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Trypsin-like protease-active extracellular protein extracts from *Porphyromonas gingivalis* ATCC 33277 induce apoptosis in bovine aortic endothelial cells

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Background and Objective: Certain virulence factors participating in periodontitis may relate to cardiovascular diseases. This study was to evaluate the pro-apoptotic effect of protein extracts from *Porphyromonas gingivalis* on bovine aortic endothelial cells (BAECs).

Material and Methods: The BAECs were exposed to trypsin-like protease-active protein extracts from *P. gingivalis*, and apoptosis was examined by Hoechst 33342 staining, DNA fragmentation assay and cleaved caspase-3 detection. When BAECs were exposed to protein extracts pretreated with trypsin-like protease inhibitor (TLCK), the apoptosis rate was evaluated by Annexin V–propidium iodide staining. To further study the potential mechanism of the pro-apoptotic effect, immunoblotting was used to detect expression of α-tubulin, integrin β1 and activated ERK1/2 in BAECs treated with protein extracts or cultured in suspension.

Results: After exposure to the protein extracts, BAECs exhibited loss of cell adhesion and apoptotic cell death. The pro-apoptotic effect could be delayed by TLCK pretreatment. In addition, BAECs treated with protein extracts showed decreased levels of α-tubulin, integrin β1 and activated ERK1/2. When BAECs were cultured in suspension, ERK1/2 activation was also inhibited, but the percentage decrease in ERK1/2 activation was less than that induced by protein extracts. Moreover, no significantly altered expression of α-tubulin was detected in suspended cells.

Conclusion: Trypsin-like protease-active protein extracts from *P. gingivalis* could induce apoptosis of BAECs. The destruction of α-tubulin and integrin β1 and decrease of ERK1/2 activation might contribute to the pro-apoptotic effect of the protein extracts.

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Periodontitis is a chronic inflammatory disease of periodontal tissues that ultimately leads to alveolar bone resorption and tooth loss. *Porphyromonas gingivalis*, a gram-negative anaerobe, has been considered as a major causative agent of periodontitis (1,2). Moreover, increasing evidence has indicated that *P. gingivalis* may contribute to the progression of cardiovascular diseases, including atherosclerosis (3–5).

P. gingivalis releases various virulence factors, including trypsin-like proteases. Gingipains, the main components of trypsin-like proteases from *P. gingivalis* (6,7), are classified into two types: the arginine-X-specific cysteine proteinases (Arg-gingipains, Rgps) and the lysine-X-specific cysteine proteinases (Lys-gingipain, Kgp) (8). Trypsin-like proteases from *P. gingivalis* were reported to be engaged in apoptosis of several types of fibroblasts and epithelial cells as well as endothelial cells (9–14). Earlier studies indicated that endothelial cell apoptosis may have a potential influence on the progression of atherosclerosis (15–17), suggesting a potential relationship of *P. gingivalis* with atherosclerosis. However, the potential mechanism of the pro-apoptotic effect of *P. gingivalis* on endothelial cells has not been fully illustrated.

Cell adhesion molecules (CAMs) mainly mediate cell-to-cell and cell-to-extracellular matrix (ECM) adhesion. They play important roles in regulating cell proliferation and apoptosis as well as cell mobility and invasion. Maintaining adhesion to ECM is crucial for most epithelial and endothelial cells to maintain sustenance. If they lose anchorage from ECM, the cells will undergo a special type of apoptosis termed anoikis (18–20). Integrins are important CAMs. They mediate certain signaling pathways through non-receptor kinases, leading to activation of some molecules facilitating cell survival, such as ERK1/2 and protein kinase B (PKB/AKT) (20). ERK1/2 plays an important role in the regulation of cellular processes such as proliferation, differentiation and survival (21,22). Earlier studies showed that proteases of *P. gingivalis* can cleave certain CAMs, such as integrins and cadherins, in endothelial cells, and induce subsequent apoptotic cell death (11–13). However, the expression of some downstream molecules, such as ERK1/2 in endothelial cells, caused by the protein extracts, has not been examined.

Besides cell attachment, integrity of cellular morphology is also crucial to cell survival (23). The interaction between microtubules and actin filaments maintains the stability of cellular morphology (23–25). Persistent destruction of microtubules or actin may lead to apoptosis (26,27). This apoptosis was recognized as amorphosis, which is another kind of apoptosis stimulated by loss of cytoskeletal architecture (23). However, the effects of protein extracts from *P. gingivalis* on the cytoskeleton of endothelial cells have not been reported.

In the present study, trypsin-like protease-active protein extracts were obtained from the culture supernatant of *P. gingivalis* ATCC 33277. The pro-apoptotic effects of protein extracts on bovine aortic endothelial cells (BAECs) were evaluated. Furthermore, the expression of α-tubulin, integrin β1 and activated ERK1/2 were examined in BAECs treated with protein extracts or cultured in suspension. These studies may help to illustrate the potential mechanism of the pro-apoptotic effects of protein extracts from *P. gingivalis*.

Material and methods

Porphyromonas gingivalis culture

P. gingivalis strain ATCC 33277, purchased from Beijing Institute of Dental Research (Capital Medical University School of Stomatology, Beijing, China) was grown in brain–heart infusion broth (Difco Laboratories, Detroit, MI, USA) supplemented with yeast extract (5 g/L), hemin (5 mg/L), vitamin K (1 mg/L), and cysteine (1 g/L) in an atmosphere of 10% H_2, 5% CO_2 and 85% N_2.

Preparation of protein extracts from *P. gingivalis*

P. gingivalis was grown to a cell density of approximately optical density at 660 nm = 1.0. At the end of incubation, the bacterial cultures were centrifuged (9400*g*, 45 min, 4°C) to remove cells, and the supernatant was filtered through a 0.45-μm-pore filter. Then with constant stirring, ammonium sulfate was added to the extracellular culture fluid to give 75% saturation. Precipitate was collected by centrifugation (9400*g*, 20 min, 4°C), resuspended in 6 mL dialysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.4), and further centrifuged (22,000*g*, 10 min, 4°C). Then, the supernatant was dialyzed against the same buffer overnight at 4°C. After dialysis, the sample was concentrated using ultracentrifugal filter devices (Millipore, Bedford, MA, USA) with a 10,000-molecular-weight-cutoff membrane. The concentrated protein extracts were further purified by centrifugation (100,000*g*, 1 h, 4°C) and stored in aliquots at −80°C.

Trypsin-like protease activity assays

Trypsin-like protease activities in these protein extracts were measured as described by Sheets *et al.* (12). To test the Rgp activity, 5 μL protein extracts were pre-incubated in 145 μL assay buffer (containing 2 M Tris–HCl, 0.1 M NaCl and 9 mM L-cysteine, pH 7.6) at 37°C for 5 min. Then, 50 μL of 4 mM *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA; Sigma, St Louis, MO, USA), the substrate for Rgp activity, was added to the reaction mixture. Immediately, absorbance at 405 nm was continuously read using a microplate reader (Bio-Rad, Hercules, CA, USA). One unit of Rgp activity was defined as the amount of enzyme releasing 1 μmol *p*-nitroanilide per minute, and calculated on maximal velocity and extinction coefficient. To test the Kgp activity, 15 μL protein extracts were pre-incubated in 135 μL assay buffer, and *N*-(*p*-tosyl)-Gly-Pro-Lys-4-nitroanilide acetate salt (Sigma) was used as the substrate. The Rgp and Kgp activities were calculated based on the mean of three measurements.

Inhibition of trypsin-like protease activities

To inhibit trypsin-like protease activitiy, *P. gingivalis* protein extracts were

pretreated for 1 h on ice with 10 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK; Sigma), a specific inhibitor of trypsin-like proteases. Then TLCK was removed from protein extracts by Microcon centrifugal filter devices with 3000-molecular-weight-cutoff membrane (Millipore).

Cell culture and treatment with protein extracts

Bovine aortic endothelial cells isolated from bovine aorta were a generous gift of Professor Yi Zhu (Department of Physiology and Pathophysiology, Health Science Center, Peking University, China). The BAECs were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 U/mL). Cells from passages 6–8 were used in this study. Near-confluent BAECs were divided into different groups. In the group treated with protein extracts, BAECs were incubated in serum-free DMEM containing 400 μg/mL protein extracts and 5 mM L-cysteine (to activate the cysteine protease activity). In the TLCK blocking group, cells were exposed to serum-free DMEM containing 5 mM L-cysteine and 400 μg/mL protein extracts pretreated with TLCK. In the control group, cells were incubated in serum-free DMEM containing 5 mM L-cysteine. Cells in various groups were incubated for the indicated times at 37°C in an atmosphere of air containing 5% CO_2.

Hoechst 33342 staining

After incubation in the presence or absence of protein extracts for 24 h, BAECs were trypsinized, washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min at room temperature. After drying on slides, cells were stained for 10 min with 5 μg/mL Hoechst 33342 (Sigma), and visualized with a fluorescence microscope at a magnification of ×400.

DNA fragmentation assay

To examine DNA laddering, 3×10^6 cells were lysed in 500 μL buffer containing 1 M Tris–HCl, 5 M NaCl, 0.5 M EDTA and 10% sodium dodecyl sulfate, followed by addition of proteinase K (100 μg/mL). After 2 h incubation at 50°C, the soluble fraction was extracted with phenol–chloroform and precipitated with ethanol. Isolated cellular DNAs or DNA markers were stained with SYBR Green, separated on a 1.5% agarose gel, and photographed under ultraviolet light.

Quantification of apoptotic cell death by flow cytometry

An Annexin V–FITC (fluorescein isothiocyanate) apoptosis detection kit (BioVision, Mountain View, CA, USA) was used to detect early and late apoptotic activities. After 24 h of incubation, cells were collected, resuspended, and stained with 5 μL Annexin V–FITC and 5 μL propidium iodide (PI) for 5 min. Then cells were collected, fixed in 4% paraformaldehyde and kept at 4°C. After washing with PBS, apoptotic cell death was detected using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and CELLQUEST analysis software (Becton Dickinson). Data were collected from a minimum of 10,000 cells per sample. The Annexin V-negative, PI-negative population was regarded as normal healthy cells, while Annexin V-positive, PI-negative cells were taken as early apoptosis and Annexin V-positive, PI-positive as necrosis/late apoptosis.

Anoikis assay

Bovine aortic endothelial cells grown to near confluency were trypsinized into single cell suspensions. Then, 1×10^6 cells were plated on 0.8% agarose-coated plates in serum-free medium as described by Orford *et al.* (28). After incubation for 24 h, cells were collected, washed in PBS, and any cell aggregates were dispersed by mild trypsinization. Cell death was determined by trypan blue exclusion analysis. Briefly, cell suspension was stained with an equal volume of 0.4% trypan blue (Sigma) and counted using a hemocytometer. Cell death rate was determined by the equation of the number of trypan-blue-positive cells divided by the number of trypan-blue-negative and -positive cells. Apoptosis was further determined by DNA fragmentation assay.

Immunoblotting for cleaved caspase-3, α-tubulin, integrin β1 and ERK1/2 activation

Cells were harvested and washed with ice-cold PBS. Whole cell lysates were prepared with protein lysis buffer containing 1% NP-40, 5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate and 1:100 dilution of protease inhibitors mixture (Sigma). After being thawed three times, the protein was collected by centrifugation (12,000*g*, 15 min), subjected to 12% (for detection of caspase-3, α-tubulin, and ERK1/2) or 8% (for detection of integrin β1) sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membrane (Millipore). After blocked with 5% non-fat dry milk for 1 h, the membrane was probed with prime antibodies in TBS-T (25 mM Tris–HCl, 125 mM NaCl, 0.1% Tween 20) containing 5% non-fat dry milk overnight at 4°C. After washing with TBS-T, the membrane was then incubated with appropriate secondary antibodies for 1 h. The immune complexes were visualized using the ECL kit (Applygen Technologies, Beijing, China) according to the manufacturer's protocol.

The polyclonal anti-cleaved caspase-3 antibodies, polyclonal anti-phosphorylated ERK1/2 antibodies and anti-total ERK1/2 antibodies were from Cell Signaling (Beverly, MA, USA). Polyclonal anti-α-tubulin antibodies and monoclonal anti-β-actin antibodies were from Santa Cruz (Santa Cruz, CA, USA). Monoclonal anti-integrin β1 antibodies were from BD Transduction Laboratories (San Diego, CA, USA). Horseradish peroxidase-conjugated secondary antibodies against rabbit (for cleaved caspase-3,

β-actin, phosphorylated ERK1/2 and total ERK1/2) and horseradish peroxidase-conjugated secondary antibodies against mouse (for α-tubulin and integrin β1) were from Zymed (Carlton Court, South San Francisco, CA, USA).

ERK1/2 activation was reflected by the relative ERK1/2 activity, and the ERK1/2 activity was determined by the ratio of phosphorylated to total ERK1/2 quantified by BandScan 4.3 software.

Statistical analysis

Experimental values from three independent experiments were expressed as means ± SEM. Difference was analyzed using unpaired Student's *t*-test or one-way ANOVA. Multiple means were compared using unpaired LSD *post hoc* or Tamhane *post hoc* test (equal variances assumed or not assumed). A value of $p < 0.05$ was considered statistically significant.

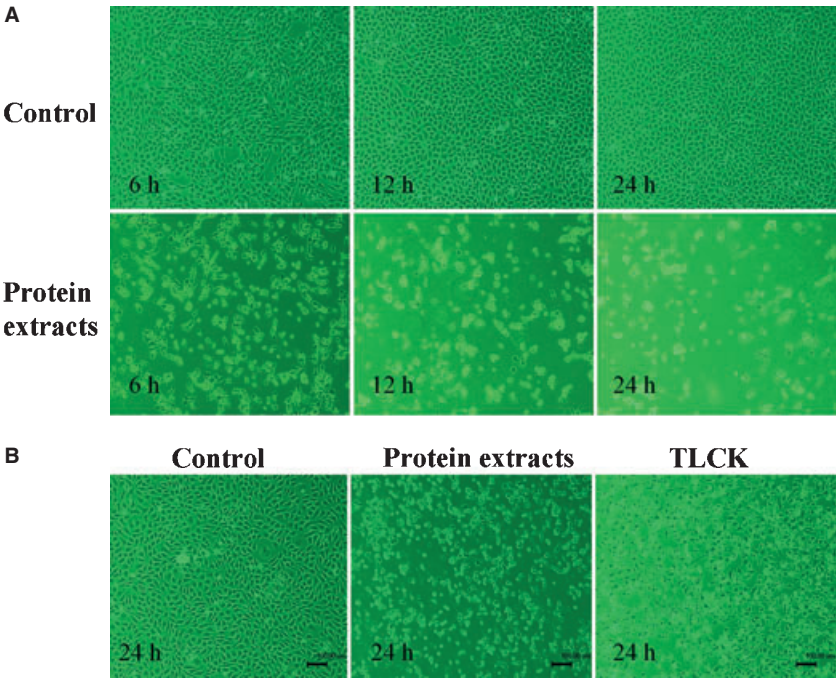

Fig. 1. Loss of adhesion in bovine aortic endothelial cells (BAECs) treated with protein extracts from *Porphyromonas gingivalis*. (A) The BAECs were treated with protein extracts (400 μg/mL) from *P. gingivalis* in the presence of 5 mM L-cysteine for 6, 12 or 24 h. (B) The BAECs were incubated for 24 h in medium containing protein extracts (400 μg/mL) with or without TLCK pretreatment. Magnification ×100. Scale bars represent 100 μm.

Results

Bovine aortic endothelial cells exhibited detachment after treatment with protein extracts

Protein extracts from *P. gingivalis* contained 73.42 U Rgp activity and 6.43 U Kgp activity per microgram. After treatment with TLCK, the Rgp and Kgp activity decreased by 96.8 and 91.14%, respectively. Bovine aortic endothelial cells exposed to protein extracts rapidly shrank and detached from the culture surface in a time-dependent manner (Fig. 1A). After exposure to TLCK-pretreated protein extracts, cell detachment from plates was significantly reduced (Fig. 1B).

Protein extracts induced apoptosis of BAECs

Apoptotic cell death was determined by Hoechst 33342 nuclear staining. Compared with the control group, the test group exhibited an increased proportion of cells with apparent morphological changes of the nucleus, with condensed and fragmented chromatin (Fig. 2A).

The DNA fragmentation assay indicated that BAECs treated with protein extracts for 24 h underwent DNA degradation and apoptotic cell death. However, formation of DNA laddering was not observed in the control group (Fig. 2B).

As shown by Western blot analysis, more cleaved caspase-3 was detected in cells exposed to protein extracts compared with that in the control group, and the activation of caspase-3 was time dependent (Fig. 2C).

Trypsin-like protease inhibitor inhibited the apoptosis induced by protein extracts in BAECs

Flow cytometry showed that BAECs exposed to protein extracts exhibited a higher percentage of early apoptotic cell death (16.33 ± 1.43%) than control cells (4.88 ± 0.42%, $p < 0.001$). However, in the TLCK group, early cell apoptosis was considerably reduced (6.03 ± 0.43%, $p = 0.001$ compared with test group), and the level was similar to that in the control group ($p = 0.228$). No significant difference of late apoptotic/necrotic cell proportion was observed among groups (control, 5.38 ± 0.81%; protein extracts, 4.69 ± 0.62%; and protein extracts + TLCK, 5.07 ± 0.86%, $p = 0.824$; Fig. 3).

Suspended BAECs showed apoptotic cell death

Trypan blue exclusion assay showed that the death rate in suspended BAECs was higher than that in the control group (12.12 ± 1.01% vs. 7.63 ± 0.44%, $p = 0.003$; Fig. 4A). Apoptosis in suspended cells was further confirmed by DNA fragmentation assay (Fig. 4B).

Decreased expression of α-tubulin, integrin β1 and activated ERK1/2 in BAECs treated with protein extracts or cultured in suspension

After BAECs were exposed to protein extracts for 6, 12 or 24 h, α-tubulin and integrin β1 were destroyed (Fig. 5A). The relative activity of ERK1/2 was reflected by the ratio of phosphorylated to total protein. After BAECs

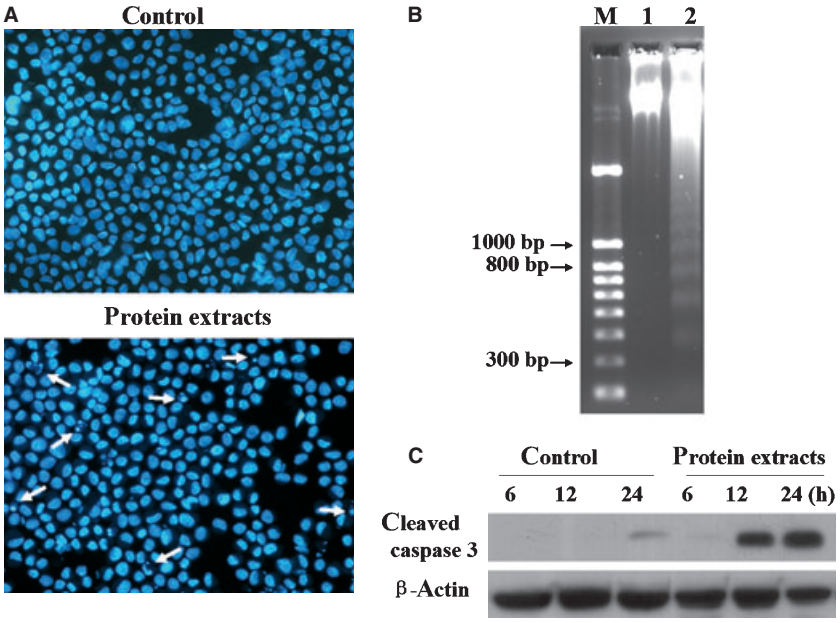

Fig. 2. Apoptosis of BAECs treated with protein extracts (400 μg/mL). (A) Representative photographs of nuclei stained with Hoechst 33342. The BAECs were exposed to protein extracts for 24 h. Arrows indicate the apoptotic cells. Magnification ×400. (B) DNA ladder formation of BAECs treated by protein extracts for 24 h. Key: M, marker; 1, control; and 2, protein extracts. (C) Effect of protein extracts on expression of cleaved caspase-3. Results represent one of three independent sets of experiments.

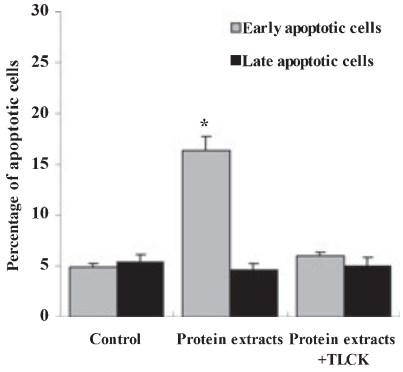

Fig. 3. Quantification of apoptotic cell death in BAECs. The BAECs were incubated in medium containing protein extracts (400 μg/mL) with or without TLCK pretreatment in the presence of 5 mM L-cysteine for 24 h. Cells were collected, stained with Annexin V and propidium iodide, and then counted with a BD FACS Calibur flow cytometer using CELLQUEST software. Data shown are means ± SEM of three independent experiments. Difference was analyzed using one-way ANOVA followed by LSD *post hoc* test (late apoptotic cells) or Tamhane *post hoc* test (early apoptotic cells). *$p < 0.05$ vs. control.

were exposed to protein extracts, the activated ERK1/2 was reduced compared with that in the corresponding control group (0.11, 0.25 and 0.15 in the protein-extract-treated group vs. 0.27, 0.61 and 1.0 in the control group at 6, 12 and 24 h, respectively; Fig. 5B).

When cultured in suspension for 24 h, BAECs exhibited no obvious destruction of α-tubulin (Fig. 6A) and showed decreased ERK1/2 activity (Fig. 6B). However, the percentage of reduction in ERK1/2 activity in suspended BAECs was significantly less than that in protein-extract-treated cells (42.73 ± 4.99% vs. 82.73 ± 4.79%, $p = 0.004$; Fig. 6C).

Discussion

In the present study, protein extracts from *P. gingivalis* ATCC 33277 destroyed the adhesion properties of BAECs and induced apoptotic cell death. When protein extracts were pretreated with TLCK, their destructive effects were greatly reduced. These results suggested that the trypsin-like protease contributed to the pro-apoptotic effect of the protein extracts. Since gingipains account for most of the trypsin-like proteases of *P. gingivalis* (6,7), the detrimental effects of protein extracts might be attributed to gingipains. The present study also demonstrated destruction of integrin β1 and α-tubulin, as well as a reduction of ERK1/2 activation in BAECs due to the effect of protein extracts from *P. gingivalis*. These phenomena may help to illustrate the potential mechanism of the pro-apoptotic effects of *P. gingivalis* on endothelial cells.

Our results confirmed that the protein extracts from *P. gingivalis* ATCC 33277 could induce apoptosis of bovine aortic endothelial cells, which was consistent with earlier studies. Sheets *et al.* reported that gingipains from *P. gingivalis* W83 induced apoptosis in bovine aortic endothelial cells (12) and this apoptosis was caspase independent (13). Kobayashi-Sakamoto *et al.* (14) reported that *P. gingivalis* cysteine proteinases induced apoptotic cell death of human microvascular endothelial cells. These studies show that the periodontal pathogen *P. gingivalis* contains components which have pro-apoptotic effects on endothelial cells. This might contribute to the association between periodontal disease and cardiovascular diseases. However, the mechanism of the pro-apoptotic effects of protein extracts from *P. gingivalis* on endothelial cells still needs to be further elucidated. In this study, we observed some phenomena that may provide some clues for explaining the mechanism.

In this study, it was found that α-tubulin was degraded in BAECs

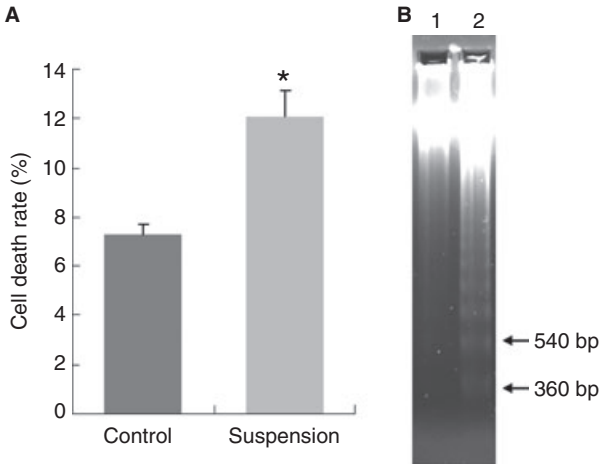

Fig. 4. Effect of loss of cell anchorage on cell viability. (A) The deaths of BAECs were compared in control cells and suspended cells. Data shown are means ± SEM of three independent experiments. Difference was analyzed using unpaired Student's *t*-test. $*p < 0.05$ vs. control. (B) Representative detection of DNA fragmentation in BAECs suspended for 24 h (key: 1, control; and 2, suspension).

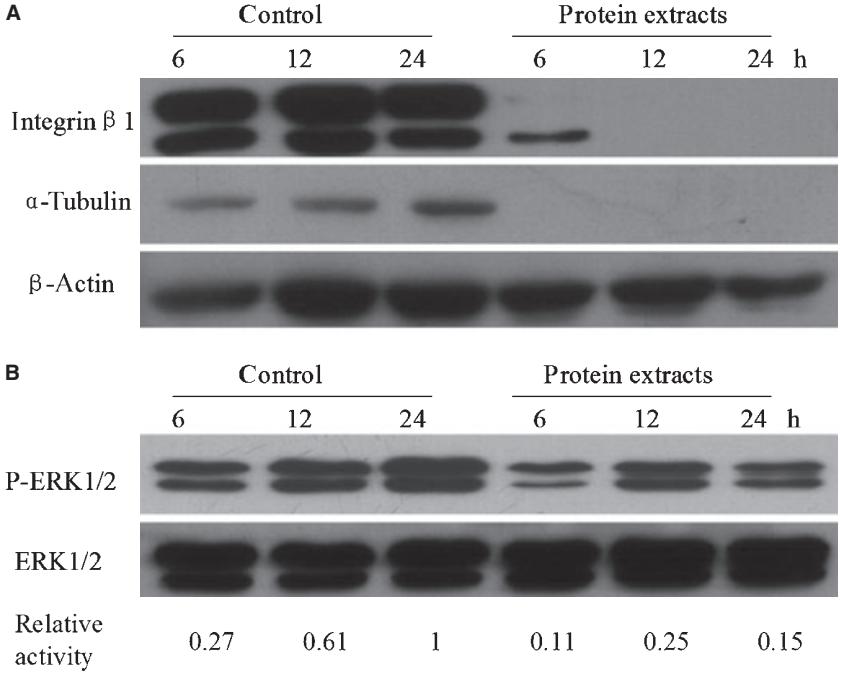

Fig. 5. Effects of protein extracts (400 μg/mL) on expression of α-tubulin, integrin β1 and activated ERK1/2. (A) Expression of α-tubulin and integrin β1 in BAECs treated with the protein extracts. (B) Activated ERK1/2 was considerably reduced compared with that in the corresponding control group. The ERK1/2 activation was reflected by relative activity determined by the ratio of phosphorylated to total protein. Activity in control cells at 24 h was set as 1. Results represent one of three independent sets of experiments.

exposed to protein extracts from *P. gingivalis*. This phenomenon has not previously been reported in the literature. α-Tubulin is a component of the microtubule, which is important for maintaining cell morphology. Loss of cell morphology may lead to cell apoptosis (23). Therefore, the degradation of α-tubulin by *P. gingivalis* protein extracts found in this study may partly account for the apoptosis of BAECs. Degradation of α-tubulin might be one mechanism of *P. gingivalis* pathogenesis. In our study, expression of α-tubulin was not altered in suspended BAECs. This excludes the possibility that degradation of α-tubulin is merely an incidental phenomenon due to loss of cell anchorage. Scragg *et al.* (29) found that gingipains could traverse the plasma membrane and localize in cytoplasm and around the nucleus. This indicated that the gingipains could enter cells and had a chance to degrade α-tubulin in cytoplasm. In addition, Sackett *et al.* (30) reported that trypsin exhibited the ability to hydrolyze α-tubulin into about 38- and 14-kDa pieces, which supports the possibility that the degradation of α-tubulin caused by *P. gingivalis* protein extracts might be enzyme-substrate dependent. It was suggested from these results that amorphosis may also participate in apoptosis of BAECs induced by the protein extracts. Nonetheless, this assumption needs further investigation in the future.

In the present study, after treatment with *P. gingivalis* protein extracts, BAECs became round and detached from culture plates, suggesting the possibility of cleavage of certain CAMs on the surface of BAECs. Our study further confirmed the destruction of integrin β1 in BAECs. This is consistent with previous studies, which indicated that gingipains from *P. gingivalis* may hydrolyze integrin β1 and other CAMs in epithelial and endothelial cells, such as cadherins, CD31 and CD99 (11–13,31,32). Moreover, Sheets *et al.* (12) suggested that one cause of the pro-apoptotic effects of gingipains on endothelial cells may be anoikis. When BAECs were cultured in suspension, the apoptotic cell death was confirmed in our study, suggesting that anoikis may partly explain the pro-apoptotic effect of the protein extracts on BAECs. Besides, as an important CAM mainly mediating attachment of cells to ECM, integrin β1, is found to be frequently expressed in the cardiovascular system and to participate in vascular development (33–35). Thus, the hydrolysis of integrin β1 by protein extracts from *P. gingivalis* might lead to the disruption of endothelium, and further influence the progression of atherosclerosis.

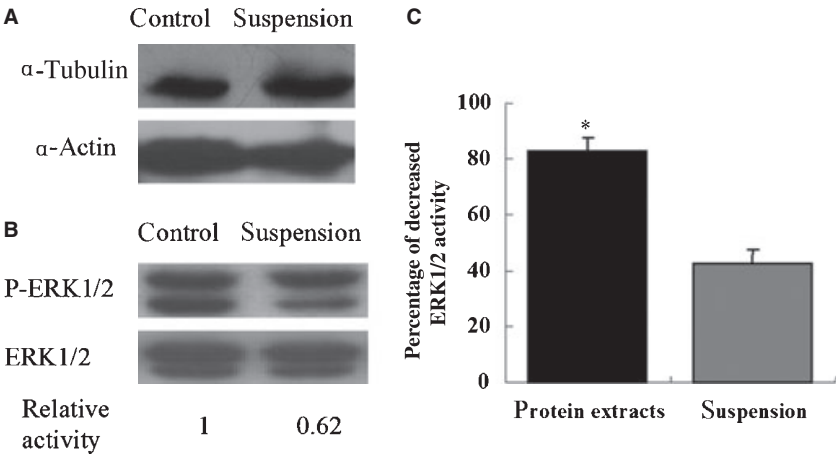

Fig. 6. Effects of cell suspension on expression of α-tubulin and activation of ERK1/2. (A) Representative expression of α-tubulin in BAECs suspended for 24 h (suspension). (B) The influence of cell suspension for 24 h on ERK1/2 activation in BAECs. The ERK1/2 activity was determined by the ratio of phosphorylated to total protein. Results represent one of three independent sets of experiments. (C) The percentage of reduction in ERK1/2 activity in BAECs treated with protein extracts and suspended cells, as calculated by the reduction in ERK1/2 activity divided by the ERK1/2 activity in control cells at 24 h in separate groups. Difference was analyzed using unpaired Student's *t*-test ($n = 3$). $^*p < 0.05$ vs. protein extracts.

In the present study, the activity of ERK1/2 was markedly decreased after BAECs were treated with protein extracts. Previous studies showed that destruction of cell anchorage may lead to suppression of ERK1/2 activation (19). However, in the present study, even though the ERK1/2 activity decreased in BAECs cultured in suspension, the ERK1/2 activity suppression was less than that in cells treated with protein extracts for the same length of time. These results indicate that the suppression of ERK1/2 activation in BAECs was not only by disruption of cell anchorage. ERK1/2 plays an important role in the regulation of cellular processes such as cell proliferation, differentiation and survival (21,22,36,37). In BAECs treated with protein extracts, the cell death rate was significantly higher than that in suspended cells, as shown by trypan blue exclusion assay (data not shown). One potential explanation is that the obvious suppression of ERK1/2 activity by protein extracts may make unanchored BAECs more sensitive to anoikis. Thus, it is possible that suppression of ERK1/2 activation may participate in the pro-apoptosis effect of the protein extracts.

The concentration of *P. gingivalis* protein extracts used in our *in vitro* study was selected according to our repeated preliminary studies (data not shown). With this concentration, the protein extracts from *P. gingivalis* showed obvious effects on BAECs. These results have been found only *in vitro*. Their activity and effects on endothelial cells *in vivo* are still undetermined. *P. gingivalis* and some other periodontal pathogens have been found in atherosclerotic plaques (38,39). Kozarov *et al*. (40) discovered viable *P. gingivalis* in an atherosclerotic plaque tissue specimen, and they estimated the presence of $\sim 1.9 \times 10^5$ *P. gingivalis* in the resected tissue. The viable *P. gingivalis* might produce toxic factors, including gingipain, and exhibit their pathogenic effects in the local area *in vivo*. However, the amount of bacteria that could injure endothelial cells still needs to be studied.

In conclusion, the present experiment showed that trypsin-like protease-active protein extracts from *P. gingivalis* induced detachment of BAECs and subsequent apoptotic cell death. We have reported, for the first time (by our knowledge), that the protein extracts from *P. gingivalis* destroyed α-tubulin, a component of the microtubule in BAECs. In addition, destruction of integrin β1 and inhibition of ERK1/2 activation in BAECs by the protein extracts were also found. These phenomena may help to explain the mechanism of the pro-apoptotic effects of *P. gingivalis* on endothelial cells.

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