Peritoneal Dialysate Effluent during Peritonitis Induces Human Cardiomyocyte Apoptosis by Regulating the Expression of GATA-4 and Bcl-2 Families

Hsin-Hui Wang [1,2*], Ping-Chun Li [3*], Hsiao-Ju Huang[4], Tzong-Yann Lee [5,6], Ching-Yuang Lin[4,7]

[1]Department of Pediatrics, Section of Nephrology, Taipei Veterans General Hospital, [2]Department of Pediatrics, Faculty of Medicine, National Yang-Ming University, Taipei,

[3]Department of Surgery, Division of Cardiovascular Surgery, China Medical University Hospital,

[4]Clinical Immunological Center, China Medical University Hospital,

[5]Department of Internal Medicine, Section of Nephrology, En Chu Kong Hospital,

[6]Department of Integrated Diagnostics & Therapeutics, National Taiwan University Hospital,

[7]College of Medicine, China Medical University

*Contributed equally to this work.

Running title: PD effluent induces cardiomyocyte apoptosis

Address for correspondence and reprint requests
Ching-Yuang Lin, M.D., Ph.D.
Clinical Immunological Center, and Division of Pediatrics Nephrology,
China Medical University and Hospital
No. 2, Yuh-Der Road, Taichung, Taiwan 40402
TEL: 886-4-2207 1501
FAX: 886-4-2207 1352
E-mail: cylin@mail.cmuh.org.tw

ABSTRACT

Cardiovascular event and infection are leading causes of death from peritoneal dialysis (PD). This study examined in vitro cellular mechanism for cardiotoxicity induced by PD-related peritonitis. Cultured human cardiomyocytes were treated with PD effluent (PDE) during peritonitis (PPDE), and effects of PPDE on cultured cardiomyocytes in terms of apoptosis, with expression its related genes assessed. Results showed PPDE treatment of cardiomyocyte leading to onset of apoptosis, as confirmed by phosphatidylserine exposure plus DNA fragmentation and damage. This apoptosis is mediated by reduced Bcl-2/Bax and Bcl-x_L/Bax ratios, as well as reduced expression of GATA-4, an important cardiomyocyte survival factor, at the level of transcription. These changes activated pro-apoptotic pathways. PPDE treatment also inhibited ERK signals, contributing to cardiotoxicity. Our findings revealed that PPDE contains potent pro-apoptotic factors that regulate expression of GATA-4 and Bcl-2 families, inducing cultured cardiomyocyte apoptosis. This pinpoints a key role of apoptosis in PD-associated cardiovascular events, along with a potential therapeutic target.

Keywords: **apoptosis, Bcl-2, cardiomyocytes, GATA-4, peritonitis**

INTRODUCTION

Cardiovascular event and infection are the first and second leading causes of death in the peritoneal dialysis (PD) populations [1-4]; both events are closely related. PD-related peritonitis is the crucial infection in PD patients [5, 7]. Peritoneal toxin should be absorbed to the systemic circulation and might induce cardiotoxicity. After an episode of severe infection in dialysis patients, risk of death from cardiovascular events is increased seven-fold for 6 months and continues to rise for up to 48 months [5, 6]. It has been considered to play a significant role in up to one sixth of patient deaths occurring during the course of PD therapy [8]. In 41.5% of patients with peritonitis-related mortality, immediate cause of death was a cardiovascular event [9]. Clinical findings indicate that a peritonitis episode may culminate in cardiovascular event [5, 8]: high incidence of peritonitis is accompanied by greater risk of death [8, 10, 11], and cardiovascular events contribute to risk of peritonitis-related death in patients undergoing PD [12-14]. However, the possible mechanisms connecting PD-related peritonitis and cardiac mortality have not been addressed.

Growing evidence implicates cardiomyocyte apoptosis as a mechanism contributing to various types of heart disease [15-17]. Cardiomyocyte apoptosis could result in a loss of contractile tissue, compensatory hypertrophy of myocardial cells, reparative fibrosis, and heart failure. In animal models, endotoxin [18, 19], exotoxin [18, 20], and inflammatory mediator [21] play important roles in cardiomyocyte apoptosis. In PD patients with infectious peritonitis, expression of inflammatory

mediators and cytokines increase in PD effluent (PDE) and correlate with treatment outcome [22-23]. Yet there are no data on effects of peritonitis PD effluent (PPDE) on cardiomyocytes viability and apoptosis.

Bcl-2 protein family members are the best characterized proteins that are directly involved in the regulation of apoptosis [24]. Bcl-2 and its closest homologues, Bcl-x_L and Bcl-w, potently inhibit apoptosis in response to many cytotoxic insults. Bax and Bak are well known proapoptotic members of the Bcl-2 protein family. Regulation of apoptosis is highly dependent on the ratio of anti-apoptotic to pro-apoptotic proteins. Conditions that induce myocardial stress cause complex alterations in levels of Bcl-2 family proteins [25].

Cardiac Bcl-2 gene expression has been shown to be regulated by GATA-4 both in vitro and in vivo [26]. GATA-4 is a transcription factor enriched in cardiac tissue that is essential for various cardiomyocyte physiological and adaptive responses. An early event in the cardiotoxicity induced by the antitumor drug doxorubicin is GATA-4 depletion, which in turn causes cardiomyocyte apoptosis [27, 28]. GATA-4 has also been shown to upregulate transcription of the anti-apoptotic genes Bcl-2 [26] and Bcl-x_L [27, 28] in cardiomyocytes, and to play a central role in regulating the survival or apoptosis of cardiomyocytes. Although previous studies have suggested the importance of apoptosis regulation and GATA-4 expression in various heart diseases, their role in PD peritonitis-related cardiotoxicity has not been elucidated.

To clarify the relationship between PD-related peritonitis and high cardiac mortality, we examined the contributions of PD-related peritonitis to cardiotoxicity. We postulated that during

PD-related peritonitis, proapoptotic pathways are activated in cardiomyocytes. To test this hypothesis, human cardiomyocytes were cultured and treated with PPDE. The possible underlying signaling pathways of cardiotoxicity induced by PPDE were examined.

SUBJECTS AND METHODS

Human cardiomyocytes culture

This research was approved by the China Medical Hospital Institutional Revien Board. Written informed consent was obtained from each individual. Human cardiomyocytes obtained from the myocardial ventricular resection specimens of patients undergoing cardiac surgery were isolated as previously described [29]. Cells were cultured for a period of eight days, and culture medium was completely replaced every three days. Cultured medium was Dulbecco's modified Eagle medium (Hyclone, UT, US) containing equal Volume of F12, 1% L-glutamine, 25% fetal bovain serum, 1% non- essential amimo acid, 1% pyruvate, 250 μl hydrocortisone, 250 μl interferrin and insulin 250 μl in each 500ml. For keeping its cardiomyocytes property, the cells were passaged before 90 percent confluent about 2-3 days.

Doxorubicin (Sigma, MO, US) (DOXO) was prepared as 50 μM stock solution in DMSO and then diluted to 0.5 and 1 μM in 1×PBS for working concentration.

We also measured electrophysiological character of cultured human cardiomyocytes, including action potential duration (APD) and peak L-Type calcium current (IcaL) [30].

Immunostaining of human cardiomyocytes and confocal microscopic imaging

To characterize cardiomyocytes in culture, cells were incubated with a desmin monoclonal antibody (mAb)(Vision Biosystems), α-sarcomeric actinin mAb (Sigma, St. Louis, MO, USA), or a rabbit

polyclonal anti-CAPON antibody (Santa Cruz Biotechnology, Inc. CA, USA), followed by a FITC-labelled IgG (Santa Cruz Biotechnology, Inc.) secondary antibody. Cells were double labeled with WGA (Invitrogen, San Francisco, USA). Staining was assessed and photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope.

Patient population and peritonitis

Turbid PPDE prior to antibiotic treatment were collected in 8 culture positive peritonitis episodes from PD patients treated in our hospital. Microorganisms were gram-positive bacteria in 5 episodes and gram-negative bacteria in 3 episodes. All patients had a Tenckhoff peritoneal catheter inserted and were treated with the standard double-bag system (Baxter Healthcare Corp., Deerfield, IL). These 5 men and 3 women had a mean age of 47.8 years. Causes of renal failure included chronic glomerulonephritis, reflux nephropathy, obstructive nephropathy and renal hypoplasia. Time on PD ranged from 10 to 56 months. Peritonitis was defined as presence of two of the following criteria: microorganisms on gram staining, subsequent positive culture of PD fluid; cloudy fluid (leukocyte count >100 cells/mL with >50% polymorphonuclear cells) and/or peritoneal inflammation symptoms. Exclusion criteria included: a) tunnel-tract or exit site infections, b) completion of antibiotic therapy for peritonitis within 28 days of study enrollment, c) the presence of peritonitis attributed to fungus or mycobacterial infection or negative culture, d) drug sensitivity showing resistance to initial antibiotic therapy, e) previous immunosuppressive therapy, f) anemia from a disorder other than chronic renal failure, and g) insulin-dependent diabetes mellitus.

Overnight dwell bags of PDE samples (SPDE) were collected also from 5 stable PD without peritonitis patients (2 men, 3 women, mean age = 50.23 ± 11.67 years) treated in our hospital. No patient was diabetic, and no peritonitis episode was noted over the past year. Causes of renal failure included chronic glomerulonephritis, obstructive nephropathy and polycystic kidney disease. Patients signed an informed consent form for these studies.

Collection of PDE

The PDE were collected as previously described [31]: PDE was centrifuged at 400 g, 4°C for 10 min. After centrifugation, supernatants were collected, refrigerated, then lyophilized and dried to powder using Freeze Dryer (Eyela, Tokyo, Japan). Powder was stored at -70°C until analysis. The lyophilized PPDE samples were reconstituted as a 625 mg/ml stock solution with 1×PBS, and filtrated by 0.45 μM filter. PPDE stock solutions were storage at $-20^{o}C$ and diluted with 1× PBS to working concentration before use.

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

Cardiomyocyte cell viability was determined by a MTT assay. In a 96-well microplate, $2.5x10^4$ cells/well were incubated in 100 μl of culture media and exposed to different concentrations of DOXO or PPDE for varying time periods. 12.5 μM MTT dye (stock ; 5 mg/ml) were added into each well and the cells were further incubated at $37^{o}C$ for 4hr. Viable cells could convert MTT dye dark blue product, the cells were lysed with dimethyl sulfoxide (DMSO) (Sigma, MO, US) and cell lysates were collected. Optical density (OD) values of the absorbance 540 nm were obtained

using a microplate reader (mode FL 331, Bio-Tek Instruments, Winooski, VT).

Apoptosis assays by flow cytometry

Apoptosis was quantitatively gauged by detecting phosphatidylserine exposure on cell membrane with Annexin V staining, as described above [32]. Cells were simultaneously stained with Annexin V-FITC (25 ng/ml; green fluorescence, R&D Systems, Minneapolis, MN, USA) and dye exclusion (propidium iodide, 20 μg/ ml, red fluorescence). Data were obtained by flow cytometry analysis with FACS-SCAN(Becton-Dickinson) FACS Canto in cell populations from which debris was gated out and analyzed.

Evaluation of apoptosis by TUNEL and DAPI staining

Cultured human cardiomyocytes were plated at approximately 2 x10^5 cells/well in 12-well plates with DOXO or PPDE and incubated at 37˚C for 24 h. Cell nuclei were stained with 4, 6-Diamidino-2-phenyindole (DAPI, Sigma) and DNA fragments labeled with AlexaFluor 488 dye-labeled anti-BrdU antibody (Apo-Brd U^{TM} TUNEL assay kit. Invitrogen). Cells were observed and photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope. An individual blinded to the experimental conditions counted at least 300 cells in 6 different high power fields for each experiment.

Comet assay

Approximately 5 x 10^3 cells/ml of cardiomyocytes were incubated with doxorubicin or PDE during

peritonitis for 24 h at 37˚C, isolated, then examined for DNA damage by Comet assay previously described [33]. Treated cells were embedded in situ in 1% agarose, then placed in lysis solution for 30 min. Cell nuclei were subsequently electrophoresed for 20 min at 1 V/cm, followed by staining with PI and visualization with a fluorescence microscope.

RNA isolation and reverse transcription

RNA was extracted from cells using RNAzol B (TEL-TEST, INC.). Resulting RNA suspension was forthwith converted to cDNA by reverse transcription, cDNA samples stored at -70°C for analysis.

Real-time PCR with SYBR green assay

Five 贡l of cDNA (1-10 ng) was mixed with SYBR green PCR core reagent or master mix reagent (Aplied Biosystems, CA, US). The thermal cycling conditions were determined according to rules of 'Thermal cycling parameters for primer optimization'. Each RNA samples was also analyzed with β-actine which serve as internal control for correcting relative specific gene expression levels. Primers were designed using Primer Express Primer Design software, as follows:

Bcl-2: sense primer: ATGTGTGTGGAGAGCGTCAA

antisense primer: ATCACCAAGTGCACCTACCC

Bcl-x_L: sense primer: ACAGCAGCAGTTTGGATGC

antisense primer: TGGGATGTCAGGTCACTGAA

Bax: sense primer: TTGGGTGAGACTCCTCAAGC

antisense primer: CACTGTGACCTGCTCCAGAA

GATA-4: sense primer: AGCTCCTTCAGGCAGTGAGA

antisense primer: CTGTGCCCGTAGTGAGATGA

β-actine : sense primer : CAGGTATGCACCCAGAGTGA

antisense : GATATGGAGAAGATTTGGCA

According to amplification plot, cycle number over the threshold means Ct value. The Ct value of non-template control is 45. The relative expression ratio among untreated RNA and different RNA samples could be calculated with 2-Ct.

Western blotting

10-50 μg protein extracts were separated by 10-12% SDS-PAGE and transferred to nitrocellulose (PVDF) membranes blocked overnight with 1×TBS buffer containing 5% skim milk. Membranes were incubated with optimal concentrations of primary antibodies: anti-GATA-4 mAb (Abcam, Abgent, San Diego, CA, USA)**,** anti-extracellular signal–regulated kinase (ERK) mAb (Cell Signaling Technology, Beverly, MA, USA)**,** and anti-β actin mAb (Sigma) in 1×TBS buffer containing 5% skim milk. Membranes were washed and then incubated with appropriate secondary antibody (goat anti-mouse mAb conjugated with HRP), and visualized by enhanced chemiluminescence ECL detection kit (Perkin Elmer, MA, US). The antibody for GATA-4 (1:100), Bcl-X_L (1:100), Bax (1:100) were purchased from Santa Cruz (CA, US) and Bcl-2 (1:1000) was

purchase from cell signaling (CA, US).

Statistics

All data are presented as mean ± standard deviation (SD). Differences between groups were analyzed by Mann-Whitney U test or ANOVA, values of $P<0.05$ considered statistically significant.

RESULTS

Characterization of human cardiomyocytes in primary culture

To characterize cardiomyocytes, muscle markers desmin and myocyte-specific protein α-sarcomeric actinin were detected (Figure 1). CAPON, recently documented as endogenous protein expressed in guinea pig cardiomyocytes, interacts with nitric oxide synthase to accelerate cardiac repolarization by inhibition of L-type calcium channels. Expression of endogenous CAPON protein in cultured cardiomyocytes was detected by immunofluorescent staining and confocal microscopy (Figure 1). Both action potential duration (APD) and peak L-Type calcium current (IcaL) were APD_{10}, APD_{50}, APD_{75} and APD_{90} : 95.4±10.6, 289.2±15.6, 308.2±15.4, and 318.4±16.4 ms, respectively, with peak IcaL density of -10.2±0.9 pA/pE at + 10mV (n=6).

PPDE induces cell death in human cardiomyocytes

Cardiac cell death is believed to play a major contributory role in development and progression of myocardial dysfunction [17]. To assess whether PPDE treatment induced cardiac cell death, cell viability were evaluated by MTT assay. Doxorubicin-induced cardiotoxicity, which has been well described [35], was used as a positive control. MTT assay showed PDE during peritonitis- and doxorubicin-induced human cardiomyocyte cell death as both dose- (Figure 2A and Table 1) and time-dependent (Figure 2B and Table. 2). When cardiomyocytes were pre-exposed to 12.5, 18, or 25 mg/ml PDE during peritonitis for 24 hr, cell viabilities were 70.6±5.7%, 58.7±9.7%, and

41.6±7.8%, respectively, all significantly lower than in cardiomyocytes without pre-treatment (P<0.05) (Figure 2A). This change was even more profound in the 48 hr treatment group (Figure 2B). When cardiomyocytes were pre-exposed to 25 mg/ml PDE from stable PD patients for 24 and 48 hr, cell viabilities were similar with cardiomyocytes without pre-treatment (data not shown).

PPDE induces apoptosis in human cardiomyocytes

The above lend substantial evidence of apoptosis playing a critical role in cardiomyocyte cell death associated with several cardiac diseases [15, 17]. To explore whether PPDE during peritonitis challenge induces human cardiomyocyte apoptosis, we assessed apoptotic cell death by flow cytometry. TUNEL staining and Comet assays were performed for determination of DNA damage. Doxorubicin, which can induce cardiomyocyte apopotosis [33], was used as a positive control. After cell incubation with 25 mg/ml PPDE peritonitis for 24 hr, apoptosis was detected by flow cytometry (Tables 1 and 2). TUNEL staining and confocal microscopy confirmed cardiomyocyte apoptosis (Figure 3A). Analyses indicated little cardiomyocyte apoptosis with non-exposed condition (control group) and exposure with 25 mg/ml PPDE from stable PD patients (Tables 1 and 2). By contrast, doxorubicin and PPDE induced apoptosis in 32.3-49.7% of human cardiomyocytes after treated for 24 hours. Finally, PPDE induced DNA damage was determined by Comet assay (Figure 3B): higher concentrations of PPDE resulted in greater numbers of damaged cells.

PPDE induced Bax increase and suppression of GATA-4 expression in human cardiomyocytes

The Bcl-2 family of proteins are key regulators of the stress-induced apoptotic pathway [25]; to determine their role in regulation of PPDE induced cardiomyocyte apoptosis, mRNA concentrations of prosurvival proteins Bcl-2 and Bcl-x_L and proapoptotic protein Bax were measured in human cardiomyocytes by quantitative real-time RT-PCR(Figure 4A). In cardiomyocytes exposed to PPDE 4 hrs, the Bcl-2/Bax and Bcl-x_L/Bax ratios were 0.30 ± 0.02 and 0.44 ± 0.02, respectively (Figure 4B). Compared to the no-exposure control group, Bcl-2/Bax and Bcl-x_L/Bax ratios dropped by 30-45% following 4 hrs of PPDE treatment (Figure 4A; $P<0.05$ versus control). Western blotting analysis for Bcl-2, Bcl-xl and Bax protein expression in the same experimental conditions obtained similar results (Figure 6A). These data indicated that PPDE treatment decreased Bcl-2/Bax and Bcl-x_L/Bax ratios, resulting in increase Bax expression in human cardiomyocytes.

Transcription factor GATA-4 has been identified as a specific myocardial survival factor which induces transcription and expression of Bcl-2 and which is associated with cell survival [27-29, 36]. To characterize mechanisms underlying PPDE activity in human cardiomyocytes, mRNA and protein expression of GATA-4 were measured. For cardiomyocytes exposed to PPDE, GATA-4 mRNA expression decreased fivefold relative to no-exposure control cells by quantitative real-time RT-PCR ($P<0.05$) (Figure 5A). Western blots of nuclear GATA-4 protein expression in PPDE exposed human cardiomyocytes also showed lower levels than the control group (Figure 5B, C), suggesting that PDE during peritonitis treatment decreases levels of GATA-4 gene expression in human cardiomyocytes.

PPDE does not contain inflammatory mediators

To evaluate whether PPDE was enriched in pro-apoptotic mediators, TRAEL, FasL, TNFα, IL-6 and IL-1 were rated by enzyme-linked immunoassay by comercial ELISA kit. Cultured supernatant from peripheral blood mononuclear cells stimulated with lippolysaccharide was used as positive control; TRAEL, FasL, TNFα and IL-1 were undetectable in 25mg/ml PPDE (data not shown). The lower limit of sensitivity was 0.70 pg/ml.

Role of ERK pathway in PPDE induced cardiotoxicity

We next examined possible signaling mechanisms regulating PPDE-induced cardiomyocyte apoptosis. The GATA-4 molecule contains putative ERK phosphorylation sites, and recent studies have shown that some survival factors [36-38] induce activity of GATA-4 via MEK/ERK-dependent phosphorylation. Therefore, we explored activity of MEK/ERK signaling pathways in PPDE treated cardiomyocytes. Figure 6 shows ERK phosphorylation significantly reduced in cells exposed to PPDE peritonitis, suggesting that PPDE inhibits the ERK signaling pathway, consistent with the idea that the ERK pathway is crucial for GATA-4 activity and cardiomyocyte survival.

DISCUSSION

Our study demonstrates that PPDE contains potent pro-apoptotic factors and causes an imbalance between proapoptotic and prosurvival pathways, inducing apoptosis in human cardiomyocytes. This study revealed a possible mechanism of PD-related, peritonitis-induced cardiotoxicity. These novel findings constitute the first direct evidence linking PD peritonitis and cardiomyocyte apoptosis. Cardiovascular events are the major cause of death in PD patients with peritonitis. Our findings demonstrate the central role of apoptosis in PD peritonitis-associated cardiovascular events, and provide an explanation for the high incidents of cardiovascular events in PD-related peritonitis.

Cardiomyocyte death is important in the pathogenesis of cardiac disease in end stage renal disease [3]. Cardiomyocyte death induces LV dilatation with compensatory LV hypertrophy, and eventually leading to systolic dysfunction. LV hypertrophy appears to be an important, independent, determinant of survival in patiens with end stage renal diseases [39]. Our study yields direct cellular evidence of PPDE from PD patients as cardiotoxic. In end-stage renal disease, cardiomyocyte death may be caused by continual LV overload, decreased large and small coronary vessel perfusion, hyperparathyroidism, and malnutrition [3]. Our data provide another possible cause of cardiac cell death in patients undergoing PD.

Recent studies have proven various cardiac pathological states associating with cardiomyocyte apoptosis [15-17]. Our study found that PPDE induced cardiomyocyte apoptosis and severity varied

when diverse pathogens induced peritonitis (data not shown). Toxicity to cardiomyocytes was most profound with exposure to Staphylococcus aureus, Staphylococcus epidermidis, and Streptococcus viridans. Gram negative pathogens such as Escherichia coli and Proteus vulgaris were less cytotoxic. Prior animal study shows both endotoxin and exotoxin exposure inducing myocardial depression [18-20]. Interestingly, pretreatment of animals or cells with lipopolysaccharide (LPS) induced LPS tolerance and largely reduced inflammation and dysfunction of the rat myocardium [40, 41]. Further study should delineate effects of pathogen-related toxins on the PPDE-induced cardiotoxicity.

A second possible cause of cardiomyocyte apoptosis is inflammatory or pro-apoptotic mediators and cytokines in PPDE. Expression of inflammatory mediators and cytokines in PPDE is reported to increase during acute peritonitis, then return to control levels [22-24]. Inflammatory mediators and cytokines have been cited as playing a role in cardiomyocyte apoptosis and clinical cardiac dysfunction [42], with TRAL, FasL, TNFα, or IL-1 undetectable in 20 mg/ml PPDE. Inflammatory mediators and cytokines in PPDE may not contribute greatly to cardiomyocyte apoptosis.

GATA-4 is a survival factor in terminally differentiated cardiac myocytes [27, 28] and may be an essential component of adaptive response of the adult heart [28]. Experiments have shown 50% reduction in GATA-4 levels impairing drug-induced stress responses, yet not interfering with normal embryonic and postnatal mouse development [28]. Our results indicate a mechanism by which PPDE inhibits transcription of the GATA-4 gene: lower GATA-4 levels in cardiomyocytes exposed to PPDE would impair their ability to respond to cardiac work or to stimuli that produce cardiac stress and then induce apoptosis. By our current findings, GATA-4 restoration or prevention

of GATA-4 depletion may prove effective cardioprotection in PD patients with peritonitis.

The essential role of GATA-4 as a survival factor may be explained, in part, by its function as an upstream activator of the antiapoptotic Bcl-2 gene family. Previous research [43] has shown that over expression of Bcl-2 attenuates myocardial apoptosis. In the present study, exposure of cardiomyocytes to PPDE caused decreases in the Bcl-2/Bax and Bcl-x_L/Bax ratios. Moreover, Bcl-2 is a major regulator of mitochondrial permeability and hence prevents activation of the "intrinsic" mitochondria-dependent apoptosis pathway [44]. The intrinsic apoptotic pathway is activated in cardiac myocytes in response to various extracellular and intracellular stimuli [44]. Our results provide evidence that activation of the intrinsic apoptotic pathway may be a possible mechanism for PPDE induced cardiomyocyte apoptosis.

Several studies have suggested that in a subset of cell types, activation of ERK can protect against pro-apoptotic stimulants [45]. However, inhibition of apoptosis by ERK depends upon cell type and stimuli. The present study found that the ERK pathway is involved in protection against PPDE-induced cultured cardiomyocyte apoptosis. Interestingly, GATA-4 contains putative ERK phosphorylation sites. Prior studies show HGF and endothelin-1 activating GATA-4 via MEK-ERK pathway-dependent phosphorylation, with dominant MEK mutant blocking HGF-induced Bcl-x_L expression in cardiac muscle cells [29, 36]. Studying phosphorylation of GATA-4 by ERK pathway will elucidate the role of MEK/ERK/GATA-4 pathway in PPDE-induced cardiomyocyte apoptosis. Apoptosis is no simple, linear chain reaction. Recent study found sustained inflammation activating multiple cell death pathways and spawns cardiomyocyte apoptosis [42]. Further research will focus

on possible extrinsic apoptotic pathway involvement and relative importance of intrinsic and extrinsic pathways in PPDE-induced cardiomyocyte apoptosis. Unlike necrosis, viewed as basically irreversible, the step-by-step nature of apoptosis suggests existence of reversible checkpoints. Our results indicate therapeutic regimens that block intrinsic apoptosis signaling pathways presumably attenuate PPDE-induced cardiotoxicity.

In conclusion, this study demonstrates for the first time that PPDE contains potent pro-apoptotic factors that regulate expression of GATA-4 and Bcl-2 families, inducing cultured cardiomyocyte apoptosis. Findings illustrate a pivotal role of apoptosis in PD peritonitis-associated cardiovascular events, explain high cardiac mortality in PD-related peritonitis, and pinpoint apoptotic events as a marker and potential therapeutic target for PD peritonitis-induced cardiotoxicity.

ACKNOWLEDGEMENT

This study was partially supported by the grant of China Medical University (CMU97-212).

REFERENCES

1. 2008. U.S. Renal Data System, USRDS 2008 Annual Data Report: Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD.
2. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. 2004. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med 351: 1296-1305
3. Parfrey PS, Foley RN. 1999. The clinical epidemiology of cardiac disease in chronic renal failure. J Am Soc Nephrol 10: 1606-1615
4. Schiffrin EL, Lipman ML, Mann JF. 2007. Chronic kidney disease: effects on the cardiovascular system. Circulation 116: 85-97
5. Bender FH, Bernardini J, Piraino B. 2006. Prevention of infectious complications in peritoneal dialysis: best demonstrated practices. Kidney Int Suppl S44-54
6. Ishani A, Collins AJ, Herzog CA, Foley RN. 2005. Septicemia, access and cardiovascular disease in dialysis patients: the USRDS Wave 2 study. Kidney Int 68: 311-318
7. Aslam N, Bernardini J, Fried L, Burr R, Piraino B. 2006. Comparison of infectious complications between incident hemodialysis and peritoneal dialysis patients. Clin J Am Soc Nephrol 1: 1226-1233
8. Fried LF, Bernardini J, Johnston JR, Piraino B. 1996. Peritonitis influences mortality in peritoneal dialysis patients. J Am Soc Nephrol 7: 2176-2182

9. Perez Fontan M, Rodriguez-Carmona A, Garcia-Naveiro R, et al. 2005. Peritonitis-related mortality in patients undergoing chronic peritoneal dialysis. Perit Dial Int 25: 274-284

10. Maiorca R, Cancarini GC, Brunori G, Camerini C, Manili L. 1993. Morbidity and mortality of CAPD and hemodialysis. Kidney Int Suppl 40: S4-15

11. Piraino B. 1998. Peritonitis as a complication of peritoneal dialysis. J Am Soc Nephrol 9: 1956-1964

12. Digenis GE, Abraham G, Savin E, et al. 1990. Peritonitis-related deaths in continuous ambulatory peritoneal dialysis (CAPD) patients. Perit Dial Int 10: 45-47

13. Firanek CA, Vonesh EF, Korbet SM. 1991. Patient and technique survival among an urban population of peritoneal dialysis patients: an 8-year experience. Am J Kidney Dis 18: 91-96

14. Lupo A, Tarchini R, Carcarini G, et al. 1994. Long-term outcome in continuous ambulatory peritoneal dialysis: a 10-year-survey by the Italian Cooperative Peritoneal Dialysis Study Group. Am J Kidney Dis 24: 826-837

15. Olivetti G, Abbi R, Quaini F, et al. 1997. Apoptosis in the failing human heart. N Engl J Med 336: 1131-1141

16. Narula J, Pandey P, Arbustini E, et al. 1999. Apoptosis in heart failure: release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy. Proc Natl Acad Sci U S A 96: 8144-8149

17. Haunstetter A, Izumo S. 1998. Apoptosis: basic mechanisms and implications for cardiovascular disease. Circ Res 82: 1111-1129

18. Natanson C, Danner RL, Elin RJ, et al. 1989. Role of endotoxemia in cardiovascular dysfunction and mortality. Escherichia coli and Staphylococcus aureus challenges in a canine model of human septic shock. J Clin Invest 83: 243-251

19. Ramana KV, Willis MS, White MD, et al. 2006. Endotoxin-induced cardiomyopathy and systemic inflammation in mice is prevented by aldose reductase inhibition. Circulation 114: 1838-1846

20. Sibelius U, Grandel U, Buerke M, et al. 2000. Staphylococcal alpha-toxin provokes coronary vasoconstriction and loss in myocardial contractility in perfused rat hearts: role of thromboxane generation. Circulation 101: 78-85

21. Mann DL. 1999. Inflammatory mediators in heart failure: homogeneity through heterogeneity. Lancet 353: 1812-1813

22. Wang HH, Lin CY. 2005. Interleukin-12 and -18 levels in peritoneal dialysate effluent correlate with the outcome of peritonitis in patients undergoing peritoneal dialysis: implications for the Type I/Type II T-cell immune response. Am J Kidney Dis 46: 328-338

23. Lai KN, Lai KB, Lam CW, et al. 2000. Changes of cytokine profiles during peritonitis in patients on continuous ambulatory peritoneal dialysis. Am J Kidney Dis 35: 644-652

24. Cory S, Adams JM. 2002. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2: 647-656

25. Bishopric NH, Andreka P, Slepak T, Webster KA. 2001. Molecular mechanisms of apoptosis in the cardiac myocyte. Curr Opin Pharmacol 1: 141-150

26. Kobayashi S, Lackey T, Huang Y, et al. 2006. Transcription factor gata4 regulates cardiac BCL2 gene expression in vitro and in vivo. Faseb J 20: 800-802

27. Aries A, Paradis P, Lefebvre C, Schwartz RJ, Nemer M. 2004. Essential role of GATA-4 in cell survival and drug-induced cardiotoxicity. Proc Natl Acad Sci U S A 101: 6975-6980

28. Suzuki YJ, Evans T. 2004. Regulation of cardiac myocyte apoptosis by the GATA-4 transcription factor. Life Sci 74: 1829-1838

29. Ancey C, Corbi P, Froger J, et al. 2002. Secretion of IL-6, IL-11 and LIF by human cardiomyocytes in primary culture. Cytokine 18: 199-205

30. Kuan-Cheng Chang, Andreas S. Barth, Tetsuo Sasano, Eddy Kizana, Yuji Kashiwakura, Yiqiang Zhang, D. Brian Foster, and Eduardo Marbán. 2008. CAPON modulates cardiac repolarization via neuronal nitric oxide synthase signaling in the heart. PNAS March 18, 2008 vol. 105 no.11 4477-4482

31. Wang HH, Lee TY, Lin CY. 2008. Integrins mediate adherence and migration of T lymphocytes on human peritoneal mesothelial cells. Kidney Int 74: 808-816

32. Chen Q, Gong B, Mahmoud-Ahmed AS, et al. 2001. Apo2L/TRAIL and Bcl-2-related proteins regulate type I interferon-induced apoptosis in multiple myeloma. Blood 98: 2183-2192

33. Kim Y, Ma AG, Kitta K, et al. 2003. Anthracycline-induced suppression of GATA-4 transcription factor: implication in the regulation of cardiac myocyte apoptosis. Mol Pharmacol 63: 368-377

34. Chang KC, Barth AS, Sasano T, et al. 2008. CAPON modulates cardiac repolarization via neuronal nitric oxide synthase signaling in the heart. Proc Natl Acad Sci U S A 105: 4477-4482

35. Shan K, Lincoff AM, Young JB. 1996. Anthracycline-induced cardiotoxicity. Ann Intern Med 125: 47-58

36. Kitta K, Day RM, Kim Y, et al. 2003. Hepatocyte growth factor induces GATA-4 phosphorylation and cell survival in cardiac muscle cells. J Biol Chem 278: 4705-4712

37. Kitta K, Clement SA, Remeika J, Blumberg JB, Suzuki YJ. 2001. Endothelin-1 induces phosphorylation of GATA-4 transcription factor in the HL-1 atrial-muscle cell line. Biochem J 359: 375-380

38. Morimoto T, Hasegawa K, Kaburagi S, et al. 2000. Phosphorylation of GATA-4 is involved in alpha 1-adrenergic agonist-responsive transcription of the endothelin-1 gene in cardiac myocytes. J Biol Chem 275: 13721-13726

39. Silberberg JS, Barre PE, Prichard SS, Sniderman AD. 1989. Impact of left ventricular hypertrophy on survival in end-stage renal disease. Kidney Int 36: 286-290

40. Barroso-Aranda J, Schmid-Schonbein GW, Zweifach BW, Mathison JC. 1991. Polymorphonuclear neutrophil contribution to induced tolerance to bacterial lipopolysaccharide. Circ Res 69: 1196-1206

41. Madorin WS, Cepinskas G, Kvietys PR. 2001. Peritonitis induces rat cardiac myocytes to promote polymorphonuclear leukocyte emigration and activate endothelial cells: effect of

lipopolysaccharide pretreatment. Crit Care Med 29: 1774-1779

42. Haudek SB, Taffet GE, Schneider MD, Mann DL. 2007. TNF provokes cardiomyocyte apoptosis and cardiac remodeling through activation of multiple cell death pathways. J Clin Invest 117: 2692-2701

43. Chen Z, Chua CC, Ho YS, Hamdy RC, Chua BH. 2001. Overexpression of Bcl-2 attenuates apoptosis and protects against myocardial I/R injury in transgenic mice. Am J Physiol Heart Circ Physiol 280: H2313-2320

44. Foo RS, Mani K, Kitsis RN. 2005. Death begets failure in the heart. J Clin Invest 115: 565-571

45. Jarpe MB, Widmann C, Knall C, et al. 1998. Anti-apoptotic versus pro-apoptotic signal transduction: checkpoints and stop signs along the road to death. Oncogene 17: 1475-1482

FIGURE LEGENDS

Figure 1. Characterization of human cardiomyocytes by immunostaining of cardiomyocyte markers. Double labeling of cardiomyocytes with WGA (red) and α-sarcomeric actinin (green) (first line)or desmin (green) (second line) or CAPON (green) (third line). Negative control using cultured human skeletal muscle cells was stained with CAPON (last line).

Figure 2. Cell viability reduced after treatment with PPDE. Human cardiomyocytes were treated with PPDE during peritonitis. Cell viability was determined by MTT assay. (A) Cells were incubated with various concentration of peritoneal dialysate effluent during peritonitis (PPDE) (12, 5, 18, 25 mg/ml) (n=8) or doxorubicin (0.5, 1 μM; Doxo) as a positive control for 24 hr. (■) treated with medium only (□) or Doxo pretreatment; (B) cell were treated with PPDE (25 ng/ml) for 24 or 48 hrs. (n=8) and controls Data are expressed as mean ± SD of 8 different PPDE. # $P<0.01$ versus medium only control, * $P<0.05$, ** $P<0.01$.

(▨: treated with PBS only) (▧: treated with medium and stable peritoneal dialysate effluent (SPDE) without peritonitis) (▩treated with medium and peritonitis peritoneal dialysate effluent (PPDE))

Figure 3. PPDE treatment induces apoptosis in cultured human cardiomyocytes. (A) APO-Brud U^{TM} TUNEL assay. Cardiomyocytes were treated with PPDE, SPDE (25 mg/ml) or Doxo (0.5 μM)

as a positive control for 24 h, then stained by the TUNEL method. Nuclei were counter stained with AlexaFluor 488dye. Confocal microscopy of cardiomyocytes showed all nuclei (blue), including apoptotic nuclei (vacuoles). (B) Cardiomyocyte DNA damage was determined by the Comet assay. Cardiomyocytes were treated with PPDE (12.5 or 25 mg/ml) or Doxo (0.5 μM) as a positive control for 24 h, and then the Comet assay was performed. (B) Western

Figure 4. (A) PPDE treatment upregulates Bax gene expression in cultured human cardiomyocytes. Human cardiomyocytes were treated with or without PPDE (25 mg/ml), and then total RNA was prepared following 4 h of treatment. Bcl-2, Bcl-x_L, and Bax mRNA expression levels in cardiomyocytes were determined by quantitative real-time RT-PCR. (n=8) Data are expressed as the mean ± SD of 8 different PPDE.* P<0.01 versus control. (B)Bcl-2/Bax and Bcl-xL/Bax ratio of experiment (A). Data are expressed as mean ± SD of 8 different PPDE. * P<0.01 versus control. ■: no treatment, medium only ; ▨: no treatment PBS only ; ▧: medium + stable PDE without peritonitis(SPDE) (treated with medium and peritonitis peritoneal dialysate effluent (PPDE)) ;

Figure 5. Effects of PPDE on expression of cardiac GATA-4 mRNA and protein. Cultured human cardiomyocytes were treated with or without PPDE (25 mg/ml), and then protein extracts and total RNA were prepared following 4 h of treatment. (A) GATA-4 mRNA expression levels in cultured human cardiomyocytes as determined by quantitative real-time RT-PCR. (B) Western blot showing GATA-4 protein levels. (C) GATA-4 protein levels in cultured human cardiomyocytes, ascertained

by densitometry. Data are expressed as mean ± SD of 8 different PPDE. * $P<0.01$ versus control.

Figure 6. PPDE treatment reduces ERK phosyhorylation, GATA4, Bcl-2, Bcl-xL expression and enhances Bax expression in cultured human cardiomyocytes. Cardiomyocytes were treated with medium only, PBS only, SPDE (25 mg/ml) for 2 and 4 hours and PPDE (25 mg/ml) for 2 and 4 hours. Cell lysates were separated by SDS-PAGE and specific monoclonal antibodies were used to detect phosphorylated and total ERK, and Bax, Bcl-2, Bcl-xL, GATA4 expression. Representative blots from 8 separate experiments were shown. Quantitative densitometry expressed as phosphorylated protein relative to total protein. Data are expressed as the mean ± SD of 8 different PPDE .* $P<0.01$ versus control. Medium only, PBS only and SPDE (25 mg/ml) in medium were used as negative controls.

Table 1. Dose Dependent manner of PPDE induced cell apoptosis in cultured human cardiomyocytes.

Treatment	% of apoptosis		
	0	12.5 mg/ml	25 mg/ml
Medium only	1.0 ± 2.7	10.4 ± 2.8	11.8 ± 3.2
PBS only	2.2 ± 3.1	10.2 ± 3.2	10.8 ± 3.6
Medium + DOXO	9.7 ± 2.4	28.5 ± 4.1*	41.2 ± 4.5*
Medium + SPDE	0.7 ± 2.5	10.2 ± 2.7	12.4 ± 3.5
Medium + PPDE	9.7 ± 2.6	32.4 ± 3.8*	48.6 ± 4.8*

Cultured human cardiomyocytes were exposed to medium only, PBS only, medium plus DOXO (0.5 μ M), medium plus SPDE (24 hours) and medium + PPDE (24hrs). Annexin V-FITC apoptosis anlysis were determined by FACS. Each value is the mean from eight independent peritonitis episodes.

*significant effect of the agonist compared with medium only control, *$p<0.05$.

Table 2. Time dependent manner of PPDE induced cell apoptosis in cultured human cardiomyocytes.

Treatment	% of apoptosis		
	0	24 h	48 h
Medium only	1.0 ± 2.7	11.5 ± 2.7	12.1 ± 3.2
PBS only	2.2 ± 3.1	10.4 ± 2.6	11.5 ± 3.4
Medium + DOXO	9.7 ± 2.4	34.8 ± 3.6*	42.2 ± 4.2*
Medium + SPDE	0.7 ± 2.5	12.2 ± 3.2	13.6 ± 3.2
Medium + PPDE	9.7 ± 2.6	48.2 ± 4.7*	57.6 ± 4.4*

Cultured human cardiomyocytes were exposed to medium only, PBS (phosphate buffer solution) only, medium plus DOXO (0.5 μM), medium + SPDE (stable peritoneal dialysate effluent) (25 mg/ml) and medium + PPDE (peritonitis peritoneal dialysate effluent) (25 mg/ml). Annexin V-FITC apoptosis anlysis were determined by FACS. Each value was the mean from eight independent peritonitis episodes.

*significant effect of the agonist compared with medium only control, *$p<0.05$.