Clusterin protects renal tubular epithelial cells from gentamicin-mediated cytotoxicity

RICHARD A. GIRTON,1 DAVID P. SUNDIN,2 AND MARK E. ROSENBERG1

1University of Minnesota, Minneapolis, Minnesota 55455; and 2Indiana University Medical Center and Veterans Affairs Medical Center, Indianapolis, Indiana 46202

Received 23 February 2001; accepted in final form 7 November 2001

Girton, Richard A., David P. Sundin, and Mark E. Rosenberg. Clusterin protects renal tubular epithelial cells from gentamicin-mediated cytotoxicity. Am J Physiol Renal Physiol 282: F703–F709, 2002. First published December 4, 2001; 10.1152/ajprenal.00060.2001.—Clusterin is a heterodimeric secreted glycoprotein that is upregulated after acute renal injury. In aminoglycoside nephrotoxicity, clusterin is induced in the tubular epithelium and increased levels are found in the urine. In this study, we developed an in vitro model of gentamicin-induced cytotoxicity in renal proximal tubule cells and tested whether clusterin protected these cells from injury. LLC-PK1 cells were incubated with varying concentrations of gentamicin in serum-free media, and cytotoxicity was quantified by lactate dehydrogenase release and confirmed by vital dye exclusion. A dose-dependent increase in cytotoxicity occurred with gentamicin concentrations up to 27 μg/ml. Clusterin decreased cytotoxicity in a dose- and time-dependent manner at 6, 12, and 24 h, whereas albumin, used as a control protein, had no effect. In contrast to the aminoglycoside model, when cells were injured by depletion of ATP, clusterin had only a minimally protective effect. LLC-PK1 cells did not express megalin, a receptor that can mediate the uptake of both clusterin and aminoglycosides into proximal tubule cells. Uptake of gentamicin into LLC-PK1 cells was observed despite the absence of megalin. In conclusion, clusterin specifically protects against gentamicin-induced renal tubular cell injury by a megalin-independent mechanism.

AMINOGLYCOSIDES CONTINUE to cause significant morbidity in clinical medicine. Despite increasing attention to appropriate dosing of aminoglycosides, the incidence of nephrotoxicity has not changed substantially in the last two decades, remaining at ~20% (18). If >14 days of therapy are required, then the incidence of nephrotoxicity approaches 50% (18). Nephrotoxicity manifests as a slow-onset, acute tubular necrosis with injury occurring primarily in proximal tubules. Because of the slow clearance of aminoglycosides from tubular cells (20), recovery from nephrotoxicity can take up to 4–6 wk, which significantly complicates patient care.

Aminoglycosides are concentrated severalfold in proximal tubular cells by pinocytosis at the luminal brush-border membrane (28, 35, 40) as well as by an undefined, quantitatively less significant basolateral mechanism (5, 6, 14). Although the polyanionic inositol phospholipids are believed to be the major membrane anionic binding sites for aminoglycosides (35), megalin, a member of the low-density lipoprotein (LDL) receptor family of glycoproteins, has been shown to mediate, in part, the cellular uptake of aminoglycosides (23). It is not known whether there are other potential mechanisms of cell entry for aminoglycosides. After endocytosis, aminoglycosides accumulate in lysosomes. These lysosomes eventually swell with excess lipid debris, giving the usual electron-microscopic appearance of myelin figures within lysosomes (15).

Several potential mechanisms of aminoglycoside-induced renal tubular cell cytotoxicity have been proposed. These include binding to phosphatidylinositol bisphosphate, which prevents its subsequent hydrolysis by phospholipase C (31). This process could interfere with potentially important signal transduction processes. Aminoglycosides may also alter inositol phospholipid distribution, with a subsequent decrease in the activity of Na-K ATPase leading to cell swelling and death (19). Furthermore, once in the cell, aminoglycosides localize to endocytic vesicles and lysosomes, where they have been shown to diminish the activity of phospholipases A and C and sphingomyelinase, eventually leading to lysosomal instability (30). Finally, aminoglycosides can localize to mitochondria, where they have been shown in vitro to competitively displace magnesium from the inner mitochondrial membrane, allowing greater monovalent cation influx, which subsequently leads to mitochondrial swelling and dysfunction (44). Although none of these effects singly accounts for all the toxicity seen with aminoglycosides, they likely all contribute to cell injury (10).

Clusterin is a unique secreted heterodimeric glycoprotein of unknown function that is widely distributed throughout the body. It circulates in the blood at concentrations ranging from 50 to 300 μg/ml (34). Clusterin is markedly induced in various models of renal tubular injury, including aminoglycoside nephrotoxicity (1, 7, 33). In these models, clusterin is synthesized...
by healthy-appearing and damaged tubular cells and is subsequently secreted into the urine. In experimental models of aminoglycoside toxicity, urinary clusterin concentrations are a marker of progressive renal injury (33). The function of clusterin in renal and other organ injury is incompletely understood, but it is believed to play a protective role by scavenging cellular debris and toxic denatured macromolecules (12, 29).

On the basis of the renal tubular cell induction of clusterin after treatment with aminoglycosides and the protective effect of clusterin in other tubular cell injury models, we hypothesized that clusterin can protect renal cells from aminoglycoside cytotoxicity. In this study, we first developed an in vitro model of gentamicin-mediated renal tubular cell injury and then used the model to show that, at physiological concentrations, clusterin can indeed protect these cells from gentamicin-mediated cytotoxicity.

MATERIALS AND METHODS

Materials. All tissue culture and laboratory reagents were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Porcine renal tubular epithelial cells (LLC-PK1) were obtained at passage 196 from American Type Culture Collection (Rockville, MD). CytoTox 96 [lactate dehydrogenase (LDH) release] cytotoxicity kits were obtained from Promega (Madison, WI). An automated plate reader with a 490-nm filter (model 550) was obtained from Bio-Rad Laboratories (Hercules, CA).

Cell culture method. Cells were cultured in DMEM supplemented with 44 mM NaHCO3, 25 mM HEPES, and 10% fetal bovine serum, and antibiotics (Pen/Strep) were added. Cells were grown to confluence in an atmosphere of 95% air-5% CO2 and passed weekly by trypsinization and a 6- to 10-fold dilution of the original cell number. Media were changed every third day. Under these conditions, the cells grow rapidly and form domes typical of this well-documented cell line. Cells were used for up to 10 passages and maintained their doming phenotype during this time.

Purification of clusterin. Clusterin was purified from fresh human sera by immunoaffinity chromatography that used the mouse anti-human clusterin mAb G7 (gift of Brendan Murphy, Melbourne, Australia) as previously described (38). Purity of the clusterin samples was documented by SDS-PAGE performed according to the method of Laemmli (17). As expected, clusterin appeared as a 40-kDa doublet under reduced conditions and an 80-kDa band under unreduced conditions (data not shown). The concentration of clusterin was measured by the Coomassie blue dye method of Bradford (3) by using bovine serum albumin (fraction V powder) as a standard.

Gentamicin cytotoxicity model. LLC-PK1 cells were grown to confluency in sterile tissue culture-treated polystyrene 96-well plates (Costar; Corning, Corning, NY), at which time the media were removed and the monolayers were washed with PBS. Controls included wells devoid of cells that were treated identically to the monolayer-containing wells. The cells were then incubated in serum-free DMEM (100 µl/well) humidified in 5% CO2 at 37°C for 48 h. All wells were then replenished with 100 µl of fresh serum-free DMEM containing gentamicin sulfate (0–27 mg/ml) and incubated for 24 h. At that point, aliquots of the media (50 µl/well) were removed and placed in the corresponding wells of a sterile 96-well “assay plate” for measurement of LDH [see Cytotoxicity assay (LDH release)]. As an additional measure of cytotoxicity, and to demonstrate the distribution of injury, we examined the cells for trypan blue exclusion. After incubation with gentamicin, the cells were washed with PBS and then stained with 0.4% trypan blue in PBS for 5 min. The monolayer was then washed twice with PBS and immediately imaged with a phase-contrast inverted microscope.

Experimental studies. To test the effect of clusterin on gentamicin-induced cytotoxicity, LLC-PK1 cells were serum starved for 48 h and then pretreated for 2 h with either chloroquine (0–100 µg/ml) or albumin (0–100 µg/ml) as a control. Gentamicin was then added to each well to achieve a final concentration of 27 mg/ml, and cytotoxicity was measured at 6, 12, and 24 h.

To test the effect of clusterin in a different model of cytotoxicity, we used the anticytotoxic agent AATP depletion, in which confluent cells in 96-well plates were pretreated for 2 h with chloroquine (0–100 µg/ml) and then incubated with anticytotoxic agent (1.5 µM) for 1 h in DMEM without serum, glucose, or glutamine, but otherwise supplemented as above. Cells were then washed with sterile PBS and subsequently incubated in the same nutrient-free media, this time free of anticytotoxic agent and supplemented with chloroquine (0–100 µg/ml), for 24 h. At the end of the incubation period, an aliquot of medium was removed from each well and used for cytotoxicity determination.

Cytotoxicity assay (LDH release). LDH release was measured colorimetrically by the CytoTox 96 nonradioactive cytotoxicity assay according to the manufacturer’s instructions. Briefly, after the plate containing the cell monolayers was centrifuged for 5 min at 250 g, a “substrate mix” (50 µl; proprietary mixture of diaphorase, lactate, and NAD+) in Tris-buffered formazan dye and Triton X-100) was added to each well of a sterile polystyrene assay plate containing 50-µl aliquots of media and was incubated in the dark at room temperature for 30 min. Control wells were included in each experiment to account for effects of medium color, dilution, and
and experimental conditions on the colorimetric assay. After color development, 1 M acetic acid (50 μl) was added to stop the reaction, and the optical density was measured in a 96-well plate reader at 490 nm. Cytotoxicity was calculated by the following formula

\[
\% \text{Cytotoxicity} = 100 \times \frac{(E - S)}{(M - S)}
\]

where E is experimental release, M is maximal release, and S is spontaneous release. On a given occasion, each experiment was done in quadruplicate, and experiments were repeated on at least two other occasions.

**Western blotting for megalin.** Cells were grown to confluency as described in Cell culture method. After reaching confluency, monolayers were washed three times with PBS, and lysates of the LLC-PK1 cells were prepared by lysing cells in SDS sample buffer (20 mM Tris, pH 8, 10% glycerol, 0.005% bromphenol blue, 2% SDS). Samples were heated at 90°C for 5 min in SDS sample buffer and electrophoresed on 4–20% SDS-PAGE gels (Novex, Carlsbad, CA) according to the method of Laemmli (17). Gels were then transferred (2 h at 12 V and 4°C) to nitrocellulose paper, and the blots were blocked overnight at 4°C in blocking buffer (50 mM Tris-HCl, 0.15 M NaCl, pH 7.4, 0.2% Tween 20, 1% BSA) and detected with primary antibody to megalin diluted 1:40,000 in Tris-saline-Tween (TST; 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4, 0.2% Tween 20). The antibody was a rabbit polyclonal antibody to a peptide from the rat cytoplasmic tail of megalin (a generous gift from Drs. Robert Orlando and Marilyn Farquhar) (13). The blots were washed with TST and detected with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin (Southern Biotechnology, Birmingham, AL) diluted 1:80,000 for 60 min at room temperature. The blots were again washed with TST and then developed by using the enhanced chemiluminescence SuperSignal West Pico system (Pierce, Rockford, IL). Rat brush-border membrane (10 μg) was run as a positive control.

**Texas red-labeled gentamicin uptake in LLC-PK1 cells.** Cells were cultured on glass coverslips in 12-well plates as described above, except they were not grown to complete confluency for imaging purposes. For experiments, media...
were aspirated, and the coverslips were rinsed three times with PBS and then incubated in media containing Texas red-labeled gentamicin (TRG; 1 mg/ml) for 1 h at 37°C. TRG was purchased from Molecular Probes (Eugene, OR), where it had been custom prepared and purified. After 1 h, cells were washed three times with cold PBS and then fixed overnight at 4°C by using 4% paraformaldehyde-PBS. The next day, coverslips were washed three times with PBS, mounted on glass slides in a 1:1 mix of PBS and glycerol containing 1% 1,4-diazabicyclo[2.2.2]octane (Sigma) and then sealed with acrylic fingernail polish. Analysis of the TRG fluorescence was examined by using a Bio-Rad MRC-1024 scanning laser confocal microscope (Bio-Rad, Richmond, CA) with a Nikon ×100 numerical aperture 1.4 Plan APO objective (Nikon, Natick, MA). Samples were illuminated by an argon-krypton laser, which used the same parameters in all cases. This laboratory has previously used all these techniques (41, 42).

Statistical analysis. All data are expressed as means ± SD. Intergroup differences were analyzed by one-way ANOVA and the Dunnett multiple comparisons test. Significance was taken to be \( P < 0.05 \).

RESULTS

To establish our in vitro model of gentamicin nephrotoxicity, LLC-PK₁ cells were incubated with varying concentrations of gentamicin for 24 h. Dose-dependent cell death occurred with a maximum of 83.82 ± 1.62% cytotoxicity by LDH release at a gentamicin concentration of 27 mg/ml (Fig. 1). These data were confirmed by using trypan blue exclusion after 24 h of exposure to gentamicin (Fig. 2). As can be seen in the figure, increasing numbers of cells were unable to exclude the vital dye because gentamicin concentration was increased. Furthermore, cell death was heterogeneous because some cells remained able to exclude the dye even at a gentamicin concentration of 27 mg/ml.

To test the protective effect of clusterin in our model, LLC-PK₁ cells were preincubated with varying concentrations of clusterin after 6 and 12 h preincubation of confluent LLC-PK₁ cells in 1.5 μM antimycin A in serum-glucose-glutamine-free media, the cells were incubated with depleted media and clusterin (0–100 μg/ml) for the following 23 h. Percentage of cytotoxicity was determined by LDH release. Error bars are SDs. \(^* P < 0.05\) vs. no clusterin.

To test whether clusterin could protect against milder degrees of gentamicin-induced injury, we examined the effects of clusterin after 6 and 12 h of incubation with gentamicin. At 24 h, clusterin offered significant partial protection of LLC-PK₁ cells from gentamicin-mediated cytotoxicity at every tested concentration (Fig. 3). Albumin had no protective effect (data not shown). This protective effect of clusterin at 24 h was dose-dependent at concentrations up to 20 μg/ml. To test whether clusterin could protect against milder degrees of gentamicin-induced injury, we examined the effects of clusterin after 6 and 12 h incubations with gentamicin (Fig. 3). As expected, shorter incubation times resulted in less cytotoxicity. A time-dependent protective effect of clusterin was observed in these shorter incubation periods because higher concentrations of clusterin were required to see protection compared with the 24-h time period.

To test whether clusterin had a similar protective effect in a different model of cell injury, antimycin A-induced cytotoxicity, a model of ATP depletion, was selected for comparison. This model consistently produced ~50% cytotoxicity when LLC-PK₁ cells were treated for 1 h with 1.5 μM antimycin A and serum-glucose-glutamine-free media, followed by 23 h of incubation in standard media (see METHODS). When varying concentrations of clusterin were added by using the same 2-h clusterin preincubation as was done with the gentamicin cytotoxicity experiments, minimal protection was observed at clusterin concentrations of 10, 50 and 100 μg/ml, but not at other concentrations (Fig. 4).

Next, we wanted to demonstrate whether clusterin’s protective effect could be by means of a megalin-mediated mechanism. Megalin is a multiligand receptor and a member of the growing LDL receptor superfamily.
Clusterin has previously been shown to be bound and taken up by megalin (16), and megalin has also been implicated as the aminoglycoside receptor in proximal tubule cells (23). As a result, we performed Western blot analysis on the cells by using a well-characterized anti-megalin antibody (13) to determine whether megalin was expressed (Fig. 5). The results demonstrated that our LLC-PK₁ cells did not express megalin compared with the positive control of isolated rat brush-border membranes.

Finally, to demonstrate that gentamicin can enter these LLC-PK₁ cells even in the absence of megalin, we examined cell uptake of TRG. After 60 min of incubation, labeled gentamicin was seen inside cells (Fig. 6).

DISCUSSION

Aminoglycoside nephrotoxicity is a significant problem that limits the clinical use of this important class of antibiotics. Insight into the mechanisms of aminoglycoside entry into tubular cells and subsequent cytotoxic events could provide important clues toward reducing the toxicity of these agents. In this study, we report on a reproducible in vitro model of gentamicin cytotoxicity that can be used to study the pathophysiology of aminoglycoside renal toxicity and to test the efficacy of therapeutic interventions. In this model, both dose- and time-dependent toxicity of gentamicin occurred.

The major finding of this study was the dose-dependent reduction of proximal tubular cell cytotoxicity by clusterin. This partial protective effect was seen with physiological concentrations of clusterin, was specific for clusterin because albumin had no protective effect, and occurred as early as 6 h after initial exposure to gentamicin. The protection was time dependent because longer incubations at lower concentrations of clusterin resulted in a protective effect, whereas shorter incubations at these same concentrations did not. Clusterin was a more potent inhibitor of toxicity in the aminoglycoside model compared with the ATP de-
pletion model, where protection was minimal and required high physiological clusterin concentrations.

The ability of clusterin to be cytoprotective is consistent with what has been seen in other models of cell injury. For example, clusterin protects renal tubular cells from oxidant stress induced by hydrogen peroxide or endogenously produced oxidants (36) and can inhibit LDL-induced lipid hydroperoxide formation and monocytic transmigration in cocultures of human artery wall cells, suggesting a protective role in atherosclerosis (25). Overexpression of clusterin protects against cell death induced by tumor necrosis factor, heat shock, and oxidants in other cell lines (11, 37, 43). Consistent with this finding is the increased cell death observed when clusterin production is inhibited by antisense transfection of prostate cells exposed to tumor necrosis factor (37). Clusterin has also been demonstrated to reduce the cytotoxicity of amyloid-β (1–40) in primary cultures of rat mixed hippocampus (2).

Clusterin may exert its protective effect by interfering with the uptake of aminoglycosides or by modulating subsequent cell injury. Megalin has been shown to be a specific receptor for clusterin and mediates clusterin uptake into renal tubular cells (16). Thus both clusterin and aminoglycosides are endocytosed by proximal tubular cells after binding to megalin. Therefore, clusterin could potentially inhibit aminoglycoside uptake by competitively binding to megalin, thereby preventing passage of the aminoglycoside from phospholipids to megalin for internalization. However, inhibition of uptake by clusterin through this megalin-dependent process was not responsible for the protective effect, because the LLC-PK₁ cells used in this study do not express megalin. Previous investigators have shown variable expression of megalin in LLC-PK₁ cells (9, 21, 26, 27). Alternatively, clusterin could inhibit uptake by binding to aminoglycosides in the extracellular space or through its ability to alter the lipid environment of the cell (4, 8). After endocytosis of aminoglycosides, we hypothesize that clusterin could influence cytoxic events through its ability to scavenge lipid byproducts or to protect the microenvironment of the cell through its ability to bind to and solubilize denatured proteins generated during cell injury (12, 29). The induction and secretion of clusterin in in vivo renal injury models (33) and the data demonstrating that clusterin functions as an extracellular chaperone (12) suggest that internalization of clusterin is not needed to explain its cytoprotective effects. An alternative explanation is that exogenously added clusterin could be internalized and exert its protective effects through an intracellular mechanism. In fact, internalization of clusterin has been demonstrated in other cells and tissues, including F9 embryonal carcinoma cells and epididymal epithelium (16, 24). In these cells and tissues, uptake is dependent on megalin, which is not expressed by our LLC-PK₁ cells. Although primarily a secreted protein, at least one truncated and apparently active form of clusterin has been identified intracellularly (32). This intracellular form of clusterin should have similar functional domains and thus potentially function as an intracellular molecular chaperone. Therefore, clusterin could act by shuttling toxic lipid products out of the cell (25).

Nephrotoxicity remains a major limitation in the clinical use of aminoglycosides. No protective agents limiting this toxicity have been identified. The ability of LLC-PK₁ cells to internalize aminoglycosides by a megalin-independent mechanism suggests megalin should not be the only target for blocking uptake. Additional studies are needed to explore a possible role for clusterin as a cytoprotective agent. The mechanism and site of action of clusterin first need to be defined. It is unclear whether clusterin is blocking uptake of aminoglycosides or acting in the extracellular compartment. If clusterin is functioning to block uptake, then it will be necessary to define its active site to develop peptides or peptide mimetics that can be delivered to the luminal side of proximal tubules. Active sites for other actions of clusterin have been defined (39). If clusterin is acting as a molecular chaperone, it may be possible to induce clusterin in tubular cells or deliver it to vulnerable cells by means of parenteral administration. The model of in vitro gentamicin toxicity we have developed offers a new system to better understand the pathophysiology of aminoglycoside nephrotoxicity and to begin to develop therapeutic agents to lessen the toxicity of these important antibiotics.

In summary, we have established an in vitro model of aminoglycoside nephrotoxicity and have demonstrated a protective effect of clusterin in this system. The reduction in cytotoxicity conferred by clusterin is independent of megalin and may be related to its ability to scavenge toxic lipid byproducts and denatured proteins.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants R01-DK-48452 (M. E. Rosenberg) and R01-DK-55527 (D. P. Sundin), National Research Service Award 5F32-DK-09777 (R. A. Girton), a National Kidney Foundation of Minnesota Young Investigator Award (R. A. Girton), and an American Heart Association Established Investigator grant (M. E. Rosenberg).

REFERENCES


