



Original Contribution

Mitochondrial superoxide production contributes to vancomycin-induced renal tubular cell apoptosis

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ABSTRACT

Vancomycin chloride (VCM), a glycopeptide antibiotic, is widely used for the therapy of infections caused by methicillin-resistant *Staphylococcus aureus*. However, nephrotoxicity is a major adverse effect in VCM therapy. In this study, we investigated the cellular mechanisms underlying VCM-induced renal tubular cell injury in cultured LLC-PK1 cells. VCM induced a concentration- and time-dependent cell injury in LLC-PK1 cells. VCM caused increases in the numbers of annexin V-positive/PI-negative cells and TUNEL-positive cells, indicating the involvement of apoptotic cell death in VCM-induced renal cell injury. The VCM-induced apoptosis was accompanied by the activation of caspase-9 and caspase-3/7 and reversed by inhibitors of these caspases. Moreover, VCM caused an increase in intracellular reactive oxygen species production and mitochondrial membrane depolarization, which were reversed by vitamin E. In addition, mitochondrial complex I activity was inhibited by VCM as well as by the complex I inhibitor rotenone, and rotenone mimicked the VCM-induced LLC-PK1 cell injury. These findings suggest that VCM causes apoptotic cell death in LLC-PK1 cells by enhancing mitochondrial superoxide production leading to mitochondrial membrane depolarization followed by the caspase activities. Moreover, mitochondrial complex I may play an important role in superoxide production and renal tubular cell apoptosis induced by VCM.

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Vancomycin chloride (VCM)¹, a glycopeptide antibiotic, is widely used for the treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA). However, nephrotoxicity is a major complaint during VCM therapy, particularly in patients receiving a high dose of VCM or VCM in combination with other antibiotics such as aminoglycoside [1–3]. Therapeutic drug monitoring (TDM) is strongly recommended to prevent VCM-associated nephrotoxicity in clinical practice, because the incidence of nephrotoxicity closely correlates with mean VCM trough concentrations of 15 µg/ml or more [3–5]. Pritchard et al. [6] reported the increased risk of nephrotoxicity with elevated VCM trough concentrations, duration of VCM therapy more than 7 days, and elevated baseline serum creatinine level. On the other hand, a trend toward decreased susceptibility to VCM in *S. aureus* has been reported [7]. A recently published guideline advocates the targeting of VCM trough level of 15 to 20 µg/ml to overcome MRSA strains with reduced susceptibility [8]. Indeed, the proportion of VCM serum trough concentrations ≥ 15 µg/ml increased

from 25.8 to 38.8%, whereas those with median VCM trough levels ≥ 20 µg/ml increased from 6.5 to 14.8% over the period of 2003–2007 [6]. However, high concentrations of VCM would also lead to an increase in the risk of nephrotoxicity [6,9,10]. The rates of observed VCM-associated nephrotoxicity are reported as 33 and 20% in those with trough levels > 20 and 15–20 µg/ml, respectively [11]. These observations suggest that TDM is one of the few effective means of prevention of VCM-associated nephrotoxicity; however, it is becoming difficult to prevent the nephropathy only by monitoring the VCM trough level or the kidney function while promoting the proper use of VCM in the reality of clinical situations.

The precise mechanisms underlying the VCM-associated nephrotoxicity remain to be clarified, although some studies in rats have shown renal tubular cell damage and the possible implication of oxidative stress in nephrotoxicity caused by VCM [12–15]. Therefore, in this study, we determined the mechanisms underlying the VCM-induced renal cell injury using a cultured renal tubular cell line, LLC-PK1 cells.

Materials and methods

Chemicals

Vancomycin chloride and α-tocopherol (vitamin E) were obtained from Wako Pure Chemicals (Osaka, Japan). Caspase inhibitors, including zDEVD-fmk (a caspase-3-specific inhibitor) and zLEHD-fmk (a

Abbreviations: VCM, vancomycin chloride; ROS, reactive oxygen species; O₂^{•−}, superoxide; SOD, superoxide dismutase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PI, propidium iodide; LDH, lactate dehydrogenase; FITC, fluorescein isothiocyanate; AMC, 7-amino-4-methylcoumarin; carboxy-H₂DCFDA, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate; PBS, phosphate-buffered saline; TDM, therapeutic drug monitoring; MRSA, methicillin-resistant *Staphylococcus aureus*.

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caspase-9-specific inhibitor), were obtained from Calbiochem (EMD Chemicals, Gibbstown, NJ, USA). Fluorescence-labeled caspase substrate Ac-LEHD-AMC for caspase-9 was purchased from Alexis Biochemicals (San Diego, CA, USA). Rotenone was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

A porcine renal tubular epithelial cell line, LLC-PK1 cells, was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in medium 199 (MP Biomedicals, Irvine, CA, USA) supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS, USA), 100 U/ml penicillin–streptomycin (Gibco, Invitrogen Corp., Carlsbad, CA, USA) in an atmosphere of 5% CO₂ in air at 37 °C. Cells were seeded on various cell culture plates described below and used for experiments on the following day, on which they reached 70–80% confluence.

WST-8 assay

Cell viability was assessed from the mitochondrial activity in reducing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) to formazan, as described previously [16]. Briefly, after treatment with VCM, cells were washed with phosphate-buffered saline (PBS). Then, the cells were incubated with 210 µl serum-free medium and 10 µl WST-8 assay solution (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) for 1 h at 37 °C in humidified air supplemented with 5% CO₂. The incubation medium was carefully taken and transferred to 96-well flat-bottom plastic plates (Nalge Nunc International, Rochester, NY, USA). The amount of formazan formed was measured from the absorbance at 450 nm with a reference wavelength of 620 nm using a microplate reader (Immuno-Mini NJ-2300; Inter Medical, Tokyo, Japan).

Leakage of lactate dehydrogenase (LDH)

Cells were seeded at a density of 3×10^4 cells/well onto 24-well plastic plates (Nalge Nunc International) and used for experiments on the following day, on which they reached 70–80% confluence. LDH leakage was expressed as the percentage of LDH released into medium compared to the total, 24 h after exposure to VCM. LDH activity was determined using an LDH assay kit (Takara Biochemicals, Osaka, Japan).

Annexin V stain and propidium iodide (PI) uptake

Annexin V stain and PI uptake were carried out using a commercial apoptosis assay kit (Mebcyto apoptosis kit; Medical & Biological Lab. Co., Ltd., Nagoya, Japan), as described previously [17]. In brief, cells were seeded at a density of 1.0×10^5 cells/well onto six-well plastic plates (Nalge Nunc International). At 24 h after seeding, the cells were incubated with VCM. The cells were washed twice with PBS and incubated for 30 min in the dark in 100 µl buffer containing 10 µl fluorescein isothiocyanate (FITC)-labeled annexin V or 5 µl PI. Cells were analyzed by a BD FACSCalibur flow cytometry system (Becton–Dickinson, San Jose, CA, USA) with excitation at 488 nm and emission using 530 nm for annexin V and 670 nm for PI. At least 10,000 cells were analyzed in each treatment. The data were analyzed using CellQuest software (Becton–Dickinson).

Assay for caspase activity

Caspase-3/7 activity was determined using the Apo-ONE homogeneous caspase-3/7 assay kit (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. The activity of caspase-9 was determined fluorometrically by the degradation of the peptide

substrate specific for caspase-9 (Ac-LEHD-AMC) as described previously [18], using the caspase activity assay kit (BioVision, Mountain View, CA, USA). Briefly, after exposure to VCM, cells were collected and centrifuged at 150 g for 10 min, and the resultant pellets were suspended in 1 ml lysis buffer (BioVision) and subjected to caspase activity assay. The reaction was started by incubating enzyme extracts with each caspase substrate for 1 h in the absence or presence of caspase inhibitors. In a set of experiments measuring caspase-9 activity, after centrifugation at 10,000 g for 10 min, the concentration of 7-amino-4-methylcoumarin (AMC) liberated into the supernatant was determined at an excitation wavelength of 380 nm and an emission wavelength of 460 nm using a fluorescence microplate reader (MTP-601F; Hitachi High-Technologies Corp., Tokyo, Japan). The protein concentration was measured using bovine serum albumin as the standard, according to the method of Bradford [19]. The caspase-9 activity was expressed as nanomoles of AMC produced per milligram of protein.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) stain

Apoptosis was assessed by TUNEL stain, as described previously [18]. Briefly, after exposure to VCM, cells were washed with PBS and fixed for 30 min at a room temperature with 4% (w/v) paraformaldehyde in PBS. The cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate solution. TUNEL stain was carried out using the Dead End fluorometric system (Promega Corp.), according to the manufacturer's instructions. The stained cells were visualized with a fluorescence microscope (BX51; Olympus, Tokyo, Japan) or quantitatively analyzed using flow cytometry (Becton–Dickinson). The flow cytometric data were analyzed using CellQuest software (Becton–Dickinson).

Mitochondrial membrane potential as measured by JC-1 staining

Changes in mitochondrial membrane potential were assessed by using the JC-1 stain, as described previously [17]. In brief, cells were cultured on eight-chamber plastic slides (Iwaki, Asahi Techno Glass Corp., Ltd., Chiba, Japan) at 3.0×10^4 cells/chamber. At 24 h after seeding, the cells were incubated with VCM for the indicated time periods. The cells were then washed with PBS and incubated with BD MitoScreen (JC-1) (Becton–Dickinson) for 1 h. Cells were visualized using a fluorescence microscope (BX51; Olympus) or quantitatively analyzed by flow cytometry (Becton–Dickinson) with excitation at 488 nm and emission using 670 nm. At least 10,000 cells were analyzed in each treatment. The flow cytometric data were analyzed using CellQuest software (Becton–Dickinson).

Release of cytochrome c from mitochondria

The release of cytochrome c from the mitochondria to the cytoplasm was assessed by using the InnoCyte flow cytometric cytochrome c release kit (Calbiochem) according to the manufacturer's instructions. In brief, cells were cultured on eight-chamber plastic slides (Iwaki) at 3.0×10^4 cells/chamber. At 24 h after seeding, the cells were incubated with VCM for 24 h. The cells were then washed with PBS and were permeabilized to enable the release of cytosolic cytochrome c, incubated on ice for 10 min, and then fixed with 4% (w/v) paraformaldehyde in PBS. The cells were washed, exposed to anti-cytochrome c antibody (for 60 min), and finally labeled with FITC-conjugated anti-IgG antibody (for 60 min). Cells were visualized using a fluorescence microscope (BX51; Olympus).

Measurement of reactive oxygen species (ROS) production

Intracellular ROS production was measured using carboxy-H₂DCFDA fluorescence labeling as described previously [17]. In brief,

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