+) rats (3 months old) were chosen for analysis, viz., 13 female (+/+) rats, 17 female (*cy*/+) rats, 6 male (+/+) rats, and 7 male (*cy*/+) rats. The animals were maintained as an inbred colony in the Animal Care Facility in Mannheim under the control of N. Gretz. They were placed in metabolic cages for 24 h to collect urine samples. In the morning, the animals were removed from the metabolic cages, and blood samples were collected under light ether anesthesia. The genotype of the rats was confirmed by histological diagnosis.

FHH rat model of focal segmental glomerulosclerosis

We used 14 rats aged 12–13 months to collect 24-h urine samples and blood samples as described above.

Tissue preparation

For histological examination, kidneys were usually immersion-fixed; alternatively, they were removed after perfusion = fixation. A midportion of each kidney was embedded in paraffin and 5-µm-thick paraffin sections were stained with hematoxylin and eosin. In addition, a midportion of the perfusion-fixed kidneys was incubated in an 18% sucrose solution (in phosphate-buffered saline) for 3 h on a shaking platform at room temperature before being snap-frozen in liquid-nitrogen-cooled isopentane. Tissues were stored at –80°C until further use.

Generation of an affinity-purified polyclonal rabbit anti-clusterin antiserum

Recombinant rat clusterin (comprising amino acids 146–361) with a T7-flag epitope at the NH_2-terminus and six histidines at the COOH-terminus was produced in *Escherichia coli* by using the prokaryotic expression plasmid pET21b (Novagen, Madison, WI). After purification over His·Bind resin (Novagen), rabbits were immunized with the recombinant protein. The same clusterin fragment was also produced in *E. coli* as a glutathione *S*-transferase (GST) fusion protein by using the prokaryotic expression vector pGEX-KG (Guan and Dixon 1991). The GST/clusterin-fusion protein was purified over a glutathione-agarose column, coupled to CNBr-Sepharose 4B (Amersham Pharmacia Biotech, Freiburg, Germany), and then incubated under constant agitation with the rabbit anti-clusterin antiserum overnight at room temperature. After the column had been washed with 10 mM TRIS-HCl pH 7.5/170 mM NaCl, with 10 mM TRIS-HCl pH 7.5/170 mM NaCl/0.02% Tween 20, with 10 mM TRIS-HCl pH 7.5/0.5 M NaCl/0.02% Tween 20, and finally with 10 mM TRIS-HCl pH 7.5/170 mM NaCl, the bound anti-clusterin antibodies were eluted with 0.2 M glycine HCl pH 2.0/0.2 M NaCl. The eluted antiserum was immediately neutralized with 1 M TRIS pH 8.8 and stored at –20°C following the addition of 0.02% NaN_3. Western blot analysis demonstrated that the affinitiy-purified antiserum reacted with clusterin, but not with the T7-flag epitope, the histidine tag, or GST (data not shown).

Western blot analysis of urine samples

Samples of urine (1 ml) were incubated with 1 ml 20% (w/v) ice-cold trichloroacetic acid for 15 min on ice and centrifuged for 10 min at 14,000 rpm and 4°C. The pellet was washed twice with 100% ethanol before being resuspended in a reducing SDS-sample buffer. Proteins were separated on a 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene-difluoride membrane. The membrane was probed with affinity-purified rabbit anti-clusterin antiserum (diluted 1:5,000) for 2 h and subsequently with horseradish-peroxidase-conjugated goat anti-rabbit IgG secondary anti-

body (diluted 1:20,000; no. A-0545, Sigma, Deisenhofen, Germany) for 1 h. Signals were detected by chemiluminescence.

Direct competitive anti-clusterin ELISA

96-well polystyrene ELISA plates (no. 655 180, Greiner Labortechnik, Frickenhausen, Germany) were coated with 1 µg recombinant T7-flag-tagged and histidine-tagged clusterin in borate-buffered saline (BBS) pH 8.5 for 3 h at 30°C. The plates were then washed three times with BBS/0.5% Tween 20 and twice with H_2O and subsequently blocked with 1% (w/v) bovine serum albumin (BSA) in BBS for 2 h at 30°C. At the same time, 100 µl each of the rat urine samples were incubated with the affinity-purified rabbit anti-clusterin antiserum (diluted 1:3,000) for 2 h at 37°C; serial dilutions of the purified T7-flag-tagged and histidine-tagged clusterin in 1% BSA/BBS served as standards. After the 96-well plates had been washed, the preincubated urine and purified clusterin samples were transferred to the plates and incubated overnight at 4°C. The following morning, the plates were washed and incubated for 1 h at 30°C with 100 µl horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:10,000 in 1% BSA/BBS. After the plates had been washed again, the bound antibody was visualized by the addition of 100 µl developing solution per well and subsequent incubation for 2 hours at room temperature in the dark; developing solution was prepared by combining 6 µl 30% H_2O_2, 360 µl tetramethylbenzidine (3 mg/ml; no. 8622, Merck, Darmstadt, Germany) dissolved in acetone, 5.64 ml 0.1 M citric acid, and 4.36 ml 0.2 M Na_2HPO_4. The reaction was stopped by the addition of 100 µl 5.3% H_2SO_4 (prepared by an 18-fold dilution of 97%–99% H_2SO_4), and the optical density was read at 450 nm on a BioRad Microplate Reader (BioRad Laboratories, Munich, Germany). Urine samples and purified clusterin samples were measured in duplicate. A standard curve was constructed from the serial dilution of the purified clusterin, and urinary clusterin concentrations were calculated from this standard curve. In the assay system used, the lower limit of detection was ~10 ng/ml, and the upper limit of quantitation was ~1 µg/ml; the inter-assay and intra-assay coefficient of variation was 6.9% and 3.1%, respectively.

Serum and urine chemistry

Plasma concentrations of creatinine and urea nitrogen, and urine concentrations of creatinine were measured on a Hitachi 717 automatic analyzer. Protein concentrations in plasma and urine were determined by using a Coomassie-Brilliant-Blue-based assay. Urinary albumin concentrations were determined by ELISA as previously described (Obermüller et al. 2001).

Statistical analysis

All values are presented as mean ± standard deviation. Analysis of variance (ANOVA) with the Bonferroni correction and a *t*-test procedure were used for statistical analysis (SAS, Cary, NC, USA). Differences were considered significant when $P<0.05$.

Results

Synthesis of clusterin after bilateral renal ischemia and in polycystic kidneys

In order to demonstrate the usefulness of our affinity-purified rabbit polyclonal anti-clusterin antiserum, we performed immunohistochemistry on post-ischemic kidney sections and on sections from polycystic kidneys. Two days after an ischemic insult, strong staining of the luminal debris in proximal tubules (Fig. 1a) and of the apical surface of distal tubular cells (not shown) was observed, similar to that described previously (Witzgall et al. 1994). In male (*cy*/+) rats, strong clusterin immunoreactivity was found predominantly in distal tubular cells (Fig. 1b), confirming previous findings obtained by in situ hybridization (Obermüller et al. 1997). These observations suggested that the measurement of urinary clusterin concentrations could be a useful laboratory marker of renal tubular injury.

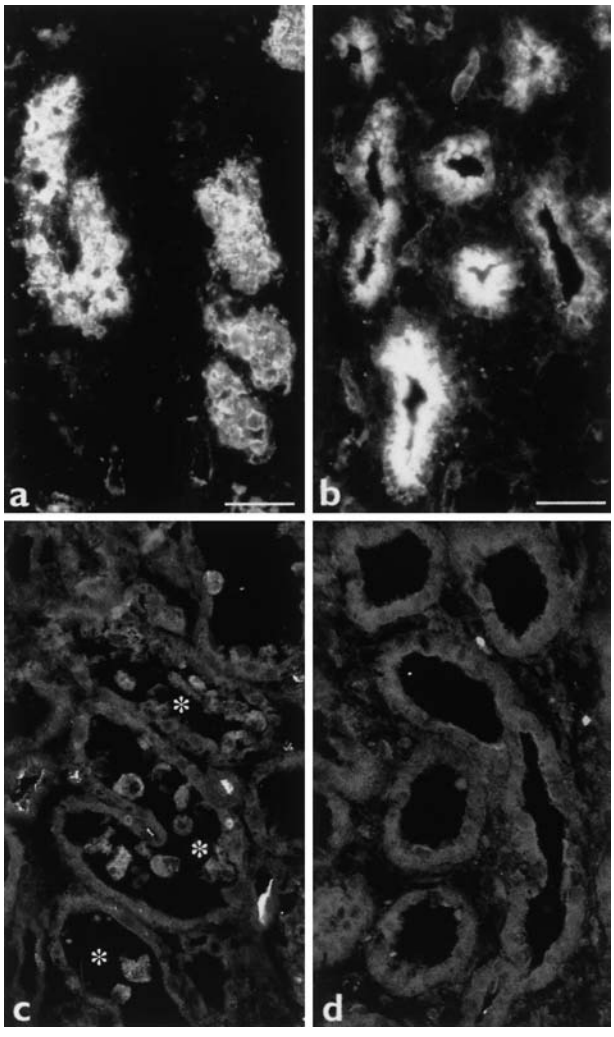


Fig. 1a–d Synthesis of clusterin after bilateral renal ischemia and in (*cy*/+) rats. Cryostat sections of perfusion-fixed kidneys were subjected to immunofluorescence with the affinity-purified anti-clusterin antiserum. **a** In several profiles in the outer stripe, which correspond to the S3-segment of the proximal tubule, strong labeling of luminal debris can be seen 2 days after the ischemic insult. **b** Distal tubular cells of male (*cy*/+) rats show prominent clusterin immunoreactivity in their apical compartment. Staining with a 1:100 dilution of the pre-immune serum showed no specific staining in either post-ischemic (**c**) or polycystic (**d**) kidneys (*asterisks* in **c** indicate tubular profiles with luminal debris). *Bars* 50 µm

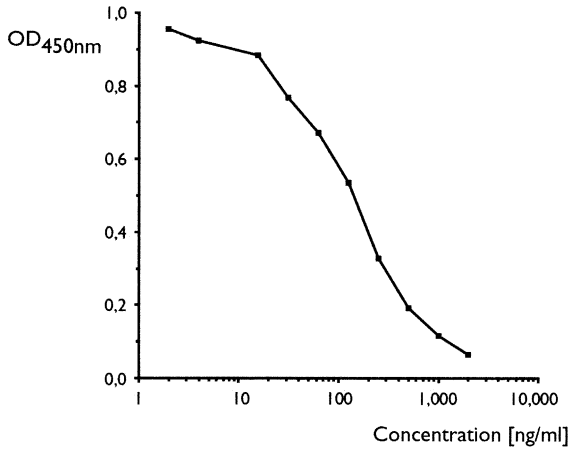


Fig. 2 Standard curve for the determination of urinary clusterin levels. Serial dilutions of purified recombinant clusterin were used in an ELISA to establish a standard curve. The useful range of the ELISA begins at a concentration of ~10 ng/ml and ends at ~1 μg/ml

Quantitation of urinary clusterin

To determine urinary clusterin concentrations, we set up a competitive single-antibody ELISA. A standard curve was established by using serial dilutions of purified clusterin (Fig. 2). As mentioned above, the lower limit of detection of clusterin was ~10 ng/ml and the upper limit of quantitation was ~1 μg/ml.

Urinary clusterin excretion after renal ischemia

In post-ischemic rats, urinary clusterin excretion was significantly increased on days 2, 3, and 4 compared with the sham-operated rats (Fig. 3a). This time-course of urinary clusterin excretion mirrored the excretion of total protein and of albumin. Total protein excretion was significantly increased on day 1 (Fig. 3b), whereas albumin excretion was significantly increased on days 2 and 3 after the ischemic insult (Fig. 3c).

Urinary excretion in (*cy*/+) rats

Male (*cy*/+) rats develop a more severe course of PKD than females (Gretz et al. 1995). We therefore measured the urinary excretion of clusterin in both genders at 3 months of age.

Whereas we did not find a significant difference in urinary clusterin excretion between 3-month-old female (*cy*/+) and (+/+) rats, 3-month-old male (*cy*/+) rats excreted significantly more clusterin than age-matched male wild-type rats and female (*cy*/+) rats, an observation consistent with the slower disease progression in female (*cy*/+) rats (Fig. 4a). The findings concerning urinary clusterin excretion were paralleled by similar results when total protein and albumin excretion were measured. The 3-month-old male (*cy*/+) rats excreted more total protein (Fig. 4b) and albumin (Fig. 4c), whereas in female (*cy*/+) rats neither the excretion of to-

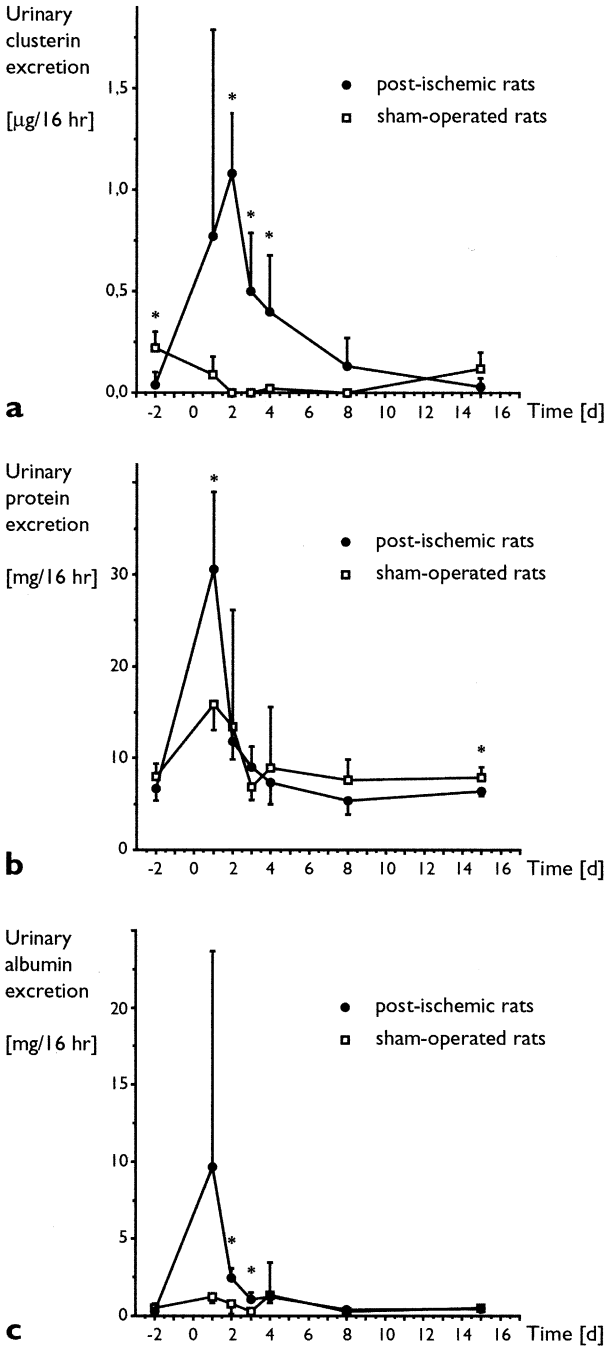
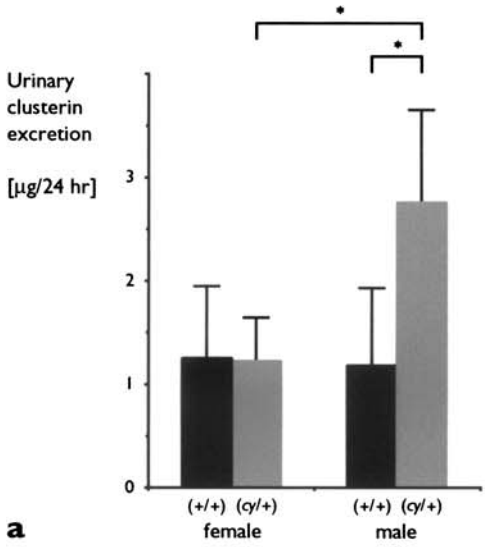
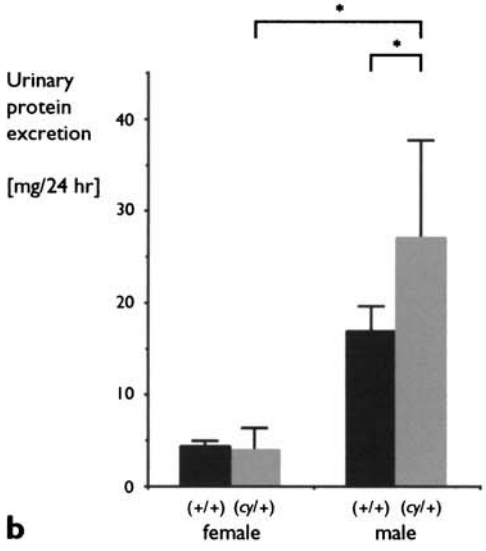
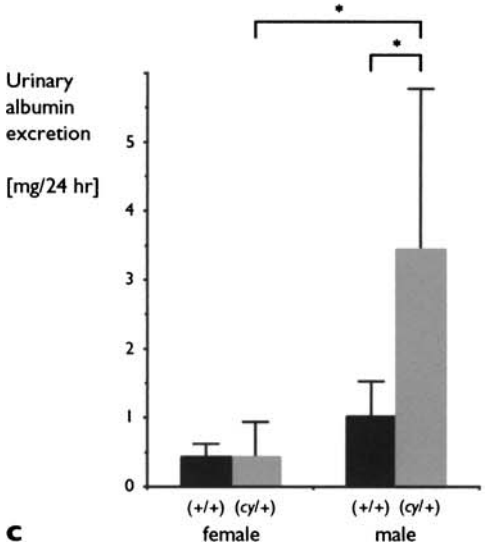


Fig. 3a–c Urinary excretion of clusterin, total protein, and albumin after bilateral renal ischemia. Two days before the ischemic insult and 1, 2, 3, 4, 8, and 15 days after ischemia for 45 min (day of ischemia = day 0), rats were placed for 16 hours in metabolic cages to collect their urine. Urinary excretion of clusterin was significantly elevated at 2, 3, and 4 days after ischemia (**a**), whereas the excretion of total protein was elevated at 1 day after ischemia (**b**), and the excretion of albumin was elevated at 2 and 3 days after ischemia (**c**). Data are given as mean ±SD (**P*<0.05)







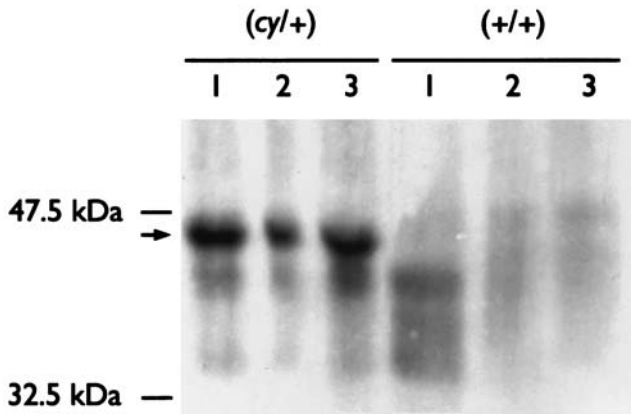


Fig. 5 Western blot analysis of urinary clusterin excretion in (*cy*/+) rats. Urine from male (*cy*/+) and (+/+) rats was subjected to Western blot analysis with the affinity-purified anti-clusterin antiserum. A band of ~45 kDa molecular mass was detected (*arrow*) only in the urine from (*cy*/+) rats; this value corresponds to the mobility described for the α-subunit of rat clusterin (Collard and Griswold 1987)

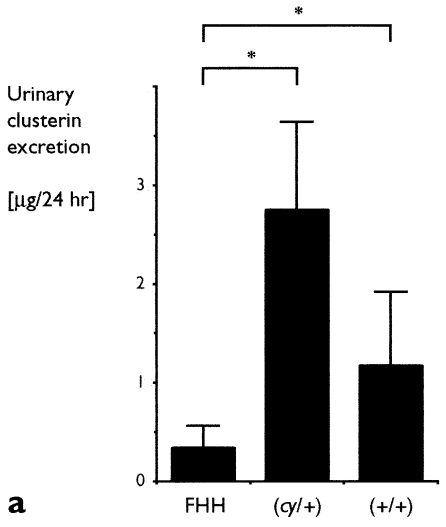
tal protein nor that of albumin was significantly different from that of age-matched (+/+) rats.

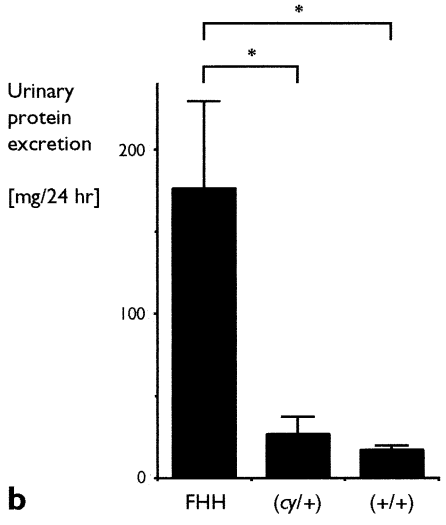
The ELISA data were subsequently confirmed by Western blot analysis. Equal volumes of urine from three male (*cy*/+) and three male (+/+) rats were precipitated with trichloroacetic acid, and the precipitated proteins were subjected to polyacrylamide gel electrophoresis under reducing and denaturing conditions. After transfer, the gel was incubated with the affinity-purified anti-clusterin antiserum resulting in the detection of a ~45-kDa clusterin band only in the urine samples from the (*cy*/+) and not from the (+/+) rats (Fig. 5).

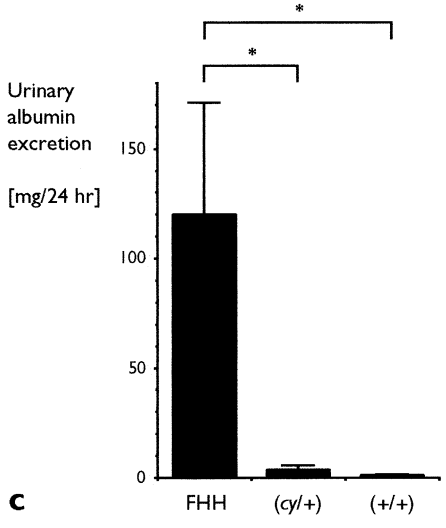
Urinary excretion of clusterin in FHH rats

Both the ischemic model of acute renal failure and PKD represent tubular injury models. Proteinuria, however, may result not only from tubular, but also from glomerular damage. Since it is sometimes difficult to differentiate between a tubular and a glomerular origin of proteinuria, we wanted to investigate whether clusterin could serve as a useful marker for tubular proteinuria. FHH rats develop focal segmental glomerulosclerosis, which is accompanied by proteinuria (Fig. 6b) and albuminuria (Fig. 6c) with levels even higher than those in 3-month-old male (*cy*/+) rats. On the other hand, urinary clusterin

Fig. 4a–c Urinary excretion of clusterin, total protein, and albumin in (*cy*/+) rats. Three-month-old (+/+) and (*cy*/+) rats were placed for 24 h in metabolic cages to collect their urine. Whereas female (*cy*/+) rats did not excrete more clusterin (**a**), total protein (**b**), or albumin (**c**) than female (+/+) rats, male (*cy*/+) rats showed significantly increased values regarding these parameters, in comparison with both male (+/+) and female (*cy*/+) rats. Data are given as mean ±SD (*P<0.05; ANOVA with Bonferroni correction)







levels of FHH rats were lower than those in 3-month-old male (+/+) rats (Fig. 6a).

Discussion

Although many functions have been assigned to clusterin, a clear consensus regarding its true role(s) has not yet emerged. In particular, it is not clear whether (and if so, in what direction) clusterin influences cell death. The recent generation of clusterin-deficient mice promises to shed some light on this question, but again the picture seems to be more complicated than expected. In autoimmue myocarditis, clusterin serves a protective function (McLaughlin et al. 2000), whereas in hypoxic-ischemic brain injury, both a negative (Han et al. 2001) and a positive (Wehrli et al. 2001) effect of clusterin have been suggested. In the kidney, clusterin is found prominently only in the early stages of nephron development (French et al. 1993; Harding et al. 1991), but it reappears after various types of acute and chronic injury (Buttyan et al. 1989; Correa-Rotter et al. 1992; Cowley and Rupp 1995; Harding et al. 1991; Nath et al. 1994; Obermüller et al. 1997; Pearse et al. 1992; Rosenberg and Paller 1991; Witzgall et al. 1994). Since urinary excretion of clusterin increases after gentamicin-induced acute renal failure (Aulitzky et al. 1992; Eti et al. 1993), we have hypothesized that it could also be used as a valuable laboratory marker of renal ischemia-reperfusion damage. Furthermore, we have tested its use in a hereditary model of renal disease, i.e., the (*cy*/+) rat model of ADPKD, which slowly develops chronic renal failure.

In post-ischemic rats, we have shown that urinary clusterin excretion peaks 2 days after the ischemic insult. This correlates well with its 24-h peak of production in the damaged S3-segment of the proximal tubule as determined by quantitative confocal laser microscopy (Witzgall et al. 1994). The S3-segment represents that portion of the nephron that is most susceptible to complete ischemia (Venkatachalam et al. 1978), and it therefore is not surprising that large amounts of clusterin can be found at this location. We believe that most of the excreted clusterin originates from the cellular debris floating in the lumen of the S3-segment, so that the 1-day delay between the peak of clusterin synthesis in the S3-segment and the urinary excretion of clusterin can be explained by the time that it takes for the debris to be flushed out from the kidney. In addition to its synthesis in the S3-segment, clusterin can also be detected in the distal tubule (Witzgall et al. 1994; this study) but this source probably only contributes to a minor portion of clusterin excretion; however, this cannot be unambigu-

Fig. 6a–c Urinary excretion of clusterin, total protein, and albumin in FHH rats. FHH rats, which suffer from focal segmental glomerulosclerosis, excrete significantly increased levels of total protein (**b**) and albumin (**c**) compared with male (*cy*/+) and (+/+) rats, but significantly less clusterin (**a**). Data are given as mean ±SD (*P<0.05; ANOVA with Bonferroni correction)

ously decided by immunohistochemistry. The time course of urinary clusterin excretion correlates well with the observed proteinuria and albuminuria, both of which peak 1 day after the ischemic insult, although urinary clusterin levels appear to be elevated for longer.

Clusterin also serves as a marker of renal damage in a chronic form of injury, i.e., the (*cy*/+) rat model of ADPKD. Here again, it reflects the severity of the disease, because the urinary excretion of clusterin is only increased in 3-month-old male (*cy*/+) and not in female (*cy*/+) rats. It is well known that male rats are more severely affected by ADPKD than female rats (Gretz et al. 1995), a finding that can also be seen in the current study. In addition to the increased excretion of clusterin, only the 3-month-old male (*cy*/+) rats suffer from pronounced proteinuria and albuminuria. Although cysts in the (*cy*/+) rat model originate almost exclusively from proximal tubules in the cortex (Cowley et al. 1993; Obermüller et al. 1997; Schäfer et al. 1994), clusterin is detectable not only in cyst wall epithelia, but also at multiple sites along the nephron both by in situ hybridization (Obermüller et al. 1997) and by immunohistochemistry (this study). The strongest clusterin immunoreactivity has been found in the distal tubule, and we therefore assume that the urinary clusterin mostly represents excreted protein and not clusterin present in detached cells as is probably the case after ischemic injury. Although at present we do not understand why the highest levels of clusterin are found in the distal tubule, we have noticed that the number of clusterin-positive distal tubules rises with increasing cyst formation. It is tempting to speculate that these distal tubular profiles belong to the same nephrons in which the cysts develop, but it was beyond the scope of the present study to prove this hypothesis.

The results from the acute and chronic renal failure models indicate that clusterin can serve as a valuable marker of tubular injury. It is unlikely that the urinary clusterin observed in these circumstances originates in the serum, since we have not detected increased excretion of clusterin in male FHH rats. The FHH rat strain is characterized by the spontaneous development of focal segmental glomerulosclerosis, a mild increase in systemic blood pressure, an elevated glomerular capillary pressure, and progressive proteinuria and albuminuria; FHH rats therefore represent a prototypical model of glomerular damage (Kriz et al. 1998). Because of alterations of the glomerular filtration barrier, these rats excrete large amounts of serum-derived total protein and albumin. Clusterin excretion in FHH rats, however, is not increased markedly, probably because of the low serum concentration of clusterin of 50–100 μg/ml (Jenne and Tschopp 1989; Murphy et al. 1988) compared with that of albumin. Therefore, the determination of urinary clusterin may be helpful for differentiating between tubular and glomerular forms of proteinuria; however, it is clear that other models of glomerular and tubular injury have to be examined to validate this hypothesis. Furthermore, urinary clusterin excretion as a marker of tubular damage should be useful independent of the site of tubular injury, because clusterin has been observed both in proximal and in distal tubules (Obermüller et al. 1997; Witzgall et al. 1994; this report).

Further studies, such as the determination of the age- and gender-dependent excretion of clusterin, are needed to determine the usefulness of a clusterin assay in a clinical setting, but a cheap and quick determination of urinary clusterin could help to screen for pre-existing tubular damage in patients undergoing potentially nephrotoxic examinations. Another possible application would be to monitor the natural course of the disease or the effectiveness of a therapeutic drug in patients with ADPKD.

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