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Original Paper

High Glucose-Mediated Cytokine Regulation in Gingival Fibroblasts and THP-1 Macrophage: a Possible Mechanism of Severe Periodontitis with Diabetes

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Key Words
Diabetes • Fibroblasts • IL-1β • Macrophages • Periodontitis • SIL-6R

Abstract
Background/Aims: Diabetic patients are susceptible to severe periodontitis, but the precise mechanism is not fully understood. Aim of this study was to explore the biological pathogenesis of severe periodontitis in diabetic patients focusing on the crosstalk of human gingival fibroblasts (HGFs) and macrophages. ***Methods:*** A total of 70 periodontitis patients with or without diabetes mellitus (DM) were enrolled, and the statistical relationships of diabetic conditions to the periodontal inflammatory parameters were examined by cross-sectional study. In *in vitro* study, HGFs cell line CRL-2014® (ATCC) and differentiated THP-1 macrophages were cultured with normal glucose (NG: 5.5 mM) or high glucose (HG: 25 mM) condition, and treated with indicated inflammatory factors such as calprotectin (CPT), interleukin (IL)-1β and IL-6. To examine the effects of HG on soluble IL-6 receptor (sIL-6R) production in THP-1 macrophages, the supernatants were collected and the sIL-6R levels were measured by ELISA. To examine the effects of HG on IL-1β or IL-6-induced matrix metalloproteinase (MMPs) production in HGFs, the supernatants were collected. Levels of MMP-1 and tissue inhibitor of MMP-1 (TIMP-1) were measured by ELISA. Finally, after conditioned medium (CM) from THP-1 macrophages cultured with NG or HG conditions was collected, HGFs were treated with the CM. The supernatants were collected 24 hours later and the levels of MMP-1 and TIMP-1 were measured. To examine the specific effects of IL-1β contained in CM on MMP-1 and TIMP-1 production in HGFs, IL-1 receptor antagonist (IL-1ra) was used. ***Results:*** There were statistical correlation between IL-1β and sIL-6R levels in gingival crevicular fluid (GCF) and HbA1c in periodontitis patients with DM (IL-1β: P=0.035, sIL-6R: P=0.040). HG and CPT significantly induced sIL-6R production in THP-1 macrophages. HG significantly enhanced IL-1β or IL-6/sIL-6R-induced MMP-1 production in HGFs. The increase of MMP-1 by both IL-1β and IL-6/sIL-6R

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was significantly inhibited by specific ERK or IκB inhibitors. Corresponding to the regulation of MMP-1 production, HG condition increased the phosphorylation of p44/42 MAPK and IκBα in HGFs treated with IL-1β or IL-6/sIL-6R. Finally, MMP-1 production in HGFs cultured with HG increased significantly by CM from THP-1 macrophages cultured with HG. The induction of MMP-1 by the CM from THP-1 macrophages cultured with HG was significantly inhibited by dose dependent of IL-1ra in HGFs cultured with HG. ***Conclusion:*** Diabetic conditions such as HG induce IL-1β and sIL-6R production from macrophages in inflammatory periodontal tissues and may exacerbate the periodontitis synergistically via MMP-1 production from HGFs.

Introduction

Diabetes mellitus (DM) is a systemic disease with several complications such as retinopathy, nephropathy and neuropathy [1]. Inflammatory periodontal disease, so-called periodontitis is also one of the diabetic complications [2]. Progression of periodontitis has been associated with poor glucose control in diabetic patients [3], although the precise mechanism remains unknown.

Interleukin (IL)-1β and IL-6 are considered to be important pro-inflammatory cytokines that elicit a wide variety of biologic activities [4], and induce the progression of periodontitis [5]. A soluble form of interleukin 6 receptor (sIL-6R), known as an IL-6 agonist, is released by proteolytic cleavage from cell surface of immune/inflammatory cells such as lymphocytes and macrophages [6]. Our recent findings showed that sIL-6R levels in gingival crevicular fluid (GCF) of inflamed sites are significantly higher than in the healthy sites in periodontitis patients [7]. Importantly, it has been reported that both IL-1β and IL-6 levels in GCF were significantly elevated in diabetic patients [8, 9]. Although synergistic effects of these cytokines may induce the severe periodontitis in diabetic patients, the relationship of diabetic conditions such as high glucose (HG) to the cytokine cascades are not fully understood.

Human gingival fibroblasts (HGFs) are the most abundant cells in periodontal connective tissues [10]. HGFs can be target of IL-6 in the presence of sIL-6R, even though HGFs do not express sufficient cell-surface IL-6R to bind appreciable levels of IL-6 [11]. Therefore, sIL-6R is an essential molecule for IL-6 signaling in HGFs. In addition, significant elevation of calprotectin (CPT), a heterodimer of S100A8 and S100A9, are found in inflamed periodontal tissues [7]. Our previous findings showed that CPT induces not only IL-6 production in HGFs [12], but also sIL-6R production in THP-1 macrophages [7], resulting in progression of periodontitis by induction of IL-6 cascades. Furthermore, vascular endotherial growth factor (VEGF) is a significant factor for diabetic complications, and is implicated in the development of neovascularization and endotherial dysfunction in diabetic retinopathy [13]. We showed that HG enhances IL-6/sIL-6R-induced VEGF production in HGFs [14]. A series of reports suggest that exaggerated response of HGF affected by both elevated glucose and inflammatory cytokines may lead to severe periodontitis.

Matrix metalloproteinases (MMPs) are important enzymes responsible for degradation of extracellular matrix (ECM) such as collagen [10]. Periodontal tissue destruction often correlates with an imbalance between MMPs and their inhibitors of MMPs (TIMPs) [15]. HGFs have important roles in gingival healing by regulating production of MMPs/TIMPs in periodontitis lesions [16]. Although there is a positive relationship between uncontrolled DM and severe periodontitis, the effects of HG on the production of MMPs/TIMPs in HGFs are unclear.

In the present study, we examined whether inflammatory cytokines in GCF have been associated with clinical parameters in diabetic patients by performing clinical cross-sectional study. Furthermore, we examined the effects of HG conditions on crosstalk between HGFs and macrophages in order to clarify the pathogenesis of severe periodontitis in diabetic patients in the viewpoint of IL-6 *trans*-signaling.

Materials and Methods

Subjects and oral/diabetic examination

A total of 70 patients with chronic periodontitis (35 males, 35 females, average age, 64.1±7.8 yr), who visited the outpatient clinic of Department of Periodontics, Tokushima University Hospital between 2015 and 2017 were enrolled in this study. Chronic periodontitis was diagnosed based on the criteria set forth by the American Academy of Periodontology [17]. Oral conditions such as number of teeth, bleeding on probing (BOP) and periodontal pocket depth (PPD) were evaluated by trained dentists, and all examiners used the same evaluation standards to reduce inter-examiner error. Patients with or without DM were recorded by a directed interview. The blood samples were collected, and casual blood sugar level (BS) and hemoglobin A1c (HbA1c) were measured immediately using checkart reader (Shionogi CO., LTD., Osaka, Japan). In order to estimate the levels of advanced glycation end-products (AGEs) accumulation in the skin, the skin autofluorescence (AF) was measured using AGE Reader (Neuroscience, Inc., Tokyo, Japan). The criteria for exclusion in this study is as follows: 1. Patients with pregnant women or in lactation. 2. Patients with systemic antibiotic intake or frequent use of anti-inflammatory drugs.

The study protocol was approved by the local ethics committee of Tokushima University Hospital (No. 2325) and the study was conducted in accordance with the principles of the Helsinki Declaration. All participants provided written informed consent.

GCF sampling and measurement of GCF sIL-6R/calprotectin levels

GCF samples were collected from 1 deepest periodontal pocket by inserting sterile 2 paper points (absorbent paper points #45, Dentsply/Maillefer, Ballaigues, Switzerland) in each patient as described in previous report [7]. IL-1β, sIL-6R and CPT levels in GCFs were measured using ELISA Kits (IL-1β, sIL-6R: R&D Systems, Minneapolis, MN; CPT: Hycult Biotech, PB Uden, Netherlands).

Reagents

Recombinant human (rh) CPT, rhIL-6, rhsIL-6R, rhIL-1β and rhIL-1 receptor antagonist (IL-1ra) were obtained from R&D Systems. Phorbol 12-myristate 13-acetate (PMA) and antibody to β-actin were obtained from Sigma (St. Louis, MO). *Porphyromonas gingivalis* (Pg) LPS was purchased from Invitrogen (Carlsbad, CA, USA). TAPI-1, inhibitor of TNF-α-converting enzyme (TACE), was purchased from Calbiochem (Darmstadt, Germany). Antibodies to phospho-p44/42 MAPK and phospho-IκBα were obtained from Cell Signaling (Beverly, MA). Mannitol was purchased from Nakarai Tesque (Kyoto, Japan).

Cell culture

The human monocytic leukemia cell line THP-1 cells (ATCC) were maintained in RPMI 1640 (GIBCO-BRL, Rockville, MD) supplemented with 10 % heat-inactivated FBS and antibiotics (Invitrogen). THP-1 cells were differentiated by 100 nM PMA, and the differentiated THP-1 cells were cultured in normal glucose (NG: 5.5 mM) or HG (25 mM glucose) condition. As an osmotic control for HG, cells were cultured in NG medium supplemented with mannitol (5.5 mM glucose + 19.5 mM mannitol).

Human gingival fibroblasts cell line CRL-2014® (ATCC) were cultured with DMEM and 10 % FBS. HGFs were also cultured with NG or HG condition, and were treated with indicated stimulations. As an osmotic control for HG, mannitol was also used.

Cell proliferation in THP-1 macrophages

To examine the effects of HG on cell proliferative activity, MTT assay was performed [12]. In short, differentiated THP-1 macrophages were cultured with NG or HG condition for 24 h and treated with CPT (50 nM) for 24 h. MTT (final concentration: 0.5 mg/ml) was added to each well and incubated for 4 h prior to the addition of 100 µl DMSO. The reaction mixture was measured fluorometrically using auto plate reader (Bio-Rad, Hercules, CA; excitation at 595 nm).

Measurement of inflammation-related proteins secreted by THP-1 macrophages or HGFs

Differentiated THP-1 macrophages of HGFs were treated with indicated conditions. Pg-LPS was used as a positive control for IL-6 production from THP-1 macrophages. Culture supernatants were collected and stocked at -80 °C. Levels of IL-6, sIL-6R, IL-1β, MMP-1, TIMP-1 and VEGF were measured using ELISA kits (R&D Systems).

Intracellular signaling

Total cellular proteins were extracted with lysis buffer (0.5 % SDS, 10 mM Tris-HCl pH 7.4, and protease inhibitor mix: Complete™ [Sigma]) after indicated stimulations. Phosphorylation of p44/42 MAPK and IκBα was examined using Western-blotting according to the previous report [11]. β-actin was used as internal control of loading proteins.

Conditioned medium (CM) preparation in THP-1 macrophages and effects of CM on MMP-1 production in HGFs

Differentiated THP-1 macrophages were cultured in RPMI 1640 with NG or HG condition for 48 h, and CM was collected. Collected medium samples were centrifuged at 3000 rpm for 10 min to remove cell debris, and filtered through a 0.45-μm filter. All CM was kept at − 80 °C, and CM diluted 20 % with DMEM was used for stimulations. Levels of MMP-1 and TIMP-1 were measured using ELISA kits (R&D Systems). To examine the effects of IL-1β contained in CM on the MMP-1 and TIMP-1 production in HGFs, rhIL-1ra (50, 500 ng/ml) was used.

Statistical analysis

Statistical significances were determined by Mann-Whitney U test and pearson's correlation coefficient test for cross-sectional clinical study, since the clinical data were not normally distributed. Next, statistical significances were determined by Student's *t*-test or ANOVA Tukey-HSD analysis for *in vitro* study. Statistical analyses were performed using JMP® 8 ver. 8.0.2 (SAS Institute Japan, Tokyo). P-values of <0.05 were considered statistically significant.

Results

A cross-sectional analysis of several parameters in patients with or without DM

As shown in Table 1, both levels of BS and HbA1c of patients with DM were significantly higher than in patients without DM (BS, P=0.0008; HbA1c, P<0.0001, Mann-Whitney U test). On the other hand, there was no significant difference between periodontitis patients with and without DM in the skin AF levels (P=0.34). In oral conditions, there was no statistical difference between periodontitis patients with and without DM in the number of teeth (P=0.89). There were also

Table 1. Comparison of several parameters between groups of periodontitis patients with or without diabetes. A total of 70 periodontitis patients was enrolled and divided into 2 groups: Periodontitis (N=37, 63.3±8.4 yr) and Periodontitis with diabetes (N=33, 64.9±7.2 yr) for cross-sectional study. Data were presented average ± SD. Levels of IL-1β, sIL-6R and CPT in gingival crevicular fluid of deepest PPD [Average PPD (mm), group of Periodontitis: 5.5±1.4, group of Periodontitis with diabetes: 5.9±1.6] were measured using ELISA kit. *P<0.05 (chi-square test or Mann-Whitney U test). N, number of subjects; DM, diabetes; PPD, periodontal pocket depth; BOP, bleeding on probing; sIL-6R, soluble form of interleukin-6 receptor; CPT, calprotectin; Skin AF, Skin autofluorescence; BS, casual blood sugar; HbA1c, Hemoglobin A1c

Parameters	Periodontitis	Periodontitis with diabetes	P-value
number of subjects (M/F)	37 (16/21)	33 (19/14)	0.23
age (yr)	63.3 ± 8.4	64.9 ± 7.2	0.55
Diabetic conditions			
BS (mg/dL)	125.9 ± 26.2	204.0 ± 104.9	0.0008*
HbA1c (%)	5.7 ± 0.4	7.6 ± 1.1	<0.0001*
Skin AF (AU)	2.2 ± 0.5	2.4 ± 0.9	0.34
Oral conditions			
number of teeth	20.5 ± 6.0	21.1 ± 5.4	0.89
ratio of 4 mm<PPD (%)	36.1 ± 33.8	46.2 ± 35.0	0.25
ratio of mobile teeth (%)	12.2 ± 19.9	29.8 ± 32.3	0.028*
ratio of BOP positive (%)	34.8 ± 29.4	49.1 ± 31.4	0.056
number of with/without 6mm>PPD	15/22	20/13	0.094
GCF IL-1β (pg)	31.9 ± 30.2	46.7 ± 38.1	0.083
GCF sIL-6R levels (pg)	84.0 ± 47.6	102.0 ± 63.7	0.36
GCF CPT levels (ng)	182.7 ± 124.7	185.6 ± 141.1	0.83

no statistical differences in the periodontal conditions: ratio of 4 mm<PPD, ratio of BOP positive and number of over 6 mm PPD (P=0.25, P=0.056 and P=0.094, respectively), although the tendency was observed. Ratio of mobile teeth in diabetic patients was higher significantly than those in non-diabetes (P=0.028). There was no significant difference in both GCF sIL-6R and CPT in the deepest PPD (P=0.36, P=0.83, respectively). Finally, our supplemental data showed that GCF IL-1β and sIL-6R levels in group of periodontitis patients with PPD greater than 6 mm were higher significantly than in periodontitis patients without PPD greater than 6 mm (P=0.0015, P=0.042, respectively), when the population was divided into 2 groups as follow: the groups of patients with or without 6mm over PPD (see Supplemental Table 1 - for all supplemental material see www.karger.com/10.1159/000494481/).

Table 2. Correlation between GCF IL-1β and sIL-6R levels and several clinical parameters. A total of 70 periodontitis patients was enrolled and divided into 2 groups: Periodontitis (N=37, 63.3±8.4 yr) and Periodontitis with diabetes (N=33, 64.9±7.2 yr) for cross-sectional study. Correlation between GCF IL-1β/ sIL-6R levels of deepest PPD and several clinical parameters were analyzed using Pearson's correlation coefficient test. r, correlation coefficient. GCF, gingival crevicular fluid; sIL-6R, soluble form of interleukin-6 receptor; PPD, periodontal pocket depth; CPT, calprotectin; skin AF, Skin autofluorescence; BS, casual blood sugar; HbA1c, Hemoglobin A1c. *, P<0.05; **P<0.01

Diagnosis	Parameters	GCF IL-1β		GCF sIL-6R	
		r	P-value	r	P-value
Periodontitis	age	0.28	0.12	0.26	0.11
	PPD	0.17	0.34	0.58	0.0001**
	GCF CPT levels	0.24	0.19	0.50	0.002**
	BS	0.09	0.65	0.14	0.43
	HbA1c	0.11	0.56	0.11	0.53
	Skin AF	0.01	0.98	0.15	0.40
Periodontitis with DM	age	0.24	0.18	0.07	0.70
	PPD	0.38	0.030*	0.47	0.006**
	GCF CPT levels	0.63	0.0001**	0.41	0.019*
	BS	0.17	0.38	0.20	0.29
	HbA1c	0.40	0.035*	0.38	0.040*
	Skin AF	0.22	0.30	0.23	0.28

Statistical associations between GCF IL-1β and sIL-6R levels and inflammation/diabetes-related factors

As shown in Table 2, there were no statistical correlation between GCF IL-1β levels and inflammatory/diabetic factors in periodontitis patients (PPD: P=0.34, GCF CPT: P=0.19, BS: P=0.65, HbA1c: P=0.56). On the other hand, there were statistical correlation between GCF IL-1β levels and inflammatory/diabetic factors in periodontitis patients with DM (PPD: P=0.030, GCF CPT: P=0.0001, HbA1c: P=0.035). Levels of GCF sIL-6R correlated statistically to the inflammatory factors in periodontitis patients with or without diabetes (Periodontitis: PPD, P=0.0001, GCF CPT: P=0.002; Periodontitis with DM: PPD, P=0.006, CPT, P=0.019). GCF sIL-6R levels correlated statistically to the values of HbA1c in periodontitis patients with DM (P=0.040, r=0.38, pearson's correlation coefficient test). GCF sIL-6R levels did not correlate to the values of other diabetic factors in periodontitis patients with DM (skin AF: P=0.28, BS: P=0.29). In the group of periodontitis patients, no significant relationships were observed between GCF sIL-6R levels and DM-related factors (skin AF: P=0.40, HbA1c: P=0.53, BS: P=0.43).

Induction of sIL-6R production in THP-1 macrophages by CPT

Although both CPT and PMA did not induce IL-6 production in THP-1 macrophages, Pg LPS increased significantly the IL-6 production in the cells (P<0.0001, ANOVA-Tukey HSD) (Fig. 1A). As shown in Fig. 1B, both CPT and PMA increased significantly sIL-6R production in THP-1 macrophages, and TAPI-1 suppressed completely the increase of sIL-6R production by CPT (P<0.0001, CPT vs. CPT+TAPI-1, ANOVA-Tukey HSD). Pg LPS did not increase the sIL-6R production in the cells.

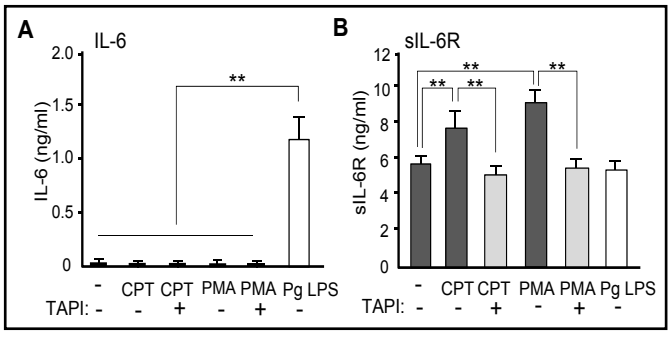

Fig. 1. Effects of calprotectin on IL-6 and sIL-6R production in THP-1 macrophages. Levels of (A) IL-6, (B) sIL-6R production in THP-1 macrophages. After cells were cultured with 5.5 mM glucose (NG) condition, the cells were treated with CPT (50 nM), PMA (100 nM) and Pg LPS (1 μg/ml) for 24 h and collected the supernatants. Both IL-6 and sIL-6R levels were measured using ELISA methods. To inhibit the tumor necrosis factor-alpha converting enzyme (TACE) activity, the cells were pretreated with the inhibitor of TACE (TAPI-1) (10 μM). Data represents as the mean ± SD from 3 independent experiments. **, P<0.01 as compared with control (ANOVA Tukey-HSD). CPT, calprotectin; PMA, phorbol 12-myristate 13-acetate; Pg LPS, Porphyromonas gingivalis lipopolysaccharide.

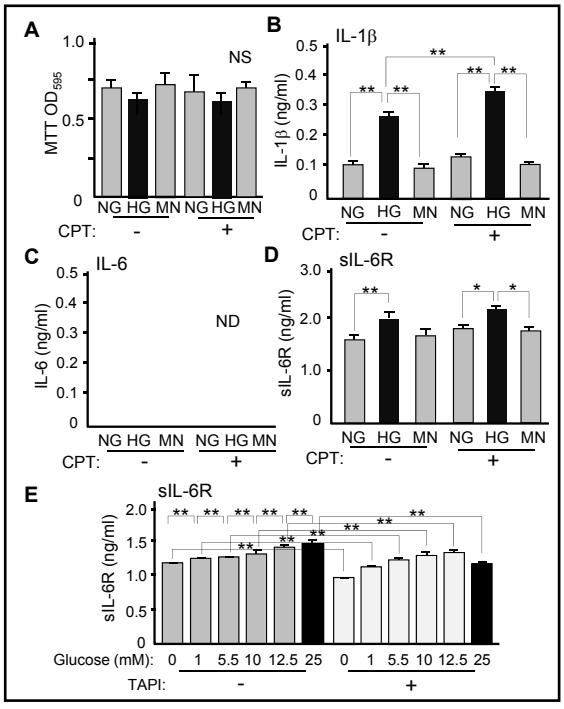

Fig. 2. Effects of high glucose on IL-1β and sIL-6R production in THP-1 macrophages. After THP-1 macrophages were cultured with 5.5 mM glucose (NG) or 25 mM glucose (HG) condition, the cells were treated with 50 nM CPT. As an osmotic control for HG, the cells were cultured with NG conditions supplemented with 19.5 mM mannitol (MN). (A) Cell proliferation. Cell proliferative activity was determined by MTT assay. Levels of CPT-induced (B) IL-1β, (C) IL-6, (D) sIL-6R production in THP-1 macrophages cultured with NG or HG conditions. After the cells were treated with CPT for 24 h and collected the supernatants. Both IL-1β and sIL-6R levels were measured using ELISA methods. (E) To inhibit TACE activity, the cells were pretreated with TAPI-1 (10 μM) for 24 h. Data represents as the mean ± SD from 3 independent experiments. CPT, calprotectin. TACE, tumor necrosis factor-alpha converting enzyme. TAPI-1, inhibitor of TACE. NS, not significant differences. *, P<0.05; **, P<0.01, ANOVA Tukey-HSD.

Effects of HG on cell proliferative activity in THP-1 macrophages

No significant differences were observed in the cell proliferation between NG and HG in THP-1 macrophages (P=0.74, ANOVA Tukey-HSD) (Fig. 2A). CPT did not induce the cell proliferative activity (NG vs. NG+CPT: P=0.99, ANOVA Tukey-HSD). The cell morphology did not change throughout the experiments.

Effects of HG on production of inflammatory cytokines in THP-1 macrophages treated with CPT

HG increased significantly IL-1β and sIL-6R production, but not IL-6 in THP-1 macrophages (IL-1β: P<0.001; sIL-6R: P=0.0067, ANOVA Tukey-HSD) (Fig. 2B-2D). CPT enhanced significantly the levels of HG-induced IL-1β production, but not sIL-6R in the cells (IL-1β: P<0.0001; sIL-6R: P=0.55, ANOVA Tukey-HSD). As shown in Fig. 2E, significant increase of sIL-6R productions were observed in THP-1 macrophages treated with glucose in a dose dependent manner, and the increase of sIL-6R productions were inhibited significantly by pretreatment of TAPI-1 (P<0.0001, Student's *t*-test).

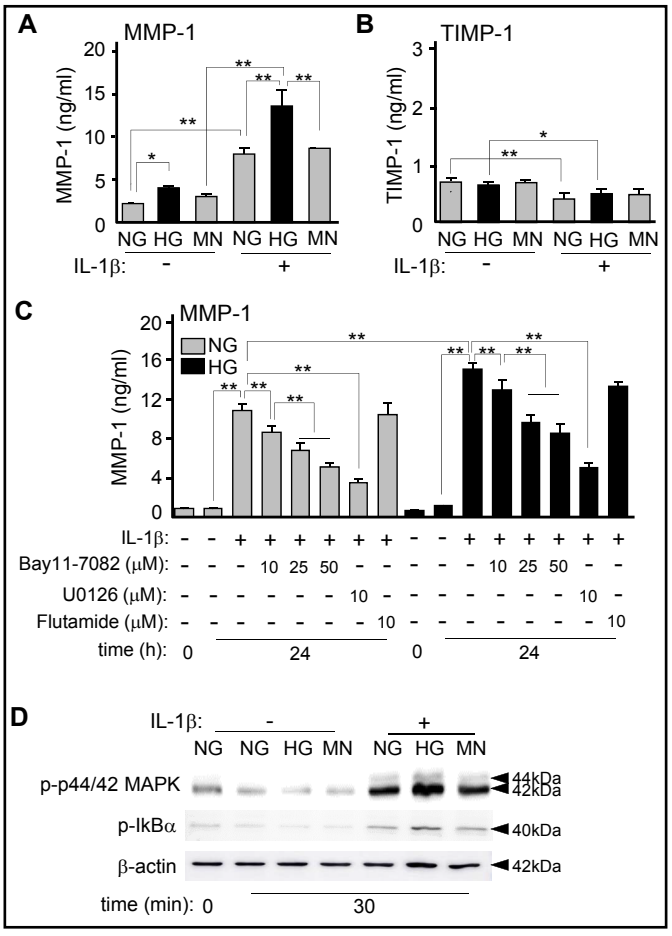

Fig. 3. Effects of high glucose on IL-1β-induced MMP-1 and TIMP-1 production in HGFs. Levels of (A) MMP-1, (B) TIMP-1 production in HGFs. After HGFs were cultured with 5.5 mM glucose (NG) or 25 mM glucose conditions, cells were treated with IL-1β (1 ng/ml) for 24 h and the culture supernatants were collected. As an osmotic control for HG, the cells were cultured with NG conditions supplemented with 19.5 mM mannitol (MN). (C) For inhibitor experiments, HGFs were pretreated with IKK inhibitor Bay11-7082 (10, 25, 50 μM) for 8 h, ERK inhibitor U0126 (10 μM), or androgen receptor inhibitor flutamide (10 μM) for 3 h and treated with IL-1β for 24 h. MMP-1 and TIMP-1 levels were measured using ELISA methods. Data represents as the mean ± SD from 3 independent experiments. (D) Enhancement of IL-1β-induced signaling in HGFs cultured with HG conditions. Cell lysates were resolved by SDS-PAGE and analyzed using Western blotting probed with antibodies against phospho-p44/42 MAPK and phospho-IκBα. Equal loading of total lysates (10 μg) was confirmed by re-probing with antibodies against β-actin. NS, not significant differences. *, P<0.05; **, P<0.01, as compared with control (ANOVA Tukey-HSD).

Enhancement of IL-1β or IL-6/sIL-6R-induced MMP-1 production in HGFs cultured with HG conditions

As shown in Fig. 3 and Fig. 4, similar results were observed in both IL-1β and IL-6/sIL-6R-induced MMP-1 production in HGFs. IL-1β and IL-6/sIL-6R significantly increased MMP-1 production in HGFs, even in NG condition (P<0.0001 vs. untreated cells, ANOVA Tukey-HSD). HG enhanced significantly IL-1β- and IL-6/sIL-6R-induced MMP-1 production in HGFs (P<0.0001 vs. NG). HG also increased MMP-1 production, even in HGFs without IL-1β or IL-6/sIL-6R stimulation. Whereas, IL-1β significantly decreased TIMP-1 production in HGFs cultured with both NG and HG condition (NG: P=0.0001; HG: P=0.027, vs. untreated cells, ANOVA Tukey-HSD) (Fig. 3B). Next, both MAPK inhibitor U0126 and NF-κB inhibitor Bay 11-7082 suppressed significantly IL-1β-mediated MMP-1 production in HGFs (NG, U0126: P<0.0001; 10 μM Bay: P<0.0001; HG, U0126: P<0.0001; 10 μM Bay: P<0.0001, ANOVA Tukey-HSD). Flutamide had no effects on the IL-1β-induced MMP-1 production in HGFs cultured with both NG and HG (NG: P=0.99; HG: P=0.76, ANOVA Tukey-HSD). As shown in Fig. 4C, the both inhibitors inhibited significantly IL-6+sIL-6R-mediated MMP-1 production in HGFs (NG, U0126: P<0.0001; 10 μM Bay: P=0.005; HG, U0126: P<0.0001; 10 μM Bay: P=0.75; 25 μM Bay: P<0.0001, ANOVA Tukey-HSD). Flutamide also had no effects on the IL-6/sIL-6R-induced MMP-1 production in HGFs (NG: P=0.99; HG: P=0.99, ANOVA Tukey-HSD). Finally, IL-1β and IL-6/sIL-6R induced phosphorylation of both p44/42 MAPK and IκBα in HGFs cultured with NG, and the increase of phosphorylation was dramatically enhanced in HGFs cultured with HG (Fig. 3D and 4D). There was no significant difference between NG and MN in induction levels of the each phosphorylation.

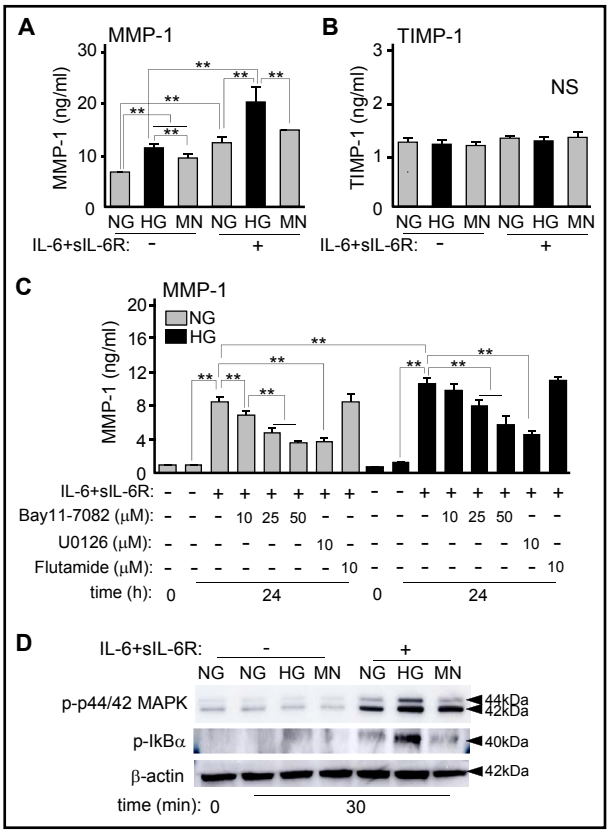

Fig. 4. Effects of high glucose on IL-6+sIL-6R-induced MMP-1 and TIMP-1 production in HGFs. Levels of (A) MMP-1, (B) TIMP-1 production in HGFs. After HGFs were cultured with 5.5 mM glucose (NG) or 25 mM glucose conditions, cells were treated with IL-6+sIL-6R (50 ng/ml each) for 24 h and the culture supernatants were collected. As an osmotic control for HG, the cells were cultured with NG conditions supplemented with 19.5 mM mannitol (MN). (C) For inhibitor experiments, HGFs were pretreated with IKK inhibitor Bay11-7082 (10, 25, 50 μM) for 8 h, ERK inhibitor U0126 (10 μM), or androgen receptor inhibitor flutamide (10 μM) for 3 h and treated with IL-1β for 24 h. MMP-1 and TIMP-1 levels were measured using ELISA methods. Data represents as the mean ± SD from 3 independent experiments. (D) Enhancement of IL-6+sIL-6R-induced signaling in HGFs cultured with HG conditions. Cell lysates were resolved by SDS-PAGE and analyzed using Western blotting probed with antibodies against phospho-p44/42 MAPK and phospho-IκBα. Equal loading of total lysates (10 μg) was confirmed by re-probing with antibodies against β-actin. NS, not significant differences. *, P<0.05; **, P<0.01, as compared with control (ANOVA Tukey-HSD).

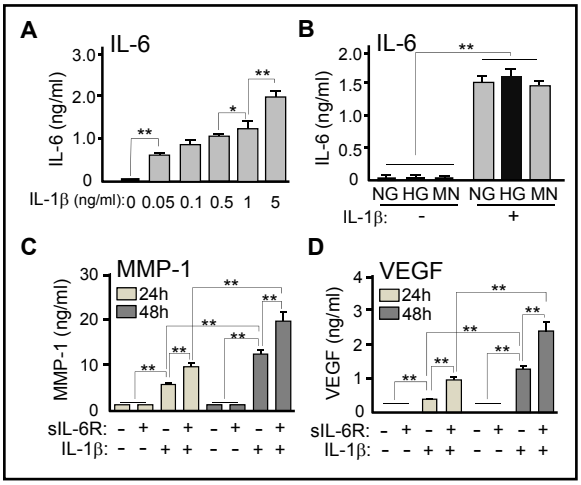

Fig. 5. Induction of MMP-1 and VEGF by co-stimulation of IL-1β and sIL-6R in HGFs. (A) IL-1β-induced IL-6 production in HGFs in a dose dependent manner. After HGFs were cultured with 5.5 mM glucose (NG) condition, cells were treated with IL-1β for 24 h and the culture supernatants were collected. IL-6 levels were measured using ELISA methods. (B) Effects of high glucose (HG) on IL-1β-induced IL-6 production in HGFs. After HGFs were cultured with NG or HG conditions, cells were treated with IL-1β (1 ng/ml) for 24 h and the culture supernatants were collected. As an osmotic control for HG, cells were cultured in NG medium supplemented with 19.5 mM mannitol (MN). Levels of (C) MMP-1, (D) VEGF production in HGFs. After HGFs were cultured with 5.5 mM glucose (NG) condition, cells were treated with combination of IL-1β (1 ng/ml) and sIL-6R (50 ng/ml) in HGFs for 24 h and the culture supernatants were collected. Data represents as the mean ± SD from 3 independent experiments. NS, not significant differences. *, P<0.05; **, P<0.01, as compared with control (ANOVA Tukey-HSD).

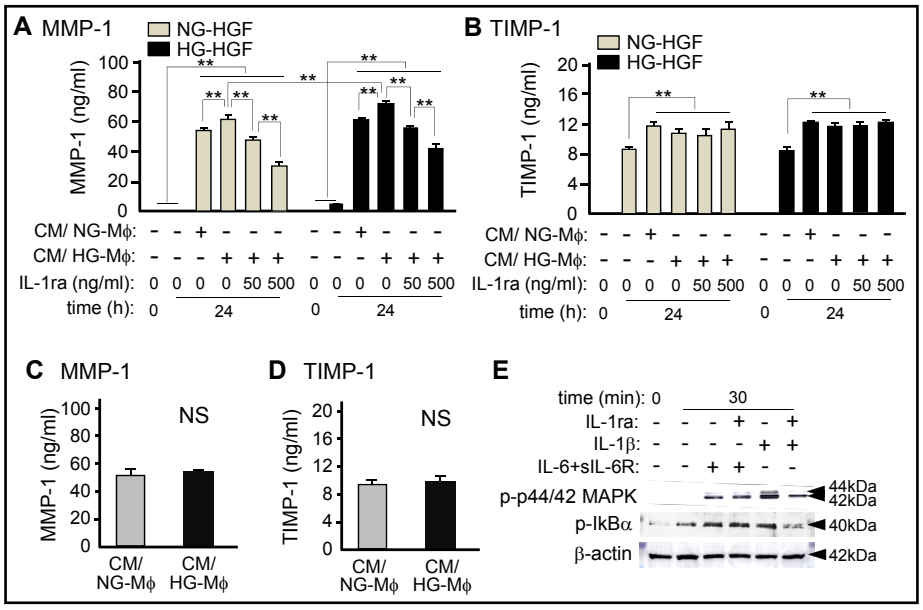

Fig. 6. Effects of MMP-1 and TIMP-1 production in HGFs treated with conditioned medium from THP-1 macrophages. Levels of (A) MMP-1, (B) TIMP-1 production in HGFs. After HGFs were cultured with 5.5 mM glucose (NG) condition or 25 mM glucose (HG), cells were treated with conditioned medium (CM) from THP-1 macrophages cultured with NG or HG conditions and the culture supernatants were collected. MMP-1 and TIMP-1 levels were measured using ELISA methods. To examine the effects of IL-1β containing in CM, HGFs were pre-treated with IL-1ra (50, 500 ng/ml). Levels of (C) MMP-1, (D) TIMP-1 contained in CM from THP-1 macrophages cultured with NG or HG conditions. Data represents as the mean ± SD from 3 independent experiments. (E) Inhibitory effects of IL-1ra (500 ng/ml) on the IL-1β signaling in HGFs. Cell lysates were resolved by SDS-PAGE and analyzed using Western blotting probed with antibodies against phospho-p44/42 MAPK and phospho-IκBα. Equal loading of total lysates (10 μg) was confirmed by re-probing with antibodies against β-actin. NS, not significant differences. *, P<0.05; **, P<0.01, as compared with control (ANOVA Tukey-HSD).

Indirect effects of IL-1β on IL-6 signaling-induced MMP-1 and VEGF production in HGFs

IL-1β increased significantly IL-6 production in HGFs in a dose dependent manner (P=0.019, 0.5 vs 1 ng/ml IL-1β; P<0.0001, 1 vs 5 ng/ml IL-1β, ANOVA Tukey-HSD) (Fig. 5A). There were no significant differences between NG and HG in 1 ng/ml of IL-1β-induced IL-6 production in HGFs (Fig. 5B). IL-1β increased MMP-1 and VEGF production in HGFs, and sIL-6R did not induce the MMP-1 and VEGF production (Fig. 5C and 5D). IL-1β-induced increase of MMP-1 and VEGF in HGFs was enhanced significantly by sIL-6R (MMP-1: 24h, P=0.0002; 48h, P<0.0001; VEGF: 24, 48h, P<0.0001, IL-1β+sIL-6R vs. IL-1β, ANOVA Tukey-HSD).

Effects of CM from THP-1 macrophages on MMP-1 production in HGFs cultured with NG or HG conditions

In HGFs cultured with NG, CM from THP-1 macrophages cultured with NG increased significantly MMP-1 production compared with untreated cells (P<0.0001, ANOVA Tukey-HSD) (Fig. 6A). CM from THP-1 macrophages cultured with HG increased significantly the MMP-1 production compared with those of CM from THP-1 macrophages cultured with NG (P=0.0026, ANOVA Tukey-HSD). Induction of MMP-1 by the CM from THP-1 macrophages cultured with HG was significantly inhibited by dose dependent of IL-1ra (P<0.0001, 0 vs. 50 ng/mL IL-1ra; P<0.0001, 50 vs. 500 ng/mL IL-1ra, ANOVA Tukey-HSD). Next, in HGFs cultured with HG, the similar responses were observed compared with those of HGFs cultured with NG. CM from THP-1 macrophages cultured with HG increased significantly the MMP-1 production in HGFs cultured with HG compared with those of HGFs cultured with NG (P<0.0001, ANOVA Tukey-HSD). The induction of MMP-1 by the CM from THP-1 macrophages cultured with HG was significantly inhibited by dose dependent of IL-1ra in HGFs cultured with HG (P<0.0001, 0 vs. 50 ng/mL IL-1ra; P=0.0001, 50 vs. 500 ng/mL IL-

1ra, ANOVA Tukey-HSD). No significant increase of MMP-1 was observed in the CM from THP-1 macrophages cultured with both NG and HG (P=0.23, Student's *t*-test) (Fig. 6C).

There were no significant differences between CM from macrophages cultured with NG and HG in TIMP-1 production of HGFs (Fig. 6B). IL-1ra had no effects on the TIMP-1 production in HGFs. Whereas, both CM increased significantly the TIMP-1 production in HGFs cultured with both NG and HG (P<0.0001, ANOVA Tukey-HSD). No significant increase of TIMP-1 was observed in the CM from THP-1 macrophages cultured with both NG and HG (P=0.46, Student's *t*-test) (Fig. 6D).

IL-1β and IL-6/sIL-6R induced the phosphorylation of both p44/42 MAPK and IκBα in HGFs cultured with NG, and the increase of phosphorylation of both p44/42 MAPK and IκBα by IL-1β, but not IL-6/sIL-6R were dramatically inhibited in HGFs pre-treated with IL-1ra.

Discussion

Periodontitis is a multifactorial inflammatory disease that causes tooth loss [18], and poor glycemic control has often been associated with severity of periodontitis clinically [3]. In our clinical study, significant progression of periodontitis was also observed in diabetic patients compared with non-diabetic patients (Table 1). Inversely, to examine whether glycemic conditions in patients with severe periodontitis are poor compared with patients with non-severe periodontitis, we performed another cross-sectional analysis (see supplemental Table). Interestingly, blood HbA1c levels in periodontitis patients with 6 mm over PPD (severe periodontitis) were higher statistically than in periodontitis patients without 6 mm over PPD (non-severe periodontitis). These bidirectional results indicate the clinical appropriateness of our database.

At first, we examined whether there were positive relationship between diabetic conditions and GCF IL-1β levels in inflamed periodontal lesions. As shown in Table 1, there was no statistical difference between non-diabetic patients and diabetic patients in GCF IL-1β levels, although the tendency was observed (P=0.083). Whereas, GCF IL-1β levels were statistically correlated to blood HbA1c levels of the diabetic patients (Table 2). Although there was no statistical difference between non-diabetic patients and diabetic patients in GCF sIL-6R levels (P=0.36), importantly, GCF sIL-6R levels were also statistically correlated to blood HbA1c levels of diabetic patients (Table 2). These clinical findings indicate diabetic conditions such as increased glycated albumin may induce IL-1β and sIL-6R production in inflamed periodontal tissues.

IL-1β and IL-6 are important pro-inflammatory cytokines in the progression of chronic inflammatory disease [19]. As shown in Fig. 1A, we demonstrated that CPT did not induce IL-6 production in THP-1 macrophages, although Pg LPS induced the IL-6 production significantly. We have considered that Pg infection has an important role in the early stages of periodontitis with or without DM, because the periodontitis is a bacterial infectious disease. Interestingly, we found for the first time that HG increased IL-1β production significantly in THP-1 macrophages, and the increase of IL-1β was more enhanced by CPT (Fig. 2B). IL-1β also induced dramatically IL-6 production in HGFs (Fig. 5A). Furthermore, we showed previously that IL-6 binding to HGFs increases significantly in the presence of sIL-6R [20]. Considering the possible source of sIL-6R in periodontitis lesions, we reported that sIL-6R production was induced by IL-6 and CPT in THP-1 macrophages [7, 21]. Importantly, we showed that CPT-mediated induction of sIL-6R inhibited significantly in THP-1 macrophages by TACE specific inhibitor, TAPI-1 (Fig. 1B). In addition, we demonstrated that HG also increased sIL-6R production significantly in THP-1 macrophages in a dose dependent manner *via* TACE activation (Fig. 2E). Franchimont et al. reported previously that IL-1/TNF-mediated induction of IL-6R shedding in osteoblastic cells is at least partly dependent on TACE activation [22]. Furthermore, Jones SA et al. reported that shedding of IL-6R was activated by the Ca^{2+} ionophore, ionomycin, and inhibited by the TAPI in THP-1 macrophages [23]. Although the effects of glucose on the Ca^{2+} mobilization are unclear, their findings encourage

our results. Because TACE levels in GCF are higher in periodontitis patients than in healthy subjects [24], sIL-6R induction by TACE may have an important role in the pathogenesis of periodontitis. A series of results suggest that shedding of IL-6R on cell surface may be induced by CPT and HG-mediated TACE activation in THP-1 macrophages.

MMP-1 plays a key role in the destruction of gingival collagen fibers because type I collagen is dominant in gingival connective tissue [25]. In addition, TIMPs are considered to be important molecules to regulate the actions of MMPs [25], and a disturbed balance of MMPs and TIMPs should be involved in the progression of periodontitis [26]. IL-1β and IL-6 may be responsible to the tissue destruction by increasing MMPs from HGFs [21]. In view of severe periodontitis in diabetic patients, therefore, we examined the effects of HG on IL-1β or IL-6-induced MMP-1 and TIMP-1 in HGFs. As shown in Fig. 3 and 4, HG enhanced significantly IL-1β or IL-6/sIL-6R-induced MMP-1 production *via* MAPKs and NF-κB pathway in HGFs. NF-κB pathway is activated by various cellular kinases including MAPKs, which are a group of signaling molecules mainly consisting of three well-characterized subfamilies: ERK1/2, JNK and p38 MAPK [27]. Our previous finding showed that HG enhances dramatically the gp130-mediated ERK pathway in IL-6/sIL-6R-treated HGFs [14]. We demonstrated that phosphorylation of IκBα was also significantly enhanced by IL-1β and IL-6/sIL-6R in HGFs cultured with HG condition. Although the enhancement of NF-κB pathway may be induced followed by ERK activation, further experiments will be needed using siRNA techniques to understand the distinct signaling pathway. IL-1β decreased significantly TIMP-1 production in HGFs, whereas IL-6/sIL-6R had no effects on TIMP-1 production in HGFs. These results suggest that the influence of MMP-1 should be enhanced dramatically in inflamed periodontal tissues of diabetic patients. HG-mediated increase of MMP-1 by IL-1β or IL-6/sIL-6R in HGFs would have an important role in the pathophysiology of severe periodontitis with DM. Recent studies reported that the MMP-1/TIMP-1 ratio in inflamed gingival tissues increased in periodontitis patients with uncontrolled glycemic conditions [28]. These clinical findings support our *in vitro* findings. Additionally, HG also increased significantly MMP-1 production in HGFs. We considered that HG affects to the cell responses by a combination of two reasons: 1. elevated glucose availability, 2. osmotic effects. We notice the significant production of MMP-1 may be, at least in part, related to the osmotic effects of HG, because the same concentration of mannitol also increased significantly the MMP-1 production even in un-treated HGFs as shown in Fig. 4A.

Next, we focused on the potential of IL-1β in periodontitis lesions. Importantly, IL-1β dramatically increased IL-6 production in HGFs (Fig. 5A). Whereas HG had no effects on the IL-1β-induced IL-6 production in HGFs. (Fig. 5B). Therefore, IL-6 cascades induced by IL-1β surrounding HGFs may be equivalent efficacy in pathogenesis of periodontitis whether the patients with DM or not. Interestingly, both MMP-1 and VEGF production were induced significantly and synergistically in HGFs co-stimulated by IL-1β and sIL-6R, even though without IL-6 (Fig. 5B and 5C). These findings suggest that sIL-6R has an important role in the induction of MMP-1 and VEGF in HGFs secondarily in response to IL-6 produced by IL-1β. IL-1β induces both direct signals of IL-1β and indirect signals of IL-6, if sIL-6R was produced by immune/inflammatory cells such as macrophages surrounding HGFs.

Finally, we examined the possibility of HGFs-macrophages crosstalk in inflamed periodontal tissues of diabetic patients. As shown in Fig. 6A, in HGFs cultured with NG, CM from THP-1 macrophages cultured with HG increased significantly MMP-1 production compared with those of NG. Furthermore, the increase of MMP-1 was enhanced significantly in HGFs cultured with HG, and the culture conditions simulate the conditions of periodontitis with DM. Since protein factors contained in CM are various and unclear, exactly, the precise mechanism of MMP-1 induction remains unknown in this culture system. However, IL-1ra, known as IL-1β antagonist, suppressed the MMP-1 production in HGFs. Therefore, we considered that the induction of MMP-1 by CM from THP-1 macrophages cultured with HG, at least in part, depends on IL-1β effects. Since rheumatoid arthritis patients have a high

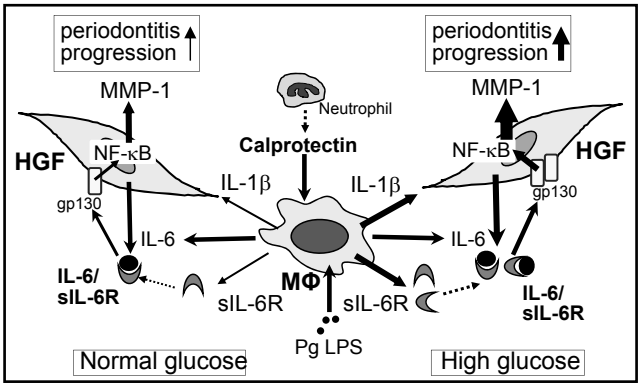

Fig. 7. Schematic representation of HG-mediated HGFs and macrophages crosstalk: A possible mechanism of severe periodontitis with diabetes. In inflamed periodontal tissues of diabetic patients, calprotectin produced by neutrophils induces sIL-6R production in macrophages. HG induces IL-1β and sIL-6R production in macrophages. In the pathogenesis of periodontitis, macrophages may be one of the key cells, because Pg LPS also induces IL-6 production. IL-1β and sIL-6R induce MMP-1 production synergistically in HGFs, because IL-1β induces dramatically IL-6 production in HGFs. Taken together, both direct effects of IL-1β on responsiveness of HGFs and indirect effects of IL-1β via activation of IL-6 signaling of HGFs by inducing sIL-6R from macrophages infiltrated in periodontitis lesion may exacerbate the pathogenesis of periodontitis synergistically in diabetic patients.

level of IL-1, clinically, the blockade of IL-1 receptors by external IL-1ra such as Anakinra modifies the disease progression [29]. IL-1ra may be a hopeful candidate for therapeutic strategy for preventing and treating in severe periodontitis with DM.

AGEs play an important role in the pathogenesis of diabetic complications. Skin AF, a marker of AGE accumulation in tissues, can be measured noninvasively using a skin AF reader [30]. Meerwaldt reported that skin AF increased statistically in diabetic patients [31]. Furthermore, Yu showed that AGEs upregulate the expression of MMP-1 in HGFs [32]. Similar to the reports, we showed that AGEs induces IL-6 production in HGFs [33]. On the other hand, our cross-sectional study showed that no statistical difference was observed between non-diabetic patients and diabetic patients in the skin AF (Table 1). We are not surprising because diabetic/glycemic conditions in the majority of patients have been well-controlled medically in the population of this study. Statistical differences should be observed between non-diabetic patients and diabetic patients in the skin AF by re-analysis using other population including patients with severe/poor diabetic conditions.

In the clinical study, although we have proposed the relationships of several glycemic factors to the GCF IL-1β and sIL-6R levels, we are only able to show associations and not causality because this was a cross-sectional analysis. However, a series of findings by both clinical and *in vitro* study suggest that diabetic conditions such as HG may induce IL-1β and sIL-6R production from macrophages in inflammatory periodontal tissues and may exacerbate the periodontitis synergistically via MMP-1 production from HGFs (Fig. 7). Enhancement of CPT or HG-induced IL-1β and IL-6 cascades in HGFs may play a key role in periodontitis progression through the crosstalk of fibroblasts-macrophages. This pathway could be an attractive target to clarify the pathophysiology of severe periodontitis with DM and contribute to establish the novel therapeutic concept in the disease.

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JHL performed *in vitro* assay, statistical analysis and wrote the article. JHL, YK, YN and TI recruited patients. JK and TN participated in data interpretation. JHL, KN and TN planned the study design. KN supervised the analysis and revised the article.

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The study protocol was approved by the local ethics committee of Tokushima University Hospital (No. 2325).

Disclosure Statement

There is no conflict of interests regarding the publication of this paper.

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