Differential Toxic Effects of Gentamicin on Cultured Renal Epithelial Cells (LLC-PK1) on Application to the Brush Border Membrane or the Basolateral Membrane

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ABSTRACT. Aminoglycoside antibiotics are generally accepted to accumulate in renal proximal tubule cells from the luminal surface and show toxic effects on the cells. The binding affinity and membrane permeability of aminoglycoside antibiotics are different at the brush border membrane (BBM) and the basolateral membrane (BLM) of proximal tubule cells. This study was performed, therefore, to investigate the differential effects of the aminoglycoside antibiotic gentamicin (GM) on cultured LLC-PK1 cells, a pig kidney proximal epithelial cell line, after addition to the BBM or the BLM side. LLC-PK1 cells were cultured on microporous membranes until forming confluent monolayers, and then GM was added to either the BBM or the BLM side. GM caused release of enzymes from the organelles, with a higher level of release observed following addition to the BBM side than that to the BLM side. Patterns of [3H]GM uptake by the cells differed in a manner dependent on whether it was added to the BBM or the BLM side. That is, the cellular uptake from the BBM side increased with incubation time, while that from the BLM side showed rapid saturation. These results suggested that aminoglycoside antibiotics show differential effects on cultured proximal epithelial cells and have differential patterns of cellular uptake when added to the BBM or the BLM side.

KEY WORDS: aminoglycoside antibiotic, basolateral membrane, brush border membrane, LLC-PK1 cell, nephrotoxicity.


The LLC-PK1 cell line was developed in 1958 by Hull and coworkers [10] from minced whole kidney tissue from a male Hampshire pig. LLC-PK1 cells have been reported to contain relatively high levels of proximal marker enzymes [26], to have fixed polarity with the microvillus membrane oriented toward the medium in culture, and to form tight junctions allowing transport of solutes (hexose, amino acid, phosphate and organic ions) and water beneath the monolayer resulting in dome formation [10, 12, 13, 22, 23]. The LLC-PK1 cell line has been suggested to be useful as an in vitro model system for evaluation of aminoglycoside antibiotic-induced nephrotoxicity [9, 28].

The nephrotoxicity of aminoglycoside antibiotics has been shown to be due to specific lesions in proximal tubular epithelial cells [11, 17, 27]. The renal pathogenesis of aminoglycoside antibiotics was attributed to their selective accumulation in proximal tubular epithelial cells [4, 21, 34]. Although aminoglycoside antibiotics gain access to proximal tubular epithelial cells via the brush border membrane (BBM) and basolateral membrane (BLM), reabsorption of the antibiotics from the BBM is thought to be the dominant route of accumulation in these cells [8, 15, 25, 29, 32, 33]. However, studies on the subcellular distribution and membrane binding of aminoglycoside antibiotics indicated that these antibiotics show greater affinity and localization to the BLM than the BBM [1, 14, 35, 36].

Therefore, this study was performed to investigate the differential effects of the aminoglycoside antibiotic gentamicin (GM) on cultured LLC-PK1 cells after addition to the BBM or the BLM side.

MATERIALS AND METHODS

Cell culture: LLC-PK1 cells (American Type Culture Collection, CRL 1392, passage 196) were obtained from Flow Laboratories (Rockville, MD, U.S.A.). Aliquots of 1.5 × 10^5 cells were plated onto microporous membranes (24.5 mm in diameter, 3.0 μm pore size) in a cell culture chamber (Coster's Transwell, Cambridge, MA, U.S.A.) in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1) containing growth supplements [30] and 5% fetal calf serum (GIBCO, Grand Island, NY, U.S.A.) in the absence of antibiotics, and incubated at 37°C in an atmosphere of 5% CO2 in air until forming confluent monolayers (7–10 days). Culture medium on each side was then replaced by fresh medium with or without GM (Sigma, St. Louis, MO, U.S.A.). The antibiotic was used at a final concentration of 1 mM in all experiments. To confirm leakage between the BBM side and the BLM side of the chamber, phenol red-free culture medium was used for addition to the BBM side. As animal and human urine contains little protein, culture medium containing no fetal calf serum was used for addition to the BBM side. The culture medium was subjected to biochemical analyses. In the present study, the cells were used between passage 202 and 217.

Biochemical analyses: The culture media were centrifuged at 1,000 × g for 5 min and the supernatants were collected. The supernatants were assayed for alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) using a Hitachi-726 automatic analyzer. N-Acetyl-β-D-glucosaminidase (NAG) was measured using p-nitrophenyl-glucosaminide as the substrate. Each enzyme activity was adjusted by subtracting the activity of the plain culture
medium.

GM uptake study: GM uptake by LLC-PK₁ cells was determined by addition of 1 mM [³H]GM (1 mCi/mg, Amersham Japan, Tokyo) to the BBM or BLM side. After incubation for 15–60 min, each side of the cells was washed 3 times with 2 ml of ice-cold phosphate buffered saline containing 0.1% GM. The microporous membrane with the cell monolayer was removed from the chamber, dissolved in liquid scintillation cocktail and counted using a Beckman LS-3800 liquid scintillation spectrophotometer. The radioactivity value was adjusted by subtracting a trace value of binding of [³H]GM to the plain microporous membrane.

Statistical analysis: Data are expressed as means ± SD. Results were analyzed by Student’s t-test.

RESULTS

As the cells were cultured on microporous membranes, preventing fluid accumulation beneath the monolayer, no dome formation [10, 12, 13, 22, 23] of LLC-PK₁ cells occurred during cultivation. A higher concentration of glucose was observed on the BLM side (data not shown). The leakage of phenol red between both sides of the monolayers was less than 1% (data not shown).

BBM enzyme release (Fig. 1): The spontaneous release of ALP, a BBM enzyme, from LLC-PK₁ cells into the culture medium was highest on the BBM side during cultivation. GM caused an increase in ALP release from the cells into the medium on the BBM side but not that on the BLM side. Addition of GM to the BBM side induced a higher level of enzyme release than that to the BLM side.

Cytoplasmic enzyme release (Fig. 2): The spontaneous release of LDH, a cytoplasmic enzyme, from LLC-PK₁ cells into the culture medium was found on the both sides during cultivation, with that into the BLM side being greater than that into the BBM side. GM caused a significant increase in LDH release into the medium on the BBM side. Addition of the antibiotic to the BBM side induced a slight increase in release of the enzyme into the medium on the BLM side.

Lysosomal enzyme release (Fig. 3): When calf serum was added to the culture medium on both sides, the level of spontaneous release of the lysosomal enzyme NAG into the medium on the BBM side was greater than that into the BLM side (data not shown). However, when calf serum was removed from the BBM side, a low level of spontaneous release of the enzyme was found on both sides. GM caused an increase in NAG release into the medium on the BBM side but not into that on the BLM side. A higher level of enzyme release was observed following addition of GM to the BBM side than to the BLM side.

Time course of [³H]GM uptake (Fig. 4): The pattern of uptake of [³H]GM by LLC-PK₁ cells was different after addition to each side. The cellular uptake from the BBM side increased with incubation time, while that from the BLM side showed rapid saturation. [³H]GM showed greater uptake into the cells from the BBM side than from the BLM side.

DISCUSSION

In this study, we confirmed the differentiated proximal epithelial properties of LLC-PK₁ cells by culturing the cells on microporous membranes. That is, the higher ALP content in the culture medium on both sides of LLC-PK₁ cells, and the higher glucose concentration on the BLM side indicated that the cells contain proximal marker enzymes [26], have fixed polarity with the microvillus membrane oriented...
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**Fig. 2.** Effects of GM on the release of LDH from LLC-PK₁ cells. The cells were cultured on a microporous membrane until reaching confluence and then treated with or without GM (⊛) for 1-3 days. GM was added to either the BBM side (▲) or the BLM side (▼) of the monolayer. The culture medium on the BBM side and the BLM side was analyzed for LDH. Each point represents the mean ± SD for 5-6 experiments. * indicates significant differences from control value; P<0.05.

**Fig. 3.** Effects of GM on the release of NAG from LLC-PK₁ cells. The cells were cultured on a microporous membrane until reaching confluence and then treated with or without GM (⊛) for 1-3 days. GM was added to either the BBM side (▲) or the BLM side (▼) of the monolayer. The culture medium on the BBM side and the BLM side was analyzed for NAG. Each point represents the mean ± SD for three experiments. * and ** indicate significant differences from control value; P<0.05 and P<0.01, respectively.

toward the medium in cell culture, and form tight junctions allowing transport of solutes and water [10, 22]. In this study, GM caused increases of enzyme release into the medium on the BBM side from the organelles (BBM, lysosome and cytoplasm) of LLC-PK₁ cells. ALP located in BBM of renal proximal epithelial cells [19]. The increase in ALP release into the medium on the BBM side of LLC-PK₁ cells may indicate impairment of the BBM of the cells by GM. These observations suggested that GM-induced increase of these enzymes into the medium on the BBM side of the cells reflects GM-induced elevation of urinary excretion of these enzymes derived from the proximal tubules [19, 24].

There is general agreement that aminoglycoside antibiotics must accumulate in proximal tubular cells in order to be nephrotoxic. It has been reported that the major uptake pathway of aminoglycoside antibiotics is across the BBM of proximal tubular cells by an endocytotic mechanism by which the antibiotics are concentrated in lysosomes of the cells and induce phospholipid accumulation and secondary lysosome formation [2, 8, 16, 29, 31]. In this study, addition of GM to the BBM side of LLC-PK₁ cells induced a higher level of enzyme release than that to the BLM side of the cells. These results support the hypothesis that aminoglycoside antibiotics induce nephrotoxicity mainly via the BBM side of...
the proximal tubular epithelium. However, we suggest that the nephrotoxicity of GM may be induced in part via the BBM side, since addition of GM into the medium on the BBM side of LLC-PK₁ cell monolayers induced a slight increase in the level of enzyme release. The distribution and binding affinity of aminoglycoside antibiotics to the BBM of proximal tubular epithelium have been reported to be greater than those to the BBM [1, 14, 35, 36]. Furthermore, Aramaki et al. [1] reported that aminoglycoside antibiotics were transported into BBM vesicles prepared from the rat kidney, while they only bound to BBM vesicles. We observed that uptake of [³H]GM from the BBM side of LLC-PK₁ cells increased with cultivation time, while that from the BLM side rapidly showed saturation. These different results suggested that GM is transported from the BBM into the cells and binds to the BBM. Accumulation of the antibiotic in the cells was suggested to be due to uptake from the BBM, and binding to the BBM may result in cytotoxicity.

The kidney removes proteins from the circulation by glomerular filtration, followed by luminal endocytosis. Proteins taken up by luminal endocytosis in renal proximal tubular cells are generally believed to be transported from endocytotic vacuoles into lysosomes to be degraded [5–7]. It has also been suggested that urinary lysosomal enzymes are normally secreted from renal proximal tubular cells by exocytosis. Lockwood and Bosmann [20] demonstrated that the rat kidney releases approximately 20% of the NAG activity per day by exocytosis into the urine. In this study, when fetal calf serum was removed from the culture medium on the BBM side of LLC-PK₁ cells, a low level of spontaneous release of NAG was found on both sides of the monolayer. Furthermore, addition of GM to the BBM side or the BLM side caused increases in NAG release into the medium on the BBM side with no increase in that of LDH at an early stage. Although the major uptake pathway of aminoglycoside anti-
biotics is across the BBM, partial uptake across the BLM has been also inferred from studies using renal cortical slices [3] and isolated perfused kidney preparations [8]. There are several mechanisms that might account for the observed release of NAG from LLC-PK₁ cells in our experiments: 1) Proteins may be taken up by endocytosis from the BBM into the lysosomes, and the cells may then release lysosomal NAG by exocytosis; 2) GM may be taken up by endocytosis from the BBM and in part from the BLM into the lysosomes, and exocytic release of NAG from the cells may increase due to the lysosomal accumulation of GM at an early stage at which the antibiotic dose not increase the release of cytoplasmic LDH; 3) GM may increase release of NAG from the cells by both exocytosis and cell damage at later stages.

Although aminoglycoside antibiotics do not accumulate in the liver, they sometimes cause elevation of serum GOT and LDH [18]. Treatment of rats with GM for 7 days caused elevation of the serum level of α-hydroxybutyrate dehydrogenase, an l-type isozyme of LDH, which is mainly located in heart and kidney (unpublished observation). In the present study, we found that GM did not cause release of ALP, a BBM enzyme, into the medium on the BLM side of LLC-PK₁ cell monolayers, but caused a higher level of LDH release into the medium on the BLM side than that on the BBM side. These observations suggested that spontaneous release of LDH across the BBM (vascular side) of LLC-PK₁ cells in part reflects the physiological serum LDH level in experimental animals and humans, and that the GM-induced increase of LDH release into the medium on the BLM side of the cells reflects elevation of serum LDH and other cytoplasmic enzymes derived from the proximal tubules.

In conclusion, the results of the present study indicated that GM shows differential effects on cultured LLC-PK₁ cells dependent on whether it is added to the BBM or the BLM side, and its toxicity is mainly mediated via the BBM when added to the BBM side. The method described here is a good model system for evaluation of the properties and mechanism of aminoglycoside antibiotic-induced nephrotoxicity.

REFERENCES

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