Higher Elastase Activity Associated with Lower IL-18 in GCF from Juvenile Systemic Lupus Patients

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Purpose: Our aim was to evaluate the expression of interleukin-18 (IL-18), interleukin-1-beta (IL-1β) and the amount of elastase activity in gingival crevicular fluid (GCF) from inflamed gingival sites in patients with juvenile systemic lupus erythematosus (JSLE), and compare these to the expression in GCF from inflamed sites in generally healthy controls. In addition, the local inflammation in periodontal tissues was related to systemic inflammation by the assessment of IL-18 levels in plasma.

Materials and Methods: GCF from 16 patients with JSLE and 14 controls were collected using a washing device. Elastase activity was measured with a specific substrate, and IL-18 and IL-1β were measured by ELISA.

Results: The percentage of visible plaque index, gingival bleeding index and attachment level were similar in JSLE and controls, while the percentage of probing depth greater or equal to 3 mm was significantly higher in the controls. The total amount of IL-1β and IL-18 in GCF were significantly decreased in JSLE, while the total amount and the percentage of free elastase activity were significantly higher in JSLE when compared with the controls. The plasma levels of IL-18 and the erythrocyte sedimentation rate were significantly higher in JSLE patients.

Conclusion: We found more active elastase in GCF from inflamed sites in JSLE patients even in the presence of significantly lower levels of IL-18 and IL-1β. The increased elastase activity suggests a hyperactivity of neutrophils in JSLE, possibly generated by a priming effect caused by the higher plasma levels of IL-18 found in these JSLE patients.

Key words: elastase, IL-18, IL1-β, juvenile systemic lupus erythematosus, periodontitis.

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Systemic lupus erythematosus (SLE) is a chronic auto-immune disease, characterised by immune responses directed to a great number of self antigens that mostly affects women in their second or third decade of life. SLE can affect various parts of the body, including joints, skin, kidney, heart, lungs, blood vessels and the brain (for review see Danchenko et al, 2006). The incidence of juvenile SLE (JSLE) is estimated between 6–20 cases per 100,000 children, and mainly occurs in girls and in children of non-Caucasian origin (Hoschberg 1997, Stichweh et al, 2004). The pathoaetiology of this chronic disease remains unclear, but genetic, immunological and environmental factors have all been implicated. Patients with SLE have been shown to have elevated plasma interleukin-18 (IL-18) concentrations (Tso et al, 2006).

IL-18, formerly called interferon (IFN)-γ-inducing factor, is a pro-inflammatory cytokine related to the IL-1 family that is produced by Kupffer cells, activated macrophages, keratinocytes, intestinal epithelial cells, osteoblasts and adrenal cortex cells (Dinarello, 1999). IL-18 is produced as a 24 kDa inactive precursor and is cleaved by the IL-1β converting enzyme (ICE, caspase-1) to generate a biologically active mature 18 kDa moiety (Ghayur et al, 1997). It plays an important role in innate immunity and it has been shown to

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induce T-helper cells type I (Th1) and Th2 cytokines, such as IL-4, IL-5, IL-10 and IL-13 (Hoshino et al, 1999). The primary functions of IL-18 include the induction of IFN-γ and tumour necrosis factor α (TNF-α) in T cells and natural killer (NK) cells (Tanaka et al, 2001), and the up-regulation of Th1 cytokines including IL-2, granulocyte macrophage colony stimulating factor (GM-CSF) and IFN-γ (Dinarello, 1999).

Human neutrophils, both in circulation and in the tissues, constitutively express the IL-18 receptor (IL-18R) (Leung et al, 2001). IL-18 induces cytokine and chemokine release from neutrophils, induces granule release and enhances the respiratory burst (Leung et al, 2001). The capacity to release cytokines and chemokines was significantly enhanced in neutrophils derived from rheumatoid arthritis sinovial fluid, indicating a differential response to IL-18 dependent upon prior neutrophil activation *in vivo*.

Activated neutrophils release elastase, a serine protease, which degrades elastin and several other functionally and structurally important proteins in the periodontium, including collagen, proteoglycans and basement membrane components (Janoff, 1985). Several studies have shown increased activity of this protease in gingival crevicular fluid (GCF) from sites of periodontitis (Overall and Sodek, 1987; Gustafsson et al, 1994; Ingman et al, 1996; Meyer et al, 1997; Figueredo and Gustafsson, 1998). Elastase is released in an active form but is normally rapidly inhibited by the protease inhibitors α-1-antitrypsin (A1AT) and α-2-macroglobulin (A2MG). Some studies have indicated that this inhibition is less effective in inflamed periodontal tissues from patients with periodontitis, thereby allowing the elastase to remain active for a longer period of time.

IL-1β, a potent pro-inflammatory cytokine, has been considered a pivotal signalling substance involved in the up-regulation of matrix metalloproteinases and down-regulation of tissue inhibitors (see Page et al, 1997, for review). Earlier studies have shown high levels of IL-1β in sites with periodontitis (Figueredo et al, 1999; Orozco et al, 2006). The role of IL-18 in periodontal disease has been less well studied. Orozo et al (2006) showed increased levels of GCF from inflamed sites of periodontitis patients.

Therefore, the aim was to evaluate the expression of IL-18 and IL-1β and the amount of elastase activity in GCF from inflamed gingival sites in patients with JSLE, and compare these to the expression in GCF from inflamed sites in generally healthy controls. In addition, the local inflammation in periodontal tissues was related to systemic inflammation by the assessment of IL-18 levels in plasma.

MATERIALS AND METHODS

Participants

The patient group comprised 16 adolescents (mean age: 15.6, SD ± 2.7 years) who were attending the Pediatric Rheumatology Clinic (NESA), Rio de Janeiro State University (UERJ), Rio de Janeiro, Brazil. Diagnosis of the patients' SLE had previously been made by the same physician in accordance with the classification of the American College of Rheumatology (Hoschberg, 1997). The median duration of disease was 4 years (range 1 to 10 years). Thirteen patients were taking medication. Fourteen individuals (mean age: 15.5, SD ± 1.5 years) with no signs of ongoing infections or inflammatory diseases were selected as controls. These persons were recruited among those visiting the clinic for an annual medical check up. The Ethics Committee of Pedro Ernesto University Hospital (UERJ, Rio de Janeiro, Brazil) and the Regional Ethics Committee in Stockholm, Sweden, approved this study. All volunteers and parents/guardians gave written consent to participate. The patients answered a questionnaire concerning their personal data.

Clinical examination

Periodontal and rheumatological clinical examinations were performed. All tooth surfaces, except for third molars, of the selected patients were examined with a Williams probe (PCP10 Color Coded Probe, Hu-Friedy, Chicago, USA), by the same calibrated examiner. The variables registered were percentage of visible plaque index (VPI) and gingival bleeding index (GBI) (Ainamo and Bay, 1975), pocket probing depth (PD) and clinical attachment level (CAL).

Clinical evaluation of rheumatological findings

The rheumatological evaluation comprised a clinical examination and the measurement of the disease activity through the Global Medical Evaluation (GME) in an analogue visual scale (ranged from inactive to severe). The Systemic Lupus International Collaborating Clinics (SLICC) evaluation and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) were used to measure SLE damage and activity, respectively. All evaluations were performed by the same paediatric rheumatologist.

The JSLE patients were subdivided according to the disease activity. A patient with a SLEDAI score differ-

ent from zero was considered to have active disease. Thirteen of the 16 participating patients took immunosuppressant drugs, prednisone, chloroquine and azathioprine, and five of them also took nonsteroidal anti-inflammatory drugs (NSAIDs).

GCF sampling method

Samples were taken from five to six deep pockets or the most inflamed sites. GCF was collected with an intracrevicular washing device modified from Salonen and Paunio (1991). The sites to be sampled were isolated with cotton rolls and dried gently with an air syringe. Supragingival plaque was carefully removed before sampling. Each pocket selected was washed five times with 5 µl of phosphate buffer saline (PBS) during continuous aspiration. The samples from the same type of site in each person were pooled, diluted with PBS to a volume of 1 ml and immediately centrifuged at 3000 g for 10 min. The supernatant was collected and frozen at -70°C, pending analysis.

For the blood samples, a total of 20 ml of venous peripheral blood was collected from each patient and control subject and stored in heparinised tubes.

Elastase activity

The granulocyte elastase substrate S-2484 (L-pyroglutamyl-L-prolyl-L-valine-p-nitroaniline, MW 445.5 Da, Heamatochrome Diagnostica, Mölndal, Sweden) was dissolved in dimethyl sulphoxide to 8 mmol/l and the working solution was made to 2 mmol/l by dilution in PBS. The alkaline phosphatase substrate, p-nitro-phenol phosphate (Janssen Chimica, Geel, Belgium), was diluted to 2.7 mmol/l in diethanolamine buffer, pH 10.0. A total of 100 µl of sample was mixed with 67 µl of substrate in a 96-well microtitre plate (Nunc Maxisorp, Nunc, Roskilde, Denmark). The mixture was incubated at 37°C and the absorbency at 405 nm was read after 2 h in a spectrophotometer (Millenia Kinetic Analyzer, Diagnostic Product Corporation, Los Angeles, CA, USA). The elastase activity was expressed in an arbitrary unit mAbs. To inhibit elastase activity, 10 µl of 0.1% A1AT was added to 90 µl of sample and incubated with agitation for 15 min at room temperature. After inhibition, the samples were tested for elastase activity, as described above. The elastase activity inhibited by A1AT was regarded as deriving from free elastase and the remaining activity as deriving from elastase bound to A2MG.

Immunological assays

In the gingival fluid, IL-1β was measured using enzyme-linked immunosorbant assay (ELISA), as reported earlier (Figueredo et al, 1999). A monoclonal antibody against IL-1β (MAB 601, R & D Systems, Minneapolis, MN, USA) diluted 1:125 in carbonate buffer was coated onto microtitre plates (Nunc Maxisorb at 4°C overnight. Samples and standards were diluted in PBS, pH 7.4. The microtitre plates were washed with PBS containing 0.05% polyoxyethylene sorbitan monolaurate (Tween® 20, Sigma Chemical, St Louis, MO, USA). After washing with PBS/Tween (4 times with 300 µl) the plates were blocked with 1% human serum albumin (HSA) for 1 hour at room temperature. The samples were washed as above and 100 µl of standard (2 pg/ml to 200 pg/ml) and undiluted samples were added respectively. The plates were incubated at 37°C for 45 min followed by washing. The detection antibody (BAF 201, R & D Systems, Minneapolis, MN, USA) was diluted 1:250 in PBS and incubated for 45 min. After washing, streptavidin diluted 1:200 was added to the plates and incubated further at 37°C for 20 min. The plates were once again washed and the undiluted substrate was added (TMB, Sigma Chemical). The reaction was stopped with 1 mol/l H_2SO_4 after 15 minutes and the absorbency was read at 450 nm in a spectrophotometer.

IL-18 was measured in GCF and in plasma with commercially available ELISA kits (IL-18, MBL, Nagoya, Japan), according to the manufacturer instructions. Total amounts of IL-1β and IL-18 were expressed as pg/ml.

Statistical analysis

The unit of analysis was the individual and the significance was set at 5%. Mann-Whitney and Spearman's correlation were applied as indicated in the text/tables. SPSS 8.0 software was used to analyse the data.

RESULTS

JSLE versus controls

The percentage of VPI, GBI and AL were similar in JSLE and controls while the percentage of PD greater or equal to 3 mm was higher in the controls ($p = 0.03$, Table 1).

Table 1 Percentage (mean [± standard deviation]) of visible plaque index (VPI), gingival bleeding index (GBI), sites with pocket depth (PD) ≥ 3 mm and sites with proximal clinical attachment loss (CAL) ≥ 2 mm in JSLE and control groups, and in the active and inactive subgroups

	VPI	GBI	PD	CAL
JSLE n = 16	33 (± 19)	33.2 (± 16)	17 (± 16)	0.2 (± 0.6)
Control n = 14	35 (± 18)	39 (± 13)	30 (± 18) *	0.2 (± 0.3)
JSLE active n = 10	41 (± 19) ***	39 (± 16) **	20 (± 16)	0.2 (± 0.5)
JSLE inactive n = 6	19 (± 9)	23 (± 11)	13 (± 16)	0.1 (± 0.3)

JSLE: juvenile systemic lupus erythematosus; AL: presence of at least 1 proximal site with AL ≥ 2 mm.
* JSLE versus control, Mann-Whitney test, $p < 0.05$; ** JSLE active versus JSLE inactive, Mann-Whitney test, $p < 0.05$; *** JSLE active versus JSLE inactive, Mann-Whitney test, $p < 0.01$.

The total amount of IL-1β and IL-18 in GCF were significantly decreased in JSLE when compared to controls ($p = 0.05$ and 0.02, respectively), while the total amount and the percentage of free elastase activity were significantly higher in JSLE when compared with controls ($p = 0.03$ and 0.001, respectively, Table 2).

The plasma levels of IL-18 and the erythrocyte sedimentation rate (ESR) were significantly higher in JSLE patients ($p = 0.04$ and 0.03, respectively, Table 2). The SLEDAI median was 4 (range from 0 to 36) and the SLICC median was 0 (range from 0 to 8). IL-18 in plasma did not correlate with ESR or with the rheumatological evaluation using the SLEDAI scale.

Active versus inactive JSLE

The percentage of visible plaque index and gingival bleeding index were higher in active compared with inactive JSLE patients, while the percentage of probing depth (PD) greater or equal to 3 mm, and attachment level (AL) greater or equal to 2 mm was similar between them (Table 1).

The total amount of IL-1β and IL-18, and the total amount and the percentage of free elastase activity in GCF were similar in active and inactive JSLE patients. The same results were observed with the plasma levels of IL-18 and ESR (Table 2).

DISCUSSION

The present study showed more unbound elastase, corresponding to still active elastase in the GCF samples from inflamed gingival sites in patients with JLSE, when compared with inflamed sites in the control patients. Elastase activity in GCF samples has been convincingly associated with inflammation and tissue destruction in periodontal disease (Figueredo and Gustafsson, 1998; Loos and Tjoa, 2005). Armitage et al (1994) showed that sites with high levels of elastase are at a significantly greater risk for progressive bone loss as assessed by digital subtraction radiography. Elastase is released from the cells in an active form but under normal circumstances the body has effective ways of inhibiting this potentially damaging enzyme. Remaining active elastase in the gingival pocket could be due to hyperactive neutrophils generating large amounts of reactive oxygen species, which can inactivate the most abundant protease inhibitor A1AT. This increased concentration of active elastase in the gingival pocket could indicate that patients with JLSE are at a greater risk of tissue degradation and in the long term, CAL.

In contrast to elastase, lower levels of IL-18 and IL-1β were observed in the patient group. Several studies have shown that IL-1β is increased in inflamed periodontal tissues as compared to controls (Figueredo et al, 1999; Faizuddin et al, 2003; Hou et al, 2003). Increased levels of IL-1β have been strongly associated

Table 2 Mean concentration of inflammatory markers in GCF and plasma (± standard deviation) in JSLE and control groups, and JSLE active and inactive subgroups

	GCF biomarkers					Plasma biomarkers	
	Total elastase (mAbs)	E-α2MG (mAbs)	Free elastase	% Free elastase	IL-1, (pg/ml)	IL-18 (pg/ml)	IL-18 (pg/ml)	ESR (mm/h)
JSLE n = 16	630 (± 598)	125 (± 72)	506 (± 540)	62 (± 30)	18 (± 17)	33 (± 38)	453 (± 302)	23 (± 16)
Control n = 14	474 (± 486)	331 (± 157)	180 (± 381)	19 (± 23)	30 (± 19)	52 (± 14)	315 (± 61)	8 (± 12)
p	NS	0.001	0.03	0.001	0.05	0.02	0.04	0.03
JSLE active n = 10	859 (± 642)	142 (± 84)	717 (± 569)	72 (± 27)	21 (± 21)	21.7 (± 24)	496 (± 364)	22 (± 18)
JSLE inactive n = 6	250 (± 233)	97 (± 33)	154 (± 236)	46 (± 20)	14 (10)	46 (±30)	375 (± 86)	25 (± 12)
p	0.05	NS	0.07	NS	NS	NS	NS	NS

E–α2MG: elastase plus α-2 macroglobulin. ESR: erythrocyte sedimentation rate. NS: not significant, Mann-Whitney test, $p \geq 0.05$.

with high neutrophil activity (Drugarin et al, 1998; Figueredo et al, 2000), whereas the role of IL-18 in periodontal disease has been less studied. Johnson and Serio (2005) evaluated IL-18 in gingival biopsies and observed higher concentrations in deeper pockets (≥ 6mm). The lower levels of IL-1β and IL-18 in GCF from JSLE patients could result from the use of various different anti-inflammatory drugs. The JSLE patients were prescribed several medications according to their clinical manifestation of the disease. Some of these medications, such as prednisone, chloroquine and azathioprine have an immunosuppressive effect (for a review, see Barrera et al, 1996). Prednisone and azathioprine have been shown to lower the concentration of IL-1β in a mouse lipopolysaccharide (LPS) induced inflammation model (Brustolim et al, 2006). Jang et al (2006) showed that chloroquine-mediated inhibition of TNF-α, IL-1β and IL-6 synthesis occurred through different modes in LPS-stimulated human monocytes/macrophages. Wozniacka et al (2006) showed that after three months of chloroquine therapy, the mean levels, of IL-6, IL-18 and TNF-α decreased significantly in the serum. Taken together, it is reasonable to propose that in the current study, the local production of IL-1β and IL-18 in the gingiva was affected by the systemic use of the anti-inflammatory drugs mentioned above.

The plasma IL-18 concentration, in contrast to that in GCF, was higher in JSLE than in controls. This might indicate that the plasma IL-18 has an effect on local neutrophil activity. This hypothesis is supported by Wyman et al (2002), who found that IL-18, even at physiological concentrations, is an effective neutrophil priming agent. Hewins et al (2006) also reported that IL-18 is likely to be important for neutrophil recruitment and priming in anti-neutrophil cytoplasmatic autoantibody-associated systemic vasculitis. Priming has been defined as the cells are 'ready to go' but awaiting further stimulus before the oxidase response is elicited. For example, only the activated cells show oxidase activity, but if the primed cell also receives an activating stimulus, the ensuing oxidase activity is greater than that in non-primed, activated cells (Hallet and Lloyds, 1995). Greater chemotaxis and degranulation have also been demonstrated when neutrophils are primed with cytokines, such as TNF-α (Bajaj et al, 1992), IL-1β (Brandolini et al, 1997) and IL-18 (Hewins et al, 2006). Therefore, it is hypothesised that the higher neutrophil activity found in JSLE might be related to some kind of pre-activation caused by the higher levels of IL-18 in the plasma.

The current study was not able to show any changes in the attachment level between JSLE and controls,

but control patients did show significantly more pocket probing depths greater or equal to 3 mm. This finding is in agreement with Mutlu et al (1993), who reported significantly lower periodontal probing depths in the patient group compared with the healthy controls. A possible explanation for this could be the use of disease-modifying drugs among the SLE patients. Only a few other studies reported the periodontal conditions of SLE patients, and to date no studies have properly described the periodontal conditions in JSLE. Nagler et al (1999) published a case report of an 18-year-old female with SLE and generalised periodontal involvement. The patient had multiple gingival recessions but no periodontal pockets or gingival bleeding. Two other cases of SLE patients with either periodontitis (Vogel, 1981) or severe gingivitis (Gonzalez-Crespo and Gornez-Reivo, 1995) have been reported.

In conclusion, the present study reports higher levels of active elastase in GCF from inflamed sites in JSLE patients in spite of significantly lower levels of IL-18 and IL-1β, suggesting a greater risk of tissue degradation and periodontal attachment loss compared with healthy juvenile controls. The local hyperactivity of neutrophils in JSLE could be somehow generated by the priming effect caused by the higher plasma levels of IL-18 found in these JSLE patients.

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Erratum to Oral Health and Preventive Dentistry Volume 5 Number 4 2007

The following figure contained an error in the printed version of Oral Health and Preventive Dentistry Volume 5 Number 4 2007. The corrected version of this article is available online at http://ohpd.quintessenz.de. Quintessence sincerely apologises to the authors and readers for this error.

In the study by Holgerson et al (Decreased Salivary Uptake of [^{14}C]-Xylitol after a Four-week Xylitol Chewing Gum Regimen), published in Oral Health Prev Dent 2007;5:313-319, the Line B of Fig 1 was omitted. The correct version is shown here. The results and conclusions of this study are not affected by this omission.

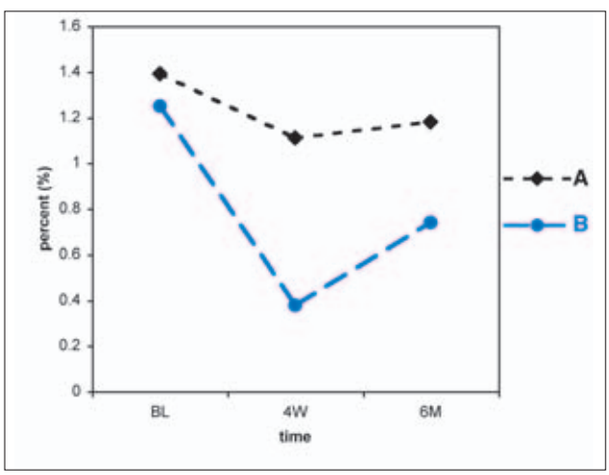

Fig 1 Proportion of salivary mutans streptococci (MS) in relation to the total viable counts (TVC) at baseline, after 4 weeks of daily chewing on xylitol-containing gums (group B) or sorbitol/maltitol control gums (group A) and 6 months after the intervention period (calculated on the original mean values of MS and TVC). One outlier was excluded due to an unrealistic high 4-week value (> 25%). There were significant differences between group A and B after 4 weeks ($p < 0.05$) and between baseline and 4 weeks in group B ($p < 0.05$).