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Resistin in gingival crevicular fluid and induction of resistin release by *Porphyromonas gingivalis* lipopolysaccharide in human neutrophils

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Background and Objective: Resistin is an adipocytokine that induces insulin resistance and is predominantly expressed in adipocytes and peripheral blood mononuclear cells. Resistin expression increases in inflammatory diseases as well as diabetes mellitus, and is upregulated by bacterial pathogens and proinflammatory cytokines. The aim of this study was to identify resistin in human gingival crevicular fluid, to compare the resistin levels in gingival crevicular fluid between subjects with and without periodontitis and diabetes mellitus and to investigate the regulation of resistin release from human neutrophils by *Porphyromonas gingivalis* lipopolysaccharide (P-LPS).

Material and Methods: Gingival crevicular fluid samples were collected from patients with chronic periodontitis ($n = 24$), patients with diabetes mellitus-related periodontitis ($n = 18$) and healthy subjects ($n = 21$). Resistin in gingival crevicular fluid was determined using western blot analysis and an ELISA kit. The glycated hemoglobin (HbA_{1c}) value was obtained from patients with diabetes mellitus-related periodontitis by a medical interview. Human neutrophils were cultured with P-LPS (0–1000 ng/mL), or incubated with inhibitors of actin or microtubule polymerization in the absence or presence of P-LPS. The medium and cellular fractions were used for determination of resistin by ELISA.

Results: The resistin level in gingival crevicular fluid from patients with periodontitis or diabetes mellitus-related periodontitis was significantly higher than that of healthy subjects. The resistin level in gingival crevicular fluid was correlated with gingival index score, but not blood HbA_{1c} value. The P-LPS increased resistin release from human neutrophils, and its induction was decreased by actin polymerization inhibitors.

Conclusion: We show, for the first time, the presence of resistin in gingival crevicular fluid. A high resistin level in gingival crevicular fluid samples from periodontitis patients may to some extent be related to P-LPS-induced resistin release from neutrophils.

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Resistin is a cysteine-rich secretory protein with 12.5 kDa molecular weight and was identified as an adipocytokine because of its expression in mouse adipose tissues and blood (1,2). In humans, resistin is detected in circulating blood, bone marrow, placenta, muscle, pancreas and synovial fluid, and it is highly expressed in monocytes/macrophages, neutrophils and lymphocytes (3–5). The resistin level in human plasma, serum or synovial fluid increases in inflammatory diseases, including atherosclerosis, inflammatory bowel diseases and rheumatic diseases (3,6). Resistin expression in peripheral blood mononuclear cells (PBMCs) is increased by interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α), and resistin was found to reduce the expression of IL-6, IL-8, IL-12, TNF-α and monocyte chemoattractant protein-1 in PBMCs or macrophages (3,5). In mice, the resistin level in blood was found to be increased in dietary and genetically induced obese mice and decreased by an anti-diabetic drug (1,2), suggesting that resistin is mainly associated with obesity and diabetes mellitus in mice. However, in humans, the relationship between resistin and obesity or diabetes mellitus has not been well elucidated. The serum resistin level in obese subjects was higher than that in lean subjects, and a positive correlation between resistin level and body mass index was observed (7). When human serum resistin levels were compared among nonobese, obese and obese diabetic subjects, there was no significant difference (8). The resistin level in human plasma was elevated in patients with diabetes mellitus, but its level in plasma and serum was not related to insulin resistance (9). The discrepancy among previous reports may be affected by ethnic differences. In the general Japanese population, Osawa *et al.* (10,11) reported that plasma resistin level was strongly associated with single nucleotide polymorphism (SNP)-358 and SNP-420, and the G/G genotype of a promoter SNP at -420 was associated with susceptibility to type 2 diabetes mellitus. In humans, resistin mainly plays a role in the modulation of inflammatory responses.

Periodontal diseases are known as an important complication of diabetes mellitus; diabetes mellitus is also an important risk factor for periodontal diseases (12,13). Saito *et al.* and Furugen *et al.* (14,15) reported that the serum resistin concentration of elderly Japanese people with periodontitis was significantly higher than that of healthy subjects and that the serum resistin level was associated with bleeding on probing, a clinical marker of periodontal inflammation. However, the existence and expression of resistin in periodontal tissues and the pathogenic role of resistin in the inflammatory responses of the periodontal diseases with or without diabetes mellitus are not well known. Recently, we found resistin in human gingival crevicular fluid from periodontitis patients using mass spectrometry (Kido J, Hiroshima Y, Bando M, Nagata T) and speculated that resistin expression may be regulated by periodontopathic bacteria in gingival crevicular fluid and periodontal tissues.

Lipopolysaccharide of *Porphyromonas gingivalis* (P-LPS) was reported to induce the expression of proinflammatory cytokines, chemokines and other inflammation-related molecules in epithelial cells, gingival and periodontal ligament fibroblasts, osteoblasts, osteoclasts and neutrophils, monocytes/macrophages and lymphocytes in periodontal tissues with inflammation (16–19), and to increase the release of calprotectin, an inflammation-related protein, from neutrophils and the calprotectin expression in monocytes (20,21). Lipopolysaccharide of *P. gingivalis* strongly aggravates periodontal diseases through multiple factors and pathogens. In contrast, LPS from *Escherichia coli* increased the expression and level of resistin in human PBMCs, macrophages or plasma (4,22–24).

In the present study, we confirmed the existence of resistin in gingival crevicular fluid and compared resistin levels in gingival crevicular fluid between subjects with and without periodontitis and diabetes mellitus-related periodontitis, and further investigated the regulation of resistin release from human peripheral neutrophils by P-LPS.

Material and methods

Collection of gingival crevicular fluid

Gingival crevicular fluid samples were collected from 63 subjects (30 men and 33 women; mean age 56.4 years; range 29–78 years) to whom the aim of the research was explained and who gave their informed consent to participate in the present study. Gingival crevicular fluid sampling was performed with the approval of the Ethics Committee of Tokushima University Hospital (approval no. 1065). The subjects were patients with chronic periodontitis (P; $n = 24$; mean age 65.7 years), patients with diabetes mellitus-related periodontitis (DM-P; $n = 18$; mean age 60.9 years) and healthy subjects without periodontitis or diabetes mellitus (H; $n = 21$; mean age 41.8 years). Periodontitis was clinically diagnosed in patients who had periodontal pockets of more than 4 mm, and diabetes mellitus was diagnosed by physicians in patients who had glycated hemoglobin (HbA_{1c}) at more than 5.8%. The gingival index score was recorded to estimate the severity of periodontal inflammation according to the standard described by Löe and Silness (25). The HbA_{1c} value (%) was obtained from patients with diabetes mellitus-related periodontitis ($n = 18$) who gave consent for a medical interview about their diabetes mellitus condition before the gingival crevicular fluid sampling. No patients had been under drug treatment for at least 6 mo before study. Exclusion criteria were aggressive periodontitis, smoking, alcoholism and/or pregnancy.

Gingival crevicular fluid was collected using paper strips (Periopaper®; Oraflow Inc., New York, NY, USA) according to our previous method (26–28). Briefly, gingival crevicular fluid sampling sites were isolated with cotton rolls, gently dried with an air syringe, and the supragingival biofilm was carefully removed. A paper strip was inserted into the orifice of a gingival crevice of < 3 mm (H group) or a periodontal pocket of more than 4 mm (P and DM-P groups) and held there for 10 s. Gingival crevicular fluid sampling was sequentially repeated

three times using three strips. Care was taken to avoid mechanical stimulation. The gingival crevicular fluid volume in a paper strip was determined using a Periotron 8000 (Harco Electronics, Winnipeg, MB, Canada). Gingival crevicular fluid was extracted from paper strips in 10 mM Tris–HCl (pH 7.4) with protease inhibitors including phenylmethylsulfonyl fluoride (35 μg/mL), leupeptin (0.3 μg/mL), pepstatin (0.25 μg/mL), *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (0.25 μg/mL) and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (0.25 μg/mL), by centrifugation. These gingival crevicular fluid samples were assayed for resistin by Western blotting and ELISA.

Preparation and culture of human neutrophils

Human neutrophils were prepared from the blood of six healthy volunteers. Blood was supplied from subjects who gave informed consent to the present study after the aim of the study was explained to them in agreement with the ethical guidelines of Tokushima University Hospital. Briefly, human neutrophils were separated from whole blood using Polymorphprep™ solution (AXIS-SHIELD PoC AS, Oslo, Norway) according to the manufacturer's instructions and seeded into culture dishes with RPMI-1640 (Sigma-Aldrich Co., St Louis, MO, USA) containing 5% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin and 50 μg/mL streptomycin. Human neutrophils (30×10^4 cells/mL) were seeded and cultured for 15–360 min with or without P-LPS (1–1000 ng/mL), or incubated with actin polymerization inhibitor (1 μM cytochalasin B or 1 μM cytochalasin D) and microtubule polymerization inhibitor (25 μM colchicine or 1 μM nocodazole) in the absence or presence of P-LPS (200 ng/mL) for 30 min at 37°C. The P-LPS was purchased from InvivoGen (San Diego, CA, USA). The property of P-LPS is mainly attributed to a specific lipid A motif, and its signal is mediated by Toll-like receptor 2 and CD14 (29,30). Cytochalasin B and nocodazole were purchased from Sigma-Aldrich Co. and cytochalasin D and colchicine from Wako (Osaka, Japan). After the culture, the cells and medium were separately collected by centrifugation at 400 *g* for 5 min at 4°C. The cultured medium (medium fraction) was mixed with protease inhibitor cocktail including phenylmethylsulfonyl fluoride (41.8 μg/mL), leupeptin (0.3 μg/mL), pepstatin (0.2 μg/mL), *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (0.2 μg/mL) and *N*-α-tosyl-L-lysine chloromethyl ketone hydrochloride (0.2 μg/mL). The cells were suspended in 10 mM Tris–HCl (pH 7.4) with protease inhibitor cocktail and destroyed by sonication (cellular fraction). The medium and cellular fractions were used for determination of resistin by ELISA.

Western blot analysis

Gingival crevicular fluid samples were collected from subjects with or without periodontitis and from patients with diabetes mellitus-related periodontitis. Human serum was prepared by centrifugation of blood collected from healthy subjects. After lyophilization, gingival crevicular fluid and serum samples were dissolved in the sample buffer including 50 mM Tris–HCl (pH 6.8), bromophenol blue, sodium dodecyl sulfate (SDS) and β-mercaptoethanol for polyacrylamide gel electrophoresis. After boiling for 5 min, the gingival crevicular fluid sample and serum (20 μg protein) were applied to 12.5% SDS polyacrylamide gel and electrophoretically separated at a constant current (20 mA); then, the proteins in the gingival crevicular fluid were electronically transferred to Polyvinylidene difluoride membrane (GE Healthcare Ltd, Amersham, Buckinghamshire, UK). The membrane was blocked using Starting Block™ blocking buffers (Pierce, Rockford, IL, USA), incubated with anti-human resistin antibody (2.5 μg/mL; BioVision, Mountain View, CA, USA) at 4°C overnight and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1/10,000 dilution; GE Healthcare Ltd) for 2 h at room temperature. The immunological signal of resistin was detected using an ECL Western Blotting Detection System (GE Healthcare Ltd) and exposed to Hyperfilm ECL (GE Healthcare Ltd).

ELISA

Resistin in gingival crevicular fluid and neutrophils was determined using Human Resistin ELISA kit (measuring range 1–50 ng/mL; BioVender Laboratory Medicine, Inc., Modrice, Czech Republic) according to the instruction manual. Briefly, the extracted gingival crevicular fluid was diluted from five- to twofold dilution with a dilution buffer in the kit and the amount of resistin determined; then, the concentration was expressed as nanograms per liter of gingival crevicular fluid volume. Resistin in the cellular and medium fractions from neutrophils was determined and its concentration expressed as nanograms per 10^4 cells.

Statistical analysis

All data analyses were performed using SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA). The statistical significance of differences in resistin level between gingival crevicular fluid samples from H, P or DM-P groups was evaluated using the Steel-Dwass test. Resistin release from neutrophils that were stimulated by P-LPS or inhibitors is expressed as the fold change from that of the control sample, and the statistical significance of their differences was determined using Student's unpaired *t*-test. The correlation between the resistin concentration in gingival crevicular fluid and gingival index or HbA_{1c} was evaluated using Spearman's rank correlation tests. A value of $p < 0.05$ was accepted as statistically significant.

Results

Identification and determination of resistin in gingival crevicular fluid

Resistin was identified in gingival crevicular fluid samples from gingival

crevices of healthy subjects and each periodontal pocket of patients with periodontitis or diabetes mellitus-related periodontitis. The band of resistin in gingival crevicular fluid was similar to that of serum (Fig. 1A). The resistin levels in gingival crevicular fluid samples from periodontitis or diabetes mellitus-related periodontitis patients appeared to be higher than those of healthy subjects.

The resistin levels in gingival crevicular fluid samples from H, P and DM-P subjects were compared (Fig. 1B and 1C). The mean amounts of resistin were 0.52 ± 0.48, 3.29 ± 3.04 and 2.01 ± 3.10 ng in H, P and DM-P groups, respectively (Fig. 1B). The resistin levels in gingival crevicular fluid samples from inflammatory pockets were significantly higher than those of samples from healthy gingival crevices, approximately five- to sixfold those of healthy subjects. The mean concentrations of resistin were 0.78 ± 0.61, 2.55 ± 2.04 and 1.84 ± 1.97 ng/μL in gingival crevicular fluid from H, P and DM-P groups, respectively, and gingival crevicular fluid volume ranged from 0.12 to 3.70 μL per site (Fig. 1C). The resistin concentrations in gingival crevicular fluid from P samples was significantly higher than those of healthy samples, but there was no significant difference between DM-P and H, or between P and DM-P.

Correlation between resistin concentration in gingival crevicular fluid and gingival index or HbA_{1c} value

The relationship between resistin concentration and severity of periodontal inflammation assessed by gingival index score or diabetes mellitus condition estimated by HbA_{1c} value was investigated (Fig. 2). The mean resistin concentrations (in nanograms per microliter of gingival crevicular fluid) in gingival crevicular fluid samples obtained from sites with each gingival index score were 0.78 ng/μL gingival crevicular fluid at gingival index = 0 (n = 21), 1.42 ng/μL gingival crevicular fluid at gingival index = 1 (n = 28), 2.91 ng/μL gingival crevicular fluid at gingival index = 2 (n = 11) and 4.57 ng/μL gingival crevicular fluid at gingival index = 3 (n = 3). The resistin concentration and gingival index score showed a slight but significant positive correlation (Fig. 2A; r = 0.53, p < 0.01). Eighteen DM-P patients had an HbA_{1c} that ranged from 5.8 to 9.3%. The resistin concentration in gingival crevicular fluid of DM-P subjects ranged from 0.25 to 7.08 ng/μL gingival crevicular fluid (mean 1.84 ± 1.97 ng/μL gingival crevicular fluid). There was no significant correlation between resistin concentration in gingival crevicular fluid and HbA_{1c} value (Fig. 2B; r = −0.13, p = 0.61).

Effect of P-LPS on resistin release from human neutrophils

The effect of P-LPS on resistin release from human neutrophils was investigated. The initial cellular contents of resistin (at time 0) in two healthy

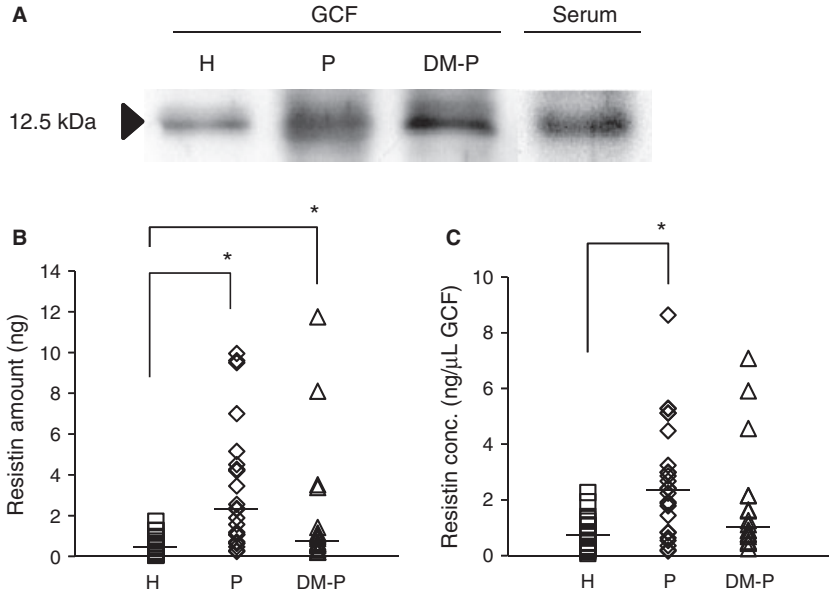

Fig. 1. Identification of resistin in gingival crevicular fluid (GCF) and comparison of resistin levels in gingival crevicular fluid samples from healthy (H) subjects and patients with periodontitis (P) or diabetes mellitus-related periodontitis (DM-P). (A) Western blot analysis of resistin in gingival crevicular fluid. Gingival crevicular fluid samples were collected from gingival crevices of H, P and DM-P subjects. The total volume of each gingival crevicular fluid sample was subjected to SDS-PAGE (12.5% gel) and western blot analysis as described in the Material and methods. Human serum was used as a positive control. The molecular weight of resistin is shown in kilodaltons on the left. (B, C) The gingival crevicular fluid samples were collected from healthy (n = 21), periodontitis (n = 24) and diabetes mellitus-related periodontitis sites (n = 18). The total resistin amount (in nanograms; B) and resistin concentration (in nanograms per microliter gingival crevicular fluid; C) in gingival crevicular fluid samples was determined by ELISA. The horizontal bars show the median values. (Steel-Dwass test; *p < 0.05).

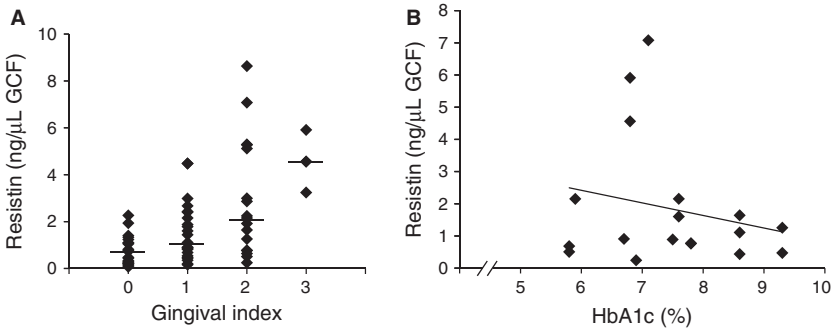

Fig. 2. Correlation between resistin concentration in gingival crevicular fluid and gingival index score (A) or glycated hemoglobin (HbA_{1c}) value in blood (B). The resistin concentration in gingival crevicular fluid samples was determined by ELISA. (A) Filled diamonds show the resistin concentration in individual gingival crevicular fluid samples (gingival index = 0, $n = 21$; gingival index = 1, $n = 28$; gingival index = 2, $n = 11$; and gingival index = 3, $n = 3$) and the horizontal bars indicate the median of concentration for each group. Correlation analysis ($r = 0.53$, $p < 0.01$). (B) The HbA_{1c} value was obtained from patients with diabetes mellitus-related periodontitis ($n = 18$). Each filled diamond shows gingival crevicular fluid resistin concentration vs. blood HbA_{1c} value. Correlation analysis ($r = -0.13$, $p = 0.61$).

persons (subject 1 and subject 2) were 1.73 and 0.85 ng per 10^4 cells, respectively (Fig. 3). When neutrophils of subject 1 and subject 2 were cultured with P-LPS (1 μg/mL) for 30 min, the resistin concentration in the cellular fraction decreased remarkably (Fig. 3A and 3C). In contrast, the concentration in the medium fraction elevated to its maximal level at 30 min and was approximately threefold that of nonstimulated cells, and then decreased after 60 min (Fig. 3B and 3D). The short-term treatment with P-LPS stimulated resistin release from human neutrophils in a dose-dependent manner (1–1000 ng/mL). At 100 and 1000 ng/mL, P-LPS significantly increased resistin release to approximately 2.7- and 4.7-fold that of nonstimulated control cultures, respectively (Fig. 4).

Effect of actin and microtubule polymerization inhibitors on resistin release

The mechanism of resistin release was investigated using actin polymerization inhibitors (cytochalasin B and cytochalasin D) and microtubule polymerization inhibitors (colchicine and nocodazole). When neutrophils were cultured with P-LPS (200 ng/mL) for

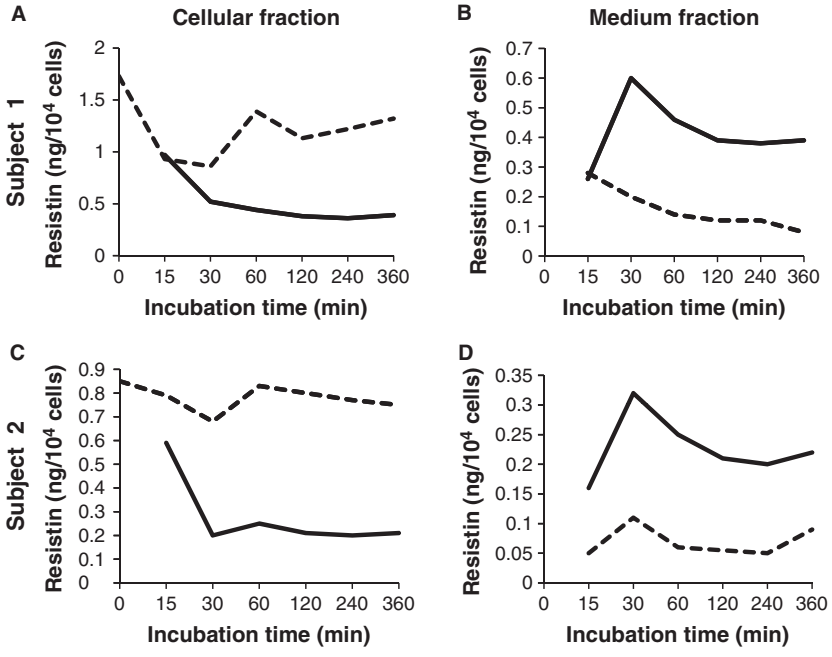

Fig. 3. Change of resistin level in human neutrophils stimulated with *Porphyromonas gingivalis* lipopolysaccharide (P-LPS). Neutrophils (30×10^4 cells/mL) from two healthy subjects (subjects 1 and 2) were incubated with or without 1 μg/mL P-LPS for 15–360 min. Resistin amounts in the cellular (A, C) and medium fractions (B, D) were determined by ELISA. Continuous lines indicate samples from neutrophils treated with P-LPS and dashed lines show samples from untreated cells.

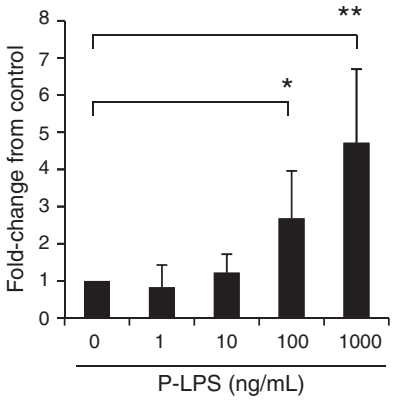

Fig. 4. Effect of P-LPS on resistin release from human neutrophils. Neutrophils (30×10^4 cells/mL) were incubated with 0–1000 ng/mL P-LPS for 30 min. The amount of resistin in the medium was determined by ELISA. Values are expressed as the fold change from the control value (P-LPS; 0 ng/mL) and means ± SD for samples from five subjects in two experiments. The control value was 0.042 ± 0.019 ng/10^4 cells. *$p < 0.05$ vs. control; **$p < 0.01$ vs. control.

30 min, resistin release was increased to about 6.7-fold the nonstimulated (control) level (Fig. 5). Cytochalasin B and cytochalasin D significantly suppressed P-LPS-induced resistin release from neutrophils to 49 and 47% of the P-LPS-stimulated level, respectively (Fig. 5). However, colchicine and nocodazole showed no significant effect on P-LPS-induced resistin release, and cytochalasin B, cytochalasin D, colchicine or nocodazole alone did not significantly affect resistin release.

Discussion

In the present study, we first showed the existence of resistin in gingival crevicular fluid and compared resistin levels among gingival crevicular fluid samples from healthy subjects and patients with periodontitis or diabetes mellitus-related periodontitis because resistin is associated with inflammation and diabetes mellitus. Furthermore, we suggested that a high level of resistin in periodontitis was due to the release from P-LPS-stimulated neutrophils. Resistin is expressed in neutrophils, monocytes/macrophages and lymphocytes and contained in circulating blood and synovial fluid (3). Gingival crevicular fluid contains many components included in serum, inflammation-related products, the degraded components of periodontal tissues and bacterial products (31–33). Resistin in gingival crevicular fluid is considered to be derived from PBMCs, neutrophils in periodontal tissues and blood. In the present study, the mean resistin concentration in gingival crevicular fluid from healthy sites was 0.52 ± 0.48 ng/μL gingival crevicular fluid (range 0.08–2.27 ng/μL gingival crevicular fluid), as shown in Fig. 1C, whereas those of serum and synovial fluid were 4.10–15.65 (8,14,15,34,35) and 3.67–5.70 ng/mL, respectively (36,37). The resistin concentration in gingival crevicular fluid was found to be remarkably higher than those of serum and synovial fluid. As gingival crevicular fluid resistin concentration is higher than that in blood, and such results are similar to those of calprotectin and TNF-α, inflammatory modulators in periodontal diseases (26,27,34,38,39), resistin may be concentrated in gingival crevicular fluid.

A change of resistin level has been reported in some diseases, including obesity, diabetes mellitus, atherosclerosis and inflammatory bowel diseases. The plasma resistin levels of diabetes mellitus patients were higher than those of healthy subjects, and serum resistin level increased in patients with type 2 diabetes and obese subjects compared with that of healthy subjects; however, it was not positively related to insulin resistance (7,9,40,41). However, human serum resistin level was not different among nonobese, obese and obese diabetic subjects (8),

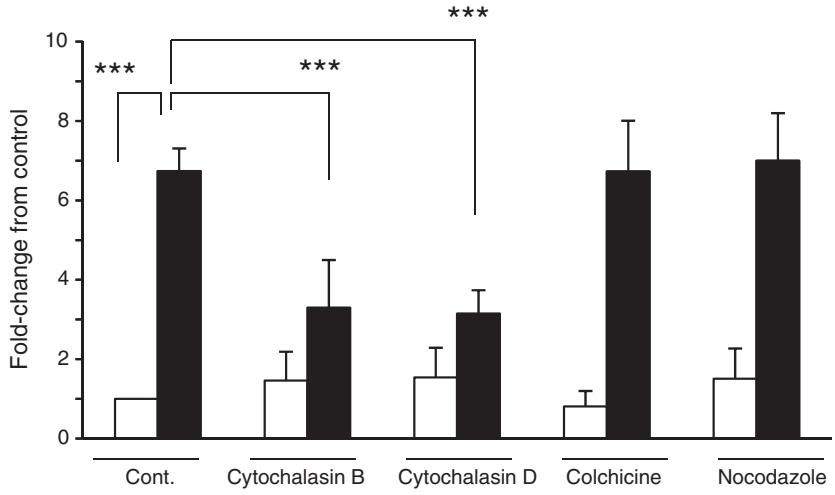

Fig. 5. Effect of cytochalasin B, cytochalasin D, colchicine and nocodazole on P-LPS-induced resistin release from human neutrophils. Neutrophils (30×10^4 cells/mL) were incubated with cytochalasin B (1 μM), cytochalasin D (1 μM), colchicine (25 μM) or nocodazole (1 μM) in the absence (open columns) or presence of P-LPS (200 ng/mL; filled columns) for 30 min. The amount of resistin released was determined by ELISA. Values are expressed as the fold change from the control value (inhibitor and P-LPS free) and means ± SD for samples from three subjects. The control value was 0.033 ± 0.012 ng per 10^4 cells. ***$p < 0.001$ vs. control or P-LPS stimulation.

showing the contrary result in terms of resistin level in diabetes mellitus patients and healthy subjects. The resistin level in human plasma was significantly associated with inflammatory markers, such as C-reactive protein level and leukocyte number (42). These reports suggest that blood resistin may play a role in inflammatory processes, but not in insulin resistance and glucose homeostasis. In the present study, the resistin levels were also increased in gingival crevicular fluid samples from periodontal pockets with inflammation in comparison to those of healthy samples and was significantly correlated with the inflammatory degree (gingival index) of periodontal tissues. However, there was no difference in gingival crevicular fluid resistin levels between periodontitis and diabetes mellitus-related periodontitis and the levels were not correlated with HbA_{1c} values. Regarding the relationship between resistin levels and HbA_{1c} levels in blood, diabetes mellitus patients with high HbA_{1c} showed a significantly higher serum resistin level than nondiabetes mellitus subjects (41,43). On the contrary, no significant association between plasma resistin and HbA_{1c} levels was observed in healthy women (44). The correlation between resistin and HbA_{1c} levels in blood was not clear, and gingival crevicular fluid resistin level is not associated with blood HbA_{1c} level, suggesting that gingival crevicular fluid resistin is positively related to inflammation of the periodontal tissues.

The resistin concentration in healthy human blood differed between individuals as follows: 3.87–7.84 ng/mL in plasma, 4.86 ± 2.90 and 6.4 ± 3.2 ng/mL in serum (15,45,46), and the resistin level in neutrophils from two healthy humans showed about twofold differences (0.85–1.73 ng per 10^4 cells; Fig. 3A and 3C). In the general Japanese population, plasma resistin level was associated with SNP genotypes in the human resistin gene (10,11), and the A allele at SNP-358 was linked with G at SNP-420 and showed a strong association with plasma resistin level (47). The resistin concentration in gingival crevicular fluid differed individually (Fig. 1C), but its level appeared not to be related to the age of the subjects (data not shown). The individual differences in resistin levels in gingival crevicular fluid may be affected by SNP genotypes of the resistin gene. In addition, resistin regulates glucose metabolism, adipogenesis and inflammatory responses by upregulating the expression of proinflammatory cytokines and chemokines, as well as nuclear factor-κB activity (3,5,48). The differences in resistin levels may affect the individual degree of inflammatory responses when bodies are attacked by pathogens. Human resistin is mainly expressed in neutrophils, lymphocytes and monocytes/macrophages; resistin expression is increased by IL-1β, IL-6 and TNF-α in human PBMCs (4,49); and LPS stimulates resistin expression and its release in human macrophages (23). In the present study, LPS from *P. gingivalis*, a periodontopathic bacterium, increased resistin release from human neutrophils. The resistin levels in serum from patients with periodontitis was significantly higher than those of healthy control subjects and associated with a clinical inflammatory marker of periodontal diseases (14,15). These results suggest that resistin is released from P-LPS-stimulated neutrophils in inflammatory periodontal tissues and exudes into gingival crevicular fluid or circulates in blood. We speculate that periodontal diseases may affect the inflammatory condition of the whole body via resistin.

Regarding resistin release from cells, resistin release from human neutrophils was significantly upregulated by short-term treatment with P-LPS (30 min incubation) as shown in Fig. 3. In contrast, resistin release was inhibited by LPS (24 h incubation) in murine adipocytes (50), and slightly increased by LPS (100 ng/mL, 2 h incubation) in human primary neutrophils (51), suggesting that the LPS response of resistin release differs with cell species and LPS treatment time. Resistin is detected in azurophilic and specific granules of human neutrophils (42,52); however, the mechanism of resistin release is not well known. The P-LPS-induced resistin release from human neutrophils was blocked by actin polymerization inhibitors (cytochalasin B and cytochalasin D), but not by microtubule polymerization inhibitors. Cytochalasins disrupt microfilament formation by blocking actin polymerization via binding to the barbed end of the actin filament (53). Cytochalasin B modulated the exocytosis of azurophil granule enzyme in phorbol myristate acetate-stimulated human polymorphonuclear leukocytes (52), and cytochalasin D inhibited the production and secretion of macrophage inflammatory protein-2 in LPS-stimulated epithelial cells (54); in addition, the release of secretory products was inhibited by a disturbance of microfilament function in salivary glands (55). Taken together, we consider that resistin is released from P-LPS-stimulated neutrophils through exocytosis of granules with resistin.

Resistin induces the expression of proinflammatory cytokines, chemokines, cellular adhesion molecules and matrix metalloproteinases in PBMCs, endothelial cells or adipocytes (3,5,56). Resistin inhibits chemotaxis and decreases the *E. coli*-induced oxidative burst of human polymorphonuclear leukocytes (57). Furthermore, resistin induces osteoclast differentiation by stimulation of nuclear factor-κB transcriptional activity (58). Although the roles of resistin in periodontitis are not yet known, resistin appears to modulate inflammation in periodontal diseases.

In summary, we found resistin in gingival crevicular fluid and the resistin level was associated with periodontitis, but not diabetes mellitus. Gingival crevicular fluid resistin may be released from neutrophis that are stimulated by periodontopathic bacteria, because P-LPS increased resistin release. We suggest that resistin modulates inflammation in periodontitis and may be a new marker of periodontitis. Further investigation of the actions of resistin on periodontal tissues, including gingival epithelial cells/fibroblasts, ligament fibroblasts, osteoblasts/osteoclasts, endothelial cells and immune

cells, is necessary to elucidate the role of resistin in periodontal diseases.

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