Effects of Arbekacin and Vancomycin on Release of Lactate Dehydrogenase and Fragmentation of DNA in LLC-PK$_1$ Kidney Epithelial Cells

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INTRODUCTION

The aminoglycoside antibiotic arbekacin and glycopeptide antibiotic vancomycin are frequently used to treat infections with methicillin-resistant *Staphylococcus aureus*. These drugs have the potential to induce nephrotoxicity (1–3). The nephrotoxicity of aminoglycoside antibiotics involves proximal tubular cells (4). Therefore, it was evaluated in LLC-PK$_1$ cells (5–7) which retain characteristics of renal tubular cells (8). Our results suggest LLC-PK$_1$ cells are useful for evaluating aminoglycoside-induced nephrotoxicity. However, little is known about the mechanism of nephrotoxicity of arbekacin.

In addition, we have investigated the nephrotoxicity and renal excretion of vancomycin (9,10). Our results suggested vancomycin is different from aminoglycoside antibiotics with respect to renal handling and its mechanism of accumulation in the kidney (9). Moreover, we previously suggested vancomycin impairs the functions of glomeruli and renal tubules (10). However, the mechanism of toxicity of vancomycin in the kidney has not been elucidated.

In contrast, cisplatin is one of the most effective antineoplastic agents, although this drug, as well as arbekacin and vancomycin, has adverse effects such as nephrotoxicity (11). The toxicity of cisplatin is predominantly observed in renal proximal tubular cells (12), and is probably due to a combination of insults including peroxidation of the cell membrane, mitochondrial dysfunction, inhibition of protein synthesis, and DNA injury (13). Recently, it was reported apoptosis, an important mechanism of cell death, as well as necrosis, are related to the mechanism of toxicity of cisplatin in renal proximal tubules (13,14).

In the present study, to clarify the mechanisms of toxicity of arbekacin and vancomycin in renal tubules, especially their association with apoptosis, the effects of these drugs on LDH release into the medium as a marker of necrosis, and on DNA ladder formation as a typical hallmark of apoptosis were examined and compared to those of cisplatin in LLC-PK$_1$ kidney epithelial cells.

MATERIALS AND METHODS

Materials

Cisplatin was purchased from Sigma Chemical Co. (St. Louis, MO). Arbekacin sulfate was provided by Meiji Seika Kaisha (Tokyo, Japan). Vancomycin hydrochloride was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of the highest purity available.

Cell Culture

LLC-PK$_1$ cells obtained from American Type Culture Collection (ATCC CRL-1392, Manassas, VA) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (GIBCO, Life Technologies, Grand Island, NY), containing 10% fetal bovine serum (Whittaker Bioproducts Inc., Walkersville, MD) without antibiotics in an atmosphere of 5% CO_2–95% air at 37°C (5). The cells were subcultured every 4–7 days using 0.02% EDTA and 0.05% trypsin. In all experiments, 60-mm dishes were seeded with 4×10^5 cells in 5 ml of complete culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum). In the present study, LLC-PK$_1$ cells were used between passages 215 and 222.

Study Protocol

To assess the toxicity of cisplatin, arbekacin and vancomycin, confluent monolayers of LLC-PK$_1$ cells grown on 60-mm dishes were treated with each drug for the indicated periods, and then photographed under phase-contrast microscopy (DIAPHOTO 300, Nikon, Japan). The final concentrations of the drugs during exposure were 0.01—1, 0.01—10, and 0.01—5 mM for cisplatin, arbekacin, and vancomycin, respectively. Control cells were incubated with culture medium without drugs in each experiment.

Lactate Dehydrogenase (LDH) Assay

To determine the LDH activity released from dying LLC-PK$_1$ cells into the medium, media were collected from cultures. The cells were washed once with ice-cold phosphate buffered saline and removed with a rubber policeman into phosphate buffered saline (2 ml per 60-mm dish). Thereafter, the media and cell samples were homogenized with a sonicator (VP-5S, TAITEC, Saitama, Japan) three times for 5 seconds each time. The LDH activity was measured using the Wróblewski-La Due method, with a kit obtained from Wako Pure Chemical Industries (Osaka, Japan).

Detection of DNA Fragmentation

DNA fragmentation was determined according to the method of Lieberthal *et al.* (13) with slight modifications. Briefly, cells were collected and lysed in buffer containing 10 mM Tris, 25 mM EDTA, and 0.5% Triton X-100 for 60 minutes

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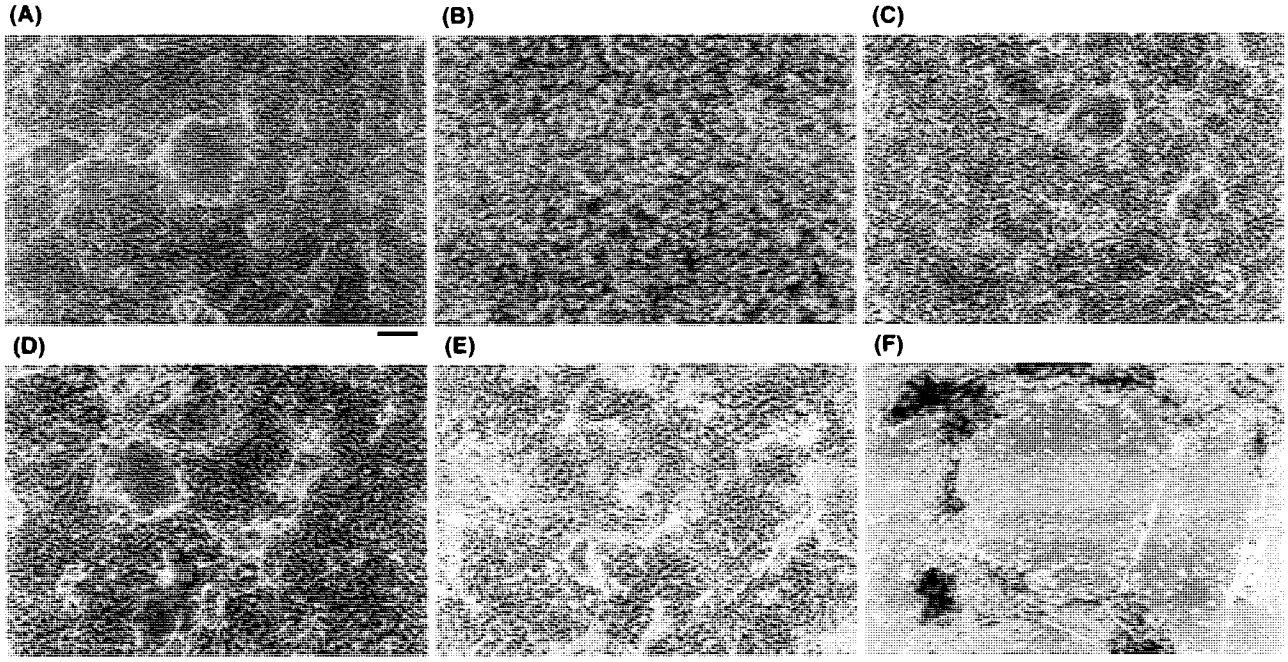

Fig. 1. Phase-contrast microscopy of LLC-PK$_1$ cells after treatment with vehicle (A), cisplatin (B), arbekacin (C) or vancomycin (D-F). LLC-PK$_1$ cells treated with drugs at 0.1 mM for 3 days were photographed under phase-contrast microscopy (A-D). LLC-PK$_1$ cells treated with vancomycin at 5 mM for 7 days were photographed under phase-contrast microscopy (E, F). Bar = 100 μm.

on ice. Cell lysates were then centrifuged at 13,000 g at 4°C for 30 minutes. Low molecular weight DNA in the supernatant was incubated with RNase (100 μg/ml) and proteinase K (100 μg/ml) at 50°C for 60 minutes. The DNA was extracted with phenol/chloroform, then 3.0 M sodium acetate, pH 5.2 (1/10 volume), and 1.0 M $MgCL_2$ (1/30 volume) were added and DNA was precipitated with ethanol. Thereafter, the DNA was resuspended in 10 mM Tris-1 mM EDTA (TE) buffer. Samples containing DNA were electrophoresed through 2.0% agarose gels in the presence of ethidium bromide, and DNA was visualized under UV illumination.

Statistical Analysis

Statistical comparisons were performed by analysis of variance (ANOVA) followed by Sheffé's test for multiple comparisons. P values of less than 0.05 (two-tailed) were considered significant.

RESULTS

Figure 1 shows micrographs of LLC-PK$_1$ cells treated with cisplatin, arbekacin, and vancomycin. Confluent monolayers of control cells exibited typical dome formation 3 days after

Table I. Effects of Cisplatin, Arbekacin and Vancomycin on LDH Release into the Medium and Total LDH Activity[a]

Drug	LDH activity	Drug concentration (mM)					
		0	0.01	0.1	1	5	10
Cisplatin	Release (%)	13.1 ± 0.8	12.2 ± 1.9	53.7 ± 2.0*	60.1 ± 1.4*	—	—
(day 1)	Total (IU/dish)	1.48 ± 0.05	1.54 ± 0.04	1.27 ± 0.12	0.58 ± 0.03*	—	—
Cisplatin	Release (%)	14.5 ± 1.6	20.7 ± 1.4	92.2 ± 0.8*	75.0 ± 2.2*	—	—
(day 3)	Total (IU/dish)	1.79 ± 0.05	1.92 ± 0.03	1.15 ± 0.04*	0.22 ± 0.02*	—	—
Arbekacin	Release (%)	15.8 ± 1.7	16.5 ± 1.2	16.9 ± 1.1	18.0 ± 0.9	19.6 ± 1.1	16.0 ± 0.9
(day 3)	Total (IU/dish)	1.35 ± 0.06	1.41 ± 0.03	1.54 ± 0.06	1.82 ± 0.10*	1.81 ± 0.06*	1.55 ± 0.05
Vancomycin	Release (%)	17.5 ± 1.4	17.0 ± 1.3	20.4 ± 1.2	17.0 ± 0.6	18.1 ± 1.1	—
(day 3)	Total (IU/dish)	1.42 ± 0.05	1.50 ± 0.05	1.65 ± 0.06	1.41 ± 0.03	1.72 ± 0.02*	—
Vancomycin	Release (%)	24.2 ± 2.6	21.9 ± 1.0	23.1 ± 1.9	19.8 ± 2.8	32.2 ± 5.7	—
(day 7)	Total (IU/dish)	1.66 ± 0.04	1.68 ± 0.02	1.64 ± 0.03	1.71 ± 0.06	1.70 ± 0.01	—

[a] LLC-PK$_1$ cells were seeded in 60-mm dishes. Four days after seeding, the culture medium was replaced with fresh medium or with that containing 0.01-1 mM cisplatin, 0.01-10 mM arbekacin or 0.01-5 mM vancomycin. LDH activities in the dishes were determined at the indicated times after treatment. Each value represents the mean ± S.E. of four dishes.
* $P < 0.05$, significantly different from the untreated control value.

treatment (Fig. 1A). The morphology of LLC-PK$_1$ cells treated with 0.1 mM cisplatin for 3 days was obviously different from that of control cells; i.e. most cells in the dish were detached from the monolayer as single cells (Fig. 1B). On the other hand, cells treated with 0.1 mM arbekacin or vancomycin for 3 days showed no marked differences in morphology from control cells (Fig. 1C and 1D). The characteristics of cells treated with arbekacin (1–10 mM) and vancomycin (1 or 5 raM) for 3 days were similar to those of control cells (data not shown). In addition, most cells treated with 5 mM vancomycin for 7 days were similar to the controls (Fig. 1E), although the monolayer became detached into the medium as a sheet (approximately 10 percent of the total area), and morphological changes were observed (approximately 5 percent of the total area) (Fig. 1F).

Table I shows LDH release and total LDH activities in LLC-PK$_1$ cells treated with cisplatin, arbekacin, and vancomycin. The LDH release from cells treated with 1 mM cisplatin for 1 day was higher than that from those treated with 0.01 or 0.1 mM cisplatin, and the release was also increased up to 3 days after treatment. The total LDH activities in cells treated with 1 mM cisplatin for 1 and 3 days were significantly decreased as compared to the respective control groups. On the other hand, there were no significant differences in LDH release in LLC-PK$_1$ cells treated with arbekacin or vancomycin for 3 days. Only slight induction of LDH release was observed in cells treated with 5 mM vancomycin for 7 days, although the LDH release in the drug-free controls tended to increase up to 7 days.

Figure 2 shows ladder formation on electrophoresis of DNA isolated from LLC-PK$_1$ cells treated with cisplatin, arbekacin, or vancomycin. The DNA from LLC-PK$_1$ cells treated with 0.1 mM cisplatin showed significant ladder formation after 1 day of treatment in both attached and floating cells (Fig. 2A). Significant ladder formation was also observed in the DNA from attached cells treated with 1, 5, or 10 mM arbekacin for 3 days, but not in that from floating cells (Fig. 2B). No effect of vancomycin treatment for 7 days was observed in attached or floating cells (Fig. 2C).

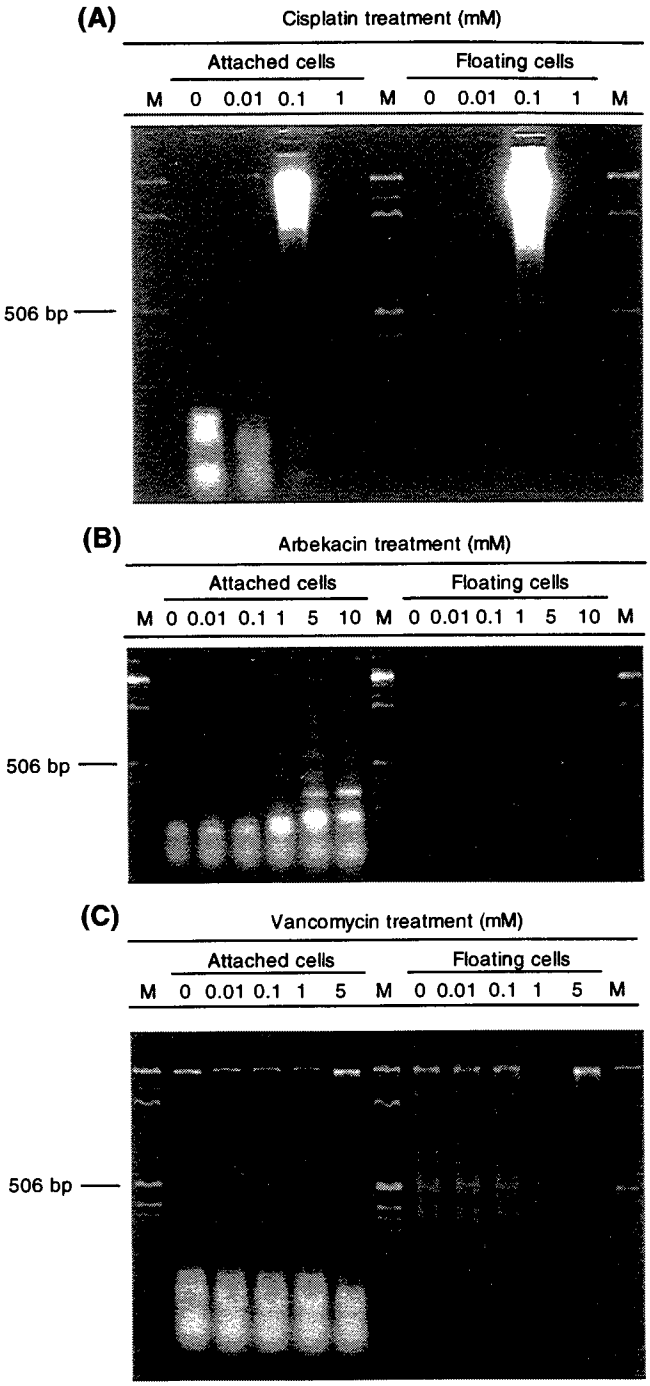

Fig. 2. Agarose gel electrophoresis of fragmented DNA extracted from attached (left) or floating (right) LLC-PK$_1$ cells. The LLC-PK$_1$ cells were treated with 0–1 mM cisplatin for 1 day (A), 0–10 mM arbekacin for 3 days (B) or 0–5 mM vancomycin for 7 days (C). M, Molecular size marker.

DISCUSSION

To evaluate the toxicity of arbekacin and vancomycin in renal tubules, the effects of these antibiotics on LLC-PK$_1$ cells were investigated microscopically as compared to those of cisplatin. In addition, LDH release into the medium and DNA fragmentation were used as markers of necrosis and apoptosis, respectively.

The LDH release increased and the total LDH activity decreased significantly in cells treated with 1 mM cisplatin, indicating that necrosis occurred (Table I). Lieberthal *et al.* (13) reported high concentrations of cisplatin induced necrosis in mouse proximal tubular cells, whereas low concentrations induced apoptosis. In the present study, DNA ladder formation as well as a significant increase in LDH release were observed in LLC-PK$_1$ cells treated with 0.1 mM cisplatin (Fig. 2A, Table 1). In addition, the morphology of most cells treated with 0.1 mM cisplatin and the exclusion of trypan blue observed by microscopy were similar to those of apoptotic cells described previously (13). Therefore, it was considered that apoptosis and necrosis occurred in the cells treated with 0.1 mM cisplatin.

DNA ladder formation was detected in attached cells treated with arbekacin (1, 5 and 10 mM), suggesting apoptosis was induced by arbekacin in LLC-PK$_1$ cells (Fig. 2B). However, DNA fragmentation induced by arbekacin was observed only in the attached cells (Fig. 2B), whereas that by cisplatin was observed in both floating and attached cells (Fig. 2A). Soler *et*

al. (15) suggested LLC-PK$_1$ cells treated with tumor necrosis factor-α can accommodate an increased frequency of apoptosis and still maintain integrity by tissue remodeling. Therefore, the number of apoptotic cells detached from monolayers would be much less in tumor necrosis factor-α-treated cells, even if apoptosis was induced. Similarly, although apoptosis was induced by arbekacin in LLC-PK$_1$ cells, neither morphological changes nor DNA fragmentation in the floating cells might be observed by tissue remodeling. In the present study, released LDH activity was not markedly increased by treatment with arbekacin, although it would be expected to increase if necrosis occurs (Table I). Therefore, our observations suggested necrosis did not occur with arbekacin treatment, at least at 0.01–10 mM for 3 days (Table I).

In the present study, we treated LLC-PK$_1$ cells with vancomycin (0.01–5 mM) for 7 days. No significant changes were observed in the LDH release or DNA pattern on electrophoresis (Table I, Fig. 2C), although slight morphological changes were observed in the cells treated with 5 mM vancomycin for 7 days (Fig. 1). These results suggested the necrosis and apoptosis were minimal in vancomycin-treated LLC-PK$_1$ cells. We reported previously that the secretion of vancomycin in renal tubules was mediated by an unknown transport system (9). Therefore, if LLC-PK$_1$ cells do not have such a transport system, the accumulation of vancomycin in the cells may be insufficient to induce necrosis and/or apoptosis. The present results are consistent with our previous observation that vancomycin impairs glomerular filtration more markedly than the renal tubular function, as compared to cisplatin (10).

In conclusion, we investigated the mechanisms of toxicity of arbekacin and vancomycin in LLC-PK$_1$ cells, as compared to that of cisplatin. Our results suggested the mechanisms of arbekacin-and vancomycin-induced toxicity in renal tubules are different from that of cisplatin. This study provided useful information for further studies of drug-induced mechanisms of toxicity in the kidneys.

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