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)-1β, IL-6 and tumor necrosis factor (TNF)-α] and matrix metalloproteinases (MMP)-1, -3 and -9 in AgP GFs. LPS upregulates IL-1β, 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-1β, 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 hyperreactivity 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...
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 (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 μg/ml streptomycin. GFs between passages 3-6 were used. The cells were treated with 1 μg/ml LPS derived from P. gingivalis (InvivoGen, cat. no. tlrl-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 (1.8x10⁵) were resuspended in 1 ml DMEM containing 5 μM 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 μl 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 (1x10⁵ per well) 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−ΔΔCq method following normalization against the expression of ACTB (21).

Measurement of various cytokine levels by ELISA. The GFs were cultured in 96-well plates (1x10⁵ per well) and then medium with or without 1 μg/ml LPS was added. The levels of IL-1β, IL-6 and TNF-α in cell culture supernatants were measured using ELISA kits (R&D Systems China Co., Ltd.; cat. no. IL-1β, 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
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 µl) 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.).

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-1β | IL-1β-forward | CTTCAGCCAATCTTTCATTGCT | 200 |
|       | IL-1β-reverse | TCGGAGATCTGAGCTGGAT  | 89  |
| IL-6 | IL-6-forward | GAGGGCTCTTCCGCAATGTA | 140 |
|       | IL-6-reverse | CCCAGTGGACAGGTCTGAC | 136 |
| TNF-α | TNF-α-forward | GTCACAGATGTGGTTCCGGAAA | 148 |
|       | TNF-α-reverse | GTCACAAATCATCAGTTTTTAGA | 127 |
| MMP-1 | MMP-1-forward | TCTGGGGAACCACTTTTCGACT | 136 |
|       | MMP-1-reverse | CACCAACGTATCAGAAAAGCAAA | 89  |
| MMP-3 | MMP-3-forward | AGTTCTTCAATCTCTACTGTGTTGCT | 148 |
|       | MMP-3-reverse | TCCCCGTATGTTACACCAATCC | 127 |
| MMP-9 | MMP-9-forward | TGACACGGCTATGTTACACTCG | 180 |
|       | MMP-9-reverse | GCCAGGGACAGTTGCTTCT | 127 |
| ACTB | ACTB-forward | AGCACAAATGAGATCAAGATCAT | 136 |
|       | ACTB-reverse | ACTCGTCATACTCTCCTGCT | 89  |

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.
The mRNA expression of TNF-α mRNA was higher in the AgP group than in the healthy group (P<0.01; Fig. 3B and D), and TNF-α expression was compared between the healthy and AgP groups (TNF-α, P<0.05; Fig. 2).

In the unstimulated cells, IL-1β was higher in the AgP group compared with the healthy group (IL-1β, P<0.05; Fig. 2). These levels were then 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 cytokines in HGFs and AgP prior to and following LPS stimulation. The mRNA expression levels (relative to the housekeeping gene) of IL-1β, 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-1β and IL-6 mRNA expression was higher in the AgP group compared with the healthy group (IL-1β, 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-1β 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).

Statistical analysis. Data represent the mean ± 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).

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-1β, IL-6 and TNF-α (Fig. 4). IL-1β and IL-6 levels in GFs unstimulated with LPS were higher in the AgP group than in the healthy group (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-1β, IL-6 and TNF-α levels in GFs were significantly higher in the AgP group than in the healthy group (IL-1β, 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-1β, 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 stimulation. The mRNA expression of genes associated with matrix degradation in HGFs and AGF with and without LPS stimulation was analyzed (Fig. 5). This analysis revealed that the expression of certain genes, such as MMP-1 and MMP-3, was significantly higher in the AgP group than in the healthy group (MMP-1, P<0.05; MMP-3, P<0.01; Fig. 5B and C). However, the expression of COL1A1 and COL3A1 was similar between the healthy and AgP groups (COL1A1, P>0.05; COL3A1, P>0.05; Fig. 5D and E).
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 (MMP-1, P<0.05; MMP-3, P<0.05; MMP-9, P<0.05; Fig. 6A, 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. 6B, D and F).

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-1β, 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-1β, 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;...
LI et al: GFS OF PATIENTS WITH AGGRESSIVE PERIODONTITIS ARE MORE SENSITIVE TO LPS STIMULATION

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 mediate 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. 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.
lipopolysaccharide. Inflammation 39: 1842-1852, 2008. Oral.
J Periodontal Res 46:
at antigens.
938-945, 2007.
Garlet GP: Destructive and protective roles of cytokines in periodontal disease. Sci Prog 78: 19-34, 1995.
periodontitis, Sci Prog 78: 19-34, 1995.
interleukin-6 (IL-6) and IL-8 secreting fibroblast subpopulations from human gingival fibroblasts. Exp Cell Res 347: 212-221, 2016.
14. 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.
15. Nicu EA, Van der Velden U, Everts V and Loos BG: Increased presence of interleukin-6- (IL-6) and IL-8 secreting fibroblast subpopulations in adult periodontitis. J Periodontol 69: 899-910, 1998.
16. Pathirana RD, O'Brien-Simpson NM and Reynolds EC: Host immune responses to Porphyromonas gingivalis antigens. Periodontol 2000 52: 218-237, 2010.
17. Wilson M: Biological activities of lipopolysaccharides from oral bacteria and their relevance to the pathogenesis of chronic periodontitis. Br J Pharmacol 153 (Suppl 1): S241-S246, 2008.
18. Dongari-Bagtzoglou AI and Ebersole JL: Increased presence of pro-inflammatory response in human gingival fibroblasts. Exp Cell Res 319: 338-347, 2008.
19. Li X, Wang X, Zheng M and Luan QX: Mitochondrial reactive oxygen species mediate the lipopolysaccharide-induced collagen breakdown during periodontitis. J Periodontal Res 37: 322-333, 2014.
20. 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.
21. 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.
22. Segueri 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.
23. 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.
24. 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.
25. Nicu EA, Van der Velden U, Everts V, Van Winkelhoff AJ, Roos D and Loos BG: Hyper-reactive PMNs in Fcγ receptor IIA 131 H/H genotype periodontitis patients. J Clin Periodontol 34: 938-945, 2007.
26. 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.