

Hyperresponsiveness of human gingival fibroblasts from patients with aggressive periodontitis against bacterial lipopolysaccharide

XUE LI, XIAOXUAN WANG and QING-XIAN LUAN

Department of Periodontology, Peking University School and Hospital of Stomatology,
National Engineering Laboratory for Digital and Material Technology of Stomatology,
Beijing Key Laboratory of Digital Stomatology, Beijing 100081, P.R. China

Received July 1, 2020; Accepted October 20, 2020

DOI: 10.3892/etm.2021.9861

Abstract. The present study aimed to investigate whether gingival fibroblasts (GFs) of patients with aggressive periodontitis (AgP) are more sensitive to lipopolysaccharide (LPS) stimulation than GFs of control subjects. AgP causes rapid periodontal destruction, including the production of cytokines [i.e. interleukin (IL)-1 β , IL-6 and tumor necrosis factor (TNF)- α] and matrix metalloproteinases (MMP)-1, -3 and -9 in AgP GFs. LPS upregulates IL-1 β , IL-6, TNF- α , MMP-1, MMP-3, MMP-9 and mitochondrial reactive oxygen species (mtROS). Fibroblasts are known to be associated with immune responses to bacterial virulence factors, but the precise mechanisms underlying this severe periodontal disease are unclear. In the present study, primary human GFs of four patients with AgP and four healthy subjects were challenged *in vitro* with LPS from *Porphyromonas gingivalis* (*P. gingivalis*). The generation of mtROS in GFs was assessed using MitoSOX Red. The expression of genes encoding inflammatory cytokines and MMPs in GFs was analyzed using reverse transcription-quantitative polymerase chain reaction, and the expression of proteins was analyzed using ELISA and Western blotting. Human GFs of patients with AgP exhibited higher

levels of mtROS, and higher mRNA and protein expression levels of proinflammatory cytokines, including IL-1 β , IL-6, MMP-1, MMP-3 and MMP-9 compared with healthy human GFs following stimulation with LPS from *P. gingivalis*. In the present study, it was demonstrated that GFs of patients with AgP display hyperactivity when challenged with LPS.

Introduction

Periodontitis is one of the leading causes of tooth loss in the adult population. This disease can be classified into various categories, and one of the most destructive amongst them is aggressive periodontitis (AgP). The incidence of AgP is lower than that of other types of periodontitis. However, it affects young individuals and may cause severe destruction of tooth-supporting structures, including tooth loss if left untreated. The current classification for diagnosing periodontal disease was established by the American Academy of Periodontology (AAP) in 1999. This classification provided strict guidelines to aid in AgP diagnosis. These include three main factors: Systemically healthy individual, rapid loss of clinical attachment and familial aggregation. AgP is characterized by generalized or localized extreme periodontal damage. It is a type of rapid and severe periodontal disease affecting systemically healthy individuals. This disease is characterized by an early age of onset, rapid rate of disease progression and aggregation of family (1).

Fibroblasts serve an important role in chronic infections, including periodontitis. Human gingival fibroblast (GF) is a functionally distinct type of fibroblast in periodontal tissues (2). GFs are subepithelial and predominantly involved in the maintenance and regeneration of periodontal tissues (3,4). The inflammatory destruction in periodontal diseases may be the result of the interactions between bacterial virulence factors and host defense mechanism (5,6). GFs respond to periodontopathic organisms or their components by initiating an inflammatory response, including the production of various proinflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)- α (7-9). The results of our previous study demonstrated that GFs may produce mitochondrial reactive oxygen species (mtROS) following stimulation

Correspondence to: Professor Qing-Xian Luan, Department of Periodontology, Peking University School and Hospital of Stomatology, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, 22 Zhongguancun Avenue South, Haidian, Beijing 100081, P.R. China
E-mail: kqluanqx1100@163.com

Abbreviations: AgP, aggressive periodontitis; GF, gingival fibroblasts; LPS, lipopolysaccharide; IL-1 β , interleukin-1 β ; mtROS, mitochondrial reactive oxygen species; MMP, matrix metalloproteinase; AGF, human GFs of patients with aggressive periodontitis; HGF, human GFs; AAP, American Academy of Periodontology; ACTB, β -actin.

Key words: gingival fibroblasts, aggressive periodontitis, lipopolysaccharide, inflammatory response, mtROS, *Porphyromonas gingivalis*

with lipopolysaccharides (LPS) of *Porphyromonas gingivalis* (*P. gingivalis*) on the tooth surface, which mediates the production of IL-1, IL-6 and TNF- α in GFs (10). Furthermore, the increased production of matrix metalloproteinases (MMPs), including MMP-1, MMP-3 and MMP-9, which degrade numerous extracellular matrix components, may be the cause of GFs contributing toward periodontal tissue destruction (11,12).

Among periodontopathic organisms, *P. gingivalis* is an anaerobic Gram-negative bacterium that appears to be associated with AgP. LPS, located on the outer membrane of the cell wall of Gram-negative bacteria, is an important bacterial surface component, and is considered to be a potent immunostimulant (13). Although periodontitis can be caused by bacteria such as *P. gingivalis*, host susceptibility is crucial to the development of the disease. The characteristics of the cells interacting with LPS from *P. gingivalis* may partly determine susceptibility. For example, mononuclear cells, neutrophils and platelets in patients with periodontitis are known to be different from the same cells in healthy individuals in their interaction with periodontal pathogens (14-18). However, little is known regarding whether differences also exist in GFs between patients with AgP and healthy subjects.

Current evidence has suggested that AgP occurs in susceptible individuals who have abnormal inflammatory/immune response to periodontal pathogens (19). Earlier studies have demonstrated that the expression levels of destruction factors associated with disease progression were higher in periodontitis tissues in patients with chronic periodontitis than in healthy subjects (20,21). At present, there is a lack of data available for comparison of cytokine profiles between GFs obtained from patients with AgP and healthy subjects. The present study tested the hypothesis that GFs in patients with AgP may be more sensitive to LPS stimulation than GFs in control subjects. The present study may elucidate the underlying mechanisms contributing toward the rapid rate of disease progression of AgP.

Materials and methods

The present study was approved by the Review Board and Ethics Committee of Peking University Health Science Center. Written informed consent was obtained from all subjects.

Cell culture and treatment. GFs derived from four patients with AgP (AGFs) and healthy subjects, who sought dental treatment at the Department of Periodontology, Peking University School and Hospital of Stomatology between January 2017 and January 2018, were used in the present study. The test group consisted of 2 males and 2 females, aged 38-50 years with a mean age of 44. The control group consisted of 2 males and 2 females, aged 32 to 40 years with a mean age of 36. Exclusion criteria included pregnancy or breastfeeding, smoking, alcohol abuse, uncontrolled diabetes and other systemic conditions that could affect the periodontal status. Healthy GFs (CGFs) were obtained from explants of human normal gingival tissues as a control group from patients seeking dental treatment at the Department of Periodontology, Peking University School and Hospital of Stomatology

between January 2017 and January 2018 (Table I; Fig. 1). GF explantation was performed as previously described (22). Individuals were designated as having AgP according to the 1999 AAP Classification of Periodontal Diseases (1). Healthy gingival tissue samples were collected from periodontal healthy groups undergoing a crown-lengthening procedure, while inflamed gingival tissue was harvested from teeth with pockets of 6 mm or more in patients with chronic periodontitis following flap debridement (22). The cells were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; HyClone; Cytiva), containing 10% (v/v) fetal bovine serum (FBS; HyClone; Cytiva) and 100 U/ml penicillin with 100 μ g/ml streptomycin. GFs between passages 3-6 were used. The cells were treated with 1 μ g/ml LPS derived from *P. gingivalis* (InvivoGen, cat. no. tlr1-pglps) for 12 h. The experiments on the 4 AgP patients and 4 healthy control subjects were repeated 5 times.

Multimodal microplate reader. Fluorescence was measured using a multimodal microplate reader (BioTek Instruments, Inc.). HGFs were trypsinized and washed with cold phosphate-buffered saline (PBS). The cells (1.8×10^5) were resuspended in 1 ml DMEM containing 5 μ M MitoSOX Red and incubated in the dark in a CO₂ incubator for 30 min. The cells were centrifuged at 130 x g for 5 min at room temperature, washed three times with PBS, and resuspended in 500 μ l PBS. The mtROS content of cells was analyzed based on the fluorescence intensity of MitoSOX Red. The mtROS content of cells was analyzed based on the fluorescence intensity of MitoSOX Red with excitation at 510 nm and emission at 580 nm.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). HGFs and AGFs were cultured in 6-well plates (1×10^5 per well) followed by the addition of medium with or without LPS. Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized using Reverse Transcription Premix (Bioneer Corporation). The thermal profile was incubation at 70°C for 5 min before chilling on ice. PCR was performed using gene-specific primers (Table II) and PCR premix (Kapa Biosystems; Roche Diagnostics). The PCR conditions were 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec (PCR machine model: Eppendorf Mastercycler X50h; Eppendorf). All reactions were performed in triplicate in two separate experiments. The relative expression levels of the targets in each sample were calculated using the comparative 2^{- $\Delta\Delta$ C_q} method following normalization against the expression of ACTB (21).

Measurement of various cytokine levels by ELISA. The GFs were cultured in 96-well plates (1×10^4 per well) and then medium with or without 1 μ g/ml LPS was added. The levels of IL-1 β , IL-6 and TNF- α in cell culture supernatants were measured using ELISA kits (R&D Systems China Co., Ltd.; cat. no. IL-1 β , 70-E-EK101B1; IL-6, 70-E-EK1061; TNF- α , EK1821), according to the manufacturer's protocols.

Western blotting. Whole protein lysates were prepared using PRO-PREP Protein Extraction Solution (Intron

Table I. Demographic characteristics of the study sample at baseline.

Sample	Age, years	Sex	Location	Treatment
Patient 1	38	Female	15, 16, 17	Flap debridement
Patient 2	43	Female	24, 25, 26, 27	Flap debridement
Patient 3	45	Male	26, 27	Flap debridement
Patient 4	50	Male	44, 45, 46, 47	Flap debridement
Healthy control 1	34	Male	16	Crown-lengthening
Healthy control 2	40	Female	36	Crown-lengthening
Healthy control 3	38	Male	25	Crown-lengthening
Healthy control 4	32	Female	26	Crown-lengthening

Table II. Primers used for gene amplification.

Gene	Primer name	Primer sequence	Primer length (bp)
IL-1 β	IL-1 β -forward	CTTCAGCCAATCTTCATTGCT	200
	IL-1 β -reverse	TCGGAGATTCGTAGCTGGAT	
IL-6	IL-6-forward	GAGGGCTCTTCGGCAAATGTA	89
	IL-6-reverse	CCCAGTGGACAGGTTTCTGAC	
TNF- α	TNF- α -forward	GCTCAGACATGTTTTCCGTGAA	140
	TNF- α -reverse	GTCACCAAATCAGCAATTGTTTGA	
MMP-1	MMP-1-forward	TCTGGGGAAAACCTTTCGACT	136
	MMP-1-reverse	CACCAACGTATTCAAAAGCACAA	
MMP-3	MMP-3-forward	AGTCTTCCAATCCTACTGTTGCT	148
	MMP-3-reverse	TCCCGTATGGTTACACCAATCC	
MMP-9	MMP-9-forward	TGTACCGCTATGGTTACACTCG	180
	MMP-9-reverse	GGCAGGGACAGTTGCTTCT	
ACTB	ACTB-forward	AGCACAATGAAGATCAAGATCAT	127
	ACTB-reverse	ACTCGTCATACTCCTGCTTGC	

IL, interleukin; TNF- α , tumor necrosis factor- α ; MMP, matrix metalloproteinase; ACTB, β -actin.

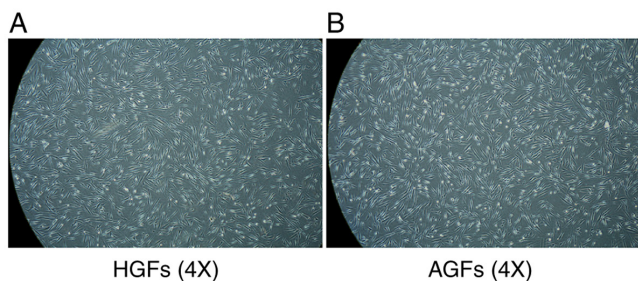


Figure 1. Images of typical morphology of GFs from AgP and from healthy subjects. (A) HGFs of passage 0. (B) AGFs of passage 0. GF, gingival fibroblasts; AgP, aggressive periodontitis; HGFs, GFs from healthy subjects; AGFs, GFs from patients with aggressive periodontitis.

Biotechnology, Inc.). The nuclear and cytoplasmic fractions were isolated using an NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Equal amounts (15 μ l) of protein, measured using the BCA protein assay (Pierce; Thermo Fisher Scientific, Inc.) were loaded into each

lane of the gel. Proteins were separated by electrophoresis on 10-12% (v/v) sodium dodecyl sulphate-polyacrylamide gels and transferred onto nitrocellulose membranes (BD Biosciences). The membranes were then blocked with 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. SW3015) for 2 h at room temperature and incubated with rabbit antibodies against MMP-1 (cat. no. Ab137332; Abcam), MMP-3 (cat. no. Ab2915; Abcam), MMP-9 (cat. no. Ab38898; Abcam) and actin (cat. no. TA-09; Abcam) at a 1:1,000 dilution for 2 h at room temperature. The membranes were then incubated with horseradish peroxidase (HRP)-coupled anti-rabbit secondary antibody (Cell Signaling Technology, Inc. cat. no. 5151, 1:2,000) at room temperature for 40 min. The membranes were then washed with TBST (20% Tween-20) 3 times, for 10 min each time. Finally, the bands were stained with an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). Rabbit anti-actin was used as a loading control (Cell Signaling Technology, Inc.). After the image was scanned, grayscale analysis was performed using Gel Image system ver.4.00 (Tanon Science and Technology Co., Ltd.).

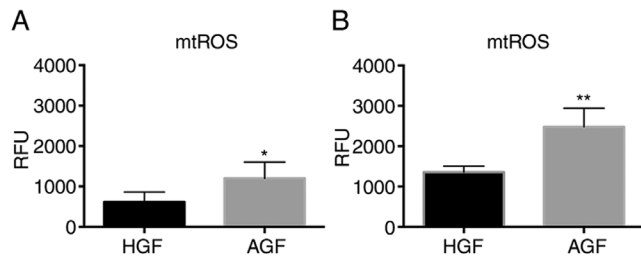


Figure 2. Level of mtROS in GFs in control subjects and patients with AgP prior to and following a 12 h challenge with LPS from *P. gingivalis*. (A) The level of mtROS in GFs of control subjects and patients with AgP prior to LPS challenge. (B) The level of mtROS in GFs of control subjects and patients with AgP following LPS challenge. * $P < 0.05$, ** $P < 0.01$ vs. HGF. mtROS, mitochondrial reactive oxygen species; GFs, gingival fibroblasts; AgP, aggressive periodontitis; LPS, lipopolysaccharide; HGFs, GFs from healthy subjects; AGFs, GFs from patients with aggressive periodontitis; RFU, relative fluorescence units.

Statistical analysis. Data represent the mean \pm standard deviation of five independent experiments. Data were analyzed using GraphPad Prism software (version 6; GraphPad Software, Inc.). Variance between the two groups was analyzed by t-test. $P < 0.05$ was considered to indicate a statistical difference. $P < 0.01$ was considered to indicate a statistically significant difference.

Results

Comparison of morphology of CGFs and AGF. The images of typical morphology of GFs from patients with AgP and from healthy subjects were observed under a microscope (Fig. 1). No difference in morphology or proliferation was identified between CGFs and AGFs.

Comparison of basal levels and augmented mtROS levels stimulated by LPS in CGFs and AGFs. The levels of mtROS in unstimulated or stimulated GFs were measured and were compared between the healthy and the AgP groups (Fig. 2). In the unstimulated cells, the mtROS level was higher in the AgP group compared with the healthy group ($P < 0.05$). In the stimulated cells, the mtROS level was significantly higher in the AgP group than in the healthy group ($P < 0.01$).

Comparison of mRNA expression levels of proinflammatory genes in HGFs and AGF prior to and following stimulation by LPS. The mRNA expression levels (relative to the housekeeping gene) of IL-1 β , IL-6, and TNF- α , which encode proinflammatory cytokines in GFs, were measured in unstimulated or stimulated GFs. These levels were then compared between the healthy and the AgP groups (Fig. 3). In the unstimulated cells, IL-1 β and IL-6 mRNA expression was higher in the AgP group compared with the healthy group (IL-1 β , $P < 0.05$; IL-6, $P < 0.05$; Fig. 3A and C), while the expression of TNF- α mRNA did not show a significant difference between the healthy and AgP groups (TNF- α , $P > 0.05$; Fig. 3E). In stimulated cells, the expression level of IL-1 β and IL-6 mRNA was significantly higher in the AgP group than in the healthy group ($P < 0.01$; Fig. 3B and D), and TNF- α mRNA was higher in the AgP group than in the healthy group (TNF- α , $P < 0.05$; Fig. 3F).

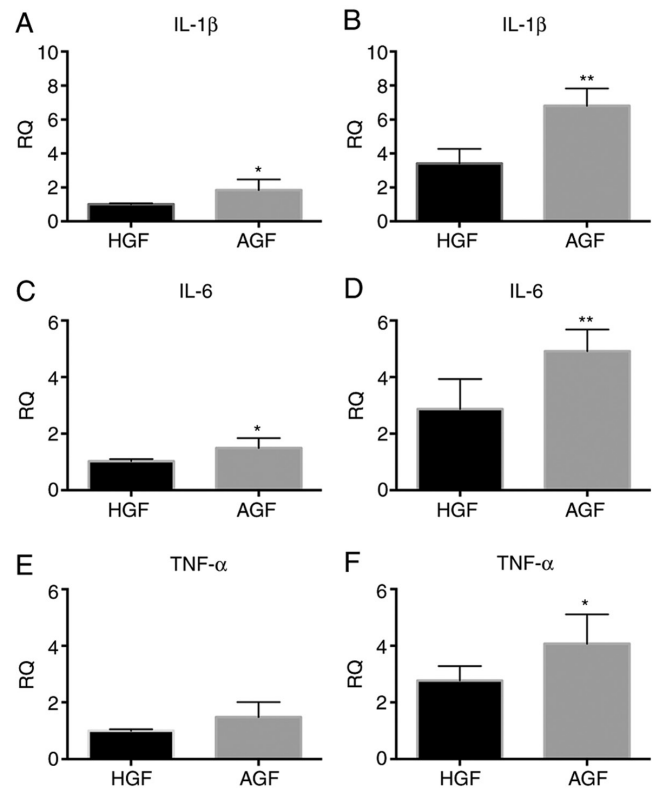


Figure 3. mRNA expression of proinflammatory cytokines, including IL-1 β , IL-6, TNF- α in control subjects and patients with AgP prior to and following a 12 h challenge with LPS from *P. gingivalis*. The mRNA expression of IL-1 β in GFs of control subjects and patients with AgP (A) prior to LPS challenge and (B) following LPS challenge. The mRNA expression of IL-6 in GFs of control subjects and patients with AgP (C) prior to LPS challenge and (D) following LPS challenge. The mRNA expression of TNF- α in GFs of control subjects and patients with AgP (E) prior to LPS challenge and (F) following LPS challenge. * $P < 0.05$, ** $P < 0.01$ vs. HGF. IL, interleukin; TNF- α , tumor necrosis factor- α ; GFs, gingival fibroblasts; AgP, aggressive periodontitis; LPS, lipopolysaccharide; HGFs, GFs from healthy subjects; AGFs, GFs from patients with aggressive periodontitis; RQ, relative quantity.

Comparison of protein levels of proinflammatory cytokines in HGFs and AGF prior to and following LPS stimulation.

To analyze whether changes in cytokines gene expression also resulted in increased protein secretion, culture supernatants of challenged and unchallenged GFs of patients and control subjects were analyzed for the presence and levels of IL-1 β , IL-6 and TNF- α (Fig. 4). IL-1 β and IL-6 levels in GFs unstimulated with LPS were higher in the AgP group than in the healthy group (IL-1 β , $P < 0.05$; IL-6, $P < 0.05$ Fig. 4A and C). However, the expression of TNF- α did not significantly differ between the healthy and AgP groups (TNF- α , $P > 0.05$; Fig. 4E). With stimulation, IL-1 β , IL-6 and TNF- α levels in GFs were significantly higher in the AgP group than in the healthy group (IL-1 β , $P < 0.01$; IL-6, $P < 0.01$; TNF- α , $P < 0.01$; Fig. 4B, D and F). There was a difference in the ratio of cytokines between GFs from AgP and GFs from healthy subjects when compared the unstimulated with stimulated states. Under the stimulated state, the ratio was higher in the AgP group than in the healthy group (IL-1 β , $P < 0.05$; IL-6, $P < 0.05$; TNF- α , $P < 0.05$; Fig. S1A-C).

Comparison of mRNA levels of genes associated with matrix degradation in HGFs and AGF with and without LPS

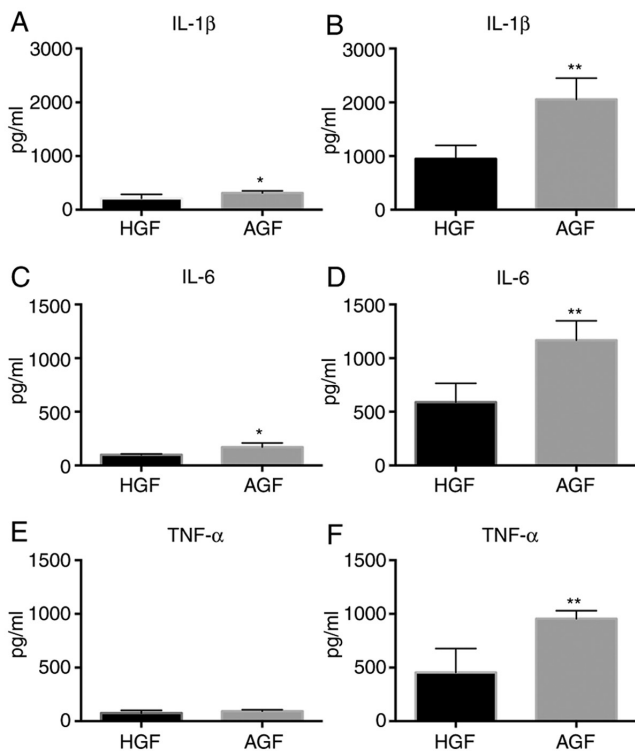


Figure 4. Protein expression of proinflammatory cytokines, including IL-1 β , IL-6 and TNF- α in control subjects and patients with AgP prior to and following 12 h challenge with LPS from *P. gingivalis*. Protein expression of IL-1 β in GFs of control subjects and patients with AgP (A) prior to LPS challenge and (B) following LPS challenge. Protein expression of IL-6 in GFs of control subjects and patients with AgP (C) prior to LPS challenge and (D) following LPS challenge. Protein expression of TNF- α in GFs of control subjects and patients with AgP (E) prior to LPS challenge and (F) following LPS challenge. * $P<0.05$, ** $P<0.01$ vs. HGF. IL, interleukin; TNF- α , tumor necrosis factor- α ; GFs, gingival fibroblasts; AgP, aggressive periodontitis; LPS, lipopolysaccharide; HGFs, GFs from healthy subjects; AGFs, GFs from patients with aggressive periodontitis.

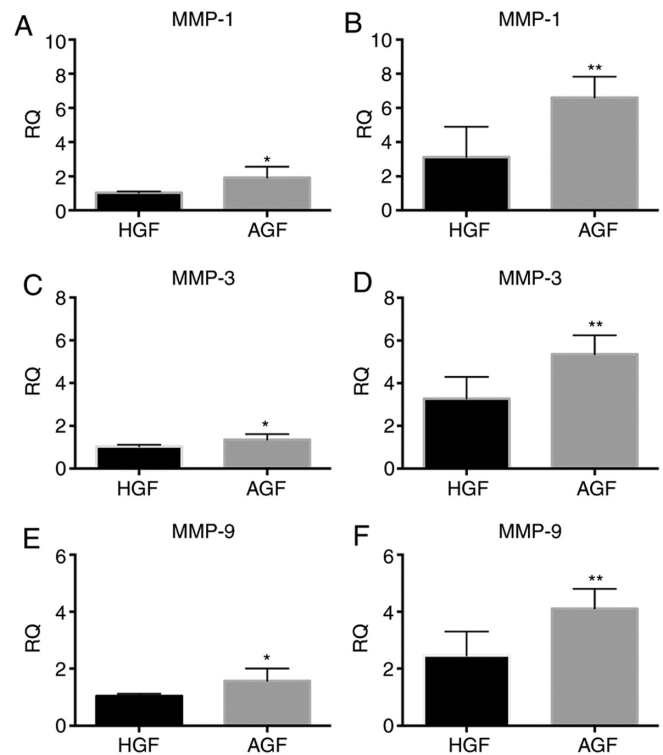


Figure 5. mRNA expression levels of MMP-1, MMP-3 and MMP-9, in control subjects and patients with AgP prior to and following 12 h challenge with LPS from *P. gingivalis*. The mRNA expression of MMP-1 in GFs of control subjects and patients with AgP (A) prior to LPS challenge and (B) following LPS challenge. The mRNA expression of MMP-3 in GFs of control subjects and patients with AgP (C) prior to LPS challenge and (D) following LPS challenge. The mRNA expression of MMP-9 in GFs of control subjects and patients with AgP (E) prior to LPS challenge and (F) following LPS challenge. * $P<0.05$, ** $P<0.01$ vs. HGF. MMP, matrix metalloproteinases; AgP, aggressive periodontitis; LPS, lipopolysaccharide; GFs, gingival fibroblasts; HGFs, GFs from healthy subjects; AGFs, GFs from patients with aggressive periodontitis; RQ, relative quantity.

stimulation. Gene expression of MMP-1, MMP-3 and MMP-9 in unstimulated or stimulated GFs was compared between the healthy and the AgP groups (Fig. 5). In the unstimulated cells, MMP-1, MMP-3 and MMP-9 mRNA expression was higher in the AgP group than in the healthy group (MMP-1, $P<0.05$; MMP-3, $P<0.05$; MMP-9, $P<0.05$; Fig. 5A, C and E). In the stimulated cells, MMP-1, MMP-3 and MMP-9 mRNA expression was significantly higher in the AgP group than in the healthy group (MMP-1, $P<0.01$; MMP-3, $P<0.01$; MMP-9, $P<0.01$; Fig. 5B, D and F).

Comparison of protein levels of genes associated with matrix degradation in GFs of HGFs and AGF with and without LPS stimulation. To investigate whether the difference was also present at the protein levels, the protein expression of MMP-1, MMP-3 and MMP-9 was measured through Western blotting. In the unstimulated cells, the levels of MMP-1 and MMP-9 were higher in the AgP group than in the healthy group ($P<0.05$; Fig. 6A-C), but there was no difference in MMP-3 expression between AgP and healthy groups ($P>0.05$; Fig. 6A and D). In the stimulated cells, the protein expression of MMP-1, MMP-3 and MMP-9 was significantly higher in the AgP group than in the healthy group (MMP-1, $P<0.01$; MMP-3, $P<0.01$; MMP-9, $P<0.01$; Fig. 6A-D).

Discussion

Human GFs are the most abundant resident cells in periodontal tissue. The continuous expression of inflammatory cytokines by GFs may be involved in the overproduction of lytic enzymes, apoptotic factors and bone-resorbing mediators, resulting in periodontal tissue destruction. Excessive production of these mediators is important for the pathogenesis and progression of periodontitis.

In the present study, it was hypothesized that as the host response is important in the pathogenesis of AgP, GFs respond differently to LPS challenge in patients with AgP than in healthy controls. Therefore, GFs of patients with AgP and healthy control subjects were challenged with LPS from *P. gingivalis*.

In the present study, AGFs exhibited higher expression of IL-1 β , IL-6, MMP-1, MMP-3 and MMP-9 without LPS than HGFs. It was not possible to verify the difference in TNF- α mRNA expression in GFs between patients with AgP and control subjects without LPS. After 12-h LPS stimulation, AGFs exhibited higher expression of IL-1 β , IL-6, TNF- α , MMP-1, MMP-3 and MMP-9 than HGFs. A previous study reported that the comparison of mRNA and protein expression of inflammatory genes was higher in inflamed GFs (IGFs;

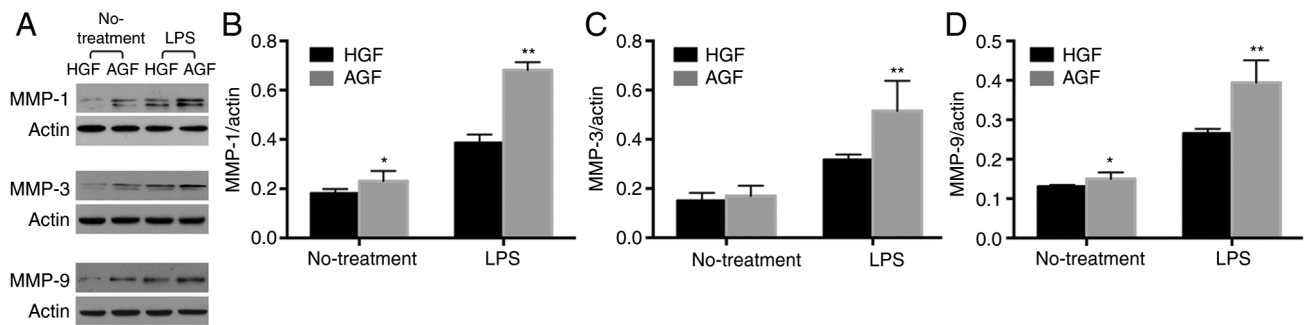


Figure 6. Protein levels of MMP-1, MMP-3 and MMP-9, in control subjects and patients with AgP prior to and following 12 h challenge with LPS from *P. gingivalis*. (A) The results of Western blotting of MMP-1, MMP-3 and MMP-9 in GFs of control subjects and patients with AgP prior to and following LPS challenge. The analysis of the relative content of (B) MMP-1, (C) MMP-3 and (D) MMP-9. * $P < 0.05$, ** $P < 0.01$ vs. treatment matched HGF. MMP, matrix metalloproteinases; AgP, aggressive periodontitis; LPS, lipopolysaccharide; GFs, gingival fibroblasts; HGFs, GFs from healthy subjects; AGFs, GFs from patients with aggressive periodontitis.

GFs isolated from patients with chronic periodontitis) than in HGFs after stimulation with *P. gingivalis* LPS (21). The present study was consistent with this previous report (21,23). To date, few studies have focused on AGFs and the present study was the first to employ *P. gingivalis* LPS to stimulate AGFs and compare the different responses in HGFs. *P. gingivalis* LPS mediates inflammation by inducing the release of proinflammatory cytokines in HGFs (24). The present study identified that the expression of inflammatory cytokines and MMPs was elevated following challenge with *P. gingivalis* LPS in AGFs. Furthermore, the differences in factors associated with inflammation were identified in GFs between the AgP and the healthy groups with/without LPS stimulation. Additionally, the differences in GFs between the AgP and the healthy groups with LPS stimulation were more significant. The results of the present study demonstrated that GFs of patients with AgP exhibit hyperreactivity in the presence of LPS. The present study has another limitation in that expression was analyzed at only one time point. It may take more time for differences in cytokine and MMP responses to become detectable. In addition to the inflammatory cytokines and MMPs, mtROS was higher in GFs in patients with AgP than in control subjects in unchallenged and challenged cells. LPS-stimulated AGFs produced inflammatory cytokines more significantly than HGFs.

MtROS are produced in the process of normal aerobic cell metabolism, serve important physiological roles in maintaining cell redox status, and are necessary for normal cellular function. They are generated as by-products of energy production, depending on the normal structure and function of mitochondrion (25). More mtROS are generated in AGFs, suggesting a possible dysfunction or a morphological change in mitochondria (26). Mitochondrial dysfunction may increase allergic airway inflammation (27), and increase inflammatory response to cytokines in normal human chondrocytes (28). In the present study, AGFs exhibited a marked increase in LPS-triggered activation of inflammatory cytokines, accompanied by MMPs release. Whether the response of GFs in AgP patients to LPS is aggravated by the increased mtROS will be a focus of our future studies.

In conclusion, the present study demonstrated that GFs of patients with AgP display hyperreactivity when challenged with LPS. Although *in vivo* analyses are required to verify these

findings, these results explain, in part, the difference in cellular responses between patients with AgP and healthy subjects. The results of the present study may help aid understanding of the pathogenesis of AgP and development of novel strategies to alleviate the inflammation. There is no clear evidence of a causal association between mtROS and aggressive periodontitis. However, increasing data have suggested that it may be an important factor in the pathogenesis of the disease.

Acknowledgements

Not applicable.

Funding

The present study was funded by the National Natural Science Foundation of China (grant no. 81271148).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QXL designed the study; XL and XW performed the experiments, analyzed the data and prepared the manuscript. QXL reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethical Committee of the School of Stomatology, Peking University (approval no. PKUSSIRB-2013017).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- No authors listed: 1999 International International Workshop for a classification of periodontal diseases and conditions. Papers. Oak Brook, Illinois, October 30-November 2, 1999. *Ann Periodontol* 4: i, 1-112, 1999.
- Buckley CD, Pilling D, Lord JM, Akbar AN, Scheel-Toellner D and Salmon M: Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol* 22: 199-204, 2001.
- Ara T, Kurata K, Hirai K, Uchihashi T, Uematsu T, Imamura Y, Furusawa K, Kurihara S and Wang PL: Human gingival fibroblasts are critical in sustaining inflammation in periodontal disease. *J Periodontol Res* 44: 21-27, 2009.
- Flavell SJ, Hou TZ, Lax S, Filer AD, Salmon M and Buckley CD: Fibroblasts as novel therapeutic targets in chronic inflammation. *Br J Pharmacol* 153 (Suppl 1): S241-S246, 2008.
- Dongari-Bagtzoglou AI and Ebersole JL: Increased presence of interleukin-6 (IL-6) and IL-8 secreting fibroblast subpopulations in adult periodontitis. *J Periodontol* 69: 899-910, 1998.
- Pathirana RD, O'Brien-Simpson NM and Reynolds EC: Host immune responses to *Porphyromonas gingivalis* antigens. *Periodontol* 2000 52: 218-237, 2010.
- Wilson M: Biological activities of lipopolysaccharides from oral bacteria and their relevance to the pathogenesis of chronic periodontitis. *Sci Prog* 78: 19-34, 1995.
- Garlet GP: Destructive and protective roles of cytokines in periodontitis: A re-appraisal from host defense and tissue destruction viewpoints. *J Dent Res* 89: 1349-1363, 2010.
- Liu J, Wang Y and Ouyang X: Beyond toll-like receptors: *Porphyromonas gingivalis* induces IL-6, IL-8, and VCAM-1 expression through NOD-mediated NF-KB and ERK signaling pathways in periodontal fibroblasts. *Inflammation* 37: 522-533, 2014.
- Li X, Wang X, Zheng M and Luan QX: Mitochondrial reactive oxygen species mediate the lipopolysaccharide-induced pro-inflammatory response in human gingival fibroblasts. *Exp Cell Res* 347: 212-221, 2016.
- Goncalves PF, Huang H, McAninley S, Alfant B, Harrison P, Aukhil I, Walker C and Shaddox LM: Periodontal treatment reduces matrix metalloproteinase levels in localized aggressive periodontitis. *J Periodontol* 84: 1801-1808, 2013.
- Seguier S, Gogly B, Bodineau A, Godeau G and Brousse N: Is collagen breakdown during periodontitis linked to inflammatory cells and expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human gingival tissue? *J Periodontol* 72: 1398-1406, 2001.
- Henderson B, Poole S and Wilson M: Bacterial modulins: A novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol Rev* 60: 316-341, 1996.
- Gustafsson A, Ito H, Asman B and Bergstrom K: Hyper-reactive mononuclear cells and neutrophils in chronic periodontitis. *J Clin Periodontol* 33: 126-129, 2006.
- Nicu EA, Van der Velden U, Everts V, Van Winkelhoff AJ, Roos D and Loos BG: Hyper-reactive PMNs in FcgammaRIIa 131 H/H genotype periodontitis patients. *J Clin Periodontol* 34: 938-945, 2007.
- Matthews JB, Wright HJ, Roberts A, Cooper PR and Chapple IL: Hyperactivity and reactivity of peripheral blood neutrophils in chronic periodontitis. *Clin Exp Immunol* 147: 255-264, 2007.
- Zhan Y, Lu R, Meng H, Wang X and Hou J: Platelet activation and platelet-leukocyte interaction in generalized aggressive periodontitis. *J Leukoc Biol* 100: 1155-1166, 2016.
- Nicu EA, Van der Velden U, Nieuwland R, Everts V and Loos BG: Elevated platelet and leukocyte response to oral bacteria in periodontitis. *J Thromb Haemost* 7: 162-170, 2009.
- Ogawa T, Ozaki A, Shimauchi H and Uchida H: Hyporesponsiveness of inflamed human gingival fibroblasts from patients with chronic periodontal diseases against cell surface components of *Porphyromonas gingivalis*. *FEMS Immunol Med Microbiol* 18: 17-30, 1997.
- Scheres N, Laine ML, Sipos PM, Bosch-Tijhof CJ, Crielaard W, de Vries TJ and Everts V: Periodontal ligament and gingival fibroblasts from periodontitis patients are more active in interaction with *Porphyromonas gingivalis*. *J Periodontol Res* 46: 407-416, 2011.
- Kang W, Hu Z and Ge S: Healthy and inflamed gingival fibroblasts differ in their inflammatory response to *Porphyromonas gingivalis* lipopolysaccharide. *Inflammation* 39: 1842-1852, 2016.
- Zdarilova A, Svobodova A, Simanek V and Ulrichova J: *Prunella vulgaris* extract and rosmarinic acid suppress lipopolysaccharide-induced alteration in human gingival fibroblasts. *Toxicol In Vitro* 23: 386-392, 2009.
- Wang PL, Ohura K, Fujii T, Oido-Mori M, Kowashi Y, Kikuchi M, Suetsugu Y and Tanaka J: DNA microarray analysis of human gingival fibroblasts from healthy and inflammatory gingival tissues. *Biochem Biophys Res Commun* 305: 970-973, 2003.
- Imatani T, Kato T and Okuda K: Production of inflammatory cytokines by human gingival fibroblasts stimulated by cell-surface preparations of *Porphyromonas gingivalis*. *Oral Microbiol Immunol* 16: 65-72, 2001.
- Kowaltowski AJ, de Souza-Pinto NC, Castilho RF and Vercesi AE: Mitochondria and reactive oxygen species. *Free Radic Biol Med* 47: 333-343, 2009.
- Yu T, Robotham JL and Yoon Y: Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. *Proc Natl Acad Sci USA* 103: 2653-2658, 2006.
- Aguilera-Aguirre L, Bacsí A, Saavedra-Molina A, Kurosky A, Sur S and Boldogh I: Mitochondrial dysfunction increases allergic airway inflammation. *J Immunol* 183: 5379-5387, 2009.
- Vaamonde-Garcia C, Riveiro-Naveira RR, Valcarcel-Ares MN, Hermida-Carballo L, Blanco FJ and Lopez-Armada MJ: Mitochondrial dysfunction increases inflammatory responsiveness to cytokines in normal human chondrocytes. *Arthritis Rheum* 64: 2927-2936, 2012.