

Original Article

Effects of *Porphyromonas gingivalis* extracellular vesicles on human periodontal ligament fibroblasts

Sheng-Gang Sang¹, Hong Rong², Ji-Bin Wang², Yi-Qiang Xie³

¹School of Tropical and Laboratory Medicine, Hainan Medical College, Haikou, China; ²Affiliated Hospital of Hainan Medical College, Haikou, China; ³Department of Traditional Chinese Medicine, Hainan Medical College, Haikou, China

Received December 4, 2013; Accepted January 19, 2014; Epub February 15, 2014; Published February 28, 2014

Abstract: The purpose of this study was to investigate the influencing mechanism of *Porphyromonas gingivalis* extracellular vesicles on human periodontal ligament fibroblasts to better understand the pathogenesis of periodontitis, the major cause of adult tooth loss. Human periodontal ligament fibroblasts were cultured and randomly assigned to a control group and an extracellular vesicles (ECV) group. The ECV group was exposed to isolated *Porphyromonas gingivalis* extracellular vesicles; the control group was not exposed. Western blotting was used to detect expression of matrix metalloproteinase 1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1), and RT-PCR was used to detect mRNA expression of *alkaline phosphatase (ALP)*. When human periodontal ligament fibroblasts were processed by *Porphyromonas gingivalis* extracellular vesicles (ECV), protein expression levels of both MMP-1 and TIMP-1 were significantly higher than that of the control group ($P < 0.05$). In contrast, ALP mRNA expression in human periodontal ligament fibroblasts processed by ECV was significantly lower than that of the control group ($P < 0.05$). *Porphyromonas gingivalis* extracellular vesicles can up-regulate expression of MMP-1 and TIMP-1 protein and ALP mRNA of human periodontal fibroblasts.

Keywords: *Porphyromonas gingivalis*, extracellular vesicles, periodontal ligament fibroblasts, matrix metalloproteinase 1 (MMP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1), alkaline phosphatase (ALP)

Introduction

Periodontitis is a chronic inflammation of the gingival and periodontal tissues and is mainly characterized by formation of inflammation of the pocket wall [1, 2]. Additionally, periodontitis causes alveolar bone resorption and gradually loosened teeth, which is the major cause of adult tooth loss [1, 2]. Current research shows that an anaerobe, *Porphyromonas gingivalis* (Pg), is a major pathogen causing periodontitis [3, 4]. The extracellular vesicle (ECV) of Pg is an outer membrane sprouting body, which is formed as follows: the bacterial outer membrane bulges outward, gradually becomes an independent component, and freely enters into the surrounding microenvironment. ECV has an integrated outer membrane, similar to its parent thallus in toxicology and immunology [5]; extracellular vesicles can reach deep periodontium due to their small size, to cause local peri-

odontal tissue destruction. Interestingly, 80% of the major toxic substances of Pg are believed to locate in extracellular vesicles [6]. In this study, human periodontal ligament fibroblasts were exposed to Pg extracellular vesicles to investigate mechanism of their effects on this tissue and the pathogenesis of periodontitis.

Materials and methods

Materials and equipment

Porphyromonas gingivalis (China Microbiological Culture Collection Center), GAM Blood-Agar-Medium (Invitrogen, Shanghai, China), CDC medium (Invitrogen), DMEM cell culture medium (Invitrogen), fetal calf serum (FCS, Invitrogen), 2.5 mg/L trypsin (Biochemistry Preparation Plant, Shanghai), anaerobic jar (800 ml/L N₂, 100 ml/L H₂, 100 ml/L CO₂), 37°C incubator, YJ-874 Clean Bench (Suzhou

ECV on human periodontal ligament fibroblasts

Purification Equipment Factory), low-temperature ultracentrifuge, freeze drying machine, CO₂ incubator (Hereaus, Germany), inverted phase-contrast microscope (Olympus, Japan), goat anti-human matrix metalloproteinase 1 (MMP-1) polyclonal antibody (Upstate Biology, USA), goat anti-human tissue inhibitor of metalloproteinase-1 (TIMP-1) polyclonal antibody (Upstate), rabbit anti-human β -actin polyclonal antibody (Sigma, USA), rabbit anti-goat secondary antibody (Sigma), goat anti-rabbit secondary antibody (Sigma), and Chemiluminescent Substrate Kit (GE, USA).

Porphyromonas gingivalis culture

Bacteria were inoculated on GAM blood-agar-medium placed in an anaerobic jar (800 ml/L N₂, 100 ml/L H₂, 100 ml/L CO₂) for culture at 37°C for 48 hours. Bacteria were then inoculated in CDC medium placed in an anaerobic jar and cultured at 37°C for 72 hours.

Extraction of Porphyromonas gingivalis membrane vesicle

Bacteria were transferred into physiological saline, washed 2 times, and centrifuged at 3000 rpm for 15 min. After discarding the supernatant, bacteria were centrifuged at 4°C at 1500 rpm for 15 min. Supernatant was concentrated 40-fold in a Minitan ultrafiltration system (molecular weight cutoff: 5000) and centrifuged at 4°C at 11000 rpm for 15 min. Supernatant was centrifuged again at 4°C and 80000 rpm for 30 min. Finally, the supernatant was discarded and the precipitate was retained.

Culturing and processing human periodontal ligament fibroblasts

Dentes premolares that were free of dental caries or periodontal disease but extracted due to orthodontic treatment were collected. Periodontal tissues of the dentes premolares were scraped under sterile conditions, then cultured and passaged via trypsin digestion. Confluent cells of the 6th generation were digested with 2.5 mg/L trypsin. Cells were counted and suspensions were diluted to 2×10⁴ cells/mL. 100 μ L were added to each well of a 96-well culture plate. Following 24 hours in culture, Supernatant and non-adherent cells were discarded. Wells were randomized into blank control group and ECV group. *Porphyromonas gingivalis* extract

was added to wells of the ECV group until the concentration reached 50 μ g/mL; plates were cultured for 48 hours.

Western blotting

100 μ L protein lysate were added to each well and incubated at room temp for 10 min. Proteins were loaded onto a polyacrylamide gel and electrophoresed for 2 hours, then transferred to a nitrocellulose membrane. Membrane was washed with PBST then sealed with 5% PBSTM for 2 hours. Primary antibody was added (1:400) and incubated overnight. Membrane was washed with 5% PBST before addition of horseradish peroxidase-labeled secondary antibody (1:2000) at room temperature for 2 hours. Membrane was again washed with PBST for 15 min. 300 μ L of each of Chemiluminescent Substrate Kit A solution and B solution were mixed and added for 1 min, then membrane was exposed, developed, and photographed. Integral optical density values of the bands were computed compared with β -actin.

RT-PCR

Total RNA was extracted from cells using Trizol reagent (TaKaRa Biotech, Dalian, China) for conventional reverse transcription. RT-PCR was used to detect the expression level of *alkaline phosphatase (ALP)* of human periodontal ligament fibroblasts. Forward primer sequence was as follows: 5'-CTT GCT GGT GGA AGG ATT CAG-3'; reverse primer sequence was as follows: 5'-GGA GCA CAG GAA GTG GCC AC-3'; the expected product was 342 bp. For β -actin, the forward primer sequence was: 5'-CAA GTA CCA GCG AGC AGC TT-3'; the reverse primer sequence was: 5'-AAA GCC GAG CTG CCA GAG TT-3'; the expected product was 216 bp. cDNA was amplified by a Mycycler PCR instrument (Sweden BIO-RED). Products were separated on 2% agarose gel. Integral optical density values of the bands were analyzed and compared with β -actin.

Statistical methods

Statistical analysis of all the results was performed using the SPSS 12.0 statistical software; all the statistical data were analyzed by t test, with $P < 0.05$ as statistically significant.

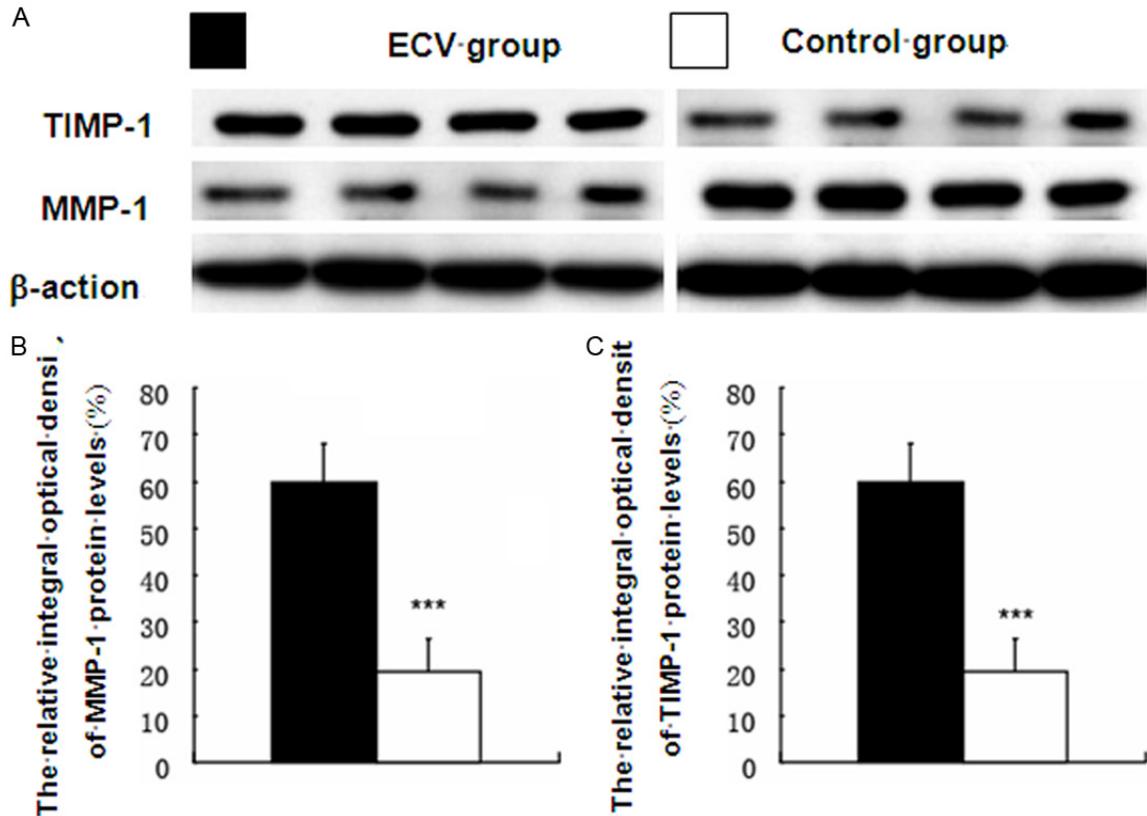


Figure 1. Effect of ECV on MMP-1 and TIMP-1 protein expression in human periodontal ligament fibroblasts. A: Western blot bands in ECV group and the control group; B: Comparison of relative integral optical density values of MMP-1 protein expression; C: Comparison of relative integral optical density values of TIMP-1 protein expression (***) ($P < 0.01$).

Results

Effect of ECV on protein expression of MMP-1 and TIMP-1 of human periodontal ligament fibroblasts

Western blotting was used to detect protein expression levels of MMP-1 and TIMP-1 in human periodontal ligament fibroblasts exposed to ECV (**Figure 1A**). Relative expression of MMP-1 in ECV-processed human periodontal ligament fibroblasts was significantly higher compared to the control fibroblasts ($P < 0.05$) (**Figure 1B**). Similarly, relative expression of TIMP-1 in ECV-exposed human periodontal ligament fibroblasts was significantly higher compared to the control group ($P < 0.05$) (**Figure 1C**).

Effect of ECV on mRNA expression of ALP in human periodontal ligament fibroblasts

To further investigate the effect of ECV on human periodontal ligament fibroblasts, we

used RT-PCR to determine mRNA expression ALP in human periodontal ligament fibroblasts (**Figure 2A**). Relative ALP mRNA expression levels in ECV-processed human periodontal ligament fibroblasts were significantly lower compared to the control group ($P < 0.05$) (**Figure 2B**).

Discussion

The main pathological changes in periodontal disease are destruction of the periodontal ligament, alveolar bone, and periodontal supporting tissues caused by external pathogenic factors [7]. Periodontal ligament fibroblasts are the main mesenchymal cells of the periodontal ligament, playing an important role in metabolism of the periodontal ligament and synthesis of collagen fibers. Recently, studies on periodontitis pathogens have shown that the main bacteria of the subgingival plaque include *Porphyromonas gingivalis*, *Prevotella intermedia*, *Spirochetes*, and other anaerobic bacteria [8, 9].

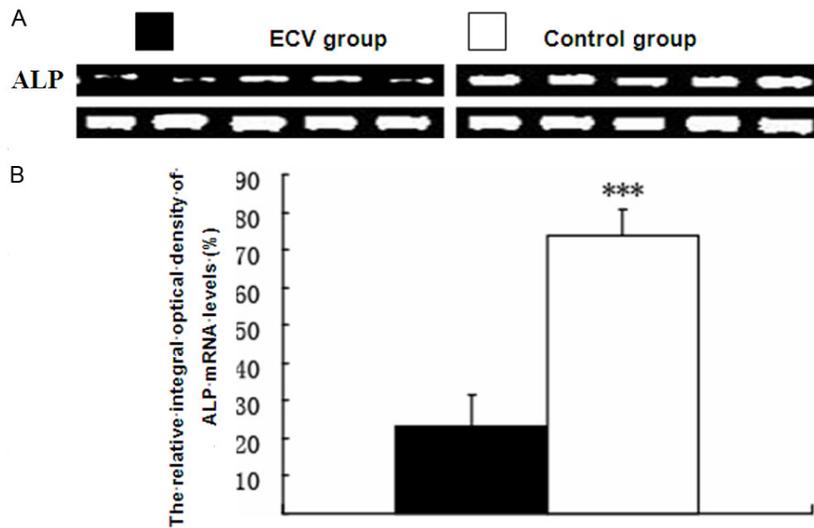


Figure 2. Effect of ECV on ALP mRNA expression in human periodontal ligament fibroblasts. A: RT-PCR bands in ECV group and the control group; B: Comparison of relative integral optical density values of ALP mRNA expression (***: $P < 0.01$).

Our study shows that *Porphyromonas gingivalis* extracellular vesicles can significantly up-regulate MMP-1 and TIMP-1 protein expression in human periodontal ligament fibroblasts. Matrix metalloproteinases are a group of zinc-dependent polypeptide enzymes, currently including 24 types, which effectively degrade the extracellular matrix (ECM) [10]. Studies have found that MMP-1, MMP-2, and MMP-3 can effectively degrade I-III and VII fiber glue [11]. Lipopolysaccharide (LPS) of Pg can cause increased protein expression of MMP-1 of gum tissues of periodontal patients and increased concentration of gingival crevicular fluid inflammatory cytokines such as IL-6 and IL-8. Further, periodontal tissue damage caused by LPS is believed to be related to over-expression of MMP-1 [12]. TIMP-1 is a natural inhibitor of MMP, reducing biological activity of MMP-1 and its ability to degrade extracellular matrix (such as collagen fiber); this maintains the metabolic balance of the extracellular matrix [13]. Our study shows that ECV can up-regulate MMP-1 expression and down-regulate TIMP-1 expression of human periodontal fibroblasts, thus disrupting the balance of MMP-1 and TIMP-1, which leads to excessive degradation of extracellular matrix. Similarly, one study has shown that LPS also can alter the balance of MMP-1 and TIMP-1 in human periodontal fibroblasts [14].

Recent work shows that periodontal ligament fibroblasts are pluripotent stem cells, mainly in-

involved in metabolism of the periodontal ligament, synthesis of collagen fibers, and other extracellular matrix. The biological activity of periodontal ligament fibroblasts is similar to osteoblasts in many respects, such as high expression of alkaline phosphatase and osteocalcin [15]. Thus, periodontal ligament fibroblasts can be differentiated into osteoblasts; these findings indicate that periodontal ligament fibroblasts may be closely involved in the metabolism of periodontal bone tissue [16]. We found that ALP

mRNA expression was reduced in ECV-processed human periodontal ligament fibroblasts, indicating that ECV can inhibit ALP synthesis in periodontal fibroblasts, thus preventing fibroblasts from differentiating into osteoblasts.

Our study has shown that *Porphyromonas gingivalis* extracellular vesicles can accelerate degradation of extracellular matrix through up-regulating MMP-1 and TIMP-1 protein expression in human periodontal fibroblasts. Additionally, by down-regulating expression of ALP mRNA, Pg ECV may prevent periodontal fibroblasts from differentiating into osteoblasts.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yi-Qiang Xie, Department of Traditional Chinese Medicine, Hainan Medical College, Haikou 571199, China. E-mail: yiqiangxie1972@126.com

References

[1] Jotwani R, Moonga BS, Gupta S and Cutler CW. Nuclear factor-kappaB p50 subunits in chronic periodontitis and *Porphyromonas gingivalis* lipopolysaccharide-pulsed dendritic cells. *Ann N Y Acad Sci* 2010; 1192: 278-285.
 [2] Kubota M, Tanno-Nakanishi M, Yamada S, Okuda K and Ishihara K. Effect of smoking on

- subgingival microflora of patients with periodontitis in Japan. *BMC Oral Health* 2011; 11: 1.
- [3] Hokamura K and Umemura K. Roles of oral bacteria in cardiovascular diseases—from molecular mechanisms to clinical cases: *Porphyromonas gingivalis* is the important role of intimal hyperplasia in the aorta. *J Pharmacol Sci* 2010; 113: 110-114.
- [4] Liu C, Hashizume T, Kurita-Ochiai T, Fujihashi K and Yamamoto M. Oral immunization with *Porphyromonas gingivalis* outer membrane protein and CpG oligodeoxynucleotides elicits T helper 1 and 2 cytokines for enhanced protective immunity. *Mol Oral Microbiol* 2010; 25: 178-189.
- [5] Kamaguchi A, Ohyama T, Sakai E, Nakamura R, Watanabe T, Baba H and Nakayama K. Adhesins encoded by the gingipain genes of *Porphyromonas gingivalis* are responsible for coaggregation with *Prevotella intermedia*. *Microbiology* 2003; 149: 1257-1264.
- [6] Tsuda K, Amano A, Umebayashi K, Inada H, Nakagawa I, Nakanishi Y and Yoshimori T. Molecular dissection of internalization of *Porphyromonas gingivalis* by cells using fluorescent beads coated with bacterial membrane vesicle. *Cell Struct Funct* 2005; 30: 81-91.
- [7] Ohyama H, Hongyo H, Shimizu N, Nishimura F, Nakagawa M, Arai H, Kato-kogoe N, Terada N, Nagai A, Takashiba S, Kurihara H, Nomura Y and Murayama Y. Clinical and immunological assessment of periodontal disease in Japanese leprosy patients. *Jpn J Infect Dis* 2010; 63: 427-432.
- [8] Souccar NM, Chakhtoura M, Ghafari JG and Abdelnoor AM. *Porphyromonas gingivalis* in dental plaque and serum C-reactive protein levels in pregnancy. *J Infect Dev Ctries* 2010; 4: 362-366.
- [9] Komiya IA, Ishihara K, Tomita S, Kato T and Yamada S. Investigation of subgingival profile of periodontopathic bacteria using polymerase chain reaction. *Bull Tokyo Dent Coll* 2010; 51: 139-144.
- [10] Steenport M, Khan KM, Du B, Barnhard SE, Dannenberg AJ and Falcone DJ. Matrix metalloproteinase (MMP)-1 and MMP-3 induce macrophage MMP-9: evidence for the role of TNF-alpha and cyclooxygenase-2. *J Immunol* 2009; 183: 8119-8127.
- [11] Okamoto K, Ishida C, Ikebuchi Y, Mandai M, Mimura K, Murawaki Y and Yuasa I. The genotypes of IL-1 beta and MMP-3 are associated with the prognosis of HCV-related hepatocellular carcinoma. *Intern Med* 2010; 49: 887-895.
- [12] Silverio-Ruiz KG, Martinez AE, Garlet GP, Barbosa CF, Silva JS, Cicarelli RM, Valentini SR, Abi-Rached RS and Junior CR. Opposite effects of bFGF and TGF-beta on collagen metabolism by human periodontal ligament fibroblasts. *Cytokine* 2007; 39: 130-137.
- [13] Poleni PE, Etienne S, Velot E, Netter P and Bianchi A. Activation of PPARs alpha, beta/delta, and gamma Impairs TGF-beta1-Induced Collagens' Production and Modulates the TIMP-1/MMPs Balance in Three-Dimensional Cultured Chondrocytes. *PPAR Res* 2010; 2010: 635912.
- [14] Cao Z, Li C and Zhu G. Inhibitory effects of bicalin on IL-1beta- induced MMP-1/TIMP-1 and its stimulated effect on collagen-I production in human periodontal ligament cells. *Eur J Pharmacol* 2010; 641: 1-6.
- [15] Yang L, Chao YL and Du L. [Study of biologic effects of simulating static magnetic field of magnetic attachment on human periodontal ligament fibroblasts]. *Hua Xi Kou Qiang Yi Xue Za Zhi* 2007; 25: 316-319.
- [16] Yu MJ, Yang PS and Ge SH. Biological effects of phenytoin on cultured human periodontal ligament fibroblasts in vitro. *Hua Xi Kou Qiang Yi Xue Za Zhi* 2008; 26: 215-218.