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Cleavage of IgG1 in gingival crevicular fluid is associated with the presence of *Porphyromonas gingivalis*

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Background and Objectives: Immunoglobulin (Ig) G1 plays an important role in the adaptive immune response. Kgp, a lysine-specific cysteine protease from *Porphyromonas gingivalis,* specifically hydrolyses IgG1 heavy chains. The purpose of this study was to examine whether cleavage of IgG1 occurs in gingival crevicular fluid (GCF) *in vivo*, and whether there is any association with the presence of *Porphyromonas gingivalis* and other periodontopathogens.

Material and Methods: GCF was obtained from nine patients with aggressive periodontitis, nine with chronic periodontitis and five periodontally healthy individuals. The bacterial loads of *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, *Prevotella intermedia* and *Tannerella forsythia* were analysed by real-time polymerase chain reaction, and the presence and cleavage of IgG1 and IgG2 were determined using Western blotting. Kgp levels were measured by ELISA.

Results: Cleaved IgG1 was identified in the GCF from 67% of patients with aggressive periodontitis and in 44% of patients with chronic periodontitis. By contrast, no cleaved IgG1 was detectable in healthy controls. No degradation of IgG2 was detected in any of the samples, regardless of health status. *Porphyromonas gingivalis* was found in high numbers in all samples in which cleavage of IgG1 was detected ($P < 0.001$ compared with samples with no IgG cleavage). Furthermore, high numbers of *Tannerella forsythia* and *Prevotella intermedia* were also present in these samples. The level of Kgp in the GCF correlated with the load of *Porphyromonas gingivalis* ($r = 0.425$, $P < 0.01$). The presence of Kgp (range 0.07–10.98 ng/mL) was associated with proteolytic fragments of IgG1 ($P < 0.001$). However, cleaved IgG1 was also detected in samples with no detectable Kgp.

Conclusion: In patients with periodontitis, cleavage of IgG1 occurs *in vivo* and may suppress antibody-dependent antibacterial activity in subgingival biofilms especially those colonized by *Porphyromonas gingivalis*.

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Periodontal disease is caused by the host inflammatory response to subgingival bacteria (1). Periodontal bacteria stimulate B-cell proliferation, predominantly through a classical antigen-specific immune response. Stimulation may also occur via B-cell superantigens or the innate immune system (2). Immunoglobulins are essential for the adaptive immune response. These specialized glycoproteins have a unique structural organization that allows

functional versatility and the recognition of 'foreign material', e.g. invading microorganisms. Bound immunoglobulin then mediates elimination of the pathogen. The variable domains within the Fab fragments promote the recognition event, whereas multiple sites at the lower hinge region and the Fc fragment initiate various effector functions such as phagocytosis, production of free oxygen radicals and activation of the classical complement pathway (3). High serum levels of IgG against periodontopathogens facilitated phagocytosis of these bacteria in an *ex vivo* study (4).

Microorganisms strongly associated with different forms of periodontitis include *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*, *Tannerella forsythia, Prevotella intermedia* and *Treponema denticola* (5). Of these, *Porphyromonas gingivalis*, which is considered a major pathogen that causes severe chronic periodontitis (6), can also be found in patients with aggressive periodontitis (7). A variety of virulence factors, including lipopolysaccharides, capsular material, fimbriae and proteases are responsible for the pathogenicity of *Porphyromonas gingivalis* (8), with proteases playing the key role. The bacterium secretes a large number of exo- and endopeptidases. The majority of the secreted proteases are cysteine proteases, including gingipains, periodontain, PrT protease and Tpr protease. Whereas arginine-specific gingipains (RgpA and RgpB) are encoded by two genes (*rgpA* and *rgpB*), the product of a single gene (*kgp*) is responsible for lysine-specific activity (9). Collectively, gingipains impair neutrophil function (10), protect the organism against complement (11), degrade the extracellular matrix (12) and bioactive proteins (13), deregulate the coagulation and kinin-generation cascades (14), alter the signalling network controlling inflammatory processes (15) and disturb the protease –protease inhibitor balance in infected periodontal tissues (16). The effect of gingipain is to sustain chronic inflammation and destruction of the periodontium [for a review see (17, 18)].

Several oral bacteria demonstrate IgG Fc-binding activity, and it is assumed that they use this ability to escape the antibody-dependent antibacterial mechanisms of the host. Indeed, Grenier and Michaud (19) report that the periodontal pathogens *Prevotella intermedia*, *Fusobacterium nucleatum*, *Parvimonas micra* and *Lactobacillus* spp. have this ability. The results of *in vitro* experiments clearly showed that *Porphyromonas gingivalis* is able to cleave the heavy chain of rabbit IgG (20). Recent detailed *in vitro* studies characterized Kgp, a protease secreted by *Porphyromonas gingivalis*, as the enzyme that specifically cleaves IgG1 and IgG3 at the hinge region (21).

Therefore, the aim of this cross-sectional study was to investigate whether cleavage of IgG1 occurs *in vivo* and, if so, whether it is associated with the presence of *Porphyromonas gingivalis* and other periodontopathogens and/or Kgp levels.

Material and methods

Subjects

Nine patients with generalized aggressive periodontitis and nine with generalized chronic periodontitis were recruited and enrolled in the study at the Department of Conservative Dentistry, University Hospital of Jena. The definitions of chronic and aggressive periodontitis were based on the classification system of the 'International Workshop for a Classification System of Periodontal diseases and Conditions' 1999 (22). Patients with chronic periodontitis were included when they showed attachment loss $\geq$ 5 mm at > 30% of sites and were aged $\geq$ 35 years. Patients with aggressive periodontitis fulfilled the following criteria: radiographic bone loss $\geq$ 50% at a minimum of two different teeth; $\geq$ 5 mm attachment loss on at least three different teeth (not first molars or incisors); and $\leq$ 35 years at the onset of disease (23). Five periodontally healthy subjects with no evidence of periodontal disease [all probing depths (PD) $\leq$ 3 mm, attachment loss = 0 mm] were recruited as controls.

Subjects with any significant systemic diseases (e.g. diabetes mellitus, cancer or coronary heart disease), those receiving antibiotic therapy within the last 6 mo and pregnant or lactating females were excluded from the study. Only non-smokers with no history of smoking were included into the study.

Ethical approval was obtained from the local ethics committee of the University of Jena and written informed consent was obtained from each subject before participation.

Clinical assessment

PD was measured using a periodontal probe (PCP-UNC 15, Hu Friedy, Leimen, Germany) at six sites per tooth. Bleeding on probing was assessed as the percentage of positive sites per subject.

Sample collection

GCF was collected at six different sites with pocket depths of $\leq$ 3.5 mm, 4–5.5 mm and $\geq$ 6 mm (two sites per depth). Thus, from each patient six samples were analysed and from each periodontally healthy subject two samples were subjected to analysis. Crevicular washes were obtained as previously described (24). A gel loading capillary tip was carefully inserted into the crevice at a level of approximately 1 mm below the gingival margin. In each case, five sequential washes with 10 μL saline (0.9% NaCl) were performed using a micropipette. The crevicular fluid was transferred into a microcentrifuge tube, immediately frozen and kept at −20°C until analysed.

Microflora

DNA was extracted from 5 μL GCF using a DNA extraction system (High Pure PCR Template Preparation Kit; Roche, Mannheim, Germany) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was then performed using a real-time rotary analyser (RotorGene 2000; Corbett Research, Sydney, Australia). The primers for *Porphyromonas*

gingivalis, *Tannerella forsythia* and *Treponema denticola* (25) and those for *A. actinomycetemcomitans* (26) have been previously described. PCR amplification was carried out in a reaction volume of 20 μL comprising 2 μL template DNA and 18 μL of reaction mixture containing 2 μL 10 × PCR buffer, 2.75 mM $MgCl_2$, 0.2 mM nucleotides, 0.5 μM each primer, 10^{-4} SYBR Green and 1 U Taq polymerase (Fermentas Life Science, St. Leon-Rot, Germany). Negative and positive controls were included in each batch. The positive control comprised 2 μL genomic DNA at concentrations ranging from 10^2 to 10^7 bacteria for the reference strains. The negative control comprised 2 μL of sterile water. Each control was added to 18 μL of reaction mixture. The cycling conditions were: initial denaturation at 95°C for 5 min, followed by 45 cycles at 95°C for 15 s, 65°C (except *A. actinomycetemcomitans*; 62°C) for 20 s using a touch-down for five cycles and 72°C for 20 s. The sensitivity and specificity of the method was checked using well-characterized bacterial strains and subgingival plaque specimens (27). Furthermore, the specificity of the amplification was assessed using melting curves. For quantification, the results from unknown plaque specimens were projected onto the counted pure culture standard curves generated for the target bacteria. The number of bacteria was classified using log stages.

Western blotting

Cleavage of IgG1 and IgG2 was examined by Western blotting. To this end, 20 μL aliquots of GCF samples were resolved by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis, thus the analysis was not normalized for the protein concentration in GCF. To avoid protein degradation during preparation (28), the samples were treated with 0.05 mM D-Phe-Phe-Arg-chloromethyl ketone (Bachem AG, Bubendorf, Switzerland), boiled in non-reducing SDS treatment buffer and then re-boiled under reducing conditions before running on 10% SDS–Tricine polyacrylamide gels and blotting onto nitrocellulose membranes. Human IgG (0.2 μg; Sigma-Aldrich, Steinheim, Germany) was used as a reference. Binding of non-specific proteins was blocked by incubation with TBS-T (20 mM Tris, pH 7.5, 0.5 M NaCl, 0.05% Tween 20) containing 5% non-fat dried milk for 1 h at room temperature. The presence of IgG1 and IgG2 was detected by incubating the membranes overnight with a mouse monoclonal antibodies (mAbs) specific for human IgG1 and IgG2 heavy chains (Zymed Labs, San Francisco, CA, USA), diluted 1 : 1000 in 1% bovine serum albumin (BSA)/T-TBS. After extensive rinsing with TBS-T, the immunoblots were incubated for 3 h with anti-goat horseradish peroxidase-conjugated secondary antibodies (Dako Deutschland GmbH, Hamburg, Germany) diluted 1 : 2000 in 1% BSA/TBS-T. The immunoblots were developed using the Amersham enhanced chemiluminescence system (GE Healthcare Life Science, Brussels, Belgium) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay

The level of the lysine-specific cysteine protease, Kgp, in the GCF was determined by an ELISA using mouse mAbs (Clone 19G8.G5.E6.C2) specific for the Kgp catalytic domain developed at the University of Georgia Monoclonal Antibody Facility with a recombinant protein as an antigen. Plates (96-well) were coated with these mAbs diluted to final concentration of 1 μg/mL in 1% BSA in phosphate-buffered saline (PBS) overnight at 4°C. After rinsing with TBS-T, the plates were blocked with 4% BSA in PBS for 2 h at room temperature and washed again. The soluble Kgp purified from *Porphyromonas gingivalis* strain HG66 was used to make a standard curve. To this end 100 μL solution of two-fold serially diluted Kgp (concentration range from 0.01 to 200 pg/μL) in 10% human pooled serum (to mimic GCF composition) was applied on the plate. In parallel GCF samples diluted 1 : 9 with 1% BSA in PBS (100 μL) were added to the wells coated with mAbs. As the negative control wells coated with isotype-matched mAbs were also processed in the same way. After 2 h incubation with gentle shaking at room temperature, the plates were washed with TBS-T and incubated for 2 h with rabbit polyclonal anti-Kgp antibodies (1 μg/mL). These antibodies were developed using native Kgp purified from strain HG66 as an antigen at the University of Georgia Animal Facility. After 2 h incubation at room temperature plates were washed with TBS-T, and treated with 100 μL of anti-rabbit horseradish peroxidase-conjugated secondary antibodies (AP307P, Chemicon Int., Billerica, MA, USA) diluted 1 : 10,000 in 1% BSA/PBS. Unbound antibodies were removed by washing with TBS-T and the peroxidase activity was determined with 3,3′,5,5′-tetramethylbenzidine as a substrate (100 μL) (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The reaction was stopped after 60 min by adding 0.18 M sulphuric acid (100 μL per well). The absorbance was then measured at 450 nm using a microplate reader. The absorbance of standard Kgp samples and analysed GCF samples was corrected for background optical density (OD) by subtraction of the equivalent readout of the negative sample. Obtained values were used to make the standard curve of pg Kgp vs. OD. The Kgp detection level of this ELISA assay was found at 0.08 pg/μL.

Statistical analysis

The clinical data were expressed as the mean ± standard deviation (SD) and analysed with the Student's *t*-test after testing the parameters for normality using the Kolmogorov–Smirnov test. For variables that did not show a normal distribution the Mann–Whitney test was used for comparison of two test groups, and correlations were determined using the Spearman rank correlation. The chi-squared test was used to compare nominal parameters. PASW 18.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses.

Results

The demographic and clinical data are presented in Table 1. Patients with periodontal disease showed significantly higher mean PD and more positive sites of bleeding on probing than periodontally healthy controls ($P < 0.05$). No difference was detected between patients with aggressive and chronic periodontitis.

Cleavage of IgG1 and IgG2

The specific cleavage of the IgG1 heavy chain at the hinge region yields a 30-kDa product. In all samples, in addition to the 30 kDa band, a stronger band of the non-cleaved heavy chain of IgG1 was visible. The periodontally healthy controls showed no evidence of any IgG1 degradation. However, cleavage of IgG1 was detected in the GCF in 10 of 18 (56%) patients with periodontitis: six of nine (67%) with aggressive periodontitis and four of nine (44%) with chronic periodontitis ($P = 0.038$). In the samples collected from the shallow pockets IgG1 cleavage was detected only very rarely. By contrast, in patients that tested positive, all pockets > 6 mm contained IgG1-derived cleavage products. The differences between the pocket depths were statistically significant ($P < 0.01$). The results are summarized in Table 2. Figure 1 shows examples of cleaved IgG1 from a patient with chronic periodontitis. Specific cleavage of IgG2 was not detected either in healthy subjects or in patients with periodontitis.

Microorganisms

Quantitative analysis of *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* and *Treponema denticola* in patients with chronic and aggressive periodontitis revealed no differences in the load of individual bacterial species between these two groups.

Conversely, sites at which specific cleavage of IgG1 (positive sites) was detected showed significantly higher loads of *Porphyromonas gingivalis* ($P < 0.001$), *Prevotella intermedia* ($P < 0.001$) and *Tannerella forsythia* ($P = 0.011$) than sites at which no cleavage (negative sites) was detected (Figure 2). When patients were infected with both *Porphyromonas gingivalis* and *Prevotella intermedia,* cleavage of the IgG1 heavy chain was observed along with further degradation products in addition to the 30-kDa product, suggesting that *Prevotella intermedia* catalyses the further cleavage of IgG1 heavy chains (Figure 3).

Table 2. Cleavage of the heavy chain in gingival crevicular fluid based on subjects

		Detection in pocket depths		
	Total	≤ 3.5 mm	4–5.5 mm	≥ 6 mm
Chronic periodontitis ($n = 9$)	4	1	2	4
Aggressive periodontitis ($n = 9$)	6	1	5	5
Controls ($n = 5$)	0	0	NA	NA

NA, not available.

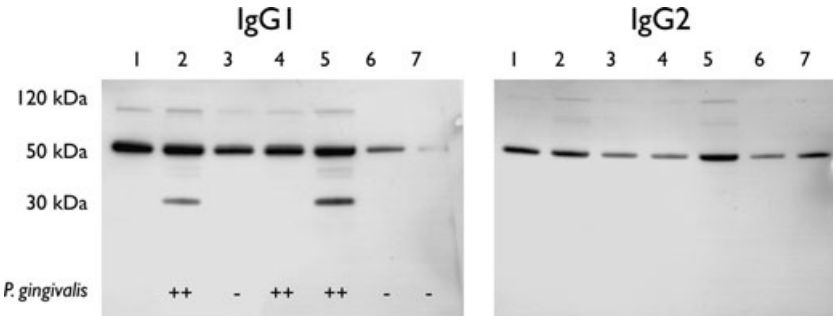

Fig. 1. Western blot of a patient infected with *Porphyromonas gingivalis*. The cleavage resulting in a product of about 30 kDa is visible in lanes 2 and 5. Lanes: 1, control (IgG); 2–7, gingival crevicular fluid; 2 and 5, probing depth (PD) ≥ 6 mm; 3 and 6, PD 4–5.5 mm; 4 and 7, PD ≤ 3.5 mm.

Lysine-specific cysteine protease (Kgp)

Kgp levels (range 0.07–10.98 pg/μL) correlated significantly with the presence of *Porphyromonas gingivalis* ($r = 0.425$, $P < 0.001$). However, Kgp was detectable in only 13 of 77 *Porphyromonas gingivalis*-positive sites. Cleavage of IgG1 was detected at all Kgp-positive sites. Although IgG1 cleavage was also observed in some samples with no detectable Kgp, the difference in IgG1 cleavage between Kgp-positive and Kgp-negative sites was highly significant ($P < 0.001$; Figure 4).

Table 1. Demographic and clinical data

	Control $n = 5$	Chronic periodontitis $n = 9$	Aggressive periodontitis $n = 9$
Age (mean ± SD) (years)	26.2 ± 1.1	59.1 ± 8.1	34.8 ± 6.5
Gender (M/F)	2 : 3	4 : 5	5 : 4
Probing depth (mean ± SD) (mm)	1.28 ± 0.29	5.45 ± 0.84*	5.86 ± 0.69*
Bleeding on probing (mean ± SD) (%)	6.43 ± 7.45	82.66 ± 17.34*	80.48 ± 18.67*

*Significantly different from control group ($P < 0.05$).

Discussion

This study identified cleavage of the IgG1 heavy chain in *Porphyromonas gingivalis*-infected periodontal pockets in patients with either aggressive or chronic periodontitis. The cleavage of IgG1 resulting in a 30 kDa product

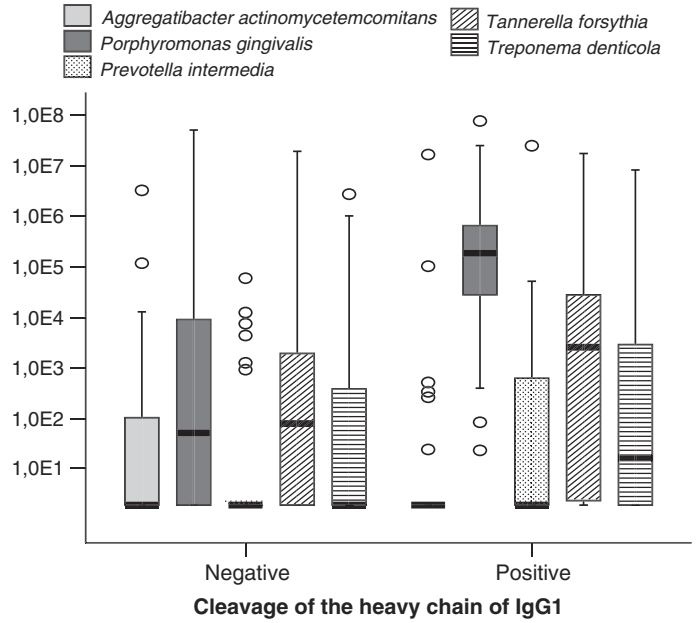

Fig. 2. Periodontopathogenic bacteria in sites with and without a cleavage of the heavy chain of IgG1. The results are presented as box plots with medians, 25th and 75th percentiles as well as whiskers and outliers (dots). Significant differences were detected for *Porphyromonas gingivalis*, *Prevotella intermedia* and *Tannerella forsythia* ($P < 0.05$).

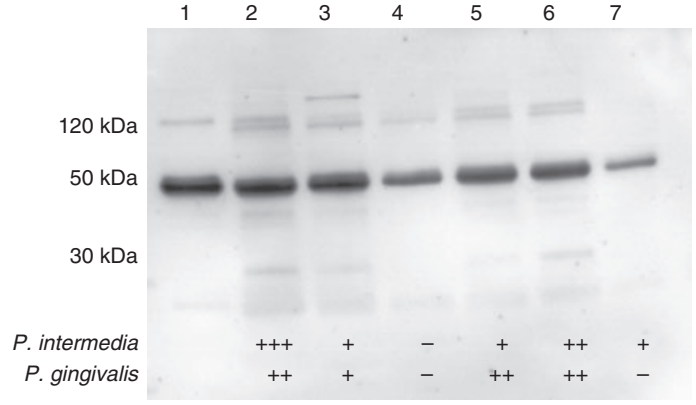

Fig. 3. Western blot of a patient infected by *Porphyromonas gingivalis* and *Prevotella intermedia*. The cleavage resulting in a product of about 30 kDa is visible as well as a further degradation, which might be associated with *Prevotella intermedia*. Lanes: 1, control (IgG); 2–7, gingival crevicular fluid; 2 and 5, probing depth (PD) $\geq$ 6 mm; 3 and 6, PD 4–5.5 mm; 4 and 7, PD $\leq$ 3.5 mm.

correlated with the presence of Kgp, a Lys-gingipain produced by *Porphyromonas gingivalis*. The concentration of Kgp in the GCF was measured using an ELISA. Specific cleavage of IgG1 was detected in all samples that were tested positive for Kgp. As cleavage of IgG1 was also detected in pockets without any measurable Kgp, it may be assumed that Kgp was present at these sites at concentrations below the detection level of the ELISA (about 0.07 pg/μL), but nevertheless still had activity against IgG1. This explanation is supported by *in vitro* finding that Kgp at concentration as low as 0.05 pg/μL can still cleave IgG1 (21). Alternatively, as argued later in the discussion some molecular variants of Kgp may not be recognized by antibodies used in this ELISA assay. Finally, it is possible that *in vivo* other proteases, e.g. from *Prevotella intermedia*, may degrade this immunoglobulin subtype. In contrast to IgG1, no IgG2-derived cleavage products were detected in keeping with this immunoglobulin resistance to proteolysis by Kgp (21) as well as to other proteases (29).

In all GCF samples, regardless of Kgp and *Porphyromonas gingivalis* levels, the substantial part of IgG1 was non-cleaved. However, this can be expected taking into account that GCF is an inflammatory exudate, which is continuously replenished with blood plasma carrying intact IgGs. Similarly, only partially cleaved IgG1 was also demonstrated in protease-rich synovial fluid in patients with rheumatoid arthritis (30). Despite only partial consumption of native IgG1 in GCF this still may have strong impact on the immune response. The majority of Kgp is outer membrane associated and the protease therefore occurs at much higher concentrations proximal to biofilm colonized with *Porphyromonas gingivalis* than in GCF. Therefore, it could be anticipated that IgG1 is very efficiently cleaved at the bacterial surface hindering IgG1-dependent complement activation and opsonophagocytosis. Such strategies to compromise host IgG effector functions by proteolysis at or proximal to the hinge region of the heavy chain of antibodies were described for tumour-associated and microbial proteases (30). Autoantibodies against IgG1 hinge are widespread, the function of these antibodies needs to be established (31). To determine the presence of these antibodies in patients with periodontitis might be an interesting topic in future research.

Porphyromonas gingivalis is clearly associated with periodontal inflammation (32) and is capable of inducing a robust serum antibody response (2). High antibody levels against *Porphyromonas gingivalis* were reported in adults with severe periodontal destruction (33). Several studies report increased levels of systemic IgG in patients with periodontitis (34–36).

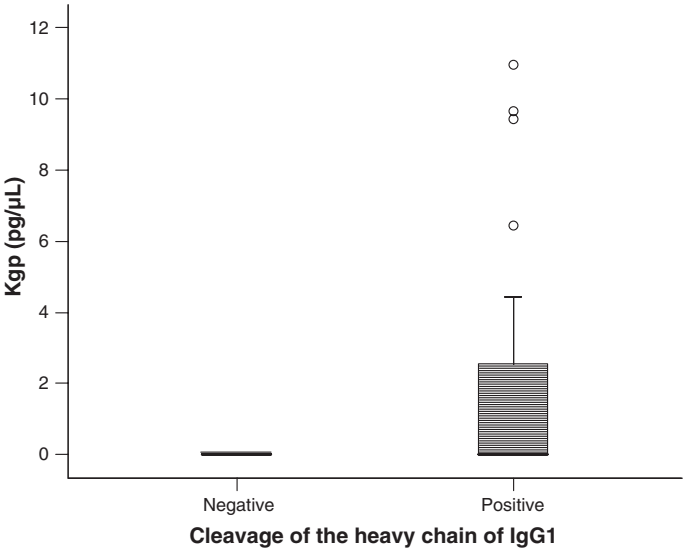

Fig. 4. Detectable level of the lysine-specific cysteine proteinase of *Porphyromonas gingivalis* (Kgp) in sites with and without a cleavage of the heavy chain of IgG1. The results are presented as box plots with medians, 25th and 75th percentiles as well as whiskers and outliers (dots). The difference between negative and positive tested sites was statistically significant ($P < 0.001$).

The IgG antibody response to *Porphyromonas gingivalis* antigens was considered beneficial for the control of *Porphyromonas gingivalis*-mediated periodontitis (37).

Proteolytic degradation of immunoglobulins is a well-known strategy used by pathogenic organisms to avoid opsonization. Accordingly, different pathogenic bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Prevotella intermedia*, *Prevotella nigrescens, Pseudomonas aeruginosa* and *Streptococcus pyogenes* produces enzymes that degrade IgA, IgG or IgM (38). For example, *Streptococcus pyogenes* secretes a cysteine protease, IdeS, which belongs to the C66 protease family and shows extremely high specificity for human IgG, cleaving a single peptide bond located in the lower hinge region (38,39). Immunoglobulins can also be degraded by streptopain/SpeB belonging to the C10 family, which is also produced in large quantities by this pathogen (40). Proteolytic cleavage of IgG by *Porphyromonas gingivalis* has been demonstrated several times, and recently gingipain K was identified as a professional IgG hydrolysing protease. IgG1 and IgG3 seem to be physiological substrates for Kgp (41).

The so-called 'gingipains', are responsible for 85% of the proteolytic activity associated with this bacterium; 46% of them are arginine-specific and 39% are lysine-specific (42). Kgp efficiently hydrolyses several human proteins, which play a key role in the maintenance of homeostasis within the periodontium, the regulation of local inflammatory reactions, and the defence against microorganisms (17, 43). These functions may explain the importance of Kgp as virulence factor produced by *Porphyromonas gingivalis* (44). In this context, it is perplexing that there are no data available regarding the *in vivo* concentration of gingipains. Calculating the Kgp concentration per site (considering the collection volume of 50 µL per washed pocket) results in a median level of 182.65 pg/site (range 3.50 –548.75 pg/site) at Kgp-positive sites. However, Kgp was detectable only in 16.9% of *Porphyromonas gingivalis*-positive sites. Interestingly, in a few cases Kgp was absent from sites infected with high levels of *Porphyromonas gingivalis*. We speculate that the lack of detection is due to unavailability of the epitope recognized by the capturing mAbs or the rabbit polyclonal anti-Kgp antibodies. These antibodies were raised against the catalytic domain alone, or against the catalytic domain-haemagglutinin-adhesion domain complex derived from strain HG66. The variability in the Kgp sequences observed between the clinical isolates and laboratory strains (45) gives credit to this explanation. Proteases are released around all types of inflammatory lesions, but they are normally inactivated within milliseconds by anti-proteases (46); however, *Porphyromonas gingivalis* seems to degrade these protease inhibitors (47) or disturb the protease–protease inhibitor balance within infected gingival tissues (13).

Immunoglobulin G is a major immunoglobulin in human serum and high titres of IgG specific for periodontopathogens (e.g. *Porphyromonas gingivalis*) are detectable in serum from patients with either aggressive or chronic periodontitis (48). Human IgG antibodies are divided into four subclasses, with IgG1 accounting for the greatest proportion of total serum IgG in adults (43–75%). IgG2 accounts for approximately 16–48% of IgG antibodies. The remaining proportion comprises IgG3 and IgG4 (49). Kinane *et al*. found a comparable subclass distribution of IgG-producing cells within the GCF, serum and biopsy tissues (50), with IgG1-producing cells being predominant in tissue or GCF, followed by IgG2-producing cells. Pietrzak *et al*. (51) analysed the IgG subclasses that react with lipopolysaccharide from *Porphyromonas gingivalis* in the serum. IgG1 was present at the highest concentration; furthermore, the results suggested a higher level of IgG1 was present in periodontitis patients infected with *Porphyromonas gingivalis* than in those without. IgG1 and IgG3 are the most effective antibody subclasses when it comes to activating the complement cascade, and IgG1 is likely directed against protein antigens (52). Thus, cleavage of IgG1 may contribute to *Porphyromonas gingivalis* ability to escape the mechanisms of innate and adaptive immunity.

Cleavage of IgG1 is not only associated with *Porphyromonas gingivalis*. A study by Gregory *et al*. (53) also analysed GCF, and contrary to our results, they often detected no intact IgG heavy chains; a result that might have been influenced by their immediate addition of reducing SDS to the samples. To avoid this artificial cleavage our samples were treated with protease inhibitors and preheated to 99°C before boiling in reducing SDS–polyacrylamide gel electrophoresis sample buffer (28). Jansen *et al*. reported that cysteine proteases produced by *Prevotella intermedia* degraded IgG within 24 h (54), supporting our findings. Further, *Tannerella forsythia* might also support the cleavage of IgG1. This bacterium contains many serine and cysteine proteases (55), yet nearly nothing was known about the function of these proteases until now. It is difficult to distinguish between the effects of *Tannerella forsythia* and *Porphyromonas gingivalis* proteases *in vivo*, because both species tend to colonize the periodontal pockets (56). Therefore, follow-up *in vitro* studies are needed to identify other proteases produced by bacteria associated with periodontitis that can cleave IgGs.

Within the limitations of this study, we have shown that cleavage of IgG1 can be detected *in vivo* and that it is associated with the presence of *Porphyromonas gingivalis* and its lysine-specific cysteine protease, Kgp. Together with proteases secreted by other bacteria, *Porphyromonas gingivalis* may suppress the adaptive immune response of patients with periodontitis.

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