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Expression of peptidylarginine deiminase-2 and -4, citrullinated proteins and anti-citrullinated protein antibodies in human gingiva

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Background and Objective: The presence of citrullinated proteins, and peptidylarginine deiminase types -2 (PAD-2) and -4 (PAD-4) in periodontal tissues, determine the presence of anti-cyclic citrullinated protein antibodies (anti-CCP) in gingival crevicular fluid (GCF) and compare the expression of these proteins between inflamed and non-inflamed sites.

Material and Methods: Tissue sections were stained using antibodies against citrullinated proteins, PAD-2 and PAD-4. RT-PCR was performed to investigate PAD-2 and PAD-4 mRNA in inflamed and non-inflamed gingival tissues. Anti-CCP antibodies in gingival crevicular fluid were detected by ELISA.

Results: Citrullinated proteins, PAD-2 and PAD-4 were detected in gingiva. There was a correlation between inflammation and expression of these proteins. mRNAs for PAD-2 and PAD-4 were detected in both inflamed and non-inflamed gingival tissues. Antibodies to CCP were found mostly in the GCF of individuals with periodontitis.

Conclusion: PAD-2 and PAD-4 (protein and mRNA) as well as citrullinated proteins are present in inflamed gingiva, and anti-CCP antibodies can be detected in the GCF of some patients. Tissue expression of citrullinated proteins and PAD increased with the severity of inflammation. The presence of anti-CCP antibodies in GCF was almost exclusive to a subset of patients with periodontitis. Increased expression of these proteins in inflamed gingiva lends support to the notion that periodontal inflammation contributes to the inflammatory burden in a similar way to rheumatoid arthritis.

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Over the past 20 years, considerable research has been undertaken investigating links between periodontitis and a number of systemic conditions of which rheumatoid arthritis (RA) is beginning to receive considerable attention (1,2). RA is a chronic inflammatory autoimmune disease characterized by persistent inflammatory infiltrate in the synovial membrane of joints, leading to joint damage, bone destruction, and disability. Formation of autoantibodies is common in RA and is thought to cause disturbance in immune regulation in patients with RA (3). Of the numerous autoantibodies present in the serum of patients with RA, rheumatoid factor and anti-cyclic citrullinated protein (anti-CCP) antibody are currently the only two routinely used

clinically as diagnostic aids (4). It has been reported that seropositivity to these antibodies often pre-dates the clinical onset of RA and may be useful predictors of disease progression (5). Of these two factors, anti-CCP is favoured as having better diagnostic sensitivity than rheumatoid factor (6).

Citrullination is a post-translational modification of proteins in which a positively charged arginine residue is converted into a neutral citrulline residue by an enzyme family known as peptidylarginine deiminases (PAD). It is a physiological process that occurs during keratinization of epithelial cells, inflammation, and apoptosis. Of the five known human PAD that citrullinate proteins, only PAD-2 and PAD-4 have been shown to be associated with RA (7,8). In addition, *in vitro* studies have shown that one of the periodontal pathogens, *Porphyromonas gingivalis*, produces a variant of PAD (sometimes referred to as *P. gingivalis* PAD or PPAD), which is capable of citrullinating proteins (9).

Of particular interest for both periodontitis and RA are the remarkable similarities of the pathologic processes involved in both diseases. For example, periodontitis and RA are considered complex diseases (10) as there are almost certainly multiple genes involved in disease susceptibility, as well as a number of environmental factors, such as smoking, which contribute to disease severity and progression. Soft and hard tissue destruction in both diseases arises from an exaggerated inflammatory response mediated by host factors. The plaque biofilm is considered the primary aetiological agent in periodontitis and there have been numerous studies suggesting a role for bacteria in RA, with some authors suggesting a role for periodontal pathogens (11,12).

The production of autoantibodies (rheumatoid factor and anti-CCP) is thought to play an important role in the pathogenesis of RA and detection of these antibodies in the serum of patients with RA is well documented. Interestingly, a role has been proposed for these same autoantibodies, with several studies reporting their presence in the serum of patients with periodontitis (11,13–16). A recent review reported the likely presence of citrullinated proteins in the gingival tissues of patients with periodontitis (17) and subsequently confirmed by immunohistochemistry (18). However, to date, no studies have reported local production of anti-CCP antibodies in the periodontium. With mounting evidence of a relationship between periodontitis and RA, autoantibodies may be a common factor in these diseases and the presence of these antibodies in the periodontium would provide evidence of this. Thus, the aim of this study was to determine if citrullinated protein, PAD-2 and PAD-4 are present in gingival tissue and anti-CCP antibodies are present in GCF and whether their expression is related to gingival inflammation. The hypothesis is that local production of citrullinated proteins, anti-CCP autoantibodies, and PAD-2 and PAD-4 in periodontal tissues provides an extra-synovial source of autoantibodies, and that expression of these proteins is increased in periodontitis.

Material and methods

Patient selection

Ethics approval was obtained from the University of Adelaide Human Ethics Research Committee and the South Australian Dental Service in accordance with the guidelines of the National Health and Medical Research Council of Australia. Informed consent was obtained before treatment and patients were given the option to withdraw from the study at any time.

Patients were excluded if there were relative or absolute contraindications to periodontal surgery, such as pregnancy, use of anticoagulants, risk of bacterial endocarditis, uncontrolled diabetes, haematological disorders, any form of neoplasm and/or chemotherapy or radiotherapy. Patients were also excluded if they had taken antibiotics, steroids, or anti-inflammatory drugs in the previous 6 mo. For this study, the exclusion of RA was essential to rule out the possibility that the presence of these proteins was not due to ongoing RA.

The study included gingival samples taken from 50 patients classified as either periodontitis ($n = 29$) or non-periodontitis cases ($n = 21$). From the non-periodontitis group histological assessment was carried out and only those samples with no inflammation were subsequently included ($n = 11$). Thus, a total of 40 patients were divided into two groups. The inflamed tissue specimens came from 29 patients receiving periodontal surgery at sites of persistent deep pocketing (probing depth > 5 mm) having previously undergone at least one course of non-surgical periodontal treatment and re-evaluation after a 3-mo healing period. Patients in this group were diagnosed with chronic or aggressive periodontitis, according to the American Academy of Periodontology 1999 classification system (19). Following histological assessment, two of the inflammation specimens were excluded because of poor histological quality. The non-inflamed specimens came from 11 patients who did not have periodontitis and required crown-lengthening surgery to remove tissue either for aesthetic purposes, or before undergoing prosthetic treatment. Following histological assessment only those specimens with a clear lack of inflammation were included in the non-inflamed group.

This strategy was one of a convenience sampling dictated by access to patients attending the clinic and the sample size had been previously determined to be sufficient for meaningful statistical analyses of the immunohistochemistry results (20–22). Sample bias was restricted by using consecutive patients who fulfilled the inclusion and exclusion criteria.

Gingival tissue collection and processing

Tissue biopsies were placed immediately into 10% normal buffered formalin solution, and stored at room temperature for 24–48 h. Samples were washed three times in phosphate-buffered saline (PBS, pH 7.2) for 30 min per wash, and then

embedded in paraffin. The tissue blocks were cut into 5-μm thick sections, which were then mounted on to 3-aminopropyltriethoxysilane-coated glass slides.

Routine histological staining

The first section from each sample underwent routine staining with haematoxylin and eosin with subsequent sections used for immunohistochemistry. The haematoxylin and eosin sections were viewed under a light microscope for histological assessment. Samples that were too small or did not contain sufficient connective tissue were excluded. Each sample was assigned an inflammatory score from 0 to 3 based on the amount of inflammatory cell infiltration, a score of 0 indicated no infiltration, a score of 1 indicated mild infiltration; a score of 2 indicated moderate infiltration, and a score of 3 indicated heavy infiltration. Two observers, who were blinded to the periodontal status of patients, allocated a score to each tissue sample according to the grading system.

Immunohistochemistry

The expression of citrullinated proteins, PAD-2 and PAD-4 in gingival tissues was investigated by immunohistochemistry using commercially available antibodies. For each of the antibodies, preliminary experiments were conducted to optimize antigen retrieval, primary antibody concentration, and detection.

Immunohistochemical detection of citrullinated proteins— To stain for citrullinated proteins, enzymatic antigen retrieval was carried out by incubating sections in the presence of Proteinase K (200 μg/mL; Abcam, Cambridge, MA, USA) for 30 min at 37°C. Following antigen retrieval, endogenous peroxidase activity was inhibited by adding 0.3% v/v H_2O_2 in 0.1% sodium azide and PBS to each section for 10 min. A blocking serum (normal horse serum; Vector Laboratories, Burlingame, CA, USA) was applied to the sections and left for 20 min to block non-specific antibody binding. The primary antibody (anti-citrulline – rabbit immunoaffinity purified IgG; Upstate, Lake Placid, NY, USA) was then incubated on the sections at a concentration of 2.66 μg/mL in PBS. Control sections, as shown in Fig. 1, were incubated with rabbit polyclonal IgG (cat. no. P0448; DakoCytomation, Carpentaria, CA, USA), at an equal concentration to the primary anti-CCP antibodies. The slides were incubated overnight at room temperature in a wet chamber. The following day, the sections were incubated with biotinylated secondary antibody (provided in the kit) in PBS in the presence of normal horse serum for 45 min. Sections were then reacted with peroxidase-labelled avidin–biotin complex (ABC reagent; Vector Laboratories) for a further 45 min.

For all proteins of interest, the protocol for colour development and

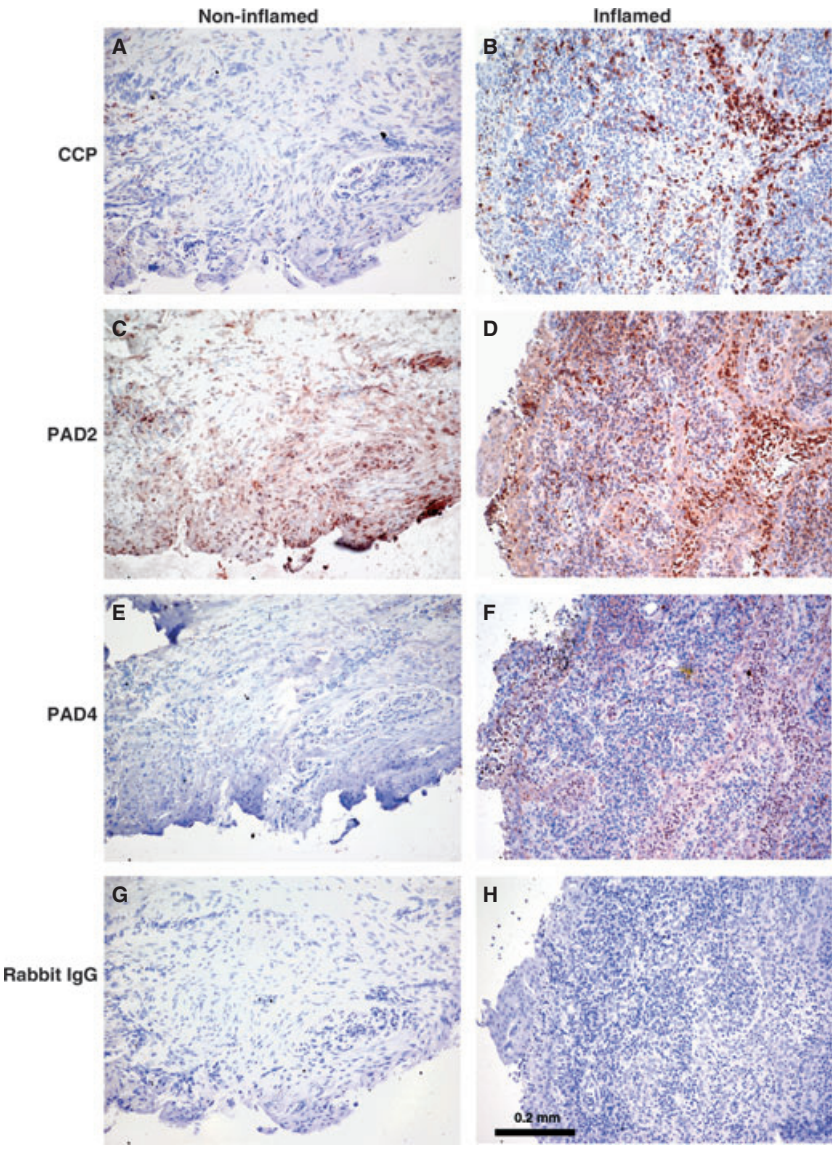

Fig. 1. (A,B) Distribution of citrullinated proteins (CCP), (C,D), peptidylarginine deiminase (PAD 2; (E,F), PAD-4 indicated by red staining in inflamed (right column) and non-inflamed (left column) human gingiva; (G,H), negative control (rabbit polyclonal IgG isotype). All sections subjected to haematoxylin counterstaining. All sections stained on same day and represent serial sections. Magnification: all micrographs shown at 100× magnification. Bar represents 0.2 mm in all panels.

counterstaining was identical. The colour reaction was developed using hydrogen peroxide as the substrate and 3-amino-9-ethylcarbazole (AEC Peroxidase Substrate Kit; Vector Laboratories) as the dye, resulting in red colouring of positively staining sections. The sections were washed three times in PBS for 5 min each, between each step, except after the addition of the blocking serum, which was shaken off before addition of the primary antibody. Following colour development and washing in deionized water, all sections were counterstained in haematoxylin for 10 s. The sections were then washed in water before being immersed in saturated lithium carbonate solution for 30 s. After washing in water, the slides were mounted with Aquatex (Merck, Whitehouse Station, NJ, USA) and 22 × 50 mm glass coverslips (HD Scientific Supplies, Wetherill Park, NSW, Australia).

Immunohistochemical detection of peptidylarginine deiminase 2— PAD-2 detection required immersion of the sections in Tris–EDTA buffer (10 mM Tris Base, 1 mM EDTA pH 9.0) heated to 95°C in a water bath for 20 min. After removal from the water bath the slides were cooled at room temperature for another 20 min. Endogenous peroxidase activity was inhibited as per the anti-citrulline protocol. Blocking serum (normal horse serum; Vector Laboratories) was added to the sections and incubated for 20 min. The primary antibody (polyclonal anti-human PAD-2 antibody; Abcam) was then added to each section at a concentration of 10 μg/mL in PBS. Control sections, as shown in Fig. 1, were incubated with rabbit polyclonal IgG (cat no P0448; DakoCytomation), at an equal concentration to the primary PAD-2 antibody (10 μg/mL). The slides were incubated overnight at room temperature in a wet chamber. The following day sections were incubated with prediluted biotinylated pan-specific universal secondary antibody (R.T.U. Vectastain Universal Quick Kit; Vector Laboratories) for 45 min. Sections were then reacted with preformed streptavidin/peroxidase complex (Vector Laboratories) for a further 45 min. Colour development, counterstaining and mounting of slides was carried out as per the anti-citrulline protocol.

Immunohistochemical detection of peptidylarginine deiminase 4— PAD-4 detection required heat-mediated antigen retrieval by immersion of the slides in sodium citrate (10 mM, pH 6.0) heated to 95°C for 20 min. The slides were then cooled at room temperature for 20 min. Endogenous peroxidase activity was inhibited and blocking serum was placed as per the anti-citrulline protocol (described above). The primary antibody (polyclonal rabbit anti-human PAD-4; Abcam) was then added to each section at a concentration of 10 μg/mL in PBS. Control sections, as shown in Fig. 1 were incubated with rabbit polyclonal IgG (cat no P0448; DakoCytomation), at an equal concentration to the primary PAD-4 antibody (10 μg/mL). The slides were incubated overnight at room temperature in a wet chamber. The following day, the sections were incubated with biotinylated secondary antibodies in PBS and in the presence of normal horse serum for 45 min. Sections were then reacted with peroxidase-labelled avidin–biotin complex (ABC reagent; Vector Laboratories) for a further 45 min. Colour development, counterstaining and mounting of slides was carried out as per the anti-citrulline protocol.

Semiquantitative analysis of immunohistochemistry

A semiquantitative analysis (SQA) for assessment of immunohistochemical staining has been used by us, and others, and described in several publications (19–21). Briefly, all slides were scanned using the NanoZoomer 2.0-HT high-resolution slide scanner and NanoZoomer Digital Pathology software (Hamamatsu Photonics, Tokyo, Japan) and the SQA was conducted to determine the proportion of cells staining positively in each section. Scores from 0 to 4 were given depending on the proportion of cells (excluding epithelial cells) staining positive for each antibody (score 0: 0–10% of the cells stain positive; score 1: 11–25% of the cells stain positive; score 2: 26–50% of the cells stain positive; score 3: 51–75% of the cells stain positive; score 4: 76–100% of the cells stain positive).

Total RNA extraction

Total RNA was extracted from inflamed ($n = 3$) and non-inflamed ($n = 1$) tissues specimens using TriReagent Solution according to the manufacturer's protocol (Ambion, Austin, TX, USA). Tissue homogenization was carried out using individual sterile pestles during this process. Total RNA was then quantified by ultraviolet spectroscopy (Nanodrop 2000; Thermo Scientific, Wilmington, DE, USA) and the quality assessed using the ratio of absorbance at 260 and 280 nm.

cDNA synthesis and real time polymerase chain reaction

One microgram of total RNA was treated with DNase 1 (Ambion, Compark Circuit, Mulgrave, Victoria, Australia) before cDNA synthesis was carried out with the SuperScript® VILO cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). Complementary DNA was diluted and products amplified with the PAD-2, PAD-4 or housekeeping gene YWHAZ primer sets detailed in Table 1 (synthesized by Geneworks, Hindmarsh, SA, Australia) (23) using Express SYBR® GreenER qPCR supermix (Life Technologies). PCR was performed using a Rotor-gene RG 3000 (Corbett Research, Mortlake, NSW, Australia) with the following cycling conditions, 3 min at 95°C, 40 cycles of (5 s 95°C, 40 s 61°C), 2 μL was analysed on a

Table 1. Primer sequences for real time-PCR (22)

PAD-2	F – ggtgggatgagcagcaagcgaatc
	R – gaacagagcgggcaggtcaatgctg
PAD-4	F – ccacacggggcaaactgtc
	R – cagcagggagatggtgaggg
YWHAZ	F – acttttggtacattgtggcttcaa
	R – ccgccaggacaaaccagtat

PAD-Peptidylarginine deiminase
PCR-Polymerase chain reaction

2% agarose gel to confirm product size.

Gingival crevicular fluid collection and processing

Gingival crevicular fluid (GCF) was collected from periodontal sites diagnosed clinically as either inflamed (periodontitis and pocketing > 6 mm; 26 sites) or non-inflamed (no bleeding on probing and pocket depths < 3 mm; 17 sites). These were the same sites used for tissue biopsies and were taken from the deepest sites. Briefly, any visible supragingival plaque at the collection site was removed to avoid plaque contamination of the paper strips. The teeth were gently washed with water and air-dried. Salivary contamination was minimized by isolation with cotton rolls and use of a saliva ejector. GCF was collected using paper strips (Periopaper™; Oraflow, Plainview, NY, USA), with one strip being used for each collection site. The paper strips were carefully inserted 1 mm into the gingival crevice, avoiding trauma to gingival tissues and allowed to remain in place for 30 s, after which they were transferred for volume measurement. The presence or absence of blood on the strips was recorded.

Volume determination was performed using a Periotron 8000™ (Oraflow). The Periopaper strips were then wrapped in foil, placed in a sterile 1-mL microcentrifuge tube, and stored at −20°C. To retrieve the GCF the Periopaper strips were placed into individual wells of a sterile 96-well microtitre plate (Flow Laboratories, McLean, VA, USA) and 225 μL of sterile PBS (pH 7.2) was added to each well. The plate was then sealed and placed on a plate shaker for 30 min at room temperature. The eluted samples were then transferred to 1.5-mL microcentrifuge tubes containing a further 225 μL of sterile PBS and stored at −20°C until analysis by ELISA (24).

ELISA detection of anti-cyclic citrullinated protein antibodies in gingival crevicular fluid

Commercially available ELISA kits were used to analyse GCF samples for the presence of anti-CCP antibodies (Diastat™ Anti-CCP FCCP200; Axis-Shield Diagnostics, Dundee, UK). As there were no commercially available ELISA kits for PAD-2 or PAD-4, GCF was not tested for the presence of either of these proteins. To determine the presence of anti-CCP antibodies the semiquantitative protocol of the Diastat Anti-CCP kit was followed. Wash buffer and reagents were prepared as per the manufacturer's instructions. Five reference standards (ranging from 0 to 100 U/mL) and positive and negative controls were run in the same plate as the GCF samples, and incubated for 60 min. Wells were then decanted and washed three times with 200 μL of wash buffer. Conjugate (100 μL) was added to each well, and the plates were incubated for 30 min. Following emptying and washing (as described above), 100 μL of substrate was added and the plates incubated for 30 min. A stop solution was added (100 μL) to each well, and the wells were gently tapped to mix. All standards and GCF samples were assayed in duplicate. A microplate reader (BioTek Powerwave; BioTek Instruments, Winooski, VT, USA) was used to read the optical density at 550 nm. Computer software (KC4; BioTek Instruments) was used to generate a weighted five-parameter logistic curve and this allowed determination of antibody activity in each sample.

Statistical analyses

A commercial statistical software package (SPSS version 11.5; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of the SQA results. For comparison of mean rank values between the inflammation and non-inflammation groups, the Mann–Whitney *U*-test was used to test the null hypothesis that there was no difference between the groups. Non-parametric data were analysed using the Fisher's exact test. Correlation between ordinal data was assessed using Kendall's τb test. All tests were two-sided, and results with $p < 0.05$ were considered statistically significant.

Results

The demographic information relating to the study sample is shown in Table 2. For immunohistochemistry the study population of 40 individuals consisted of 20 men and 20 women, mean age 55.5 ± 2.44 for the inflammation group and 52.6 ± 3.7 years for the non-inflamed group. The

Table 2. Patient demographics and clinical parameters measured

	Total	Periodontitis	Non-periodontitis
Number	50	29	21
Age (years)[a]	48.3 ± 20.4	55.6 ± 13.1*	38.3 ± 24.3
Male	29/50 = 58%	18/29 = 62%	11/21 = 52%
Female	21/50 = 42%	11/29 = 38%	10/21 = 48%
PD (mm)[a]	5.08 ± 2.41	6.55 ± 1.72**	3.05 ± 1.63
Recession (mm)[a, b]	0.54 ± 1.34	1.10 ± 1.32**	−0.24 ± 0.94
BoP positive	27/50 = 54%	22/29 = 75.9%**	5/21 = 23.8%
Smoking history[c]	11/50 = 22%	9/29 = 31.0%	2/21 = 9.5%
GCF volume (μL)[a]	0.36 ± 0.44	0.45 ± 0.54	0.24 ± 0.22

[a]Scores represent mean ± SD.

[b]Negative recession was recorded where the gingival margin was coronal to the cemento-enamel junction, in cases of gingival enlargement.

[c]Patients were categorized as 'smokers' if they were current smokers or ex-smokers, or 'non-smokers' if they had never smoked. Further categorization by length of smoking habit, time of quitting, or pack years resulted in a number of very small groups.

*Statistically significant difference compared to non-periodontitis group, $p < 0.02$ Mann–Whitney *U*-test.

**Statistically significant difference compared to non-periodontitis group, $p < 0.0001$ Mann–Whitney *U*-test.

inflamed specimens were obtained from 29 patients with chronic periodontitis. This group had a mean pocket depth of 6.55 mm (± 0.32 mm). The non-inflamed specimens were obtained after histological assessment of 21 non-periodontitis samples from 11 patients (two men and nine women) with a mean pocket depth of 2.64 ± 0.28 mm. The histological level of inflammation in the samples was graded (Table 3). In the inflamed samples, 37% had mild inflammation, 41% had moderate inflammation and 22% had severe inflammation. A medical history was recorded for each patient and patients were not excluded from the study on the basis of their medical history. A list of medical conditions reported by the subjects is shown in Table 4. Of note is that none of the subjects was reported to have RA.

The expression of citrullinated proteins in gingival connective tissue was generally localized to the cytoplasm of the fibroblasts, endothelial cells and infiltrating inflammatory cells (Fig. 1). The levels of expression appeared to be associated with histological diagnosis (Fig. 2). The inflamed samples had a mean SQA score higher than the non-inflamed samples (1.8 ± 0.24 vs. 0.9 ± 0.21). This difference was statistically significant ($p = 0.04$).

Staining for PAD-2 also appeared to be widely distributed, as noted for the citrullinated proteins, in the inflamed samples (Fig. 1). The mean SQA score (Fig. 2) was higher in the inflamed specimens than the non-inflamed samples (2.76 ± 0.29 vs. 1.54 ± 0.28) and this difference was highly significant statistically ($p = 0.002$).

The SQA for PAD-4 (Fig. 2) indicated that its expression was higher in the inflamed samples than non-inflamed tissues, with a highly significant difference observed between these groups (1.88 ± 0.32 vs. 0.18 ± 0.18, $p = 0.0001$).

The SQA scores for all proteins were also analysed for any correlation between the level of inflammation and protein expression within the tissues. Figure 2 shows the distribution of SQA scores for each protein, categorized by the level of inflammation, with an apparent trend towards increasing expression of each of the proteins with higher levels of inflammation. Correlation between infiltration score and CCP SQA was moderate but highly significant ($r = 0.394$, $p = 0.006$). There was also moderate correlation between infiltra-

Table 3. Analysis of inflammatory status of tissue specimens from inflamed and non-inflamed sites

Inflammation score	Inflamed	Non-inflamed
Score 0	0	11
Score 1 (mild)	10	0
Score 2 (moderate)	11	0
Score 3 (severe)	6	0
	27[a]	11

[a]Two periodontitis samples excluded following histological assessment.

Table 4. Systemic medical conditions and medications reported by subjects

	Inflammation $n = 29$ (%)	Non-inflammation $n = 11$ (%)
Hypertension	9 (31)	4 (36)
Hypercholesterolaemia	6 (21)	0 (10)
Osteoarthritis	2 (7)	1 (9)
Diabetes type 2	3 (10)	0 (0)
Gout	2 (7)	0 (0)
Thyroidectomy	1 (3)	0 (0)
Asthma	1 (3)	1 (9)
Depression	1 (3)	0 (0)
Fibromyalgia	1 (3)	0 (0)
Osteoporosis	1 (3)	0 (0)
Rheumatoid arthritis	0 (0)	0 (0)

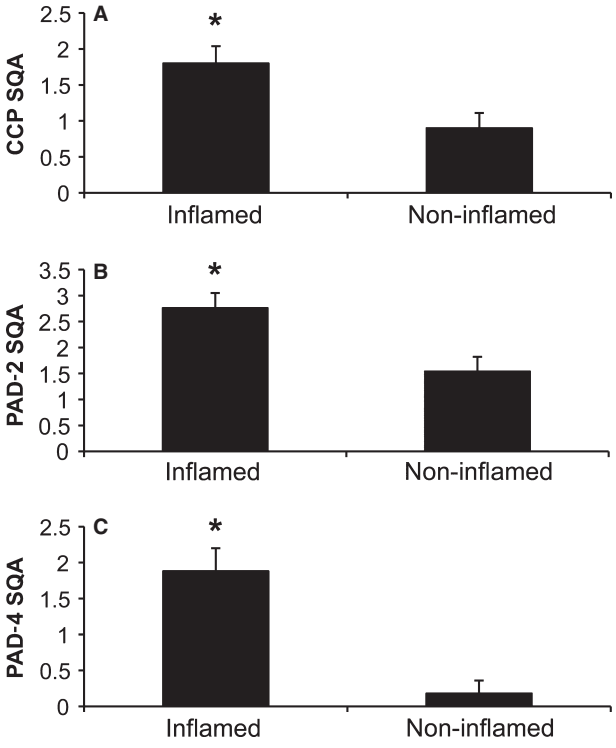

Fig. 2. Semiquantitative analysis (SQA) of immunostaining in inflamed and non-inflamed human gingiva for (A), citrullinated proteins (CCP); (B), peptidylarginine deiminase (PAD) 2 and (C), PAD-4. * $p < 0.05$.

tion and PAD-2 SQAs that approached statistical significance ($r = 0.380$, $p = 0.010$). A positive and highly significant correlation was observed between cell infiltration score and PAD-4 SQA ($r = 0.565$ and $p = 0.0001$).

When SQA results for the three proteins were analysed for correlation with clinical parameters, there were positive and significant correlation between pocket depth and PAD-2 SQA ($r = 0.387$, $p = 0.006$) and between pocket depth and PAD-4 SQA ($r = 0.463$, $p = 0.001$) but not between pocket depth and CCP SQA. PAD-2 SQA and PAD-4 SQA were also correlated significantly with bleeding on probing ($r = 0.540$, $p = 0.001$ and $r = 0.530$, $p = 0.001$ respectively).

To assess local production of PAD-2 and PAD-4, RT-PCR assessment of tissue homogenates for expression of mRNA for PAD-2 and PAD-4 was carried out (Fig. 3). Expression of mRNA for PAD-2 and PAD-4 was noted in both inflamed and non-inflamed tissues. Owing to low sample numbers these were not subjected to statistical analysis.

In testing for anti-CCP antibodies in the GCF, two distinct groups of readings were noted: those that were clearly negative, and those that scored between 1.7 and 5.3 U/mL (Fig. 4). While most of the positive samples were from the periodontitis group (nine of 11, 82%) there was no statistically significant correlation between GCF positivity for anti-CCP and periodontitis or non-inflamed ($p = 0.097$).

Discussion

Citrullination occurs in a range of normal physiological functions, including apoptosis and terminal differentiation of the epidermis. However, it also has been associated with a number of pathological conditions, including RA, multiple sclerosis and Alzheimer's disease (25). This process is mediated through enzymatic conversion of arginine residues to citrulline via the action of PAD enzymes. In this cascade, proteins become antigenic through the conversion of arginine to citrulline by deimination enzymes such as PAD (26). The accumulation of proteins such as fibrin within the synovium and their prolonged and complex degradation, which includes citrullination, leads to exposure of new epitopes to immunocompetent cells within the synovium (26). In this context, anti-CCP antibodies have a high predictive value for the onset of RA several years before it is evident clinically and they are associated with more severe clinical outcomes (6). Moreover, the presence of both RF and anti-CCP autoantibodies is highly predictive of severe and progressive RA (5).

Interestingly, citrullinated proteins and PAD-4 are not present in synovial tissues prior to disease onset (27). However, as inflammation precedes disease onset in RA, the expression of both citrullinated proteins and PAD-4 increase in the synovium (28). This suggests that citrullination in the joint may be a consequence of RA rather than a cause but also suggests that anti-CCP antibodies produced outside the joints may be involved in the progression of RA. In the present study, citrullinated proteins were detected in gingival tissues at both inflamed and non-inflamed sites with higher expression being noted with increasing inflammation, which is in agreement with reports of the expression of these proteins in inflamed synovial tissues (27–29). This indicates that citrullination of proteins occurs in the gingival tissues during periodontal inflammation and raises the possibility that the periodontium may be an extra-articular site of citrullinated proteins. Furthermore, autoantibodies to these citrullinated proteins may be formed in the periodontium, eventually contributing to the RA disease process.

It is important to note that the presence of autoantibodies does not necessarily induce autoimmune diseases, and some of these antibodies could arise as part of the natural antibody response by B cells. Such natural antibodies produced by B cells are generally poly-reactive, have a low avidity and are increased in patients with periodontitis. Hence, in future studies it will be important to show the quality and quantity of anti-CCP antibodies induced in periodontitis tissue.

In RA there is no correlation between titres for serum anti-CCP antibodies and citrullinated protein expression in the synovial tissue (30). There is, however, a correlation between anti-CCP levels in serum and synovial fluid. Anti-CCP antibodies represented a higher proportion of IgG in the synovial fluid than serum,

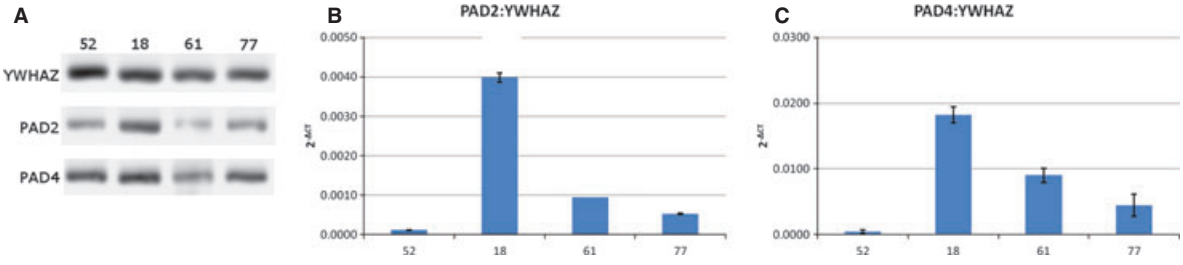

Fig. 3. (A) Bands indicate peptidylarginine deiminase (PAD) 2 and PAD-4 mRNA extracted from non-inflamed and inflamed gingival tissue biopsy samples. (B,C) Expression of PAD-2 and PAD-4 mRNA normalized to YWHAZ. Sample description: 52, no inflammation, 18, 61, and 77 inflamed tissues.

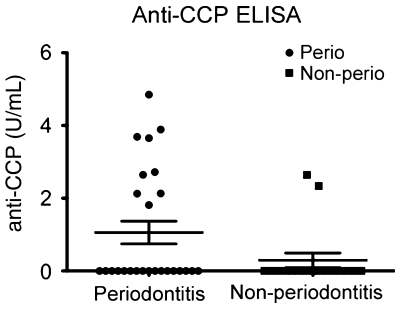

Fig. 4. Distribution of gingival crevicular fluid for anti-citrullinated proteins (anti-CCP) antibodies in periodontitis and non-periodontitis sites. Error bars represent SEM.

suggesting that local production in the synovium is likely. In our study, we demonstrated the presence of anti-CCP antibodies in GCF and this presumably results, at least in part, from a local production of CCP as judged by the expression of mRNA for PAD-2 and PAD-4 in these tissues. It has been shown that anti-CCP antibodies are quite specific for RA, and the prevalence of seropositivity in healthy subjects is very low (0–2%) (31). According to the self-reported medical history data we collected, none of the subjects in our study had RA. The presence of anti-CCP antibodies in the GCF of patients in our study may indicate early or impending RA, although it seems unlikely that this would explain such a high percentage of positive samples, considering that prevalence of RA is widely reported as being about 1% (32). Alternatively, it may support the suggestion that anti-CCP antibodies are involved in the periodontitis disease process. The presence of anti-CCP antibodies in serum has been reported in a percentage of patients with other (non-RA) inflammatory diseases, such as psoriatic arthritis (33), systemic lupus erythematosus (34) and Sjögren's syndrome (35,36).

Several studies have reported the presence in serum of anti CCP antibodies in chronic and aggressive periodontitis vs. healthy patients (14–16,37). Therefore, in this study it was not necessary repeat this because the principal aim of this proof of principle study was to determine the local presence and production of these antibodies in inflamed gingival tissues. Clearly future studies correlating levels of anti-CCP in gingival tissues, GCF and serum are needed.

Notwithstanding the need to determine the quality and quantity of antibody response for anti-CCP antibodies in patients with periodontal disease, the finding of anti-CCP antibodies in inflamed gingival tissues in this study leads us to speculate a role for this process in RA. We hypothesize that priming anti-CCP antibodies may be produced during the development of periodontitis. If a later event such as joint inflammation results in citrullination then, in a primed individual, the subsequent antibody response could be very robust. This fits well with the so-called two-hit model proposed for the development of chronic inflammatory disorders such as RA (37,38). Such a response is in keeping with recent observations that experimental animals with a pre-existing chronic infection or periodontitis develop experimental arthritis at a faster and more pronounced rate than non-primed animals (39–41).

To date, no studies analysing anti-CCP antibodies in GCF have been published; however, several studies have reported their presence in the serum of patients with periodontitis (15,16). Seropositivity for anti-CCP antibodies has been noted in a small proportion (< 10%) of non-RA patients with generalized aggressive periodontitis, while healthy controls and patients with chronic periodontitis or gingivitis were all seronegative. In a recent study, a small group of patients with chronic periodontitis was examined for the presence of anti-CCP antibodies in serum, and only one patient of 20 was found to be seropositive (36). A study of patients with RA and chronic periodontitis found an association between seropositivity for anti-CCP antibodies and increased severity of periodontitis (14).

Both PAD-2 and PAD-4 have been isolated in inflammatory cells, which is consistent with them having an important role in the development of RA (7,8). In the present study both PAD-2 and PAD-4 were detected in inflamed gingival tissue samples, which is consistent with a report published recently (18). In the present study, significant correlation was found between expression of PAD-2 or PAD-4 and inflammation. These results support the findings of a previous study examining the expression of PAD in synovial tissue biopsies from patients with RA and non-RA controls in which the expression of PAD was not exclusive to RA tissues. However, the expression of both PAD-2 and PAD-4 showed significant correlation with the level of inflammation (8). Studies in animal models have also reported increased expression of PAD-4 in more severe inflammation (27).

Staining for PAD-4 in our study consisted of a mixture of nuclear and cytoplasmic staining mostly in mononuclear cells; however, endothelial cells and fibroblasts also stained positively. This is consistent with previous reports of PAD-4 expression in other tissues, including RA synovium (8,42) and various tumours (43,44).

Although the expression of PAD by PPAD has been well documented (9) this was not examined in the present study because of the current lack of availability of commercial antibodies to PPAD. While citrullinated proteins were located in the gingival tissues, and PAD-2 and PAD-4 (both protein and mRNA) were also detected, it cannot be assumed that no other PADs were involved in the citrullination process. *P. gingivalis* is strongly associated with periodontitis (45) and may infiltrate the gingival tissues (46). Thus, PPAD could be present in inflamed periodontal tissues. Further research is required to determine if PPAD is present in the periodontium, and whether it is involved in citrullination of proteins in the gingival tissues.

Anti-CCP antibodies were detected in GCF and the majority of the positive readings (nine of 11) came from periodontitis sites. While the statistical analysis did not reveal a significant correlation between clinical diagnosis (periodontitis or gingivitis) and GCF positivity, the fact that the positive

samples were almost exclusively from the periodontitis sites suggests there may be some connection. One explanation for these findings (anti-CCP antibodies in some but not all periodontitis and gingivitis sites) is that the PADs and citrullinated proteins are involved generically in tissue inflammation in the gingival tissues, regardless of periodontitis or gingivitis status, whereas the autoantibodies to citrullinated proteins may only be produced in a certain susceptible subset of patients. It has previously been suggested that chronic periodontitis is actually a 'constellation' of several different polygenetic and polymicrobial disease variants with similar clinical presentation (47). If true, the fact that not all of the patients with periodontitis were positive for either of these antibodies could be indicative of a role for these antibodies in a particular subset of patients with periodontitis.

In this study coexisting medical conditions were reported (Table 3) but due to very low numbers, an assessment of correlations between other systemic inflammatory conditions and the gingival tissues could not be performed. However, given that citrullination is known to occur in many other chronic inflammatory conditions the findings of this study confirm the induction of these molecules in chronic inflammation. Because of the very common occurrence of gingivitis (it may be present in up to 90% of the adult dentate population) (48), the finding of citrullination in inflamed gingival tissues is interesting and indicates that gingivitis may have the potential to produce citrullinated proteins which may serve as autoantigens in individuals susceptible to developing rheumatoid arthritis.

In conclusion, this study has demonstrated that citrullinated proteins, PAD-2 and PAD-4 are present in inflamed periodontal tissues and that anti-CCP antibodies can be detected in the GCF of some patients. Tissue expression of citrullinated proteins and PAD appeared to increase with the severity of localized inflammation. The presence of autoantibodies to CCP in the GCF was almost exclusive to a subset of patients with periodontitis and did not appear to be related to localized inflammation. These findings indicate local production of these proteins in the periodontium. The presence of these proteins in the periodontium, combined with their known importance in the development and progression of RA, is further evidence of similarities and possible linkage of these two diseases.

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