Cultured Human Proximal Tubule Cells as a Model for Aminoglycoside Nephrotoxicity*†

MARY ANN SENS, M.D., PH.D.,
GORDON R. HENNIGAR, M.D.,
DEBRA J. HAZEN-MARTIN, PH.D.,
JOHN G. BLACKBURN, PH.D.,
and DONALD A. SENS, PH.D.

DEPARTMENTS OF PATHOLOGY AND PHYSIOLOGY
MEDICAL UNIVERSITY OF SOUTH CAROLINA,
CHARLESTON, SC 29425

ABSTRACT

Despite numerous clinical and animal studies, the initial injury and pathogenesis of aminoglycoside nephrotoxicity remains unclear. To compliment and extend existing research avenues, a cell culture model system representative of the human proximal tubule (HPT) was tested to determine its applicability for use in studies assessing aminoglycoside-induced cellular toxicity. For this determination, the proximal tubule cell cultures were exposed to increasing concentrations of streptomycin and monitored for cell death and light and electron microscopic changes under both confluent (resting) and subconfluent (actively-dividing) culture conditions. Confluent cultures exposed to streptomycin were also assessed for possible alterations in transport activities by monitoring the electrical properties of the cells through Ussing chamber analysis. Both the confluent and subconfluent cultures demonstrated concentration-dependent toxicity to streptomycin. Ultrastructural analysis disclosed that both actively-dividing and stationary cultures contained "myeloid bodies" within the cytoplasm, consistent with those known to occur in vivo. In studies relating cell numbers to the dosage and time of exposure to streptomycin, the confluent cultures demonstrated an "insult-recovery" period at toxic, but sub-lethal, concentration, again correlating to the known in vivo experience with this class of antibiotics. The subconfluent cultures demonstrated increased resistance to the toxic effects of streptomycin, again mimicking the clinical experience with aminoglycoside toxicity. Chamber analysis, at a streptomycin dose well below the toxic level, indicated changes in the transport activities of these cultured cells. It is proposed

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† Send reprint requests to Mary Ann Sens, M.D., Ph.D., Division of Cellular Biopathology, Department of Pathology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425.
that the use of cultured proximal tubule cells could be a useful model system to extend current research avenues assessing the mechanism of aminoglycoside nephrotoxicity.

**Introduction**

The nephrotoxic potential of the aminoglycoside antibiotics is well documented in the literature, and clinical evidence of nephrotoxicity occurs in approximately 10 percent of patients receiving these antibiotics.\(^2,10,16\) It has been demonstrated that the initial insult affects the renal proximal tubules as evidenced by the early histopathologic lesions largely confined to this site.\(^8,11\) Further studies have confirmed the early ultrastructural appearance of cytoplasmic "myeloid bodies"\(^11\) in the proximal tubule cells along with alterations in tubular function\(^13\) and release of tubule proteins into the urine\(^1,17\) as a consequence of aminoglycoside administration. The myeloid bodies have also been reported to be excreted into the urine by aminoglycoside-treated animals,\(^18\) and many researchers believe that these bodies are the earliest morphological evidence of aminoglycoside nephrotoxicity. Thus, available evidence strongly suggests that the renal proximal tubule is the primary site affected in aminoglycoside-induced nephrotoxicity.

Despite the localization of pathologic damage to the proximal tubules in both clinical and animal model studies, the initial injury and mechanism of cellular toxicity remains unclear. While many studies have attempted to delineate the initial biochemical event leading to aminoglycoside nephrotoxicity and renal failure, no agreement has been reached on which alterations are the primary or secondary events mediating the toxic response. A cell culture model could prove helpful in the definition of the mechanism of cellular toxicity. A valid cell culture model would serve to remove as many secondary complications as possible, i.e., glomerular involvement, tubular obstruction, vascular compromise, and systemic complications such as electrolyte imbalances and infections. As such, the present study examines a culture system based on the human renal proximal tubule grown in serum-free medium as a possible model for use in the study of aminoglycoside nephrotoxicity.\(^3,5\) For these initial studies, the aminoglycoside streptomycin was chosen for characterization of the model system since it is not concentrated in the kidney cortex under in vivo conditions, as are other aminoglycoside antibiotics,\(^12\) and does not have multiple isomeric forms like gentamicin. Although differences in the nephrotoxic potential of the aminoglycosides have been shown to exist, both clinical and experimental observations have strongly suggested that a similar mechanism of nephrotoxicity exists for this entire antibiotic class.\(^4,9,21\) The results of this study will demonstrate that exposure of cultured proximal tubule cells to streptomycin results in cellular changes similar to those documented to occur in the in vivo setting.

**Materials and Methods**

**Reagents**

Dulbecco's modified Eagles' (DME), Ham's F-12 growth medium, fetal calf serum, and trypsin versene (0.05 percent, 0.02 percent) were obtained* as were the serum-free growth medium components.† Aminoglycoside antibi-
otics‡ and Bovine type I collagen§ were also obtained. Other items used included Millicell HA filters,‖ tissue culture plasticware of the Corning trademark and reagents from the preparation of routine solutions,** and twelve-well cell culture plates.** The water utilized in all reagent and media preparation was obtained from a Milli Q system.¶

Tissue Culture

Cultures of human proximal tubule (HPT) cells were grown as described previously.³,⁵ Briefly, the cells were grown from explant fragments of human renal cortex in a defined growth medium consisting of a 1:1 mixture of DME and F-12 supplemented with selenium (5 ng per ml), insulin (5 µg per ml), transferrin (5 µg per ml), hydrocortisone (36 ng per ml), triiodothyronine (4 pg per ml), and epidermal growth factor (10 ng per pml). Subculture of these cells produced a uniform population of epithelial cells that formed domes at confluency. Enzyme histochemical profiles of the cells for acid phosphatase, alkaline phosphatase, glucose 6-phosphate, β-glucoronidase, nonspecific esterase, 5' nucleotidase, dinucleotide phosphate (NAD) diaphorase, reduced nicotinamide-adenine dinucleotide phosphate (NADP) diaphorase, succinic dehydrogenase and carbonic anhydrase all exhibited consistent reactivities. Staining of the cells for keratin, Factor VIIIIR:Ag, and Tamm-Horsfall proteins were uniformly negative. These results parallel the enzyme profile within the proximal tubule and are not consistent with an origin from other parts of the nephron nor of the interstitial cells of the kidney. Additionally, both the electrical properties and ultrastructural profiles of the cells were supportive of an origin from the human proximal tubule.

For the measurement of cell growth and ultrastructure, cultures of HPT cells at passages 3–4 were grown and passaged without antibiotics for two passages and then subcultured into 12-well plates, with each well containing two ml of growth medium. Confluent or resting culture experiments were performed following a 1:2 subculture. In these studies, the cell numbers remained stable in the control cultures throughout the experimental course. Subconfluent studies were performed following a 1:5 subculture, and the control wells demonstrated an exponential growth phase during the experimental course. Following the appropriate subculture, the cultures were fed fresh growth medium containing a given concentration of streptomycin. All concentrations were performed in triplicate. The concentrations utilized were: 6500, 5000, 3500, 2500, 1250, 750, 500, 250, 100, 50, 25, and 5 µg per ml. The cells were fed fresh growth medium every three days.

Throughout the experimental course (two weeks for confluent cultures, three weeks for subconfluent cultures), the cells were monitored using an Olympus IM inverted microscope. Every third day, cell counts were obtained using the Olympus inverted microscope interfaced via a Hitachi video camera to the Zeiss Videoplan, a computer-linked image analysis system. Random fields of cells were selected and the cells in the fields counted using the mouse attachment and digitizing tablet. For each concentration, a minimum of 20 fields was selected and counted for each well at each time point. All concentrations were tested in triplicate. The criterion for acceptance of individual time points was the finding of no statistical difference in cell numbers within the triplicate samples at each con-

‡ U.S. Biochemical, Cleveland, OH.
§ Collagen Corp., Palo Alto, CA.
‖ Millipore Corp., Bedford, MA.
¶ Fisher Scientific Corp., Atlanta, GA.
** CoStar, Cambridge, MA.
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concentration. All statistical analyses were performed utilizing the Videoplan software and included the Student's two-sided t-test as well as basic statistical methods such as mean, standard deviation, and standard variance.

ELECTRON MICROSCOPY

Throughout the time course of the experiment, triplicate cultures were processed for electron microscopy every third day. Monolayers were fixed in situ with 2.5 percent glutaraldehyde in a pH 7.4, 0.1 M phosphate-buffered saline at room temperature for one hour followed by routine dehydration and embedding in Epon 812. After polymerization, the cultureware plastic was removed. Ultrathin sections were examined after uranyl acetate-lead citrate staining in a JEOL 100S electron microscope. For each concentration of streptomycin, two to four areas of each culture well were randomly selected for embedding. From each resulting Epon block, separate grids were processed which contained multiple cell profiles.

The resulting electron micrographs were quantitated utilizing the Videoplan image analysis system. The cell area, cell nuclear area, myeloid body number and area, vacuolar area and number, and the cytoplasmic area were determined. For each concentration and day examined, at least 50 cell profiles were obtained. The software for the measurement and corresponding statistical analysis was obtained from Zeiss. The total myeloid body area, the total vacuolar area, and the cytoplasmic area of each cell present in the electron micrographs were then measured with the semi-automated Zeiss IBAS image analysis system. Additional statistical measurements (mean, weighted mean, and percent of control) were performed on the IBM AT using the dBASE III Plus and Systat software packages.

ELECTROPHYSIOLOGY

For electrophysiologic characterization, the cells were plated at a 1:1 subculture ratio onto collagen-coated (bovine collagen, type I) Millicell HA filters utilizing standard subculture procedures. The cells were fed fresh growth medium every three days and utilized for electrophysiological analysis following 10 days in culture. The confluent filters were placed into a locally-constructed Ussing chamber and bathed with tissue culture fluid aerated with five percent CO₂ in air. The exposed filter area was one square centimeter. The pH and temperature of the bathing solution were maintained at 7.4 and 37°C, respectively. Streptomycin was added to the growth medium bathing the filter preparations four days after subculture.

The electrical characteristics of the cells were measured with a locally-constructed voltage clamp. Potential difference (PD) and short-circuit current (Isc) were measured directly. The sample was maintained under open circuit conditions except during the measurement of short-circuit current. The resistance (R) was determined from the potential produced by passing a constant current pulse (10μA a 200msec duration) across the filter preparation. The time constant for the epithelial preparation was 30msec.

The electrical measurements were made with commercial calomel electrodes. Agar saturated with potassium chloride was used to connect the electrodes to the Ussing chamber. Electrode polarization potentials and fluid resistances were measured and compensated. The filters (no cells) did not contribute to any of the electrical measurements. Transepithelial potential differences are referenced to the “contra-luminal” side of the cell monolayer. All measurements were taken after the electrical parameters had stabilized at peak values (within
30 min). The Student's t-test was utilized to assign significance between groups. The filter preparations were stained with hematoxylin and orange G and examined by light microscopy for confluency and damage at the conclusion of each experiment.

Results

CONCENTRATION STUDIES

As seen in figure 1, increasing the growth medium concentration of streptomycin produced a corresponding decrease in the number of viable HPT cells. Although the results depicted are for confluent cultures of HPT cells, the subconfluent cultures yielded equivalent results. In both cases, the effects of streptomycin were minimal, and statistically, no difference from control cells was observed until the dose reached 500 μg per ml. When analyzed on the last day of culture growth, all cultures exposed to streptomycin at concentrations in excess of 500 μg per ml demonstrated statistically significant (p < 0.01) reductions in cell growth compared to controls receiving no streptomycin. The highest concentration of streptomycin tested (6,500 μg per ml) produced large decreases in cell numbers; however, even this concentration was not 100 percent lethal to the HPT cells. The response of the HPT cells to a specific concentration of streptomycin was time-dependent and is discussed separately later.

TIME COURSE OF CONFLUENT CULTURES EXPOSED TO STREPTOMYCIN

Different time-dependent patterns of streptomycin toxicity were demonstrated for different concentrations of the antibiotic (figure 2). These patterns could be condensed into five general classes of cell response. The first pattern, demonstrated at very low streptomycin concentrations (50 μg per ml and below) exhibited no significant differences in cell growth from control cells unexposed to streptomycin, and the results were superimposable upon one another. The second pattern, noted at streptomycin concentrations between 100 and 500 μg per ml, demonstrated an initial minimal decrease in cell numbers early in the time course of exposure followed by a full recovery to control values by the end of the experimental time course. The third pattern, noted at streptomycin concentrations of 750 and 1250 μg per ml, demonstrated a large initial decrease in cell numbers followed by a recovery phase notable for a large sustained increase in cell proliferation. The cells in the recovery phase never approached control values. This was most likely due to a cell culture artifact and not an acquired inability of the cells to obtain control values. This artifact resulted from the large initial cell decrease in cell numbers which produced a sparse culture and resulted in new proliferation occurring as isolated clones of cells. As these clones enlarged during recovery,
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Figure 2. The effect of various streptomycin concentrations on the growth and survival of confluent human proximal tubule cells over time. Pattern one demonstrates the minimal effects of streptomycin on HPT cells and cannot be distinguished from (and is superimposed on) the pattern of control cells. This pattern occurs between 5 and 50 µg per ml of streptomycin (square = 50 µg per ml). Pattern two occurs within the concentration range of 100 to 500 µg per ml (circle = 250 µg per ml). Pattern three occurs within the concentration range of 750 and 1250 µg per ml (triangle = 750 µg per ml). The fourth pattern occurs at a streptomycin concentration of 2500 µg per ml (sideways triangle). The last pattern occurs at streptomycin concentrations between 3500 and 6000 µg per ml (open squares = 3500 µg per ml).

Time Course of Subconfluent Cultures Exposed to Streptomycin

The subconfluent cultures were placed into culture in such a way that they would be able to undergo active cell division during the early portion of the experimental time course of exposure to various streptomycin concentrations. The results of this study are presented in figure 3 and demonstrated that cell proliferation was influenced by streptomycin in a dose-dependent manner. Streptomycin concentrations of 50 µg per ml and below had no effect on the ability of HPT cells to undergo proliferation, with the results being superimposable with control cells. Thereafter, as the streptomycin concentration was increased, there was a dose-dependent inhibition of cell growth. This inhibition of cell
growth was noted to occur with exposure to as little as 100 μg per ml of streptomycin, with inhibition increasing with streptomycin concentration until complete inhibition of HPT cell growth occurred at a concentration of 5000 μg per ml. This is in marked contrast to the results obtained on confluent cultures, where marked decreases in cell numbers were noted to occur as a result of exposure to identical streptomycin concentrations.

On subconfluent cultures, no cell death was noted to occur over that of control cells until a streptomycin concentration of 6500 μg per ml was obtained. Light microscopic examination of the growth-inhibited cultures demonstrated no increase in the number of detached cells over control cultures, indicating that the growth inhibition noted was not a result of cell death and proliferation occurring simultaneously. In contrast, subconfluent cultures exposed to 6500 μg per ml of streptomycin demonstrated a large population of detached cells consistent with cell toxicity. Thus, the results suggest that the HPT cells respond to streptomycin exposure differently depending on whether the cells are quiescent or actively dividing.

**Electron Microscopy**

Ultrastructural examination of the HPT cells exposed to streptomycin clearly revealed the presence of numerous "myeloid bodies" within the cytoplasm (figure 4a). As depicted, these myeloid bodies were clearly noted within three days of exposure to 250 μg per ml of the drug; however, even lower concentrations could be noted to form repeatedly limited numbers of these structures. Higher magnification revealed a close similarity in the structure of these myeloid bodies to that seen in vivo (figure 4b). In both confluent and subconfluent studies, both the number and the percentage of cytoplasmic area occupied by these bodies increased directly with increasing streptomycin concentration and time of exposure. For HPT cells exposed to high concentrations of streptomycin, the cytoplasm of the cell could be noted to be largely replaced by the large number of myeloid bodies (figure 5). The mean area of the myeloid bodies did not vary significantly with streptomycin concentration or time of exposure to a given concentration; however, a slight increase was noted at the highest concentrations tested. This slight increase may reflect coalescence of bodies or simply the difficulty in delineating single myeloid bodies with the densely packed cytoplasm as seen in figure 5.

Routine visual examination of the electron micrographs was also strongly suggestive that subconfluent and confluent HPT cells exposed to equal concentrations of streptomycin accumulated myeloid bodies with differing efficiency. For this reason, the numbers of myeloid bodies within the cytoplasm of the exposed confluent and subconfluent HPT cells were quantitated over time utilizing image analysis technique. The results of this analysis clearly demonstrated that, when exposed to equal concentrations of streptomycin, subconfluent HPT cells accumulated far fewer myeloid bodies than HPT cells maintained in a non-dividing state (figure 6). This finding was repeatable regardless of the streptomycin concentration to which the cells were exposed. Other than the finding of myeloid body formation, ultrastructural examination revealed no other remarkable findings attributable to streptomycin exposure. At high levels of exposure, necrotic cell profiles were found with frequency; however, features observed were those normally found in degenerating cells. Lower concentrations of streptomycin produced no ultrastructural differences from control cells. Likewise, there was no increase in the
number of myeloid bodies formed by control cells during the time course of examination.

**Electrophysiology**

Confluent monolayers of HPT cells were grown on permeable supports such that their electrophysiologic properties could be assessed using Ussing chamber analysis. For these studies, the HPT cells grown on the filter were exposed to either 250 or 125 μg per ml of streptomycin for a six-day period, placed in the Ussing chamber, and the potential difference (PD), short circuit current (Isc) and resistance (R) determined. The results of this determination demonstrated that exposure to 250 μg per ml of streptomycin caused a significant reduction in PD, Isc and resistance (table 1). An identical determination at a lower concentration of streptomycin (125 μg per ml) demonstrated a similar trend, but was not statistically significant. Exposure of control cells (previously unexposed to streptomycin) to 250 μg per ml of streptomycin in the Ussing...
chamber bathing solutions did not alter the electrophysiological parameters over a 2-hour period of exposure. These results would suggest that relatively low doses of streptomycin are capable of altering the transport activities of HPT cells.

Discussion

The administration of streptomycin to cultures of HPT cells in serum-free medium evokes many of the changes which might be expected to occur from the knowledge gained from the use of aminoglycoside antibiotics in vivo. The first similarities between this model system and the in vivo situation could be appreciated from the growth curves of HPT cells exposed to streptomycin. Both clinical and animal model studies have demonstrated that the initial renal dysfunction elicited by the aminoglycosides may reverse itself despite continued administration of the drug. For confluent HPT cells exposed to toxic, but sublethal, concentrations of streptomycin, initial decreases in cell number occurred which temporally corresponded to the onset of renal dysfunction and early focal necrosis which is noted to occur in the clinical setting. Furthermore, with continued exposure to streptomycin, a “recovery” phase of the HPT cells was clearly evident and mimicked the clinically documented resolution of renal dysfunction even with continued aminoglycoside administration. This correlates temporally with the initiation of tubular epithelial regeneration visible on renal biopsy of patients receiving aminoglycoside therapy. The “insult-recovery” phase is concentration-dependent in the clinical setting, in vivo animal models and in the in vitro model discussed here.

In both clinical and animal model studies, it has been inferred that actively dividing tubular cells undergoing recovery from previous aminoglycoside exposure were more resistant to the toxic effects of continued exposure to the aminoglycosides. Likewise, it has also been noted in both clinical and animal studies that younger subjects were more resistant to the toxic effects of aminoglycosides. In the present study, actively dividing HPT cells were likewise clearly more resistant to the effects of streptomycin than stationary cultures. A comparison of the confluent (resting) cultures with the subconfluent (dividing) cultures demonstrated that the concentrations of streptomycin needed to produce a decrease in cell number were much higher for the actively-dividing cells. In fact, the only subconfluent cul-

| TABLE I |
| The Effect of Streptomycin on the Electrical Characteristics of Human Proximal Tubule Cell Monolayers |

<table>
<thead>
<tr>
<th>Potential Difference (mV)</th>
<th>Short Circuit Current (μA)</th>
<th>Resistance (KΩ cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-1.65±/-0.13</td>
<td>8.15±/-1.11</td>
</tr>
<tr>
<td>Streptomycin (250 μg/ml)</td>
<td>-0.92±/-0.14*</td>
<td>2.05±/-0.39*</td>
</tr>
<tr>
<td>Streptomycin (325 μg/ml)</td>
<td>-1.31±/-0.19+</td>
<td>6.14±/-0.97+</td>
</tr>
</tbody>
</table>

Compared to control, the * indicates values significant to p < 0.001, the + indicates values not significantly different.
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The concentration of streptomycin necessary to produce alterations in HPT cells cannot easily be compared to the in vivo situation. This reflects both the relatively low nephrotoxic clinical effects of streptomycin and the different endpoints used to express nephrotoxicity in vivo for related antibiotics. In vivo, nephrotoxicity is marked by alterations in renal function and culminates in acute renal failure. Yet even with complete clinical renal failure, the focal nature of acute tubular necrosis (ATN) is usually apparent on biopsy early in the course of the disorder, with variable numbers of tubular cells actually necrotic. Rarely, even in severe ATN, is there total necrosis and loss of the entire tubular epithelium. This focal toxicity in vivo renders judgment of what to define as a toxic concentration of streptomycin in cell culture very difficult. This is further complicated by the fact that in vivo experiments and clinical studies invariably utilize drug dosage or serum concentration to correlate nephrotoxicity. Rarely is the toxicity correlated with the concentration of the drug at the local area of the proximal tubule, and this local concentration may be markedly different from the serum concentration. The ability to bathe cells in defined concentrations of aminoglycoside antibiotics is one of the strengths of this proposed cell culture model system.

Additionally, the necrosis of the tubular cells in vivo may be secondary to renal failure itself and the systemic complications (ischemia, DIC, electrolyte imbalance, etc.) that ensue. Since secondary systemic complications are removed from the cell culture studies, higher concentrations of the agent may be necessary to produce in vitro necrosis. Such an argument would be supported by the electrophysiological alterations noted for HPT cells exposed to streptomycin. At relatively low concentrations of streptomycin, both potential difference and short circuit current were altered in the HPT cells, results indicative of changes in the ion transport properties of the cells. These changes occurred at streptomycin concentrations that had very minimal toxic effect on the HPT cells as regards growth and necrosis. Thus, the different endpoints
in each system render comparison of the concentrations needed to produce nephrotoxicity tenuous.

Lastly, the concentrations of streptomycin necessary to produce electrophysiological alterations in HPT cells are close to those normally present in many cell culture growth formulations. This suggests that research endeavors utilizing cultures of renal epithelial cells for the study of transport phenomena should use some caution in data interpretation if streptomycin is a standard component of the growth medium. Thus, the present study suggests that cultured HPT cells could provide a promising avenue to compliment and extend the present research efforts seeking to define the cellular aspects of aminoglycoside nephrotoxicity.

Acknowledgments

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References