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Porphyromonas gingivalis-related cardiac cell apoptosis was majorly co-activated by p38 and extracellular signal-regulated kinase pathways

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Background and Objective: Little is known about the pathogenesis of apoptosis caused in cardiac tissues by periodontitis pathogens. The purpose of this study was to determine the related effect of periodontal pathogen *Porphyromonas gingivalis* on cardiac cell apoptosis.

Methods: DNA fragmentation, nuclear condensation and activated apoptotic caspases were measured by agarose gel electrophoresis, nuclear DAPI (4′,6-diamidine-2-phenylindole dihydrochloride) stain and western blotting analysis following the surrounding medium of *P. gingivalis* and/or pre-administration of SB203580 (p38 inhibitor), U0126 [mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor], LY294002 [phosphoinositide 3-kinase (PI3K) inhibitor], cyclosporine A (CsA: calcineurin inhibitor), and Sp600125 [c-Jun N-terminal kinase (JNK) inhibitor] in cultured cardiac H9c2 cells.

Results: The surrounding medium of periodontal pathogen *P. gingivalis* increased DNA fragmentation, nuclear condensation and the activated apoptotic caspase-3, -8, and -9 proteins in H9c2 cells. DNA fragmentation and nuclear condensation of H9c2 cells treated with *P. gingivalis* medium were completely blocked by SB203580 plus U0126 and were decreased after pre-administration of SB203580 only, U0126 only, LY294002, CsA, but were increased by Sp600125.

Conclusion: Our findings suggest that the development of cardiac cell apoptosis can be directly induced by *P. gingivalis* medium. *Porphyromonas gingivalis*-related H9c2 cell apoptosis was mainly co-activated by p38 and ERK pathways and may be involved in death receptor-dependent (caspase 8) and mitochondria (caspase 9)-dependent apoptotic pathways. *Porphyromonas gingivalis*-related cardiac cell apoptosis was also partially mediated by PI3K or calcineurin signaling pathways, whereas the JNK pathway might play a protective role in *P. gingivalis*-related cardiac cell apoptosis.

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The accumulation of epidemiologic, *in vitro*, clinical and animal evidence suggests that periodontal infection may be a contributing risk factor for heart diseases (1). Periodontitis pathogens may disseminate through the blood to infect the vascular endothelium and contribute to the occurrence of atherosclerosis and risk of myocardial ischemia and infarction (2). *Porphyromonas gingivalis*, a major pathogen of chronic adult periodontitis, exhibits several potential virulence properties that could play a role in the development of cardiovascular disease as mediators of low density lipoprotein oxidation, foam cell formation, and rupture of atherosclerotic plaque (3). *Porphyromonas gingivalis* was demonstrated to adhere and to invade bovine aortic and heart endothelial cells (4). However, little is known about whether the pathogenesis of cardiac cell apoptosis is associated with the periodontitis pathogen *P. gingivalis*.

Apoptotic cell death plays a critical role in a variety of cardiovascular diseases, including myocardial infarction, heart failure, and atherosclerosis (5). Apoptosis is characterized by DNA fragmentation, nuclear condensation, and activation of the cysteine-dependent aspartate-specific proteases (caspase) family (6). Members of the caspase family of cysteine proteases have been firmly established to play key roles in signal transduction cascades that culminate in apoptosis. Caspases are normally expressed as inactive precursor enzymes that become activated during apoptosis (7). Caspase-8 and caspase-9 appear to be the apical caspases activated in death receptor-dependent and mitochondrial-dependent apoptotic pathways, respectively, and caspase-3 mediates both pathways (8). The occurrence of apoptosis has been reported to contribute to the loss of cardiac cells in cardiomyopathy, and is recognized as a predictor of adverse outcomes in subjects with cardiac diseases or heart failure (9). Cardiac cell apoptosis may occur in a variety of diseases, such as hypertension, myocardial infarction, endocrine disorders, toxicants, and bacterial endocarditis (10, 11).

A number of studies have elucidated molecules responsible for the development of cardiac apoptosis, including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and calcineurin pathway (8, 12). The best characterized MAPK cascades signaling pathway consists of a sequence of successively acting kinases that ultimately result in the dual phosphorylation and activation of the p38 MAPK cascade (p38), the extracellular signal-regulated kinase (ERK), and the c-Jun N-terminal kinase (JNK) (8, 13). MAPK pathways are key factors in host signaling events and can also play important roles in the internalization of pathogenic bacteria *P. gingivalis* in gingival epithelial cells (14). Activation of p38 MAPK plays an important role in apoptotic cell death, such as that inhibiting p38 MAPK attenuated post-ischemic reperfusion apoptosis in cardiac cells (15). The JNK pathway appears to be activated by oxidative stress and is associated with cardiac cell survival or apoptotic activity (16). PI3K, representing a family of intracellular signaling proteins, controls a variety of important cellular functions such as proliferation and apoptosis and is involved in the pathogenesis of numerous diseases, including heart failure (17). Calcineurin has been reported as a critical mediator for cardiac hypertrophy, ischemia-induced heart, and cardiac myocyte apoptosis (18–20). Transgenic mice that express the activated forms of calcineurin in the heart did develop cardiac hypertrophy and heart failure that mimic human heart disease (19). However, it is unclear what signaling pathways mediate *P. gingivalis*-related cardiac cell apoptosis.

The aims of the current study are first to determine the effect of periodontal pathogen *P. gingivalis* on cardiac cell apoptosis, second to clarify the possible signaling pathways involving in *P. gingivalis*-related cardiac cell apoptosis, and third to evaluate whether *P. gingivalis*-related cardiac cell apoptosis involves death receptor-dependent and/or mitochondrial-dependent apoptotic pathway. We hypothesized that periodontal pathogen *P. gingivalis* or its metabolic byproducts may lead to cardiac cell apoptosis.

Material and methods

Porphyromonas gingivalis medium preparation

Porphyromonas gingivalis obtained from American Type Culture Collection (ATCC 33277) was maintained on brain heart infusion enriched with vitamin K3 (menadione, 0.5 mg/l) and hemin (5 mg/l) in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2 at 37°C. The turbidimetric method was used to evaluate bacterial cultures with 2 McFarland standard [6×10^8 colony-forming units (CFUs)], the upper aqueous layer was collected following centrifugation at 10,000 *g*, filtered through a 0.22 μm filter, and stored at −80°C. The upper aqueous layers of *P. gingivalis* bacterial mediums were diluted to 100-fold, 10-fold, and two-fold.

Cell culture and inhibitors

H9c2 cardiac cells were obtained from American Type Culture Collection (ATCC CRL-1446) and were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 mM pyruvate in humidified air (CO_2 5%) at 37°C. H9c2 cells were cultured in serum-free medium for 12 h, and then treated with control (serum-free medium), SB203580 (10 μM; p38 inhibitor), U0126 (10 μM; MAPK ERK1/2 kinase inhibitor), LY294002 (10 μM; PI3K inhibitor) SP600125 (10 μM; JNK inhibitor), and cyclosporine A (CsA, 0.5 μM; Calcineurin inhibitor) for 1 h and post-treated with *P. gingivalis* two-fold, 10-fold, or 100-fold diluted bacterial mediums. The incubation was continued for another 24 h, and then the cells were harvested and extracted for the analysis.

DNA fragmentation

H9c2 cells were lysed in 50 μl of lysis buffer (50 mM Tris-HCl pH 7.4, 20 mM EDTA, 1% IGEPAL-630) followed by incubation with 1% sodium dodecyl sulfate and 5 μg/ml RNase (Roche

Molecular Biochemicals, Mannheim, Germany) for 2 h at 56°C and 2.5 g/ml proteinase K (Roche) for 2 h at 37°C, and only fragmented DNA was extracted. DNA was ethanol-precipitated and finally resuspended in distilled water. The fragmented DNA was electrophoretically fractionated on 1.5% agarose gel and stained with ethidium bromide.

Nuclear condensation stained with DAPI (4′,6-diamidine-2-phenylindole dihydrochloride)

H9c2 cells grown on a 6 mm plate were washed twice with phosphate-buffered saline and fixed for 30 min in 4% buffered paraformadelhyde. The cells were then stained with 1 μg/ml 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI, Roche) for 30 min. The stained cells were examined with fluorescence microscopy. The nuclei condensation was expressed by percentage of cell death and expressed by relative percentage of *P. gingivalis* induced cell death.

Western blot analysis

Cultured H9c2 cells were scraped and washed once with phosphate-buffered saline. Cell suspension was then spun down, and cell pellets were lysed for 30 min in lysis buffer [50 mM Tris pH 7.5, 0.5 M NaCl, 1.0 mM EDTA pH 7.5, 10% glycerol, 1 mM beta-mercaptoethanol (BME), 1% IGEPAL-630 and proteinase inhibitor cocktail tablet (Roche)] and spun down 9000**g** for 10 min. Then, the supernatants were transferred to new eppendorf tubes for western blot analysis. Proteins from H9c2 cell line were then separated in 12% gradient sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Nonspecific protein binding was blocked in blocking buffer (5% milk, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) and blotted with specific antibodies of activated form of caspase 3, caspase 8, caspase 9 and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as indicated for each experiment in the blocking buffer at 4°C overnight. Densitometric analysis of immunoblots was performed using AlphaImager 2200 digital imaging system (Digital Imaging System, San Leandro, CA, USA).

Experimental protocols

Protocol 1 — To assess whether the surrounding medium of periodontitis pathogen *P. gingivalis* induces myocardial cell apoptosis, DNA fragmentation was measured three times by agarose gel electrophoresis in cardiac H9c2 cells treated with serum-free medium (control), 100-fold diluted, 10-fold diluted, and two-fold diluted surrounding mediums of *P. gingivalis* for 24 h.

Protocol 2 — To clarify the possible signaling pathway of periodontitis pathogen *P. gingivalis* medium induced myocardial cell apoptosis, DNA fragmentation was measured three times by agarose gel electrophoresis in cardiac H9c2 cells with pre-administration of control (serum-free medium) or various signaling pathway inhibitors (SB203580, U0126, SB203580 plus U0126, LY294002, SP600125, and cyclosporine A) for 1 h and with post-administration of serum-free medium (control), and bacterial medium containing 10-fold diluted *P. gingivalis* medium for 24 h. Two-fold diluted *P. gingivalis* medium was also treated.

Protocol 3 — To clarify the possible signaling pathway *P. gingivalis* induced cardiac cell apoptosis, nuclei condensation was measured by DAPI (1 μg/ml) staining in cardiac H9c2 cells with pre-administration of control (serum-free medium) or various signaling pathway inhibitors (SB203580, U0126, SB203580 plus U0126, LY294002, SP600125, and cyclosporine A) for 1 h and with post-administration of serum-free medium (control), and bacterial medium containing 10-fold diluted *P. gingivalis* medium for 24 h. Two-fold diluted *P. gingivalis* medium was also treated.

Protocol 4 — To evaluate whether *P. gingivalis*-related cardiac apoptosis involved death receptor-dependent and/or mitochondria-dependent apoptotic pathway, the activated form of caspase 8 (death receptor dependent), caspase 9 (mitochondria dependent), and caspase 3 (both death receptor and mitochondria dependent) were measured twice by western blotting analysis in cardiac H9c2 cells treated with either serum-free medium (control) or 10-fold diluted *P. gingivalis* medium for 0.5 h, 1 h and 2 h.

Statistics

The nuclei condensation of cardiac H9c2 cells was compared among groups treated with either control, 10-fold diluted *P. gingivalis* medium, and various pre-treated signaling pathway inhibitors using one-way analysis of variance (ANOVA) with pre-planned contrast comparison with control group and 10-fold diluted *P. gingivalis* medium group. In all cases, a difference at $p < 0.05$ was considered statistically significant.

Results

Apoptosis measured by DNA fragmentation was induced by *Porphyromonas gingivalis* medium

DNA ladder formation revealed that cardiac H9c2 cells underwent DNA fragmentation and apoptosis on exposure to 100-fold diluted, 10-fold diluted, and two-fold diluted surrounding medium of *P. gingivalis*, compared to the serum-free control (Fig. 1).

Porphyromonas gingivalis induced DNA fragmentation in H9c2 cell was activated by p38, extracellular signal-regulated kinase, phosphoinositide 3-kinase, and calcineurin pathway

Cardiac H9c2 cells underwent DNA fragmentation on exposure to 10-fold diluted *P. gingivalis* medium and more DNA fragmentation on exposure to two-fold diluted *P. gingivalis* medium compared with serum-free control (Fig. 2). The 10-fold diluted *P. gingivalis* medium induced DNA fragmentation was completely blocked by SB203580 (p38 inhibitor) plus U0126 (ERK inhibitor) and partially

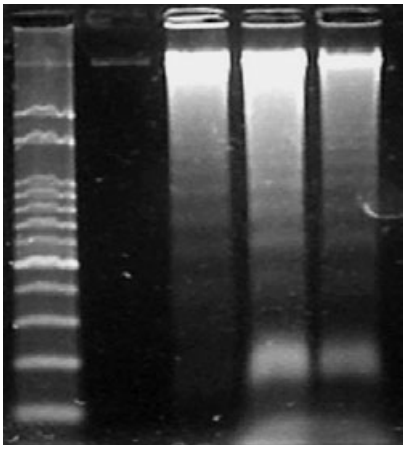

Fig. 1. Representative DNA fragmentation was measured by agarose gel electrophoresis in cardiac H9c2 cells treated with Markers (M), serum-free medium (Con: control), and 100-fold diluted (100 × P.g), 10-fold diluted (10 × P.g), and two-fold diluted (2 × P.g) surrounding medium of *Porphyromonas gingivalis* (6×10^8 CFU/ml) for 24 h.

attenuated by SB203580 (p38 inhibitor), U0126 (ERK inhibitor), LY294002 (PI3K inhibitor), or cyclosporine A (calcineurin inhibitor). In contrast, DNA fragmentation induced by 10-fold diluted *P. gingivalis* medium was enhanced by SP600125 (JNK inhibitor) (Fig. 2).

Porphyromonas gingivalis medium induced apoptosis measured by condensed nuclei with DAPI staining was mediated by p38, extracellular signal-regulated kinase, phosphoinositide 3-kinase, and calcineurin pathway

The condensation of nuclei stained with DAPI, indicating cell apoptosis, was significantly ($p < 0.01$) increased after administration of 10-fold diluted *P. gingivalis* medium (11.32%) and was further increased after administration of two-fold diluted *P. gingivalis* medium (17.10%) compared with control (3.64%) (Fig. 3). The increased condensed nuclei (11.32%) induced by 10-fold diluted *P. gingivalis* medium in H9c2 cells were significantly ($p < 0.01$) reduced after pre-administration of SB203580 (p38 inhibitor, 7.25), U0126 (ERK inhibitor, 5.05%), LY294002 (PI3K inhibitor, 7.02%), or cyclosporine A (calcineurin inhibitor, 7.50%), but were enhanced by SP600125 (JNK inhibitor). The increased condensed nuclei (11.32%) induced by 10-fold diluted *P. gingivalis* medium in H9c2 cell were totally blocked (0.96%) after pre-administration of the combination of SB203580 (p38 inhibitor) and U0126 (ERK inhibitor) (Fig. 3).

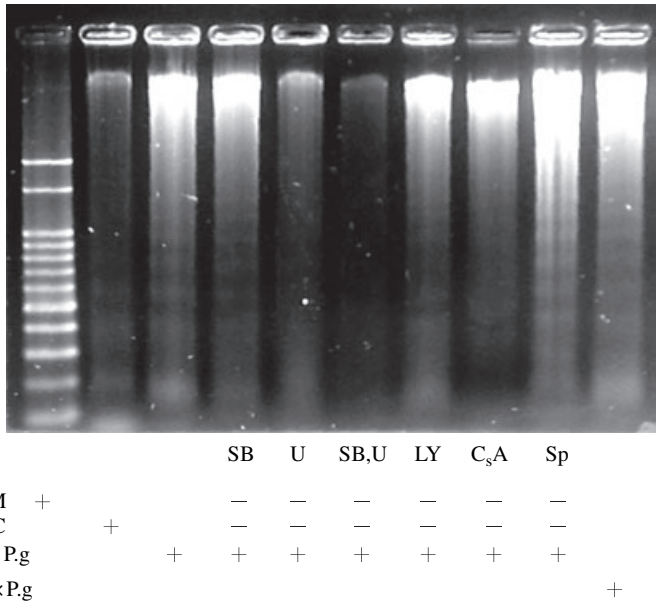

Fig. 2. Representative DNA fragmentation was measured by agarose gel electrophoresis in cardiac H9c2 cells treated with serum-free medium (C: control), and 10-fold diluted (10 × P.g) and two-fold diluted (2 × P.g) surrounding medium of *Porphyromonas gingivalis* (6×10^8 CFU/ml) for 24 h. H9c2 cells were pre-treated with one of the following signaling pathway inhibitors, either SB203580 (SB: p38 inhibitor; 10 μM), U0126 (U: MAPK ERK1/2 kinase inhibitor; 10 μM), SB203580 plus U0126 (Sb, U), LY294002 (LY: phosphoinositide 3-kinase inhibitor; 10 μM), cyclosporine A (CsA: calcineurin inhibitor; 0.5 μM), or SP600125 (Sp: Jun N-terminal kinase inhibitor; 10 μM) for 1 h and post-treated with 10-fold diluted (10 × P.g) surrounding medium of *P. gingivalis* for 24 h.

Apoptotic caspase protein activity was induced by *Porphyromonas gingivalis* medium

Western blot analysis revealed that the activated forms of caspase 3, 8, and 9 protein products were increased after administration of 10-fold diluted *P. gingivalis* medium for 0.5 h, 1 h, and 2 h (Fig. 4).

Discussion

Major findings

Our main findings can be summarized as follows.

(i) Periodontal pathogen *P. gingivalis* medium increased DNA fragmentation, nuclear condensation and activated caspase-3, -8, and -9 protein activities in H9c2 cells, which strongly suggests that *P. gingivalis* medium directly induced cardiac apoptosis and *P. gingivalis* medium induced cardiac cell apoptosis may be involved in death receptor-dependent (caspase-8) and mitochondrial (caspase-9)-dependent apoptotic pathways.

(ii) The increased DNA fragmentation and nuclear condensation of H9c2 cells treated with *P. gingivalis* medium were completely blocked by SB203580 (p38 inhibitor) plus U0126 (MAPK ERK1/2 kinase inhibitor), which suggests that *P. gingivalis*-related cardiac cell apoptosis was mainly co-activated by p38 and ERK pathways.

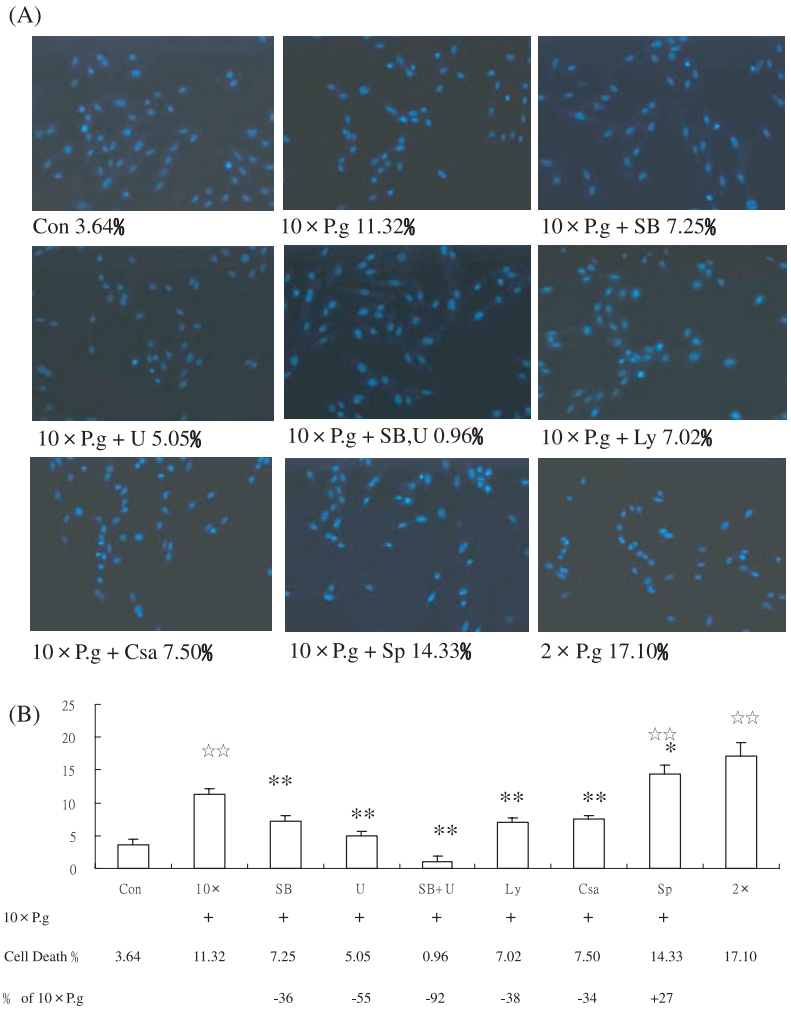

Fig. 3. (A) Representative nuclei condensation expressed by percentage of cell death (nuclei condensation) was measured by DAPI (4,6-diamidino-2-phenylindole, 1 μg/ml) staining in cardiac H9c2 cells treated with either control (Con, upper left panel), 10-fold diluted *Porphyromonas gingivalis* medium (10 × P.g, upper middle panel), pre-treated SB203580 (p38 inhibitor; 10 μM) plus post-treated 10 × *P. gingivalis* medium (10 × P.g + SB; upper right panel), pre-treated U0126 (MAPK ERK1/2 kinase inhibitor; 10 μM) plus post-treated 10 × *P. gingivalis* medium (10 × P.g + U; middle left panel), pre-treated SB203580 and U0126 plus post-treated 10 × *P. gingivalis* medium (10 × P.g + SB, U; middle middle panel), LY294002 (phosphoinositide 3-kinase inhibitor; 10 μM) plus post-treated 10 × *P. gingivalis* medium (10 × P.g + Ly; middle right panel), pre-treated cyclosporine A (calcineurin inhibitor; 0.5 μM) plus post-treated 10 × *P. gingivalis* medium (10 × P.g + CsA; lower left panel), pre-treated SP600125 (Jun N-terminal kinase inhibitor; 10 μM) plus post-treated 10 × *P. gingivalis* medium (10 × P.g + Sp; lower middle panel), two-fold diluted *P. gingivalis* medium (2 × P.g; lower right panel). All signaling pathway inhibitors were pre-treated for 1 h and *P. gingivalis* media (10 × and 2 ×) were treated for 24 h. % represents the percentage of cell death (nuclei condensation). (B) $^{**}p < 0.01$, significant different of percentage cell death (nuclei condensation) between *P. gingivalis* medium and control; $^{*}p < 0.05$, $^{**}p < 0.01$, significant different of percentage cell death (nuclei condensation) between *P. gingivalis* medium and pre-treated signaling pathway inhibitors plus *P. gingivalis* medium.

(iii) DNA fragmentation and nuclear condensation of H9c2 cells treated with *P. gingivalis* medium were partially decreased after pre-administration of SB203580 (p38 inhibitor), U0126 (MAPK ERK1/2 kinase inhibitor), LY294002 (PI3K inhibitor) and CsA (calcineurin inhibitor), but was increased by Sp600125 (JNK inhibitor). This suggests that p38, ERK, PI3K, or calcineurin pathways were partially involved in *P. gingivalis*-related cardiac cell apoptosis and that the JNK pathway might play a protective role in *P. gingivalis*-related cardiac cell apoptosis.

Experimental limitation

Porphyromonas gingivalis, a gram-negative anaerobe, is involved in the pathogenesis of periodontal disease and plays important roles in eliciting cellular responses in various ways (21). *Porphyromonas gingivalis* possesses a variety of virulence factors, including gingipains, fimbrillin peptides, capsule polysaccharides, lipopolysaccharides, haemagglutinating and haemolysing activities, toxic products of metabolism, outer membrane vesicles, and other enzymes (22). Lipopolysaccharide from gram-negative bacteria induced cardiac apoptosis and decompensated heart failure (23, 24), even when low dose was applied (25). Except lipopolysaccharide, the effects of most virulence factors associated with *P. gingivalis* on cardiac muscle, to our knowledge have not been investigated. Because of the widespread virulence factors, the surrounding medium of *P. gingivalis* can produce cardiac cell apoptosis via various virulence factors or metabolic byproducts. Therefore, we have to add a note of caution that any effect noted in the present investigation cannot be isolated to any specific virulence factors or any metabolic byproducts. Nevertheless, the goal of the present study was to differentiate the effects of *P. gingivalis* medium acting on cardiac cells and to clarify possible pathways. Clearly, additional experiments using a reductionist approach, such as investigating gingipains only or lipopolysaccharides only, will be required in order to identify which specific virulence factors or metabolic byproducts direct cause myocardial cell apoptosis.

Porphyromonas gingivalis and cardiac abnormality

Apoptosis is characterized by DNA fragmentation, nuclear condensation,

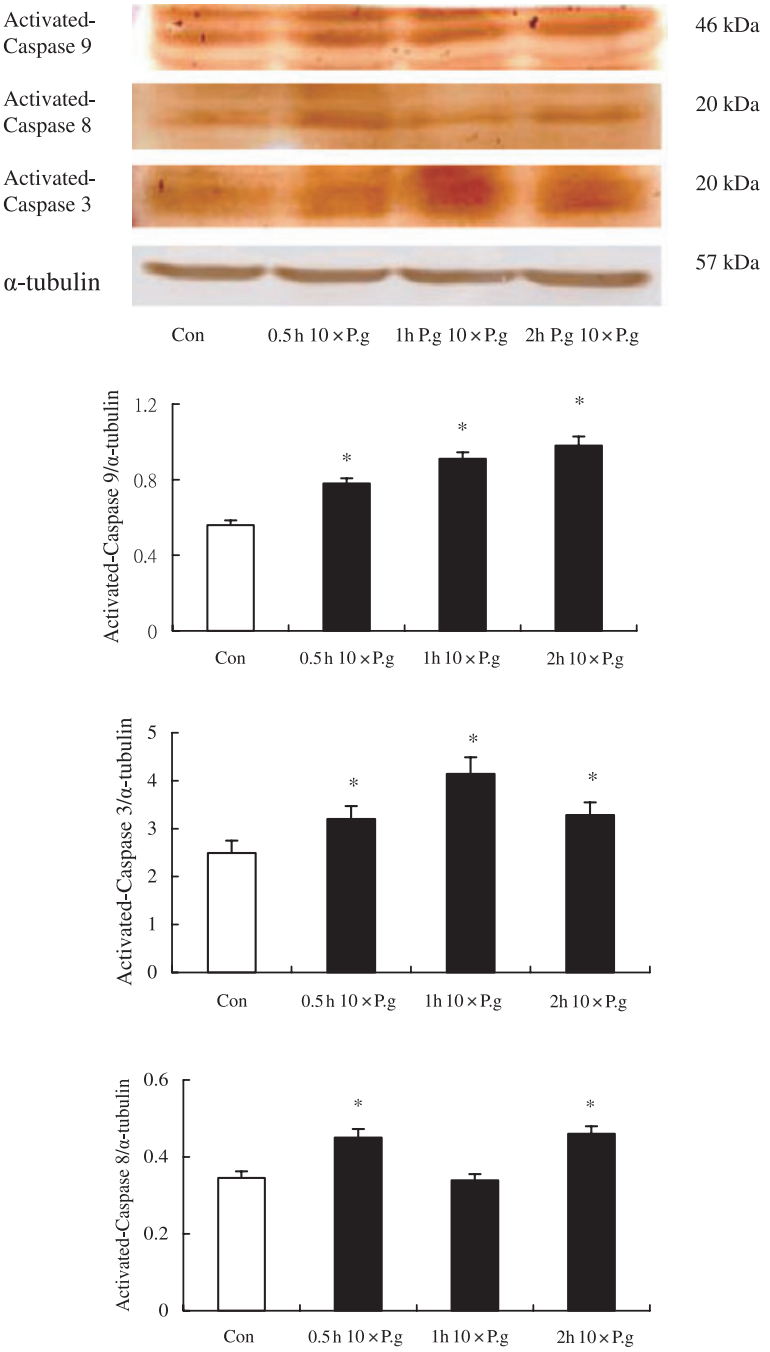

Fig. 4. The representative protein products of caspase 9, caspase 8, and caspase 3 in activated form were measured by western blotting analysis in cardiac H9c2 cells treated with either control (Con), 10-fold diluted *Porphyromonas gingivalis* medium (10 × P.g) for 0.5 h, 1 h and 2 h. A relative quantification on the basis of α-tubulin (40 μg) was applied. Bars represent the relative quantification on the basis of α-tubulin measured by densitometric analysis and indicate mean values ± SD. $P \leq 0.05$, significant differences between control (Con) and 0.5 h, 1 h or 2 h 10 × *P. gingivalis* (10 × P.g).

and activation of the caspase family of proteases (6). Caspases are normally expressed as inactive precursor enzymes that become activated during apoptosis (7). Caspase-8 and caspase-9 proteins appear to be the apical caspases activated in death receptor-dependent and mitochondrial-dependent apoptotic pathways, respectively, and caspase-3 is a common component to mediate both pathways for cleavage of DNA fragmentation and contribute apoptosis (8). Our finding shows that periodontal pathogen *P. gingivalis* medium increased DNA fragmentation, nuclear condensation and caspase-3, -8, and -9 protein activities in H9c2 cells, which suggests that *P. gingivalis* medium induced cardiac cell apoptosis. The observation of increased caspase-3, -8, and -9 in H9c2 cells treated with *P. gingivalis* medium suggests that *P. gingivalis* medium induced cardiac cell apoptosis may be mediated by caspase-8 and caspase-3 related death receptor apoptotic pathways and by caspase-9 and caspase-3 related mitochondrial-dependent apoptotic pathways. Further studies are required to clarify the detailed mechanism of *P. gingivalis* medium induced death receptor or mitochondrial-apoptotic pathways. The role of *P. gingivalis* on cardiac apoptosis, to our knowledge, has not been previously reported. Therefore, no relative studies linking between *P. gingivalis* and cardiac apoptosis can be referred to as support for our findings. Some non-cardiac studies indicated that *P. gingivalis* itself did affect the vascular system, such as coronary arteries (3, 26). *Porphyromonas gingivalis* was suggested to be involved in atherosclerotic lesion formation (26) and was found in the aorta of some cardiovascular patients with open-heart surgery (27), the latter of which suggests that periodontal pathogen *P. gingivalis* did enter the vascular system and might be involved in cardiovascular diseases (27). Most cross-sectional, prospective epidemiological studies, animal model studies, and cellular studies have demonstrated that periodontal disease (*P. gingivalis*) may be associated with endothelial cells, atherosclerosis formation, and coronary heart disease (1–4, 26, 27). Myocardial ischemia and infarction are often preceded by acute thromboembolic events induced by periodontal disease, such as *P. gingivalis* (2). It is still an unknown issue whether the 'post-ischemia cardiac apoptosis' or 'post-infarction cardiac apoptosis' in patients with coronary artery disease will be synergistically enhanced by some virulence factors or

metabolic byproducts of *P. gingivalis*. The current findings provide a potential link between *P. gingivalis and* myocardial apoptosis, suggesting some virulence factors or metabolic byproducts of *P. gingivalis* might directly target myocardial cells and might directly involve apoptosis related heart diseases. Of course, further animal or clinical studies are required to clarify the direct role of virulence factors or metabolic byproducts of *P. gingivalis* on cardiac apoptosis or heart failure.

Pathways of *Porphyromonas gingivalis*-related cardiac cell apoptosis

The increased DNA fragmentation and nuclear condensation of H9c2 cells treated with *P. gingivalis* were completely blocked by SB203580 (p38 inhibitor) plus U0126 (MAPK ERK1/2 kinase inhibitor). This finding suggests that *P. gingivalis*-related cardiac cell apoptosis is mainly co-activated by p38 and ERK pathways. *Porphyromonas gingivalis* lipopolysaccharide was reported to interfere with salivary mucin synthesis through inducible nitric oxide synthase activation by ERK and p38 kinase (28). The dynamic complex balance between ERK, JNK, and p38 pathways may be important in determining whether a cell survives or undergoes apoptosis (29). However, p38 and/or ERK appear to be involved in different apoptotic pathways in cardiac cells by various stimuli and may interact with complex signal transduction network (8). No evidence is currently available as to why *P. gingivalis*-related cardiac cell apoptosis was completely blocked by p38 inhibitor plus MAPK ERK1/2 kinase inhibitor. Therefore, further study focusing on p38 and ERK pathways is critical for clarifying the possible therapeutic mechanism for *P. gingivalis*-related cardiac apoptosis and cardiac disease.

The current findings indicate that *P. gingivalis*-related H9c2 cell apoptosis was partially mediated by p38, ERK, PI3K, and calcineurin signaling pathways, suggesting that *P. gingivalis*-related cardiac cell apoptosis might be mediated by multiple signaling pathways. Activation of the MAPK family plays a key role in the pathogenesis of various processes in the heart, e.g. myocardial apoptosis and heart failure (30). The ERKs are strongly activated by growth factors and physical stress, whereas JNKs and p38 can be activated by various cell stresses, such as metabolic stress or protein synthesis inhibitors, UV radiation, heat shock, cytokines, and ischemia (30) Herein, our findings imply that ERK, p38 and PI3K in cardiac cells can be activated by one more cell stress, i.e. periodontal infection, and mediate apoptotic activity in cardiac cells. Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase involved in a wide range of cellular responses to calcium mobilizing signals and has been implicated as a mediator in the signaling pathways to the myocardial apoptosis (31). Calcineurin was found to dephosphorylate Bad, a pro-apoptotic member of the Bcl-2 family, thus enhancing Bad heterodimerization with Bcl-xL and promoting apoptosis (32). Furthermore, a previous study showed that myocardial failure was caused by other bacterial infection (*Streptococcus viridans*) and dramatic changes in intracellular calcium metabolism were found in the heart (33). Little is known so far about the calcineurin activity affected by *P. gingivalis*. Because *P. gingivalis* possesses a variety of virulence factors, any virulence factor or its metabolic byproducts may directly or indirectly contribute to cardiac cell apoptosis via certain signaling pathways. However, responses of virulence factors in different cellular types appear to be quite different. For example, lipopolysaccharide, one of the virulence factors in *P. gingivalis*, up-regulated TNF-alpha production in macrophages via JNK, p38 MAPK, and NF-kappaB (34). Although *P. gingivalis* lipopolysaccharide activates p38 kinase in human monocytes, it does not activate p38 or ERK kinase in endothelial cells (35). In contrast, JNK inhibitor further enhances *P. gingivalis* induced DNA fragmentation and nuclear condensation, and the JNK pathway might play a protective role in *P. gingivalis*-related cardiac cell apoptosis. A previous report showed that activation of c-Jun N-terminal kinase promotes survival of cardiac myocytes after oxidative stress and suggested that JNK activation plays a protective role in cardiac apoptosis (16). Therefore, we can generally conclude that p38, ERK, PI3K, and calcineurin signaling pathways may be possible candidates involving *P. gingivalis*-related myocardial cell apoptosis, whereas the JNK pathway might play a protective role in *P. gingivalis*-related cardiac cell apoptosis.

Further hypotheses for clinical application

Our findings can provide a future hypothesis that myocardial cell apoptosis may potentially gradually occur in patient with periodontitis infected by *P. gingivalis*, which might be mediated by p38, ERK, PI3K, and calcineurin signaling pathways. In order to prevent potential cardiac diseases, such as heart failure, in periodontitis patients infected by *P. gingivalis*, these patients should ideally maintain excellent oral hygiene and suppress *P. gingivalis* infection as low as possible. Our findings further proposed that a combination of p38 and ERK signaling pathways inhibitors may be a good candidate for developing a future therapeutic approach to prevent *P. gingivalis*-related cardiac apoptosis and/or heart failure. Of course, further clinical studies are needed to confirm our hypotheses for clinical application.

Acknowledgements

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