J Clin Periodontol 2005; 32: 1047–1054 doi: 10.1111/j.1600-051X.2005.00832.x

Journal of Clinical Periodontology

Imbalance between soluble tumour necrosis factor receptors type 1 and 2 in chronic periodontitis

Ikuyo Ikezawa, Hideaki Tai, Yasuko Shimada, Yasutaka Komatsu, Johnah C. Galicia and Hiromasa Yoshie

Division of Periodontology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Ikezawa I, Tai H, Shimada Y, Komatsu Y, Galicia JC, Yoshie H. Imbalance between soluble tumour necrosis factor receptors type 1 and 2 in chronic periodontitis. J Clin Peridontol 2005; 32: 1047–1054. doi: 10.1111/j.1600-051X.2005.00832.x.

Abstract

Background: Soluble types of tumour necrosis factor (TNF) receptors type 1 and 2 modulate the TNF-α-mediated inflammatory responses in chronic periodontitis (CP).

Objectives: This study investigated the levels of TNF-α, soluble TNF receptor type 1 and 2 in gingival crevicular fluid (GCF) and serum of healthy subjects and CP patients.

Materials and Methods: Thirty-eight sera and 73 GCF samples were collected from 16 healthy subjects and 22 CP patients. GCF was collected from probing pocket depth (PPD) $\leqslant 3$ mm sites of healthy subjects, PPD $\leqslant 3$, 4–6 and $\geqslant 7$ mm sites of CP patients. The levels of TNF-α, soluble TNF receptor type 1 and 2 in the serum and GCF were quantified by enzyme-linked immunosorbant assay.

Results: The total amounts of TNF-α, soluble TNF receptor type 1 and 2 in GCF significantly elevated with increasing PPD in both site-based ($p < 0.05$) and subject-based ($p < 0.05$) analyses. However, their levels progressively diverged as the pocket depths increased, with the soluble TNF receptor type 2 level being comparatively lower than type 1. On the other hand, soluble TNF receptor type 2/type 1 ratios in GCF decreased as the severity of periodontitis increased ($p < 0.0001$).

Conclusion: The imbalance between soluble TNF receptor type 1 and 2 levels in GCF could be related to CP severity.

Keywords: chronic periodontitis; gingival crevicular fluid; serum; soluble tumour necrosis factor receptor; tumour necrosis factor-α

Accepted for publication 8 June 2005

Tumour necrosis factor-α (TNF-α) is a proinflammatory cytokine produced by a wide spectrum of cells. TNF-α was detected in gingival crevicular fluid (GCF) of periodontally diseased sites, and was suggested to be found in disease sites prior to clinically observable disease (Rossomando et al. 1990). It also has been detected in gingival tissues from periodontitis-affected sites of humans, and known to induce tissue destruction and bone resorption (Wilton et al. 1992, Page et al. 1997).

TNF-α mediates its diverse biologic effects by binding into two high-affinity cell surface receptors: TNF receptor 1 (TNFR1, p55 TNFR) and TNF receptor 2 (TNFR2, p75 TNFR). Both types of TNFRs are expressed by almost all cell types, including macrophages, lymphocytes, neutrophils and fibroblasts (Aderka 1996). TNFR1 and R2 have differences in both their extracellular and cytoplasmic domains and thus are functionally distinct. TNF signalling to cells is largely mediated by TNFR1, while the main function of TNFR2 is thought to enhance this activity by binding TNF and then passing it onto the TNFR1, i.e. ligand passing.

Thus, TNFR2 would increase the sensitivity of a cell to TNF stimulation and enhance the TNFR1-mediated response. Soluble forms of TNF receptors are generated by proteolytic cleavage of the extracellular domain of TNFR1 and R2. This soluble TNFR1 and R2 (sTNFR1 and R2) can compete for TNF with the cell surface receptors and block the TNF-α activity (Aderka 1996). Thus, the role of TNFR1 and R2 is not limited to signal transduction but includes extracellular regulatory functions affecting TNF bioavailability. The potential biological and clinical significance of these receptors has been reported in inflammatory, infectious, malignant and autoimmune disorders such as systemic lupus erythematosus and rheumatoid arthritis (RA) (Cope et al. 1992, Hayashi 2003).

With regard to periodontal disease, it has been reported that sulcular epithelial cells, monocyte/macrophage-like cells, fibroblasts and endothelial cells express TNFR1 and R2 as well as TNF-α (Tervahartiala et al. 2001). Assuma et al. (1998), Graves et al. (1998) and Delima et al. (2001) indicated that sTNFR2 significantly reduced the loss of connective tissue attachment and the loss of alveolar bone in experimental periodontitis. In addition, Ohe et al. (2000) reported that both TNF-α and interleukin-1β (IL-1β) upregulated the gene expression of TNFR2 and did not affect that of TNFR1. These cytokines upregulated the release of sTNFR2 from human gingival fibroblasts but not that of sTNFR1. These findings suggest that TNFR2 and its soluble products may modulate TNF-α-mediated inflammatory responses in periodontal disease. Recently, we have reported that the genetic polymorphism of the TNFR2 gene is associated with severe chronic periodontitis (CP) in Japanese. The frequency of the TNFR2 (+587G) polymorphic alleles are significantly increased in Japanese nonsmoking patients with severe CP (Shimada et al. 2004). However, there are no reports yet on the relationship between TNF-α, sTNFR1, R2 and the severity of periodontitis in vivo.

Therefore, in this study, we examined the levels of TNF-α, sTNFR1 and R2 in GCF and serum of healthy subjects and patients with CP. Then, we investigated the relationship between the levels of these cytokines in periodontal health and disease.

Material and Methods

Subjects

In total, 16 healthy subjects and 22 untreated patients with moderate to severe CP were recruited for this study. They had been referred to the Periodontal Clinic of the Niigata University Medical and Dental Hospital, and agreed to participate in this research.

Subjects were selected from both genders, with more than 20 teeth present, and with no relevant systemic illness, pregnancy or recent medication. CP patients had not used mouthrinses or pulsed oral irrigators on a regular basis. All subjects were Japanese and nonsmokers. None of them had received any periodontal therapy within the preceding 3 years. The study was approved by the Institutional Review Board of the Niigata University Faculty of Dentistry, and in accordance with the Helsinki declaration, a written informed consent was obtained from every participant before inclusion in the study.

Clinical assessments

All subjects were evaluated clinically and radiographically at the first visit to assess the following periodontal measurements: number of teeth, probing pocket depth (PPD), clinical attachment level (CAL), bleeding on probing, alveolar bone loss (BL), plaque index (PlI) and gingival index (GI). Six sites were examined on each tooth: mesio-buccal, buccal, disto-buccal, disto-lingual, lingual and mesio-lingual. One calibrated examiner took all measurements and recorded the results. Full-mouth radiographs were evaluated by two calibrated readers to examine alveolar bone levels on the mesial and distal aspects of each tooth. BL was measured from the cemento-enamel junction to the root apex at an arbitrary point on a radiograph, and expressed as a percentage of the total root length (Schei et al. 1959).

Collection of serum and GCF

Serum and GCF sampling was performed before commencing the initial periodontal treatment. Serum was obtained from clotted venous blood samples by centrifugation at $2000 \times g$ for 20 min. and then stored at -80°C until use.

A total of 73 sites, consisting of 16 sites from 16 healthy subjects and 57 sites from 22 CP patients, were selected on the basis of clinical examination. The PPD sites were grouped into $\leqslant 3$, $= 4$–6 and $\geqslant 7$ mm. One representative site per group was selected from each patient. Among the CP patients, 13 had PPD of $\leqslant 3$ to $\geqslant 7$ mm, six had PPD of $\leqslant 3$ to 4–6 mm and three had PPD of 4–6 to $\geqslant 7$ mm. We were not able to measure the GCF levels in the $\leqslant 3$ mm pockets of the last three patients mentioned above because these pockets were located in the posterior areas of the upper and lower jaws that were continuously flooded with saliva. To avoid contamination that could compromise our results, these pockets were excluded. This method was also used in choosing the appropriate representative site per PPD group.

After isolating the tooth with a cotton roll, supragingival plaque was removed with curettes, without touching the marginal gingiva. The crevicular site was then gently dried with an air syringe and the GCF was collected with Periopaper filter paper strips.

The strips were placed into the pocket until mild resistance was sensed and left in place for 30 s. Samples visually contaminated with blood or diluted by saliva during the sampling time were discarded and after 30 s, a new strip was inserted until no contamination was visible. The procedure was repeated four times in the same site. The volume of GCF was measured by means of a pre-calibrated Periotoron 6000 (HARCO Electronics Ltd., Winnipeg, Canada).

After recording the measurements, they were converted into actual GCF volumes using a calibration graph. The GCF samples were placed in tubes with a transport medium containing 200 μl of phosphate-buffered saline without calcium chloride and magnesium chloride with 0.5% bovine serum albumin. The tubes were vortexed for 10 min. and then centrifuged for 10 min. at $10{,}000 \times g$. Samples were stored at -80°C.

Measurement of TNF-α, sTNFR1 and R2 in GCF and serum

Levels of TNF-α, sTNFR1 and R2 in serum and GCF samples were determined by using a commercially available enzyme-linked immunosorbant assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN, USA). These are standard ''sandwich'' ELISAs and were performed according to the manufacturer's instructions using human recombinant standards. These assays have a lower limit of detection for TNF-α at 0.5 pg/ml, and 80 pg/ml for sTNFR1 and R2.

Concentrations of the cytokine and its soluble receptors in 200 μl sample were determined by generation of a standard curve for comparison. We reported the results of GCF data as the total amount of cytokine and concentrations in picogram per site.

Concentrations (picograms/microlitre) of the cytokine and its soluble receptors in GCF were calculated from the volume of GCF estimated from the calibration unit reading according to the following formula (Vernal et al. 2004):

$$\text{Concentration} = \text{total amount (pg)} / \text{GCF volume } (\mu\text{l})$$

Statistical analysis

All data analyses were performed using a statistical package (Stat View J-4.5

application program, SAS Institute Inc., Cary, NC, USA).

Differences in clinical parameters of each subject, concentrations and ratios of TNF-α, sTNFR1 and R2 in serum between healthy subjects and CP patients were compared by using the Mann–Whitney *U*-test.

Analyses of the clinical parameters of each site and of the total amounts and concentrations of TNF-α, sTNFR1 and R2 and sTNFR2/R1 ratio in GCF were performed in the PPD ⩽ 3 mm sites of healthy subjects and in the PPD ⩽ 3, 4–6 and > 7 mm sites of CP patients using Kruskal–Wallis test. Moreover, intra-group analysis was performed using the Mann–Whitney *U*-test and adjusted by Bonferroni's correction in order to compensate for the multiple comparisons.

Spearman's rank correlation analysis was used to analyse the correlations among the total amount of TNF-α, sTNFR1 and R2 in GCF, between the total amount of sTNFR1 and R2 and PPD of sampling sites and between sTNFR2/R1 ratio and PPD of sampling sites.

Subject-based analysis was also carried out to further clarify the relationship between the mean BL and mean PPD (representing the individual periodontal status) of each CP patient, and their individual sTNFR1 and R2 levels and sTNFR2/R1 ratio in the GCF of each sampled site. The sites were grouped into three: PPD ⩽ 3 mm, PPD = 4–6 mm and PPD ⩾ 7 mm. The mean sTNFR1 and R2 of each subject was computed, and the results were then correlated with the corresponding mean PPD. A significant association was noted ($p < 0.05$) after using Spearman's rank correlation.

Results

Clinical characteristics of healthy subjects and CP patients

The clinical parameters of healthy subjects and patients with CP are presented in Table 1.

There were significant differences between healthy subjects and CP patients in all clinical parameters except age.

Table 1. Clinical parameters of healthy subjects and patients with chronic periodontitis

	Healthy ($n = 16$)	Chronic periodontitis ($n = 22$)	*p*-value
Age (years)	52.4 ± 3.1	57.6 ± 1.7	NS
Male/female	4/12	3/19	–
Mean PPD (mm)	2.3 ± 0.1	3.4 ± 0.2	< 0.0001
Mean CAL (mm)	2.4 ± 0.1	3.9 ± 0.2	< 0.0001
Mean BL (%)	10.8 ± 0.4	30.5 ± 1.9	< 0.0001
Mean PlI	0.4 ± 0.0	0.7 ± 0.1	0.0003
Mean GI	0.2 ± 0.0	0.9 ± 0.1	< 0.0001

Values represents the mean ± SE.
PPD, probing pocket depth; CAL, clinical attachment level; BL, bone loss; PlI, plaque index; GI, gingival index; NS, not significant.

Levels of TNF-α, sTNFR1 and R2 in serum

Table 2 shows the levels and ratios of TNF-α, sTNFR1 and R2 in serum of healthy subjects and CP patients. There were no significant differences between healthy subjects and CP patients in the levels of TNF-α, sTNFR1 and R2 in serum. As the biological activity of TNF-α appears to be related to the ratio of its soluble receptors in vivo (Rooney et al. 2000), we calculated the sTNFR1/TNF-α, sTNFR2/TNF-α and sTNFR2/R1 ratios. No significant differences in sTNFR1/TNF-α, sTNFR2/TNF-α ratios were found between the two groups. The sTNFR2/R1 ratio was lower in the CP patients but the difference was not significant ($p = 0.051$).

Table 2. Concentrations and ratios of TNF-α, sTNFR1 and R2 in serum of healthy subjects and patients with chronic periodontitis

	Healthy ($n = 16$)		Chronic periodontitis ($n = 22$)		*p*-value
	mean ± SE	median (IR)	mean ± SE	median (IR)	
TNF-α (pg/ml)	1.7 ± 0.2	1.6 (0.5)	1.8 ± 0.2	1.7 (0.6)	NS
sTNFR1 (pg/ml)	871.4 ± 66.7	787.0 (298.5)	934.9 ± 37.9	965.0 (156.0)	NS
sTNFR2 (pg/ml)	1687.3 ± 139.6	1506.0 (434.5)	1587.8 ± 97.0	1486.0 (491.0)	NS
sTNFR1/TNF-α ratio	541.2 ± 40.1	533.5 (190.4)	596.4 ± 49.8	574.4 (213.7)	NS
sTNFR2/TNF-α ratio	1055.1 ± 89.6	993.7 (411.6)	1042.0 ± 122.2	898.7 (607.5)	NS
sTNFR2/R1 ratio	1.9 ± 0.1	2.0 (0.4)	1.7 ± 0.1	1.8 (0.6)	NS

SE, standard error; IR, inter-quartile range; TNF-α, tumour necrosis factor-α; sTNFR1, soluble tumour necrosis factor receptor 1; sTNFR2, soluble tumour necrosis factor receptor 2; NS, not significant.

Table 3. Clinical parameters and GCF volume in each group

	Healthy ($n = 16$)	Chronic periodontitis			*p*-value		
		PPD ⩽ 3 mm ($n = 19$)	PPD = 4–6 mm ($n = 22$)	PPD ⩾ 7 mm ($n = 16$)	PPD ⩽ 3 mm *versus* PPD = 4–6 mm	PPD = 4–6 mm *versus* PPD ⩾ 7 mm	PPD ⩽ 3 mm *versus* PPD ⩾ 7 mm
PPD (mm)	2.7 ± 0.1	2.9 ± 0.1	5.0 ± 0.2**	7.9 ± 0.3**	< 0.0001	< 0.0001	< 0.0001
CAL (mm)	2.6 ± 0.1	3.4 ± 0.2**	5.4 ± 0.3**	8.5 ± 0.5**	< 0.0001	< 0.0001	< 0.0001
PlI	0.0 ± 0.0	0.6 ± 0.2*	1.0 ± 0.2**	1.1 ± 0.1**	NS	NS	NS
GI	0.0 ± 0.0	0.6 ± 0.1**	1.3 ± 0.1**	1.5 ± 0.1**	0.0003	NS	< 0.0001
BL (%)	8.1 ± 1.3	19.7 ± 3.4**	30.5 ± 3.3**	57.2 ± 5.1**	0.0096	0.0002	< 0.0001
BOP	0/16	3/19	16/22	9/16	–	–	–
GCF volume (μl)	0.8 ± 0.1	1.1 ± 0.1	2.3 ± 0.3**	3.3 ± 0.3**	0.0007	NS	< 0.0001

Values represent the mean ± SE. PPD, probing pocket depth; CAL, clinical attachment loss; PlI, plaque index; GI, gingival index; BL, bone loss; BOP, bleeding on probing; GCF, gingival crevicular fluid; NS, not significant.
*$p < 0.0125$; **$p < 0.0025$ (compared with the healthy group).

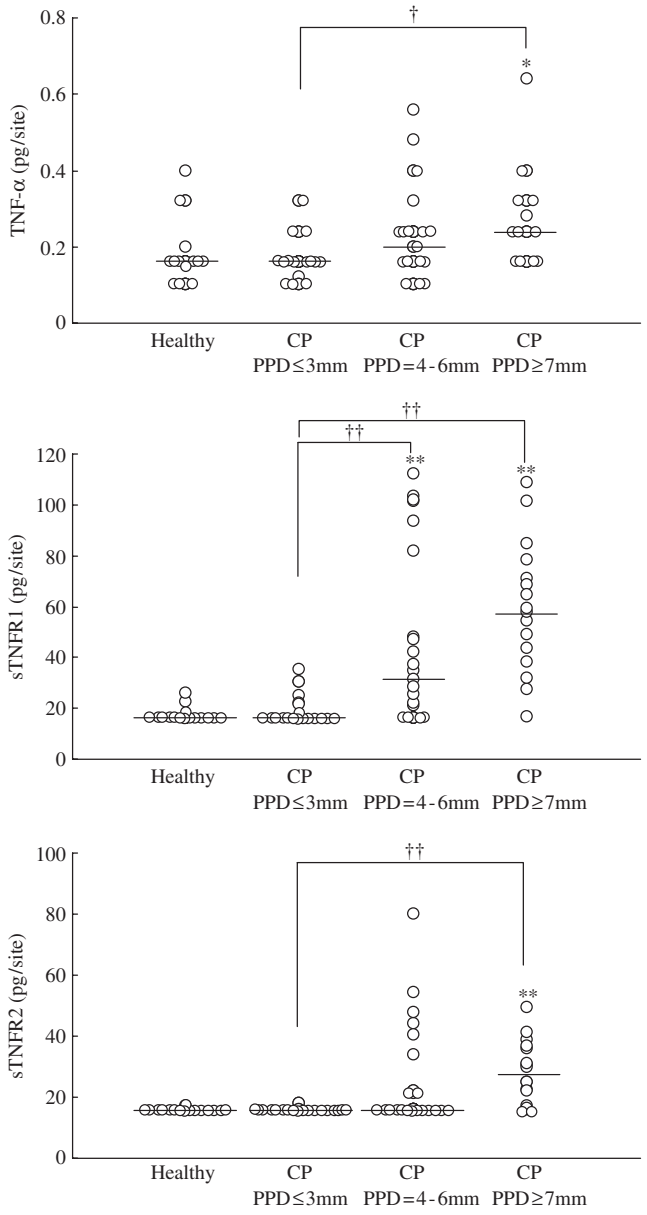

Fig. 1. Dot plots showing the total amounts of tumour necrosis factor-α (TNF-α), sTNFR1 and R2 in gingival crevicular fluid in healthy subjects with probing pocket depth (PPD) ⩽ 3 mm sites and in chronic periodontitis (CP) patients with PPD ⩽ 3 mm, PPD = 4–6 mm and PPD ⩾ 7 mm sites. The horizontal bars show the median value (enclosed in parentheses here) in picogram/site: TNF-α in healthy = 0.16 (0.05) PPD ⩽ 3 mm = 0.16 (0.09), 4–6 mm = 0.22 (0.08) and ⩾ 7 mm = 0.24 (0.16); sTNFR1 in healthy = 16.0 (0.0), PPD ⩽ 3 mm = 16.0 (0.0), 4–6 mm = 33.1 (65.6) and ⩾ 7 mm = 58.4 (33.7); sTNFR2 in healthy = 16.0 (0.0), PPD ⩽ 3 mm = 16.0 (0.0), 4–6 mm = 16.0 (18.0) and ⩾ 7 mm = 27.3 (16.7). Statistical analyses were performed with the Kruskal–Wallis test. Each two groups were compared using the Mann–Whitney *U*-test and adjusted by Bonferroni correction in order to compensate for the multiple comparisons (compared with the healthy group ($^{*}p < 0.0125$, $^{**}p < 0.0025$); $\dagger p < 0.0125$, $\ddagger p < 0.0025$).

Sites of GCF sampling

In total, 73 sites were included in this study. Clinical parameters and GCF volume of PPD ⩽ 3 mm sites of healthy subjects and PPD ⩽ 3, 4–6 and ⩾ 7 mm sites of CP patients are outlined in Table 3. GCF volume is expressed as the total volume collected for 30 s × 4 times. Clinical parameters significantly increased with increasing PPD (Kruskal–Wallis test: PPD, $p < 0.0001$; CAL, $p < 0.0001$; PII, $p < 0.0001$; GI, $p < 0.0001$; BL, $p < 0.0001$). Similar results were obtained in GCF volumes where the lowest amount was found in PPD ⩽ 3 mm sites of healthy subjects and highest in PPD ⩾ 7 mm of CP patients (Kruskal–Wallis test, $p < 0.0001$). However, there was no difference between the PPD ⩽ 3 mm sites of healthy subjects and CP patients.

Levels of TNF-α, sTNFR1 and R2 in GCF samples

Site-based analysis

A positive correlation was observed between the total amounts of TNF-α and sTNFR1 and between the total amounts of TNF-α and sTNFR2 in GCF samples (Spearman's rank order: $r_s = 0.42$; $p = 0.0004$, $r_s = 0.42$; $p = 0.0004$, respectively). Moreover, there was a highly significant correlation between the total amount of sTNFR1 and R2 in GCF samples (Spearman's rank order: $r_s = 0.87$; $p < 0.0001$).

Total amounts of TNF-α, sTNFR1 and R2 in GCF samples of healthy and diseased sites are presented in Fig. 1. All of them showed statistical differences between the PPD ⩽ 3 mm sites of healthy and CP subjects, and the PPD = 4–6 and ⩾ 7 mm sites of CP patients (Kruskal–Wallis test: TNF-α, $p = 0.013$; sTNFR1, $p < 0.0001$; sTNFR2, $p < 0.0001$). However, the difference in the total amount of TNF-α, sTNFR1 and R2 in PPD ⩽ 3 mm between healthy and CP patients was not significant. CP intra-group comparisons revealed that the difference in their total amount steadily rose with increasing pocket depths. TNF-α level showed a 1.3- and 1.6-fold increase in the sites with PPD = 4–6 and PPD ⩾ 7 mm compared with the site with PPD ⩽ 3 mm, respectively.

The same could be observed in the sTNFR1 and R2 levels, wherein PPD = 4–6 mm and PPD ⩾ 7 mm showed a sharp 2.4- and 3.0-, and 1.6- and 1.7-fold increase, respectively.

The concentrations of TNF-α, sTNFR1 and R2 in GCF samples of healthy and diseased sites are presented in Table 4. The levels of TNF-α and sTNFR2 between the healthy sites and the PPD = 4–6 mm of CP patients, and between the healthy sites and ⩾ 7 mm sites of CP patients showed statistical differences (Kruskal–Wallis test: TNF-α, $p = 0.0002$; sTNFR2, $p < 0.0001$). Intra-group analyses revealed that TNF-α and sTNFR2 levels in CP patients were significant in the PPD ⩽ 3 mm *versus* PPD ⩾ 7 mm only.

Table 4. Concentrations of TNF-α, sTNFR1 and R2 in GCF of each group

	Healthy ($n = 16$): median (IR)	Chronic periodontitis: median (IR)			*p*-value		
		PPD $\leqslant$3 mm ($n = 19$)	PPD = 4–6 mm ($n = 22$)	PPD $\geqslant$7 mm ($n = 16$)	PPD $\leqslant$3mm *versus* PPD = 4–6 mm	PPD = 4–6 mm *versus* PPD $\geqslant$7 mm	PPD $\leqslant$3mm *versus* PPD $\geqslant$7 mm
TNF-α (pg/μl)	0.32 (0.25)	0.15 (0.07)	0.11 (0.13)*	0.08 (0.04)**	NS	NS	0.0011
sTNFR1 (pg/μl)	27.0 (17.3)	18.8 (8.0)	20.0 (8.9)	17.5 (5.4)	NS	NS	NS
sTNFR2 (pg/μl)	24.9 (17.5)	18.8 (8.8)	11.6 (9.1)**	8.2 (3.4)**	NS	NS	0.0003

IR, inter-quartile range; TNF-α, tumour necrosis factor-α; sTNFR1, soluble tumour necrosis factor receptor 1; sTNFR2, soluble tumour necrosis factor receptor 2; NS, not significant.
*$p < 0.0125$; **$p < 0.0025$ (compared with the healthy group).

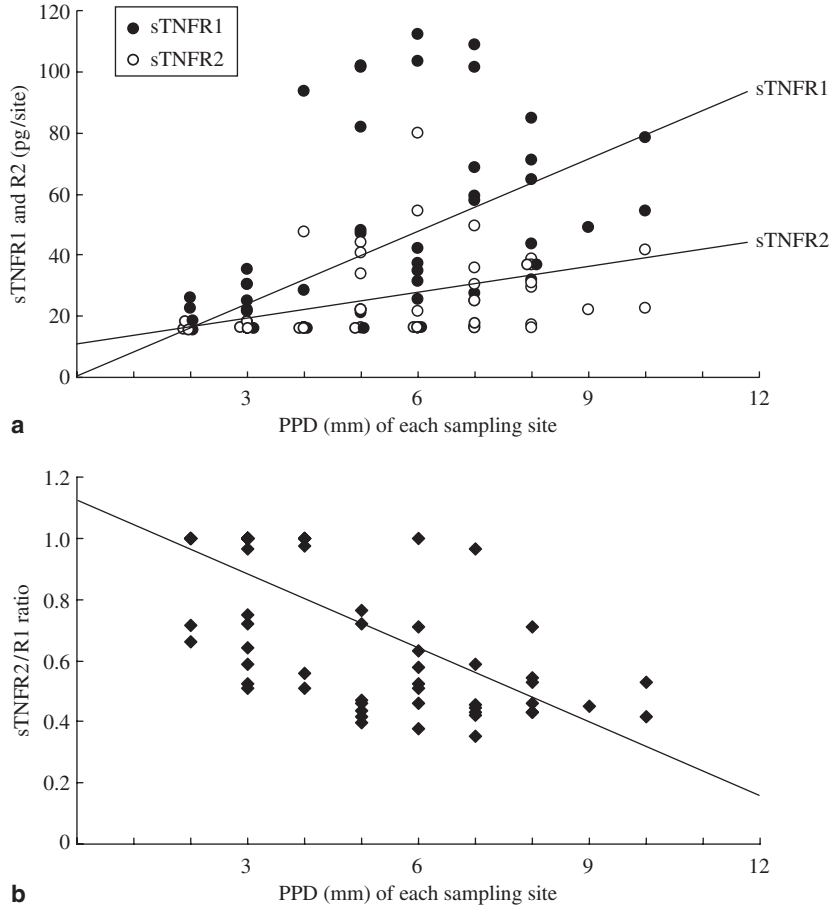

Fig. 2. (a) Correlation between probing pocket depth (PPD) (mm) of each sampling site and sTNFR1 and R2 in gingival crevicular fluid (GCF). Statistical analysis was performed using Spearman's rank order (sTNFR1, $r_s = 0.72$; $p < 0.0001$, sTNFR2, $r_s = 0.63$; $p < 0.0001$). (b) Correlation between PPD (mm) of each sampling site and sTNFR2/R1 ratio in GCF. Statistical analysis was performed using Spearman's rank order ($r_s = -0.71$, $p < 0.0001$).

We analysed the relationship between sTNFR1 and R2 and PPD (Fig. 2a). The GCF levels of sTNFR1 and R2 significantly increased with increasing pocket depths (Spearman's rank order R1: $r_s = 0.72$, $p < 0.0001$; R2: $r_s = 0.63$, $p < 0.0001$). However, their levels progressively diverged as the pocket depths increased, with the sTNFR2 level being comparatively lower than sTNFR1. The imbalance between the two sTNFRs was therefore clearly demonstrated in this study. We then calculated the correlation between sTNFR2/R1 ratio and PPD (Fig. 2b). The sTNFR2/R1 ratio significantly decreased as the PPD values increased (Spearman's rank order: $r_s = -0.71$, $p < 0.0001$).

Subject-based analysis

As host response is a critical factor in periodontitis, we tried to analyse the association between the severity of periodontitis represented as the mean BL and mean PPD, and levels of TNF-α, sTNFR1 and R2 and sTNFR2/R1 ratio in the GCF of each CP patient. We also wished to determine whether variations of their levels within the same PPD in different subjects would correlate with the mean BL and mean PPD.

The correlation between the mean BL of each patient and total amounts of TNF-α, sTNFR1 and R2 and sTNFR2/R1 ratio in the GCF of each CP patient's sampling site grouped into PPD $\leqslant$ 3 mm, PPD = 4–6 mm and PPD $\geqslant$ 7 mm was not significant in all of the sampling site groups. On the other hand, significant results were obtained between the mean PPD and the total amounts of sTNFR1 and R2 in the 4–6 mm site group (Fig. 3) (Spearman's rank order R1: $r_s = 0.55$, $p = 0.012$; R2: $r_s = 0.69$, $p = 0.002$) as well as in the $\geqslant$7 mm site group (Spearman's rank order R1: $r_s = 0.74$, $p = 0.004$; R2: $r_s = 0.53$, $p = 0.04$, respectively). The GCF levels of sTNFR1 and R2 progressively diverged as the mean PPD increased, with the sTNFR2 level being comparatively lower than sTNFR1. The ratios of TNFR2/R1 in the 4–6 and $\geqslant$7 mm site groups decreased as the mean PPD increased.

However, the correlations were not significant. To confirm the effect of sTNFR1 and R2 total amounts and ratio in GCF on an individual basis, we computed the mean sTNFR1 and R2 of each subject (collection method is described in ''Materials and Methods'') and tried to correlate the results with the corresponding mean PPD (Fig. 4). Highly significant results were obtained both in the sTNFR1 and R2 levels and

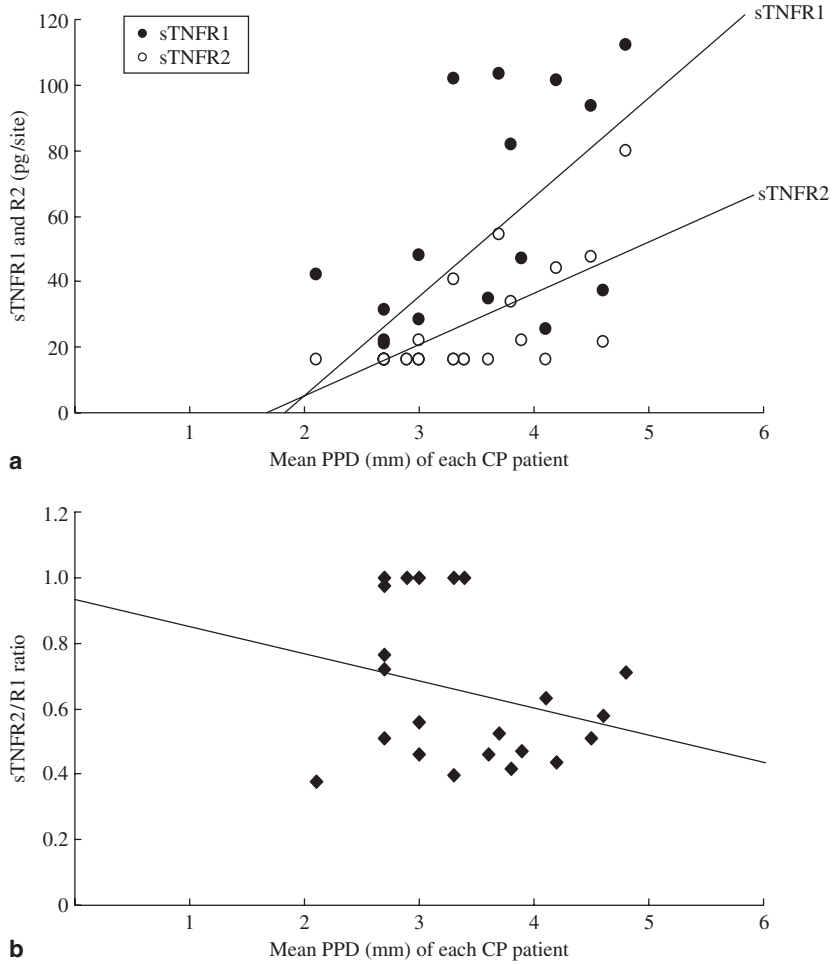

Fig. 3. (a) Correlation between sTNFR1 and R2 in gingival crevicular fluid (GCF) from probing pocket depth (PPD) = 4–6 mm site group and mean PPD of each in chronic periodontitis (CP) patient. Statistical analysis using Spearman's rank order (sTNFR1, $r_s = 0.55$; $p = 0.012$, sTNFR2, $r_s = 0.69$; $p = 0.0015$). (b) Correlation between sTNFR2/R1 ratio in GCF from the PPD = 4–6 mm site group and mean PPD of each CP patient.

ratio and mean PPD (Spearman's rank order R1: $r_s = 0.78$, $p < 0.0001$; R2: $r_s = 0.78$, $p < 0.0001$; R2/R1 ratio: $r_s = -0.71$, $p < 0.0001$).

Discussion

In this study, we investigated the relationship among TNF-α, sTNFR1 and R2 in GCF and serum samples of healthy subjects and CP patients.

We analysed the total amount and concentration (in picograms) of TNF-α, sTNFR1 and R2 in GCF collected from four consecutive insertions of paper points at 30 s each in the same site (Table 4, Fig. 1). Collecting a standard amount of GCF is essential to express the results as concentration because GCF volume is very small and exhibits wide variations. However, several studies have indicated that the expression of GCF data as the total amount per standardized sampling time is a more sensitive way than reporting them as concentration, and should be used when estimating periodontal disease activity (Lamster et al. 1986, Tsai et al. 1995).

The total amounts of TNF-α, sTNFR1 and R2 in GCF significantly elevated with increasing PPD values ($p < 0.05$) (Fig. 1). The measurement of sTNFR1 and R2 levels in GCF of periodontitis patients has not yet been reported. There were several reports concurrent with our data concerning TNF-α level (Lee et al. 1995, Engebretson et al. 1999, Gamonal et al. 2003), which was present in very low concentrations in the GCF (Rossomando et al. 1990, Rossomando & White 1993). In this study, we detected the level of TNF-α in GCF using high sensitive ELISA with the minimum detectable by dose set at 0.5 pg/ml. On the other hand, the levels of sTNFR1 and R2 in GCF were detected by normal ELISA kit measuring down to 80 pg/ml. The levels of sTNFR1 and R2 in GCF significantly correlated with the level of TNF-α ($r_s = 0.42$, 0.42, respectively). Our results support the possibility that measurement of sTNFR1 and R2 instead of TNF-α in GCF may be a useful marker for diagnosis of and monitoring periodontal disease activity. However, further studies are needed to confirm our findings.

Joshipura et al. (2004) have measured sTNFR1 and R2 levels in serum of 468 men who were free of cardiovascular disease, diabetes and cancer. There was no difference in the soluble receptor levels between subjects with and without periodontitis. In our serum data, there was also no difference in levels of TNF-α, sTNFR1 and R2 between healthy subjects and CP patients (Table 2). However, the sTNFR2/R1 ratio in serum was lower but not significant ($p = 0.051$) in CP patients than in healthy subjects, and their ratio in GCF significantly decreased as the PPD values increased (Fig. 2b). GCF is a complex mixture of substances derived from serum, leucocytes, structural cells of the periodontium and oral bacteria. Serum will provide the basic cytokine profile of gingival tissue and crevicular fluids, as there is a constant flow of fluid from the blood into the gingival crevice (Uitto 2003). Therefore, sTNFR2/R1 ratio in the serum might reflect their ratio in the GCF of periodontitis patients as well.

Regarding the inter-relationship between the levels of TNF-α and sTNFRs in the local area of inflammatory diseases, Rooney et al. (2000) reported their levels in synovial fluid samples from 45 children with juvenile chronic arthritis. They indicated that TNF-α, sTNFR1 and R2 levels in synovial fluid were higher in the severe (polyarticular) group than in the mild (pauciarticular) group. In periodontitis, our findings also exhibited elevated TNF-α, sTNFR1 and R2 levels in GCF of severe periodontal pocket sites compared with healthy sites and the sTNFR2/R1 ratio in GCF of periodontitis significantly decreased as the PPD values increased (Figs 1 and 2).

Interestingly, the sTNFR2/R1 ratio in synovial fluid of arthritis seemed to decrease in the severe group as well. Thus, it seems that juvenile chronic arthritis, a destructive inflammatory dis-

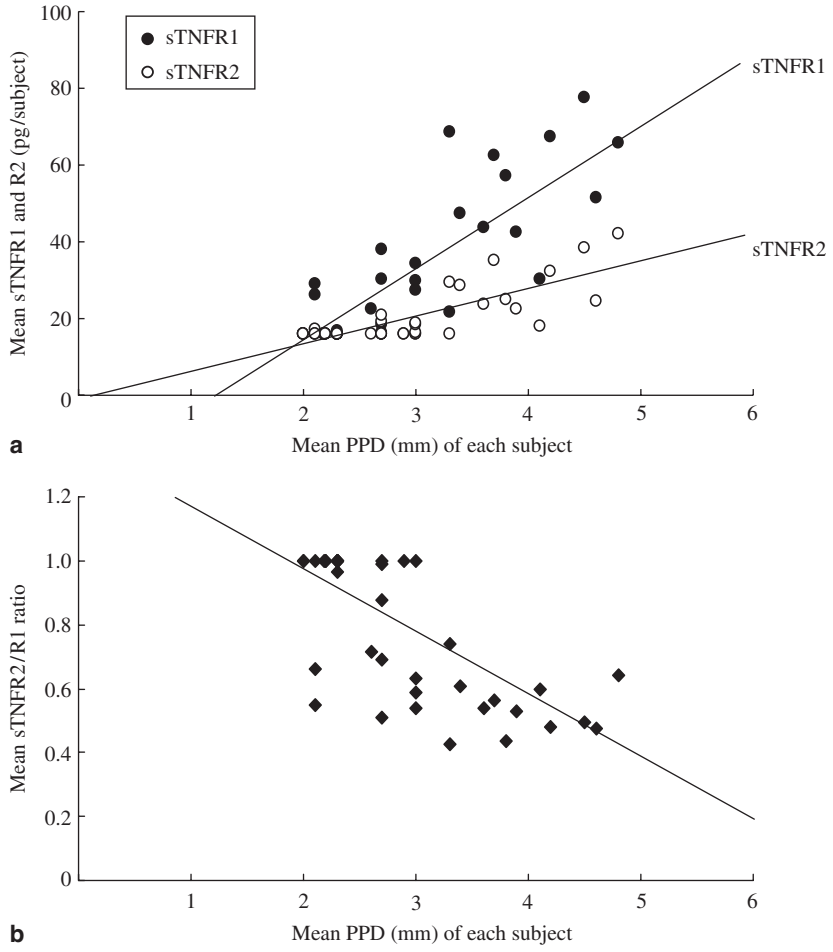

Fig. 4. (a) Correlation between the mean sTNFR1 and R2 in gingival crevicular fluid (GCF) and the mean probing pocket depth (PPD) of each patient. Statistical analysis using Spearman's rank order (sTNFR1, $r_s = 0.78$; $p < 0.0001$, sTNFR2, $r_s = 0.78$; $p < 0.0001$). (b) Correlation between the mean sTNFR2/R1 ratio in GCF and the mean PPD of each patient. Statistical analysis was performed using Spearman's rank order ($r_s = -0.71$; $p < 0.0001$).

ease of the joint, has the same local inflammatory response as periodontitis.

We have indicated that the GCF levels of sTNFR1 and R2 significantly rose with the increasing PPD. However, their levels progressively diverged as the pocket depths increased, with the sTNFR2 level being comparatively lower than sTNFR1 (Fig. 2a). We also revealed that the sTNFR2/R1 ratio significantly decreased as the PPD values increased (Fig. 2b). Therefore, the imbalance between the two sTNFRs in GCF could be associated with periodontitis lesions. The two sTNFRs were proven to neutralize the activity of TNF-α by competition with the membrane-bound TNFRs and act as antagonists (Aderka 1996). In particular, sTNFR2 would suppress periodontal attachment loss and alveolar bone destruction in experimental periodontitis (Assuma et al. 1998, Graves et al. 1998, Delima et al. 2001), and the release of sTNFR2 from human gingival fibroblast under inflammatory conditions may contribute to the inactivation of circulating TNF-α, leading to the downregulation of periodontal disease (Ohe et al. 2000). These findings imply that an insufficient increase of sTNFR2 level in GCF compared with elevated sTNFR1 level may result in a decreased sTNFR2/R1 ratio, which in turn could lead to the progression of periodontitis. This suggests the possibility that the imbalance between the two sTNFRs could be resolved by supplementing the insufficient sTNFR2 in the gingival tissues and GCF as an adjunctive therapy, resulting in the improvement of periodontitis.

To examine whether individual levels of TNF-α and sTNFRs in GCF reflect the general periodontal status, a subject-based correlation analysis was carried out. As shown in Fig. 3, the mean PPD and the sTNFR1 and R2 were positively correlated in both the 4–6 and the $\geqslant 7$ mm sites. Furthermore, the sTNFR1 and R2 levels and ratio showed similar results as the site-based analysis. It could also be noted that pockets with similar depths do not necessarily produce the same amount of sTNF receptors. These findings reflect the differences in periodontal disease activity not only locally within the GCF-sampling sites but also generally on an individual basis (Fig. 4). Such differences could result from individual host immune responses as modulated by cellular components, proteolytic host cell enzymes, inflammatory mediators and others in periodontitis-affected sites between subjects. But considering that our results revealed that there was a correlation between the decreasing sTNFR2/R1 ratio and increasing mean PPD, it could be hypothesized that although personal differences do occur in the production of the sTNF receptors in periodontal pockets, their ratio could possibly predict the susceptibility to and progression of periodontitis.

In RA, it is known that the disease state is modulated by IL-1, TNF-α and TNF-α antagonists (e.g. sTNFR1 and R2) can arrest the disease in both animal and human studies (Moreland 1998). Etanercept, a recombinant sTNFR2: Fc fusion protein, has demonstrated excellent safety and efficacy in large-scale, randomized, double-blind, placebo-controlled trials of patients with RA and juvenile RA (Garrison & McDonnell 1999).

Therefore, it is suggested that a recombinant sTNFR2 or etanercept could one day be applied to treat periodontal diseases. However, the method, timing and duration of administration suitable for periodontal therapy and potential untoward side effects would need to be investigated in order to make use of such cytokine antagonists practical.

In conclusion, our study has revealed that TNF-α, sTNFR1 and R2 in GCF are significantly increased in CP and the sTNFR2/R1 ratio decreases with increasing PPD values. The imbalance between sTNFR1 and R2 levels in GCF could be related to the severity of CP. A large prospective study would be needed to establish the relationship between TNF-α, sTNFR1 and R2, and disease activity of periodontal disease.

Acknowledgements

This study was supported by Grant-in Aids for Scientific Research (16,390,612 17,791,553) from the Ministry of Education, Science Sports and Culture of Japan, and the fund for promotion of science from Tanaka Industries Co. Ltd. (Niigata, Japan).

References

Aderka, D. (1996) The potential biological and clinical significance of the soluble tumor necrosis factor receptors. *Cytokine and Growth Factor Reviews* **7**, 231–240.

Assuma, R., Oates, T., Cochran, D., Amar, S. & Graves, D. T. (1998) IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. *The Journal of Immunology* **160**, 403–409.

Cope, A. P., Aderka, D., Doherty, M., Engelmann, H., Gibbons, D., Jones, A. C., Brennan, F. M., Maini, R. N., Wallach, D. & Feldmann, M. (1992) Increased levels of soluble tumor necrosis factor receptors in the sera and synovial fluid of patients with rheumatic diseases. *Arthritis and Rheumatism* **35**, 1160–1169.

Delima, A. J., Oates, T., Assuma, R., Schwartz, Z., Cochran, D., Amar, S. & Graves, D. T. (2001) Soluble antagonists to interleukin-1 (IL-1) and tumor necrosis factor (TNF) inhibits loss of tissue attachment in experimental periodontitis. *Journal of Clinical Periodontology* **28**, 233–240.

Engebretson, S. P., Lamster, I. B., Herrera-Abreu, M., Celenti, R. S., Timms, J. M., Chaudhary, A., Giovine, F. & Kornman, K. (1999) The influence of interleukin gene polymorphism on expression of interleukin-1β and tumor necrosis factor-α in periodontal tissue and gingival crevicular fluid. *Journal of Periodontology* **70**, 567–573.

Gamonal, J., Sanz, M., O'Connor, A., Acevedo, S., Suarez, I., Sanz, A., Martinez, B. & Silva, A. (2003) Delayed neutrophil apoptosis in chronic periodontitis patients. *Journal of Clinical Periodontology* **30**, 616–623.

Garrison, L. & McDonnell, N. D. (1999) Etanercept: therapeutic use in patients with rheumatoid arthritis. *Annals of the Rheumatic Diseases* **58**, 165–169.

Graves, D. T., Delima, A. J., Assuma, R., Amar, S., Oates, T. & Cochran, D. (1998) Interleukin-1 and tumor necrosis factor antagonists inhibit the progression of inflammatory cell infiltration toward alveolar bone in experimental periodontitis. *Journal of Periodontology* **69**, 1419–1425.

Hayashi, H. (2003) Serum tumor necrosis factor alpha and its soluble receptor levels in patients with systemic lupus erythematosus. *The Journal of Tokyo Medical University* **61**, 248–254.

Joshipura, K. J., Wand, H. C., Merchant, A. T. & Rimm, E. B. (2004) Periodontal disease and biomarkers related to cardiovascular disease. *Journal of Dental Research* **83**, 151–155.

Lamster, I. B., Oshrain, R. L. & Gordon, J. M. (1986) Enzyme activity in human gingival crevicular fluid: considerations in data reporting based on analysis of individual crevicular sites. *Journal of Clinical Periodontology* **13**, 799–804.

Lee, H.-J., Kang, I.-K., Chung, C.-P. & Choi, S.-M. (1995) The subgingival microflora and gingival crevicular fluid cytokines in refractory periodontitis. *Journal of Clinical Periodontology* **22**, 885–890.

Moreland, L. W. (1998) Soluble tumor necrosis factor receptor (p75) fusion protein (ENBREL) as a therapy for rheumatoid arthritis. *Rheumatic Diseases Clinics of North America* **24**, 579–591.

Ohe, H., Takashiba, S., Naruishi, K., Chou, H.-H., Yamada, H., Nishimura, F., Arai, H. & Murayama, Y. (2000) Tumor necrosis factor-α (TNF-α)-induced and interleukin-1β (IL-1β)-induced shedding of TNF receptors from gingival fibroblasts. *Journal of Interferon and Cytokine Research* **20**, 1077–1082.

Page, R. C., Offenbacher, S., Schroeder, H. E., Seymour, G. J. & Kornman, K. S. (1997) Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontology 2000* **14**, 216–248.

Rooney, M., Varsani, H., Martin, K., Lombard, P. R., Dayer, J.-M. & Woo, P. (2000) Tumor necrosis factor alpha and its soluble receptors in juvenile chronic arthritis. *Rheumatology* **39**, 432–438.

Rossomando, E. F., Kennedy, J. E. & Hadjimichael, J. (1990) Tumor necrosis factor alpha in gingival crevicular fluid as a possible indicator of periodontal disease in humans. *Archives of Oral Biology* **35**, 431–434.

Rossomando, E. F. & White, L. (1993) A novel method for the detection of TNF-alpha in gingival crevicular fluid. *Journal of Periodontology* **64**, 445–449.

Schei, O., Waerhaug, J., Lovdal, A. & Arno, A. (1959) Alveolar bone loss related to oral hygiene and age. *Journal of Periodontology* **30**, 7–16.

Shimada, Y., Tai, H., Endo, M., Kobayashi, T., Akazawa, K. & Yamazaki, K. (2004) Association of tumor necrosis factor receptor type 2+587 gene polymorphism with severe chronic periodontitis. *Journal of Clinical Periodontology* **31**, 463–469.

Tervahartiala, T., Koski, H., Xu, J.-W., Häyrinen-Immonen, R., Hietanen, J., Sorsa, T. & Konttinen, Y. T. (2001) Tumor necrosis factor-α and its receptors, p55 and p75, in gingiva of adult periodontitis. *Journal of Dental Research* **80**, 1535–1539.

Tsai, C.-C., Ho, Y.-P. & Chen, C.-C. (1995) Levels of interleukin-1β and interleukin-8 in gingival crevicular fluids in adult periodontitis. *Journal of Periodontology* **66**, 852–859.

Uitto, V.-J. (2003) Gingival crevice fluid – an introduction. *Periodontology 2000* **31**, 9–11.

Vernal, R., Chaparro, A., Graumann, R., Puente, J., Valenzuela, A. M. & Gamonal, J. (2004) Levels of cytokine receptor activator of nuclear factor kb ligand in gingival crevicular fluid in untreated chronic periodontitis patients. *Journal of Periodontology* **75**, 1586–1591.

Wilton, J. M. A., Bampton, J. L. M., Griffiths, G. S., Curtis, M. A., Life, J. S., Johnson, N. W., Powell, J. R., Harrap, G. J. & Critchley, P. (1992) Interleukin-1 beta (IL-1β) levels in gingival crevicular fluid from adults with previous evidence of destructive periodontitis. *Journal of Clinical Periodontology* **19**, 53–57.

Address:
Hiromasa Yoshie
Division of Periodontology
Niigata University Graduate School of Medical and Dental Sciences
5274, Gakkocho-Dori 2-ban-cho
Niigata 951-8514
Japan
E-mail: yoshie@dent.niigata-u.ac.jp

Clinical relevance

Scientific rationale: Periodontitis is modulated by TNF-α and TNF receptors; however, no studies on their GCF levels in humans have been reported as yet. In animal model, soluble TNF receptor 2 inhibited periodontal destruction.

Principal findings: GCF levels of soluble TNF receptors 1 and 2 significantly rose with increasing probing depths but receptor 2 level was lower, indicating that the imbalance between these receptors could be associated with the clinical conditions of periodontitis.

Practical implications: Soluble TNF receptors, particularly soluble TNF receptor type 2/type 1 ratio, may be new candidates as periodontal disease activity markers.