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Original Article

Antibiotics induce apoptosis of human peritoneal mesothelial cells

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SUMMARY: The peritoneal mesothelial cell is a critical component of the peritoneal membrane. The intraperitoneal use of several antibiotics to treat bacterial peritonitis is current clinical practice. Our previous study showed that cephalothin (CPL) and cefotaxime (CFT) have cytotoxic effects on human peritoneal mesothelial cells (HPMC), however, the exact mechanism of cytotoxicity has not been elucidated. In the present study, flow cytometry, TdT-mediated dUTP nick-end labelling (TUNEL) staining and electron microscopy were used to detect the apoptosis of HPMCs. Immunofluorescent staining was used to evaluate the cytochrome c distribution pattern. Western blotting was used to assess apoptotic signalling proteins. We found that CPL (0.5 mg/mL) and CFT (1 mg/mL) induced apoptosis of HPMCs, whereas cefazolin (0.5 mg/mL) and ceftriaxone (0.5 mg/mL) failed to induce apoptosis of HPMCs. While the DNA content of CFT- or CPL-treated cells was reduced, as determined by flow cytometry, cefazolin and ceftriaxone had no such effect. The CFT- or CPL-treated cells displayed the features of apoptosis both under the electron microscope and by using TUNEL staining. However, cefazolin and ceftriaxone produced the same result as the medium controls. Furthermore, CFT and CPL increased the expression of Bax and p53, and caused the translocation of cytochrome c from the mitochondria to the cytoplasm. The HPMC treated by CFT but not by CPL induced the cleavage of procaspase-3 to form active caspase-3. In conclusion, cefotaxime and cephalothin induce apoptosis of HPMCs *in vitro*. Signal transduction may be through the mitochondrial pathway.

KEY WORDS: **antibiotics, apoptosis, p53, peritoneal mesothelial cell, signalling pathway.**

INTRODUCTION

Continuous ambulatory peritoneal dialysis (CAPD) is successfully used as one of the maintenance regimens of end-stage renal disease. The peritoneal mesothelial cell (PMC) covers the entire peritoneal cavity surface and is a critical component of the peritoneal membrane.[1] It performs many important functions, which are all needed to maintain the peritoneal membrane as a dialysing organ. Peritoneal mesothelial cells form a non-adhesive surface to facilitate movement between apposing tissues. Injuries or infections within the peritoneal cavity cause fibrin to form, and adhesion formation was found to be inversely correlated with the fibrinolytic activity of the peritoneum.[2] The PMC modifies the release of substances such as plasminogen activator and its inhibitor,[3] and its fibrinolytic activity is the key factor to prevent the formation of fibrous adhesions.[4] Another important function of mesothelial cells involves their role in the host defense mechanism. During peritonitis, bacteria and peritoneal macrophages activate PMCs, which release chemokines[5] and control the peritoneal immune response by releasing prostaglandins and cytokines such as IL-6, IL-8, MCP-1 and RANTES.[6] The PMCs also serve as antigen-presenting cells to activate helper T-cells.[7] Therefore, the integrity of peritoneal mesothelial cells directly affects the success of CAPD.

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Although the PMC is crucial for CAPD, CAPD itself has detrimental effects on PMCs. Peritoneal membranes undergo many structural and functional changes in patients who received long-term CAPD treatment.[8] One of the most important changes is peritoneal fibrosis. The pathogenesis of peritoneal fibrosis is not clearly understood, but the cytokines released during bacterial peritonitis may be one of the causes.[9] The dialysate[10–12] and drugs[13] administered into the peritoneal cavity during CAPD have toxic effects on PMCs. The high glucose content in the dialysate affects PMCs via various pathways and results in extracellular matrix accumulation.[14] These facts may partly explain that the severity of peritoneal fibrosis correlates with the duration of CAPD.[15] Nevertheless, the severity and frequency of bacterial peritonitis during CAPD are more predictable of long-term membrane survival.[16]

The most frequent complication of CAPD is bacterial peritonitis. The number of PMC in dialysate effluent increases during the episode of bacterial peritonitis.[17] This implies that the exfoliation of the mesothelium is aggravated during the peritonitis, and that the number of PMC in the peritoneum is decreased. As stated before, PMCs play an important role in peritoneal immune response. Therefore, the use of any agents that might be harmful to the PMCs should be avoided during the course of peritonitis. The use of antibiotics via the intraperitoneal route is the current recommended treatment for bacterial peritonitis.[18] It is therefore important to know whether these antibiotics have adverse effects on PMCs. Our previous study showed that cephalothin, cefotaxime, cefamandole, and cefuroxime, at the concentrations of treating CAPD peritonitis via the intraperitoneal route, have cytotoxic effects on human PMCs.[19] Cephalothin (CPL) and cefotaxime (CFT) are the two most obvious antibiotics to be cytotoxic to PMCs, but the exact mechanism of this cytotoxicity has not been elucidated. The present study is designed to clarify the cytotoxic mechanism of these two antibiotics in human PMCs.

Two modes of cell death have been differentiated: apoptosis and necrosis. Apoptotic cell death is an active process under molecular control.[20,21] The regulatory process of apoptosis is now a matter of great concern. Apoptotic cells detach from the culture substrata separately and undergo cellular and nuclear shrinkage, but necrotic cells swell and detach by the sheet.[22] According to our previous observations by light microscopy, cells undergoing antibiotic-induced cytotoxicity have the characteristic appearance of apoptotic cells. Therefore, we wanted to examine whether antibiotics might induce apoptosis of PMCs. In the present study, we demonstrated that CFT and CPL induced human PMC apoptosis, as opposed to cefazolin and ceftriaxone, which did not induce human PMC apoptosis. Furthermore, we also studied the signalling pathway of the apoptosis induced by CFT and CPL.

METHODS

Materials

Trypsin-EDTA, RPMI-1640 medium, Proteinase K, and trypan blue were obtained from Invitrogen Life Technologies (New York, NY, USA). Culture flasks and plates were purchased from Corning (Corning, NY, USA) and precoated with Vigtrogen 100 (Celtrix Laboratory, Palo Alto, CA, USA) before cells were loaded into them. Fetal bovine serum (FBS) was obtained from Biochrom KG (Berlin, Germany). Triton X-100, bovine serum albumin, penicillin, streptomycin, insulin, propidium iodide, cefazolin, ceftriaxone, CFT, and CPL were purchased from Sigma (St Louis, MO, USA). Monoclonal antibodies to cytochrome c, Bax, and Bcl-2, and horseradish peroxidase-labelled goat antimouse immunoglobulin G (IgG) were purchased from Becton Dickinson Transduction Laboratories (San Diego, CA, USA). Monoclonal antibody to caspase 8 was supplied by Cell Signalling Technology Inc. (Beverly, MA, USA). Monoclonal antibody to p53 was bought from Oncogene Research Products (Boston, MA, USA). Monoclonal antibody to caspase 3 was from the Imgenex Corporation (San Diego, CA, USA). Fluorescein diisothiocyanate (FDIC)-labelled goat $F(ab')_2$ fragment to mouse IgG was purchased from DAKO (Glostrup, Denmark). Agents used for western blot analysis were from Bio-Rad Laboratories Inc. (Hercules, CA, USA) unless otherwise specified.

Human peritoneal mesothelial cell culture

Specimens of human omentum were obtained from abdominal surgical procedures with the informed consent of the donors. These patients underwent elective abdominal surgery and the omentum was normal. The method of omentum enzymatic disaggregation was used as previously described.[23] Briefly, a piece of omentum was washed in sterile phosphate buffered saline (PBS) threefold and then incubated with 15 mL of trypsin-EDTA (0.125%) for 20 min at 37°C with continuous rotation. After incubation, the omentum and the suspension were centrifuged at 50 *g* for 5 min at 4°C. The supernatant was discarded and the cell pellet was washed once in RPMI-1640 medium containing 20% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and insulin (30 μg/mL). After another centrifugation under the same conditions, the cells were resuspended in the same medium and seeded into a 75-cm^2 flask. In 2–4 days, the cells became confluent and were subcultured with medium containing 10% FBS. The cells were initially bipolar or multipolar but became cobblestone-like in appearance upon confluence. By using the immunofluorescence method, HPMC were identified by the presence of vimentin and cytokeratin, and the absence of desmin and factor VIII-related antigen. All experiments listed below were performed in passage 1–3 cells and repeated at least threefold using cells from different subjects.

Flow cytometry

Human peritoneal mesothelial cells were cultured to subconfluence in a 50-cm^2 dish with 10% FBS-containing RPMI. The media were then changed to 2% FCS-containing RPMI with and without antibiotics (cefazolin (0.5 mg/mL), ceftriaxone (0.5 mg/mL), CFT (1 mg/mL), or CPL (0.5 mg/mL)). After incubations for 40, 48, and 56 h, the cells (including those cells suspended in the medium) were harvested by using trypsinization. Cells were resuspended in PBS at a concentration of 1×10^6/mL and fixed in 2 mL methanol for 30 min at 4°C. After HPMC were fixed, the mixture was incubated in 0.5 mL of propidium iodide solution (0.05 mg/mL in 3.8 mol/L Na citrate) and 0.5 mL of RNAse A (0.5 mg/mL) at room temperature for 30 min. Finally, the

HPMC were resuspended in 1 mL PBS and analysed by using flow cytometry (FACScan; Coulter, Fullerton, CA, USA) according to the manufacturer's instructions. The cells in the subdiploid peak were considered as apoptotic.[24]

In situ detection of apoptosis

Human peritoneal mesothelial cells were cultured to subconfluence in a four-chamber slide with 10% FBS-containing RPMI. The media was then changed to 2% FBS-containing RPMI with and without antibiotics (cefazolin (0.5 mg/mL), ceftriaxone (0.5 mg/mL), CFT (1 mg/mL), or CPL (0.5 mg/mL)). After incubation for 48 h, the cells were fixed by 4% paraformaldehyde. Apoptotic cells were detected by using a fluorescence *in situ* cell-death detection kit (Roche Molecular Biochemicals, Mannheim, Germany). This kit uses terminal deoxynucleotidyltransferase (TdT) for the incorporation of labelled nucleotides (or the TUNEL (TdT-mediated dUTP nick-end labelling) technique) into DNA strand breaks *in situ*. Fluorescein labels incorporated in nucleotide polymers are detected and quantified by using fluorescence microscopy. For evaluation by fluorescence microscopy, an excitation wavelength of 455 nm and detection wavelength of 510 nm are used. Apoptosis is defined by morphological criteria. Cells containing fragmented nuclear chromatin will exhibit bright green nuclear staining.[25]

Transmission electron microscopy

Human peritoneal mesothelial cells were cultured to subconfluence in a 50-cm^2 dish with 10% FBS-containing RPMI. The medium then was changed to 2% FBS-containing RPMI with and without antibiotics (cefazolin (0.5 mg/mL), ceftriaxone (0.5 mg/mL), CFT (1 mg/mL), or CPL (0.5 mg/mL)). After incubation for 48 h, the cells (including cells suspended in the medium) were trypsinized and fixed. The cells were then processed for examination under the electron microscope.

Western blotting

Human peritoneal mesothelial cells were cultured to subconfluence in a 50-cm^2 dish with 10% FBS-containing RPMI. The media was then changed to 2% FBS-containing RPMI with and without antibiotics (CFT (1 mg/mL) or CPL (0.5 mg/mL)). After incubations for 40, 48, and 56 h, the cells (including cells suspended in the medium) were lysed by ice-cold lysing solution (65 mmol/L Tris base, pH 8.0, containing 154 mmol/L NaCl, 1 mmol/L EDTA, 1% (octylphenoxy)polyethoxyethanol, 1 mmol/L phenylmethanesulfonyl fluoride, leupeptin (1 μg/mL), pepstatin (1 μg/mL), aprotinin (1 μg/mL), and 0.25% Na deoxycholate). Samples were rotated for 15 min at 4°C and then centrifuged at 12 000 *g* for 5 min at 4°C. The supernatant was recovered, and protein concentration was measured by using the bicinchoninic acid assay (Bio-Rad), with bovine serum albumin as the standard. Samples were incubated for 5 min at 95°C in loading buffer (12 mmol/L Tris-HCl, pH 6.8, with 25% glycerol, 2% sodium dodecyl sulfate, 14.4 mmol/L 2-mercaptoethanol, and 0.1% bromophenol blue), and 50 μg of protein were loaded on SDS-polyacrylamide gels of different percentages (and exclusion limits) corresponding to the molecular weight of the target proteins. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane by electroblotting. The membrane was blocked in 1% BSA/0.05%Tween/PBS solution overnight at 4°C. Mouse antihuman monoclonal antibodies to Bcl-2, Bax, p53, caspase 8 or caspase 3 were used as primary antibodies. A horseradish peroxidase-labelled goat antimouse IgG was used as a secondary antibody. Blots were developed by incubation in a chemiluminescence substrate and were exposed to X-ray films.

Immunofluorescent stain

Human peritoneal mesothelial cells were cultured to subconfluence in a four-chamber slide with 10% FBS-containing RPMI. The media were then changed to 2% FCS-containing RPMI with and without antibiotics (CFT (1 mg/mL) or CPL (0.5 mg/mL)). After incubation for 48 h, the cells were fixed by using 4% paraformaldehyde. Cells were permeabilized with 0.2% Triton X-100 and then blocked by 10% FBS in PBS. Cells were incubated with mouse antihuman cytochrome c antibody and then with FDIC-labelled antimouse IgG antibody. Slides were observed under a fluorescence microscope, using an excitation wavelength of 455 nm and a detection wavelength of 510 nm.

Statistical analysis

All data are expressed as mean ± SEM. The comparison of drug effects was conducted by using a Student's *t*-test. A *P*-value less than 0.05 was considered to be significant.

RESULTS

To correlate the clinical situation, the antibiotics were tested *in vitro* by adding the same concentrations as suggested by the Ad Hoc Advisory Committee on Peritonitis Management[18,26] to the dialysate. According to our previous study,[19] CFT (1 mg/mL) and CPL (0.5 mg/mL) have cytotoxic effects on HPMCs. Cefazolin, which is the same as CPL, is a first generation cephem, and was chosen to compare against CPL. Ceftriaxone, which is the same as CFT, is a third generation cephem, and was chosen to compare against CFT. Both cefazolin (0.5 mg/mL) and ceftriaxone (0.5 mg/mL) are not toxic to HPMC according to our previous report.[19] After incubating with CFT for 48 h or CPL for 40 h, the DNA content of HPMCs became noticeably reduced, whereas cefazolin or ceftriaxone did not have such an effect. To determine the amount of DNA in each cell, flow cytometry was used with propidium iodide staining. The percentages of subdiploid (apoptotic) cells over time are shown in Table 1. In addition to the flow cytometry technique, the antibiotics-induced apoptosis could also be demonstrated by using TUNEL staining. The nuclei of HPMCs displayed bright green fluorescence after 48 h of CFT or CPL treatment, but cefazolin- or ceftriaxone-treated cells showed faint fluorescent staining of the nuclei, similar to the medium controls (Fig. 1). After 48 h of CFT or CPL treatment, the apoptotic features (such as condensation of the nuclear chromatin, wrinkling of the nuclear membrane, dilation of endoplasmic reticulum, and relatively normal structure of the mitochondria) can also be verified by using electron microscopy (Fig. 2). The cells treated with cefazolin or ceftriaxone for 48 h had the same result as the medium

Table 1 Percentages of human peritoneal mesothelial cells (HPMC) in sub-G1 group (apoptosis) after treatment with cefazolin, ceftriaxone, cefotaxime or cephalothin for various time periods

	Hours after incubation		
	40	48	56
Control	1.8 ± 0.4	2.6 ± 0.6	3.3 ± 0.6
Cefazolin (0.5 mg/mL)	1.8 ± 0.5	2.7 ± 0.5	2.8 ± 0.5
Ceftriaxone (0.5 mg/mL)	1.7 ± 0.4	2.6 ± 0.3	2.3 ± 0.7
Cefotaxime (1 mg/mL)	6.6 ± 1.5	11.6 ± 2.4*	13.2 ± 2.5*
Cephalothin (0.5 mg/mL)	8.2 ± 1.0*	14.6 ± 1.7*	23.8 ± 4.0*

Data are expressed as mean ± SEM. $n = 3$; $^*P < 0.05$ versus the control.

controls, which did not display ultrastructural changes of apoptosis. All these data indicated that CFT (1 mg/mL) and CPL (0.5 mg/mL) could induce the apoptosis of HPMCs, whereas cefazolin or ceftriaxone failed to induce apoptosis of HPMCs.

After the findings of HPMC apoptosis were confirmed, we further studied the signalling pathway of apoptosis induced by CFT and CPL. First, we examined several proteins involved in apoptosis by Western blotting. The protein level of Bcl-2 did not alter, but Bax and p53 expression increased after CFT or CPL treatment (Fig. 3). The procaspase-3 (32 kDa) expression did not change significantly. While the active forms of caspase-3 (17 and 12 kDa) were produced after the treatment with CFT, the CPL treatment did not generate the same active form (Fig. 4). The expression of the caspase 8 active form did not change after CFT or CPL treatment (Fig. 5). Second, the results of immunofluorescence staining could discern a change in the distribution of cytochrome c after treatment with CFT or CPL. Cytochrome c is generally found normally distributed within the mitochondria, and therefore under normal conditions, the mitochondria are stained while the nuclei remained unstained (Fig. 6a). In contrast, after the treatment of HPMC with CFT or CPL, cytochrome c was released into the cytoplasm and the whole cells, including the nuclei, were covered by fluorescent stain (Fig. 6b,c).

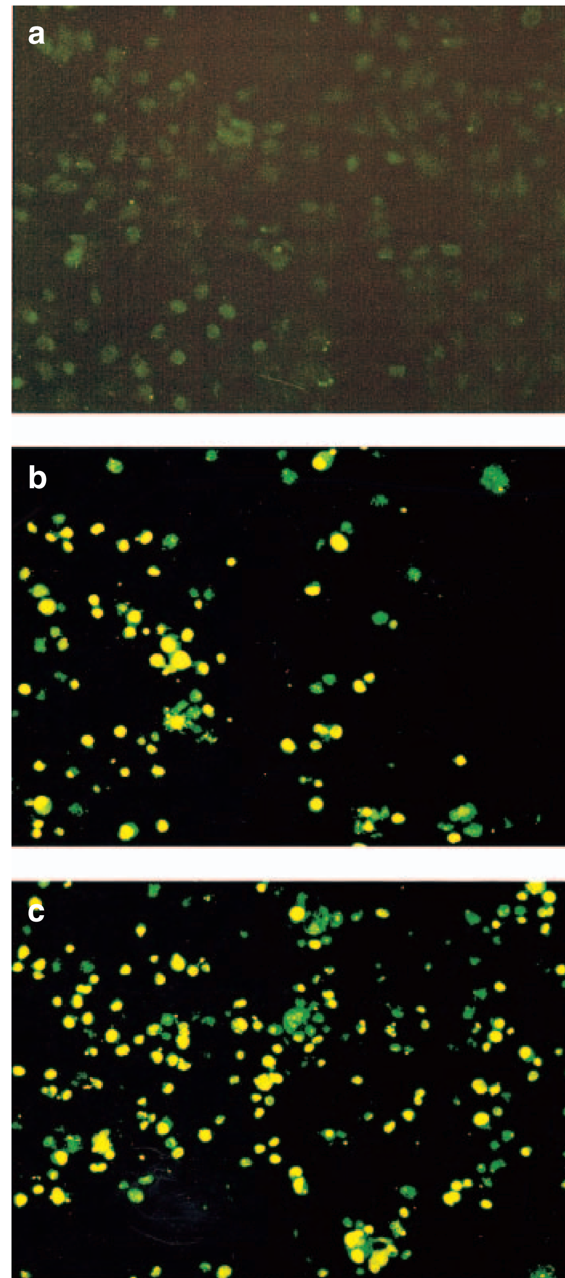

Fig. 1 Antibiotic-induced apoptosis was demonstrated by the TdT-mediated dUTP nick-end labelling (TUNEL) staining. The control cells showed faint fluorescent staining of the nuclei (a). The nuclei of the human peritoneal mesothelial cells (HPMC) displayed bright green fluorescence after 48 h of exposure to (b) cefotaxime (CFT; 1 mg/mL) or (c) cephalothin (CPL; 0.5 mg/mL) treatment. The cells treated by cefazolin (0.5 mg/mL) or ceftriaxone (0.5 mg/mL) for 48 h showed the same results as the control (data not shown).

DISCUSSION

It is well known that peritoneal dialysate[10,12] and drugs[13] used in CAPD have adverse effects on HPMCs. However, there are few studies examining the induction of apoptosis by these agents in HPMC.[11,27] The present study demonstrated that CFT and CPL induced HPMC apoptosis *in vitro*, whereas cefazolin and ceftriaxone did not. When we consider the important functions of the HPMC, with the same antimicrobial activity, cefazolin

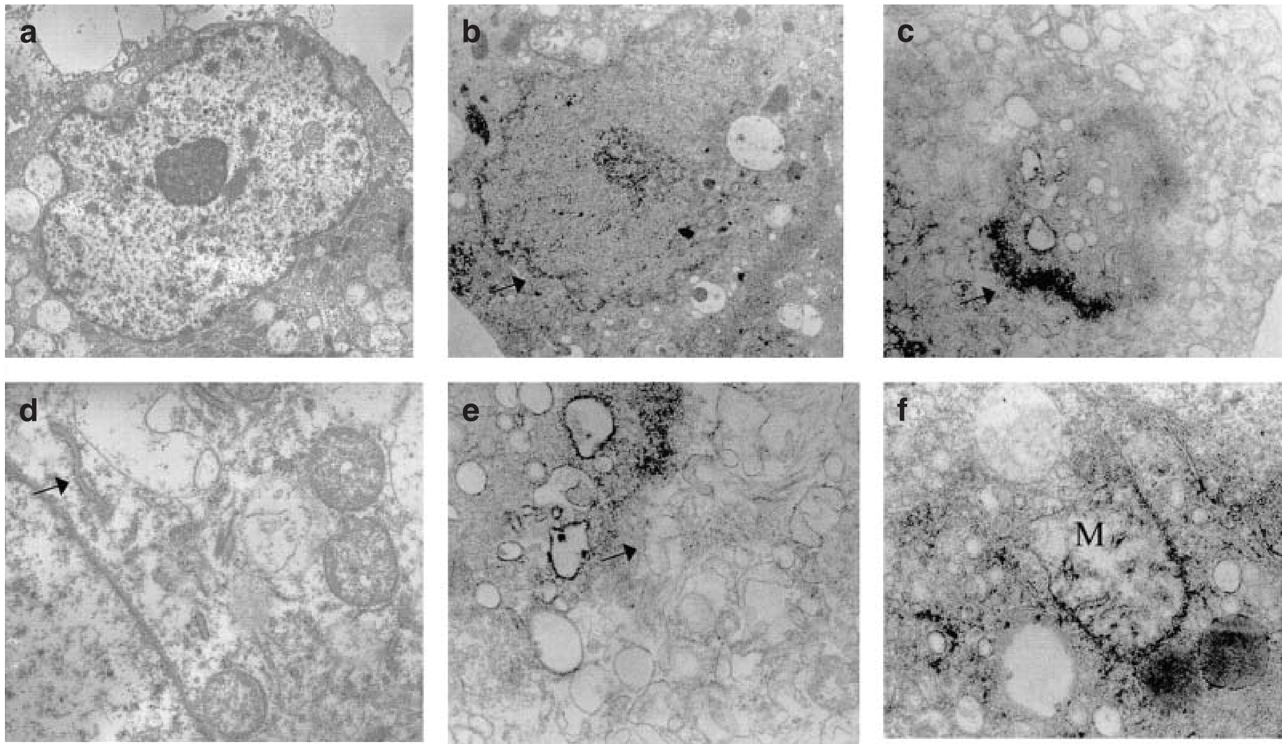

Fig. 2 After a 48 h incubation with and without cefotaxime (CFT; 1 mg/mL; the same picture was noted after treatment with cephalothin (CPL; 0.5 mg/mL)), the human peritoneal mesothelial cells (HPMC) were examined by electron microscopy. The control cell displayed a (a) normal nucleus and an endoplasmic reticulum (arrow in Fig. 2d). The CFT-treated cells showed wrinkling of the nuclear membrane (arrow in Fig. 2b), condensation of the nuclear chromatin (arrow in Fig. 2c), dilation of the endoplasmic reticulum (arrow in Fig. 2e), and the relatively normal structure of the mitochondria (M in Fig. 2f).

and ceftriaxone would be the better choices with regards to the treatment of bacterial peritonitis via the intraperitoneal route. At present, no animal or clinical studies have been conducted to compare the differences regarding the short- or long-term membrane functions between the two groups of antibiotics. Our study warrants an *in vivo* research regarding the relationship between the cytotoxicity of antibiotics and peritoneal membrane function.

The signalling pathway of apoptosis on HPMC has not been studied. In general, the signalling pathway of apoptosis is complex, but can be divided largely into a Fas/Fas ligand and a mitochondrial pathway (reviewed in[21]). The tumour suppressor p53 is a transcriptional factor that is involved in apoptosis partly by inducing Bax expression.[28,29] Bax is a death promoter, which is neutralized by heterodimerization with Bcl-2. Bax translocates into the outer mitochondrial membrane and then a leakage of cytochrome c from the mitochondria into the cytosol follows.[30] Caspase-9 and caspase-3 are activated sequentially, and this event then leads to the breakdown of chromosomal DNA. In the present study, we first demonstrated that CFT and CPL induced the apoptosis of HPMC at the concentration that was administered intraperitoneally to our CAPD patients. It was then found that the CFT and CPL increased the expression of p53 and Bax, while the expression of Bcl-2 was only slightly changed. We also demonstrated that cytochrome c was released from the mitochondria into the cytoplasm. After treatment of CFT or CPL, caspase 8 was not activated; this precluded the Fas/Fas ligand pathway involvment. All these data support the hypothesis that the signalling of apoptosis induction by CFT and CPL is through the mitochondrial pathway.

Caspases belong to a family of cysteine proteases. They are essential components of a proteolytic cascade that is involved in the execution stages of apoptosis. Our data regarding caspase-3 activation by these two antibiotics were different. Cefotaxine clearly induced the expression of an active form of caspase-3, whereas CPL did not. Although caspase-3 was thought of as the common executive protease during apoptosis, several studies have demonstrated that caspase-3 is dispensable in apoptosis (reviewed in[31]). Recently, the study of caspase-3-deficient cells showed that caspase-3 deficiency does not affect Bax-induced apoptosis.[32] Our data on CPL-induced apoptosis further confirmed that apoptosis may occur without caspase-3 activation.

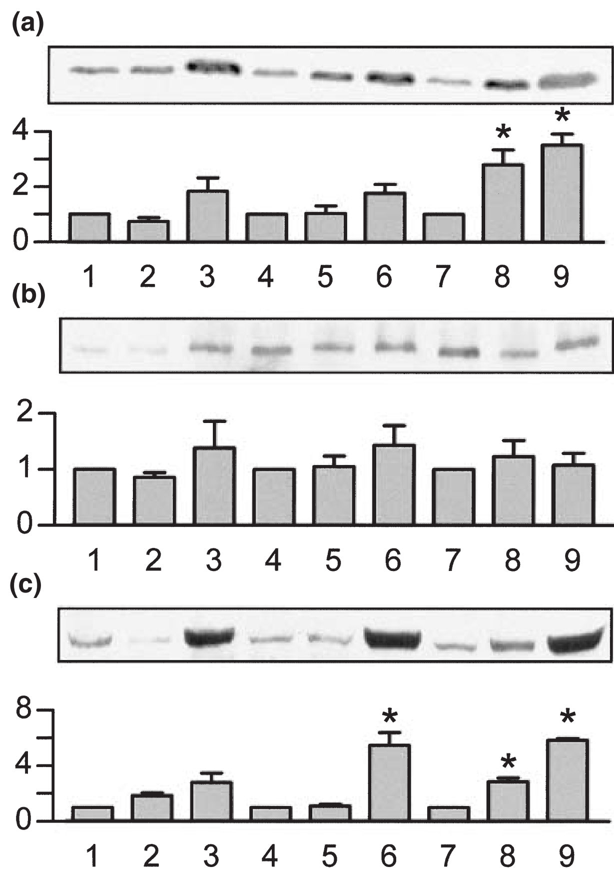

Fig. 3 After 40–56 h of incubation with cefotaxime (CFT; 1 mg/mL) or cephalothin (CPL; 0.5 mg/mL), the protein expressions of (a) Bcl-2, (b) Bax, and (c) p53 were examined by using western blotting. Lanes 1, 2 and 3 represent the control, CFT and CPL treatment for 40 h, respectively. Lanes 4, 5 and 6 represent the control, CFT and CPL treatment for 48 h, respectively. Lanes 7, 8 and 9 represent the control, CFT and CPL treatment for 56 h, respectively. Data are expressed as mean ± SEM. $n = 3$, $^{*}P < 0.05$ versus the control.

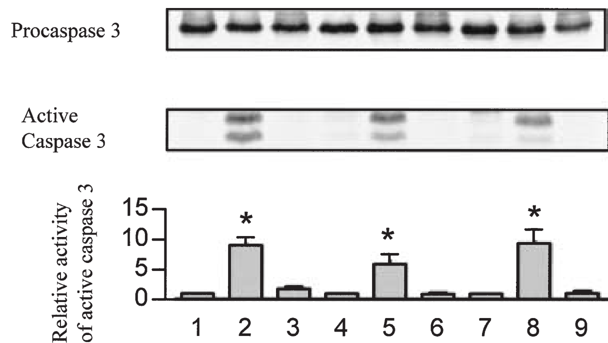

Fig. 4 After 40–56 h of incubation with cefotaxime (CFT; 1 mg/mL) or cephalothin (CPL; 0.5 mg/mL), the protein expressions of procaspase 3 (32 kDa) and active forms of caspase 3 (17 and 12 kDa) were examined by using Western blotting. Lanes 1, 2 and 3 represent the control, CFT and CPL treatment for 40 h, respectively. Lanes 4, 5 and 6 represent the control, CFT and CPL treatment for 48 h, respectively. Lanes 7, 8 and 9 represent the control, CFT and CPL treatment for 56 h, respectively. Data are expressed as mean ± SEM. $n = 3$, $^{*}P < 0.05$ versus the control.

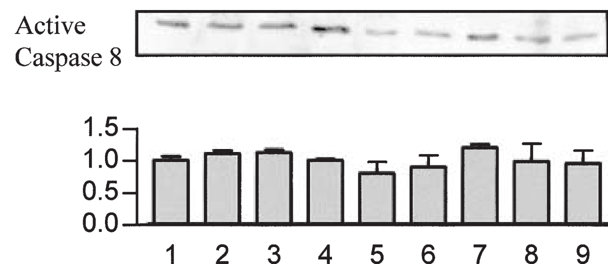

Fig. 5 After 40–56 h of incubation with cefotaxime (CFT; 1 mg/mL) or cephalothin (CPL; 0.5 mg/mL), the protein expressions of active forms of caspase 8 (18 kDa) were examined by using western blotting. Lanes 1, 2 and 3 represent the control, CFT and CPL treatment for 40 h, respectively. Lanes 4, 5 and 6 represent the control, CFT and CPL treatment for 48 h, respectively. Lanes 7, 8 and 9 represent the control, CFT and CPL treatment for 56 h, respectively. Data are expressed as mean ± SEM, $n = 3$.

To the best of our knowledge, there is no previous report on the cytotoxicity of CFT and CPL. In the literature, cephaloridine is the most frequently studied cephalosporin known to induce toxicity. Cephaloridine has been shown to deplete renal glutathione[33] and reduce tubular cell numbers and mitochondrial carnitine transport (i.e. it is toxic to mitochondrial fatty acid metabolism).[34] Our data suggested that CFT and CPL induced apoptosis by affecting mitochondria. It is reasonable to propose that CFT and CPL might affect mitochondrial redox status. The effects of CFT and CPL on mitochondrial oxidative and fatty acid pathways in HPMC warrant further study.

In conclusion, cefotaxime and cephalothin induce apoptosis of human peritoneal mesothelial cells *in vitro*. The signalling transduction may be through the mitochondrial pathway.

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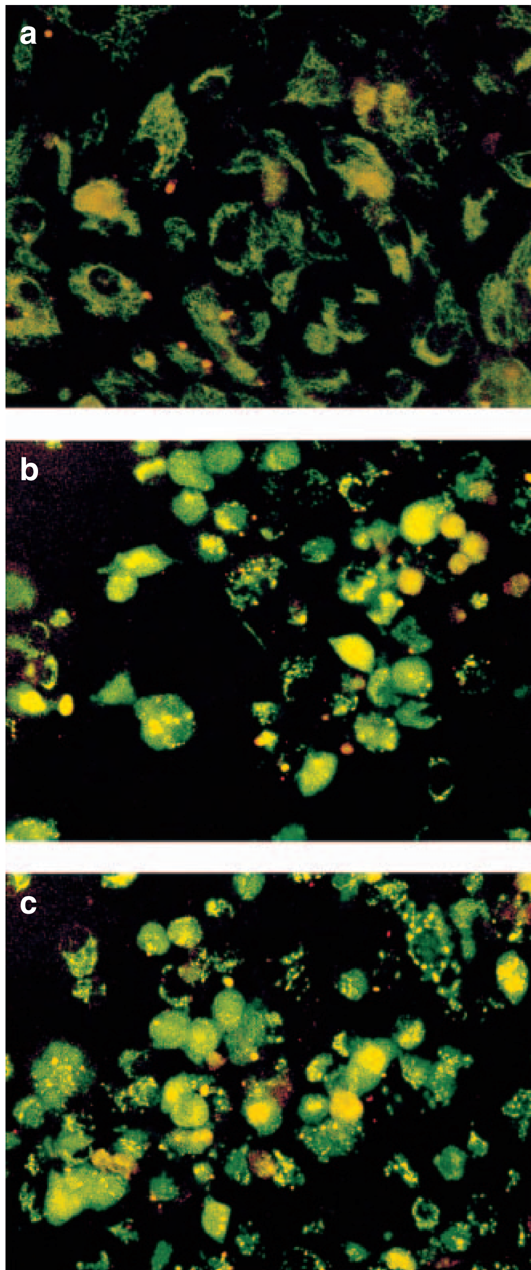

Fig. 6 Distribution of cytochrome c was changed after treatment with cefotaxime (CFT) or cephalothin (CPL). The fluorescent stain remained in the mitochondria while failing to stain the nuclei of control cells (a). After the treatment of human peritoneal mesothelial cells (HPMC) with (b) CFT (1 mg/mL) or (c) CPL (0.5 mg/mL) for 48 h, cytochrome c was released into the cytoplasm, causing the fluorescent stain to cover the whole cells, including their nuclei.

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