Polymyxin B Induces Apoptosis in Kidney Proximal Tubular Cells

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The nephrotoxicity of polymyxins is a major dose-limiting factor for treatment of infections caused by multidrug-resistant Gram-negative pathogens. The mechanism(s) of polymyxin-induced nephrotoxicity is not clear. This study aimed to investigate polymyxin B-induced apoptosis in kidney proximal tubular cells. Polymyxin B-induced apoptosis in NRK-52E cells was examined by caspase activation, DNA breakage, and translocation of membrane phosphatidylserine using Red-VAD-FMK [Val-Ala-Asp(O-Me) fluoromethyl ketone] staining, a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, and double staining with annexin V-propidium iodide (PI). The concentration dependence (50% effective concentration [EC₅₀]) and time course for polymyxin B-induced apoptosis were measured in NRK-52E and HK-2 cells by fluorescence-activated cell sorting (FACS) with annexin V and PI. Polymyxin B-induced apoptosis in NRK-52E cells was confirmed by positive labeling from Red-VAD-FMK staining, TUNEL assay, and annexin V-PI double staining. The EC₅₀ (95% confidence interval [CI]) of polymyxin B for the NRK-52E cells was 1.05 (0.91 to 1.22) mM and was 0.35 (0.29 to 0.42) mM for HK-2 cells. At lower concentrations of polymyxin B, minimal apoptosis was observed, followed by a sharp rise in the apoptotic index at higher concentrations in both cell lines. After treatment of NRK-52E cells with 2.0 mM polymyxin B, the percentage of apoptotic cells (mean $\pm$ standard deviation [SD]) was 10.9% $\pm$ 4.69% at 6 h and reached plateau (>80%) at 24 h, whereas treatment with 0.5 mM polymyxin B for 24 h led to 93.6% $\pm$ 5.57% of HK-2 cells in apoptosis. Understanding the mechanism of polymyxin B-induced apoptosis will provide important information for discovering less nephrotoxic polymyxin-like lipopeptides.

Gram-negative pathogens, in particular *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, that are resistant to most current antibiotics present a significant problem globally (1, 2). Unfortunately, the development of new antibiotics to treat infections caused by these problematic pathogens has decreased precipitously over the last two decades (2). As a consequence, “old” polymyxins have been revived as a last-line therapy (3, 4). Two polymyxins, namely, polymyxin B and polymyxin E (synonym, colistin), have been available clinically since the late 1950s but were abandoned in the 1970s due to their potential for nephrotoxicity (5, 6). There is little doubt that there is an association between polymyxin therapy and nephrotoxicity (7–9), and recent clinical studies have shown that the incidence rate is up to 60%, depending on the definition of nephrotoxicity (7, 10–12). Unfortunately, the mechanism(s) of polymyxin-induced nephrotoxicity is not clear (13).

Acute tubular necrosis and increased serum creatinine concentrations have been reported associated with polymyxin-induced nephrotoxicity (8, 14). Cumulative dose- and duration-dependent increases of serum creatinine have been observed in rats (15) and humans (16, 17) after intravenous administration of colistin methanesulfonate (CMS), an inactive prodrug of colistin. Pharmacokinetic studies indicated that both polymyxin B and colistin undergo very extensive net tubular reabsorption from tubular urine back into blood in the kidney (18, 19). Furthermore, colistin-induced tubular apoptosis in rats was reported, and coadministration of antioxidants appeared to be protective against colistin-induced nephrotoxicity (20–22). Therefore, it is very likely that polymyxin-induced nephrotoxicity is related to kidney tubular cell apoptosis. Apoptosis, also known as programmed cell death (23), is characterized by a series of events, including activation of a family of cysteine-containing aspartate-directed pro-

teases known as caspases (24, 25), condensation and fragmentation of nuclei (26, 27), mitochondrial alterations (28, 29), translocation of membrane phosphatidylserine (30, 31), formation of apoptotic bodies (26), and cell death (32). The mechanism(s) of polymyxin B-induced apoptosis has not been investigated. In the present study, we investigated the characteristics of polymyxin B-induced apoptosis in cultured rat and human kidney proximal tubular cells.

MATERIALS AND METHODS

Reagents. Polymyxin B (sulfate; catalog number 81334; lots 12168230506110 and BCBD1065V; minimum potency, 6,500 IU/mg, which is greater than the USP specification of not less than 6,000 IU/mg) and staurosporine were purchased from Sigma-Aldrich (NSW, Australia). A stock solution of 40 mM polymyxin B in Milli-Q water was prepared and sterilized with a syringe filter (Millex-GV; 0.22 μ m; Millipore). Staurosporine stock solution (1 mM) was prepared in sterile dimethyl sulfoxide (DMSO) (American Type Culture Collection [ATCC], Manassas, VA, USA) and used as a positive control.

Cell culture. Rat (NRK-52E) and human (HK-2) kidney proximal tubular cells (ATCC, Manassas, VA, USA) were employed. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was utilized for NRK-52E cells. HK-2 cells were cultured in

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keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (BPE) (0.05 mg/ml) and human recombinant epidermal growth factor (EGF) (5 ng/ml). All components of the growth medium were purchased from Invitrogen (Life Technologies, Victoria, Australia). NRK-52E (0.5×10^5 cells/ml) and HK-2 (0.25×10^5 cells/ml) cells were seeded in 12-well plates or 8-well chamber slides in growth medium at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h and 48 h, respectively. The medium was then removed by aspiration, and the cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4; Invitrogen). The treatments described below were then conducted in DMEM supplemented with 0.1% FBS for NRK-52E cells and in K-SFM supplemented with BPE (0.05 mg/ml) and EGF (5 ng/ml) for HK-2 cells.

Assessment of polymyxin-induced caspase activation, DNA damage, and membrane translocation of phosphatidylserine. Initially, activation of caspases, an essential step for the execution of apoptosis (24, 25), was examined using a CaspGLOW red active caspase staining kit (BioVision, Milpitas, CA, USA) (33). Briefly, NRK-52E cells were cultured in 12-well plates and incubated with or without polymyxin B (1.0 mM) for 24 h and then treated with Red-VAD-FMK [Val-Ala-Asp(O-Me) fluoromethyl ketone] (1:3,000) in DMEM at 37°C for 30 min. Activated caspases were detected by laser scanning microscopy (a Nikon A1-R confocal microscope with NIS-Element imaging software) using excitation/emission wavelengths of 561 nm and 570 to 600 nm. Staurosporine (1.0 μM)-treated cells were employed as the positive control. As activated caspases trigger caspase-activated DNase (CAD), an enzyme responsible for the fragmentation of DNA (34, 35), we further detected the DNA damage in polymyxin B-treated NRK-52E cells by *in situ* detection of DNA fragments using a TUNEL Universal Apoptosis Detection kit (GenScript, Piscataway, NJ, USA). In the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, NRK-52E cells after incubation for 24 h in the presence or absence of polymyxin B (1.0 mM) on chamber slides (Nunc Lab-Tek Chamber Slide system; 8 wells on Permanox; Sigma-Aldrich) were fixed in 4% paraformaldehyde, followed by incubation for 10 min at 25°C with blocking solution (3% hydrogen peroxide in methanol). Then, the cells were permeabilized with 0.1% (vol/vol) Triton X-100 in aqueous 0.1% (wt/vol) sodium citrate, followed by incubation with TUNEL reaction mixture containing 45 μl equilibration buffer, 1 μl biotin-11-dUTP, and 4 μl terminal deoxynucleotidyl transferase (TdT) for 1 h at 37°C. The cells were incubated with streptavidin-horseradish peroxidase (HRP) solution for 0.5 h at 37°C, followed by incubation with 3,3'-diaminobenzidine (DAB) substrate and 0.3% hydrogen peroxide in PBS at 25°C for 10 min in the dark. Cells treated with 20.0 U/μl DNase I for 20 min were employed as the positive control. The samples were visualized using a Nikon A1-R confocal microscope with NIS-Element imaging software.

Membrane translocation of phosphatidylserine, another consequence of caspase activation (30, 31), was measured to assess polymyxin B-induced apoptosis in rat and human kidney proximal tubular cells by double staining with annexin V and PI. This was conducted using an Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen) as described previously, with minor modifications (36, 37). Both NRK-52E and HK-2 cells were cultured in 12-well plates and incubated with or without polymyxin B (1.25 mM for NRK-52E cells and 0.5 mM for HK-2 cells) for 24 h. Staurosporine (1.0 μM) was employed as the positive control to induce apoptosis. After incubation, cells in plates were centrifuged ($150 \times g$; 5 min), and the supernatant was discarded; then, PBS (pH 7.4) was added and the plates were centrifuged again ($150 \times g$; 5 min). The PBS was discarded, and the cells were detached from the plates using 300 μl of trypsin-EDTA solution (0.25% and 0.05% for NRK-52E and HK-2 cells, respectively; Invitrogen) for ~3 min at 37°C. The trypsin was inactivated with 1.0 ml of DMEM for NRK-52E cells and K-SFM for HK-2 cells. The cell suspension was centrifuged ($450 \times g$; 5 min) in 1.5-ml tubes, and the supernatant was discarded. The cell pellets were resuspended in ice-cold PBS (pH 7.4) and centrifuged ($450 \times g$; 5 min). The supernatant was discarded, and the cell pellets were resuspended in 100 μl of ice-cold 1×

annexin-binding buffer. Five microliters of Alexa Fluor 488-conjugated annexin V and 1.0 μl of PI (100 μg/ml) were added to the cell suspension and incubated in the dark for 15 min at room temperature. Then, 0.4 ml of ice-cold 1× annexin-binding buffer was added, and induction of apoptosis was monitored immediately by fluorescence-activated cell sorting (FACS) (FACSCanto II; Becton-Dickson, CA, USA). Fluorescence emission was measured at 530 and 575 nm using an excitation wavelength of 488 nm. Annexin V-positive/PI-negative and annexin V-positive/PI-positive cells were considered early and late apoptotic cells, respectively, and both were counted as total apoptotic cells (38). The percentage of apoptotic cells in the total number of cells is designated the apoptotic index.

Assessment of the concentration dependence and time course of polymyxin-induced apoptosis. NRK-52E and HK-2 cells were cultured in 12-well plates and incubated with and without polymyxin B (final concentrations, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, and 4.0 mM for 24 h for NRK-52E cells and 0.125, 0.25, 0.375, and 0.5 mM for 16 h for HK-2 cells) to evaluate concentration-dependent apoptosis. The concentration of polymyxin B required to induce 50% of maximal apoptosis (50% effective concentration [EC₅₀]) was calculated by fitting a Hill function, with basal response to account for the low degree of apoptosis in the absence of drug, using unweighted nonlinear least-squares regression analysis in GraphPad Prism (V5.0; GraphPad Software, San Diego, CA, USA). Time-dependent induction of apoptosis was measured in the presence of polymyxin B (2.0 mM) at 1, 6, 12, 16, 20, and 24 h for NRK-52E cells and 0.5 mM polymyxin B at 6, 12, 16, and 24 h for HK-2 cells. Cells treated with staurosporine (1.0 μM) were employed as the positive control to induce apoptosis. Induction of apoptosis was measured by FACS as described above. All experiments were conducted in three independent replicates, and data are presented as means ± standard deviations (SD).

RESULTS

Unlike the untreated negative-control cells (Fig. 1A), pancaspase activation, a key characteristic of early-stage apoptosis, was observed in NRK-52E cells after 24 h of incubation with 1.0 mM polymyxin B, similar to the staurosporine-treated positive-control cells (Fig. 1B and C). DNA breakage, usually followed by activation of caspases, was also observed from *in situ* TUNEL staining; compared to the untreated control without TUNEL-positive nuclei (Fig. 2A), polymyxin B-treated NRK-52E cells showed dark-brown TdT-labeled nuclei (Fig. 2B), an important biochemical hallmark of apoptosis, similar to the nuclei of the DNase I-treated cells (Fig. 2C). Both the pancaspase activation and TUNEL assays indicated that polymyxin B induced apoptosis in rat kidney tubular cells. Subsequently, externalization of phosphatidylserine was examined in polymyxin B-treated NRK-52E and HK-2 cells. The untreated control NRK-52E cells showed minimal labeling ($8.63\% \pm 0.9\%$) (Fig. 3A) with annexin V and annexin V-PI, compared to the labeling of the cells treated with polymyxin B ($72.6\% \pm 7.9\%$) (Fig. 3B) and staurosporine ($93.7\% \pm 1.4\%$) (Fig. 3C); the corresponding viability data are presented in Fig. 3D to F.

The concentration-dependent apoptosis induced by polymyxin B is shown in both NRK-52E and HK-2 cells (Fig. 4). Polymyxin B displayed EC₅₀ (95% confidence interval [CI]) values of 1.05 (0.91 to 1.22) mM for NRK-52E cells after 24 h of incubation and 0.35 (0.29 to 0.42) mM for HK-2 cells after 16 h of incubation. After 24-h polymyxin B treatment with 2.0 mM for NRK-52E cells and 0.5 mM for HK-2 cells, the percentages of apoptotic cells were >80% for both cell lines (Fig. 4). Therefore, these concentrations of polymyxin B were employed for examination of time-dependent induction of apoptosis by polymyxin B (Fig. 4). The time dependence of polymyxin-induced apoptosis was also examined

FIG 1 Confocal microscopic visualization (magnification, $\times 20$) of caspase activation in NRK-52E cells using Red-VAD-FMK staining. (A) Control cells. (B) 1.0 mM polymyxin B. (C) 1.0 μ M staurosporine.

by FACS (Fig. 5). Minimal induction of apoptosis was observed at 6 h in NRK-52E cells treated with 2.0 mM polymyxin B, followed by rapid induction of apoptosis until 24 h, when a plateau was reached. Compared to NRK-52E cells, HK-2 cells appeared more susceptible to polymyxin B treatment. At 0.5 mM polymyxin B, rapid induction of apoptosis was observed up to 16 h with no substantial increase of the apoptotic index after 16 h. Similar time courses of apoptosis were also observed with human kidney proximal tubular HK-2 cells after treatment with a range of concentrations (0.125 to 1.0 mM) of polymyxin B (data not shown). The apoptotic indices of the untreated control cells of both NRK-52E and HK-2 did not change over the same period. Similar changes in viability were observed for both NRK-52E and HK-2 cells (data not shown).

DISCUSSION

Based upon our recent pharmacokinetic/pharmacodynamic studies, the concentrations of both polymyxin B and colistin in plasma in many patients may be suboptimal with the currently recommended dosage regimens (19, 39–41). Administration of doses higher than those in the approved product information for polymyxins may be required to achieve optimal clinical efficacy; however, the potential for nephrotoxicity is a major dose-limiting factor (19, 39, 42). Our recent study in rats showed that colistin induced apoptosis in the kidney (21). The mechanism(s) of polymyxin-induced nephrotoxicity is poorly understood. The aim of

the present study was to investigate polymyxin B-induced apoptosis in rat and human kidney proximal tubular cells.

Extensive tubular reabsorption of polymyxins was reported previously (18, 19, 43). This may lead to accumulation of polymyxins in kidney tubular cells, thereby predisposing to nephrotoxicity. The proximal tubule is the most common site of nephrotoxicity induced by many other toxicants (44–46). Therefore, immortalized tubular cell lines originating from rat and human kidney tissues are widely utilized for *in vitro* evaluation of the nephrotoxicity of drugs (44, 47, 48). A recent study using the porcine renal proximal tubular cell line LLC-PK1 showed that polymyxin B induced $\sim 50\%$ necrosis at 0.5 mM and did not cause apoptosis when measured with the DNA-staining reagent 4',6'-diamidine-2'-phenylindole (DAPI) (49). Unfortunately, DAPI is not specific for apoptosis measurement (50). Additionally, a low level of expression of unidentified transporter(s) responsible for polymyxin B uptake could be a potential explanation for the lack of apoptosis in LLC-PK1 cells after treatment with 0.5 mM polymyxin B (51). In the present study, NRK-52E rat kidney proximal tubular cells were first examined, as our previous polymyxin pharmacokinetic and nephrotoxicity studies were conducted in rats (15, 18, 22). Importantly, the concentration- and time-dependent apoptosis induced by polymyxin B in NRK-52E cells was also observed in the human kidney proximal tubular cell line HK-2 (Fig. 4 and 5).

FIG 2 Immunohistochemical images of apoptotic nuclei (arrows) in NRK-52E cells treated with vehicle (control) (A), 1.0 mM polymyxin B (B), and 20.0 U/ μ l DNase I (C).

FIG 3 Double staining with annexin V and PI in NRK-52E cells. (A) Control cells. (B) 1.25 mM polymyxin B. (C) 1.0 μM staurosporine. In each panel, the upper left quadrant represents cells stained by annexin V (early-apoptotic cells), the bottom right quadrant represents cells stained by PI (necrotic cells), the upper right quadrant represents cells stained by both annexin V and PI (late-apoptotic cells), and the bottom left quadrant represents cells not stained by annexin V or PI (viable cells). (D to F) Viability data for panels A to C. (D) Control cells. (E) 1.25 mM polymyxin B. (F) 1.0 μM staurosporine. The error bars represent standard deviations (SD).

Positive labeling with Red-VAD-FMK of NRK-52E cells exposed to polymyxin B showed the presence of activated caspases (Fig. 1). Activation of caspases is a common phenomenon in apoptosis of kidney tubular cells due to nephrotoxic injury (52, 53). Activation of caspases can be triggered by two potentially interacting and reversible pathways mediated by mitochondria (intrinsic) and cell surface death receptor (extrinsic) (54). Polymyxins are known to interact with phospholipids of membranes and strongly bind with mitochondria in mammalian cells (55, 56). Conceivably, activation of caspases by polymyxin B could be me-

diated by intrinsic and/or extrinsic pathways of apoptosis, and this warrants further investigation. Activated caspases are also essential for the regulation of CAD, a cytosolic endonuclease responsible for the DNA breakage activity that propagates apoptotic cell death (54). Therefore, in the present study, DNA breakage was investigated using an *in situ* TUNEL assay to detect the free ends of DNA after breakage, one of the important biochemical characteristics of apoptosis (57, 58). Dark-brown TdT-labeled nuclei were detected in the polymyxin B-treated cells, providing evidence for apoptosis (Fig. 2). This observation is consistent with the reported

FIG 4 Apoptotic indexes of NRK-52E (A) and HK-2 (B) cells as a function of the polymyxin B concentration (24 and 16 h, respectively). Means $\pm$ SD ($n = 3$) are shown.

FIG 5 Apoptotic indexes of NRK-52E (A) and HK-2 (B) cells incubated with polymyxin B (2.0 and 0.5 mM, respectively) for different times (mean $\pm$ SD; $n = 3$). ▲, polymyxin B treatment; ●, control treatment with vehicle only.

colistin-induced apoptosis in rat kidneys (21). For further investigation of polymyxin B-induced apoptosis, we utilized double staining with annexin V and PI. Apoptotic cells have externalized membrane phosphatidylserine; therefore, annexin V, a calcium-dependent phospholipid-binding protein that binds to phosphatidylserine with high affinity, serves as an excellent quantitative apoptotic marker using FACS (59). Polymyxin B treatment (1.25 mM for 24 h) caused apoptosis in NRK-52E cells (annexin V-positive and annexin V-PI-positive cells; Fig. 3B), consistent with the results obtained with the pancaspase and TUNEL assays (Fig. 1 and 2). Furthermore, polymyxin B treatment led to rapid transition of the NRK-52E and HK-2 cells from early apoptosis to late apoptosis, as shown by the colabeling with both annexin V and PI. A key finding of the present study was that all three different assays, i.e., activation of caspases (Fig. 1), DNA damage (Fig. 2), and translocation of membrane phosphatidylserine (Fig. 3), confirmed that polymyxin B induced apoptosis in kidney tubular cells.

Using the quantitative FACS method, we revealed that polymyxin B induced apoptosis in a concentration- and time-dependent manner in both NRK-52E and HK-2 cells (Fig. 4 and 5). This finding is in line with the cumulative dose- and time-dependent kidney proximal tubular injury reported recently after intravenous administration of CMS in rats (15, 22) and humans (17). Additionally, dose-dependent kidney toxicity was also observed after polymyxin B treatment in rats (14). In the present study, the EC_{50} values of polymyxin B (0.35 and 1.05 mM for HK-2 and NRK-52E cells, respectively) are higher than the concentrations of colistin reported recently in the homogenate of kidney tissue (i.e., ~ 0.1 mM) from rats with histological evidence of nephrotoxicity following several days of treatment with colistin (21). There are at least two potential explanations for this apparent discordance. First, the tissue concentration is an average value in the tissue homogenate that very likely does not represent the concentration of polymyxins within kidney tubular cells after extensive reabsorption. Second, in the cultured cells, uptake of polymyxins may be limited by the magnitude of the expression of an as-yet-unidentified transporter(s) compared to that in kidney tissue. In fact, there may be no disagreement between the EC_{50} values observed here and the concentration of polymyxins in rat kidney tissue homogenates (21). As demonstrated in the cell culture studies reported here, the extent of cell toxicity as measured by the apoptotic index is the result of the combination of time and concentration. In the cell culture studies, the incubation period was 16 or 24

h for HK-2 and NRK-52E cells, respectively, whereas *in vivo* (in rats or patients) nephrotoxicity may result from several days of exposure to concentrations that may be somewhat lower than those causing toxicity after 16 or 24 h in cell culture.

In the present study, the different EC_{50} s of polymyxin B (0.35 and 1.05 mM for HK-2 and NRK-52 cells, respectively) between the human and rat cell lines suggest that polymyxin B-induced apoptosis is cell line dependent (Fig. 4 and 5). Clearly, our study highlights the need for further investigations into the mechanisms of polymyxin-induced apoptosis and its association with nephrotoxicity due to polymyxin treatment.

Conclusion. To our knowledge, this is the first study to reveal that polymyxin B induces apoptosis in rat and human kidney proximal tubular cells in a concentration- and time-dependent manner. Understanding the molecular mechanisms of polymyxin-induced apoptosis and nephrotoxicity may provide invaluable information for developing a novel therapeutic strategy using nephroprotectants and for discovering less nephrotoxic polymyxin-like lipopeptides for treatment of infections caused by the very problematic multidrug-resistant Gram-negative pathogens.

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