Bone resorbing activity and cytokine levels in gingival crevicular fluid before and after treatment of periodontal disease


Abstract

Background: The aim of the present study was to investigate bone resorption activity (BRA), interleukin-1α (IL-1α), IL-1β and interleukin-1 receptor antagonist (IL-1ra) in gingival crevicular fluid (GCF) in sites with no signs of periodontal disease and in sites with horizontal or angular loss of periodontal bone. These assessments were performed before and after periodontal treatment.

Methods: GCFs were collected from 10 individuals with filter strips from two healthy sites and four sites with deep pathological periodontal pockets, two of which showed horizontal bone loss and two with angular bone loss. All diseased pockets were treated with flap surgery and systemic Doxyferm®. Twelve months later GCF was collected again and treatment outcome evaluated. BRA in GCFs was assessed in a bone organ culture system by following the release of 45Ca from neonatal mouse calvariae. The amounts of IL-1α, IL-1β and IL-1ra in GCFs were quantified by enzyme-linked immunosorbent assay (ELISA).

Results: Treatment resulted in reduction of pocket depths with 3.5 ± 0.5 mm in sites with angular bone loss and 2.8 ± 0.3 mm in sites with horizontal bone loss. Initially, BRA, IL-1α, IL-1β and IL-1ra were significantly higher in GCFs from diseased sites compared with healthy sites. No differences in BRA and cytokine levels were seen between GCFs from pockets with horizontal and angular bone losses. The levels of IL-1α, IL-1β and IL-1ra were significantly reduced after treatment of diseased pockets. Pocket depths were significantly correlated to BRA only in pre-treatment sites with angular bone loss. BRA was correlated to IL-1α, IL-1β, but not to IL-1ra, in diseased sites with angular bone loss, before and after treatment. The reductions of BRA in the individual sites, seen after treatment, were not correlated to the reductions of IL-1α, IL-1β or IL-1ra.

Conclusions: These data show that BRA and cytokine levels are increased in GCFs from sites with periodontal disease and that periodontal treatment results in reduction of the cytokines. Our findings further indicate that IL-1α and IL-1β play important roles for the BRA present in GCFs, but that other factors also contribute to this activity.

Key words: bone resorption; inflammation; interleukin-1; periodontal disease

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and activity is required. Inflamed gingival tissue contains numerous types of inflammatory mediators, some of which are able to stimulate bone resorption and/or degradation of extracellular matrix. Several molecules present in inflammatory process have been found to stimulate bone resorption in vitro and in vivo, including interleukin-1 (IL-1), IL-6, IL-11, IL-17, oncostatin M (OSM), leukemia inhibitory factor (LIF), tumour necrosis factor (TNF-α, TNF-β), macrophage colony-stimulating factor (M-CSF), bradykinin, thrombin and prostaglandins (PGs) (Martin et al. 1998, Lerner & Lundberg 2002, Horowitz & Lorenzo 2002). The most potent proinflammatory cytokines stimulating bone resorption are interleukin-1α (IL-1α) and IL-1β (Martin et al. 1998, Horowitz & Lorenzo 2002). The IL-1 cytokine gene family has three members, IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra), which are glycoproteins of approximately 17 kDa and which are the products of three different genes. Two different receptor proteins recognising IL-1α, IL-1β and IL-1ra have been identified (Dinarello 1997a). Type I receptors are responsible for signal transduction and cell activation, whereas type II receptors, either membrane bound or soluble, have little or no agonist activity and therefore act as decoy receptors (Dinarello 1997b). Thus, the IL-1 receptor activity is decreased both by the type II receptors, which act as neutralising decoy receptors, and by IL-1ra, which binds to the IL-1 receptors, and thereby block IL-1 activation (Dinarello 1998).

IL-1 is a pleiotropic cytokine having multiple biological activities including stimulation of osteoclast recruitment and activation. IL-1 also stimulates fibroblast production of matrix metalloproteinases important for the degradation of non-mineralised extracellular tissue (West-Mays et al. 1995). In addition, IL-1 up-regulates expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, which makes it possible for the leukocytes to bind to and penetrate through the vascular wall into the area of inflammation (Scholz et al. 1996).

Mononuclear leukocytes are the cells mainly responsible for IL-1 production in inflammatory processes, but this cytokine can also be produced by a large variety of cells including gingival fibroblasts (Yucel-Lindberg et al. 1995).

Several studies have reported increased levels of inflammatory mediators such as IL-1 and prostaglandin E2 (PGE2) in gingival crevicular fluids (GCFs) from diseased sites with periodontal bone loss compared with healthy ones (Masada et al. 1990, Shapira et al. 1994, Rasmussen et al. 2000, Engebretson et al. 2002). Furthermore, GCF from diseased sites has been shown to stimulate bone resorption in vitro to a higher degree than GCFs from healthy sites (Lerner et al. 1998, Rasmussen et al. 2000) and one important factor responsible for this bone resorbing activity (BRA) seemed to be IL-1α.

The loss of bone tissue surrounding the tooth can either be of a horizontal or angular type. A pocket with an angular bone loss appears to be more prone to progression (Papapanou & Wennström 1991). Following non-surgical treatment of diseased pocket sites, probing pocket depth is less reduced over time in sites with an angular type of bone loss compared with pockets showing horizontal bone loss (Ehnevid & Jansson, 1998). Mechanisms responsible for the differences in progression between diseased sites with angular or horizontal bone loss are not known, but one possibility may be that differences in inflammatory mediators and BRA are involved. The aims of the present study were (i) to perform an intradividual study of the levels of IL-1α, IL-1β, IL-1ra and BRA activity in GCFs from healthy and diseased sites presenting horizontal or angular bone loss; (ii) to study the effect of periodontal treatment on the levels IL-1α, IL-1β, IL-1ra and BRA in GCFs; (iii) to analyse if there is any correlation between BRA and levels of IL-1α, IL-1β, IL-1ra and (iv) to investigate if BRA activity and levels of IL-1 in GCF can be related to probing pocket depth.

Materials and Methods

Materials

*Modification of minimal essential medium (MEM) was purchased from Gibco, Life Technologies Ltd, Paisley, UK; Whatman 3MM chromatography paper from Whatman Lab. Sales Ltd, Maidstone, UK; *[^3]Ca* from DuPont de Nemours, Brussels, Belgium; the ELISA kits for human IL-1α, IL-1β and IL-1ra from R&D Systems Europe Ltd, Abingdon, UK. Indomethacin was a kind gift by Merck, Sharp and Dohme BV, Haarlem, the Netherlands.

Subjects and site selection

The study group consisted of 10 adult patients, two women and eight men, mean age 51 (46–66 years) with moderate-to-advanced periodontitis; six were smokers. All patients were referred to the Department of periodontology at Givle county hospital for periodontal treatment. Prior to referral, the patients had received periodontal treatment by dental hygienist, including hygiene instruction, scaling and root planing without showing clinical signs of healing. To be included in the study, at least four sites with ongoing periodontal disease were required. Two of these sites had to present horizontal bone loss and two sites angular bone loss. Furthermore, two sites with no signs of periodontal disease were also required. Sites with alveolar bone loss >2 mm measured from cementoenamel junction to the alveolar crest on bitewing radiographs were regarded as marginal bone loss (Källenstal & Månsson 1989) and if the bone presented an intrasosseous defect ≥3 mm it was regarded as being of an angular type. Bone loss of a horizontal or angular type and the corresponding pocket depth ≥5 mm with bleeding on probing or pus was regarded as a site with ongoing disease. A site with bone loss, not exceeding 2 mm, pocket depth <3 mm and no bleeding on probing was regarded as a healthy site. From each patient, GCFs were harvested from two diseased sites with horizontal bone loss, two diseased sites with angular bone loss and two healthy sites, before and 1 year after treatment. All patients were treated with open flap surgery by the same surgeon and systemic antibiotics, Doxyferm® (Nordic Drugs, Limhamn, Sweden) 100 mg/day was administered for 15 days. After surgery, the patients were instructed to rinse twice a day with Corsodyl® (Glaxo SmithKline HB, Möln达尔, Sweden) 0.2% for 3 months. Professional hygiene procedure was performed every fortnight during the rinsing period. One year after treatment, GCFs were collected in nine of the subjects from the same sites that were selected prior to treatment and pocket depth at the sample sites was measured. After initial examination, one of the patients moved to Africa and could not further participate in the study. One
tooth with angular bone loss had to be extracted before the follow up examination due to acute infection. None of the patients was suffering from systemic illness. The patients had not received periodontal treatment or antibiotics within the preceding 6 months or any anti-inflammatory drugs 3 weeks prior to the study. All patients were informed and gave their consent to participate in the study. The study protocol was approved by the ethics committee for human research of Umeå University.

GCF sampling and eluation
GCFs were sampled using a filter paper technique. The paper strips were obtained by cutting Whatman 3 MM chromatography paper, to a dimension of 2 × 8 mm (Griffiths et al. 1988). Prior to sampling, supragingival bacterial plaque was removed from the tooth by cotton pellet and water. The area was isolated with cotton rolls and saliva ejector, to avoid saliva contamination and the gingival surface gently dried using an air syringe. A paper strip was inserted into the gingival crevice until mild resistance was felt and left in place for 30 s. The strip was then eluted with 200 μl of sterile NaCl as previously described (Rasmussen et al. 2000).

Bone resorption bioassay
Bone resorption was quantified by analysing the release of calcium from cultured neonatal mouse calvariae. The percentage release of 45Ca from pre-labelled bones was assessed by analysing the release of 32P from bones dissected from mice that at an age of 1–2 days were injected with 1.5 μCi 32P. Five days later, the mice were sacrificed and six bone fragments were micro-dissected from the parietal part of the calvariae (Ljunggren et al. 1991). Then the bones were preincubated in α-modification of MEM (α-MEM) containing 0.1% albumin and 1 μM indomethacin (Lerner 1987). After 18–24 h, the bones were extensively washed and placed in multiwell dishes containing 1.0 ml α-MEM (added 0.1% albumin), with or without sterile filtered GCF eluate. The bones were incubated for 96 h at 37 °C in a humidified atmosphere of 5% CO2 in air. At the end of the culture period, the amounts of isotope in bones and in media were analysed using a scintillation counter and the percentage release of isotope was calculated (Lerner 1987). The percentage release data were recalculated and the results expressed as percent of control, which was set at 100%. This allowed for accumulation of data from several experiments. The ethical committee at Umeå University approved the animal procedure.

Portions of GCF from healthy sites and from sites with either vertical or horizontal bone loss were diluted in α-MEM and assayed for bone resorbing activity. Each GCF sample was tested for its effect on bone resorption in five different bone fragments and the average of the percentage release in these five bones was compared with the average percentage release in five bones cultured in basic medium without any GCF (controls).

Enzyme-linked immunosorbent assay (ELISA) of IL-1α, IL-1β and IL-1ra
The IL-1α and IL-1β concentrations in GCF eluates were assessed by using commercially available ELISA kits. These assays detect levels of IL-1α and IL-1β as low as 0.3 pg/ml. According to the manufacturer, the assays do not show any significant cross-reactivity to a wide spectrum of different related cytokines. The concentrations of IL-1ra in GCF eluates were analysed using a commercially available ELISA kit and by following the instructions supplied by the manufacturer. The sensitivity of this assay is 14 pg/mL.

Statistical analyses
Data were collected and transferred to a spreadsheet program on a personal computer. For data analysing StatView 5.0 for windows was used. The values for BRA activity and IL-1 levels showed large standard deviations and skewed distribution. Wilcoxon’s signed rank test was used to investigate differences of BRA and levels of IL-1α, IL-1β and IL-1ra between diseased sites with different types of bone loss and healthy sites, further more to analyse changes of these parameters in the individual site before and after treatment. Bonferroni’s correction for multiple testing was taken into account where appropriate. The non-parametric Spearman coefficient for ranked data was used to analyse correlations between BRA activity and IL-1 levels and between BRA activity, IL-1 levels and probing pocket depth before and after treatment. p-Values less than 0.05 were considered to indicate statistical significance.

Results
In the initial analysis using GCFs harvested before treatment, the GCF eluates were tested in a series of different concentrations (0.01–1%) and the GCFs from diseased sites caused concentration-dependent stimulation of 45Ca release (data not shown), in agreement with previous observations (Lerner et al. 1998, Rasmussen et al. 2000). The GCF eluates harvested before treatment from sites with horizontal and vertical bone loss caused a 1.81- and 1.96-fold stimulation, respectively, of 45Ca release when tested at a concentration of 1% (Fig. 1a). There was no statistically significant difference between GCFs from sites with vertical and horizontal bone losses. In agreement with these data, there was likewise no significant difference between the bone resorbing activity in GCFs from sites with horizontal and vertical bone losses when assayed at a concentration of 0.3% (data not shown). GCFs from clinically healthy sites did not cause any stimulation of 45Ca release (Fig. 1a).

Of the 20 sites assayed for BRA before treatment, 18 healthy sites, 18 sites with horizontal bone loss and 17 sites with angular bone loss were assayed for BRA 12 months after

<table>
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<th>Table 1. Pocket depth before and after treatment</th>
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<td>vertical bone loss (mm) ± SE</td>
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<tr>
<td>horizontal bone loss (mm) ± SE</td>
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<td>initial</td>
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<td>8.1 ± 0.6</td>
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<td>4.7 ± 0.4</td>
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<td>3.8 ± 0.2</td>
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<td>difference initial – after treatment</td>
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<td>3.5 ± 0.5***</td>
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treatment. Of the 18 sites with horizontal bone loss, 15 showed a reduction of BRA after treatment, one exhibited increased BRA and two did not show any difference (Fig. 1b). In the 17 sites with angular bone loss, 13 sites showed a reduction of BRA after treatment and four an increase (Fig. 1c). When the 45Ca release values before and after treatment were compared in the both types of diseased sites, there was statistically significant difference between pre- and post-treatment values only for those with horizontal bone loss ($p<0.01$) (Figs. 1b,c). In healthy sites, no significant stimulation of 45Ca release was seen, neither before nor after treatment (Fig. 1d).

The amount of IL-1α in GCFs before and 12 months after treatment was statistically significant for both diseased sites ($p<0.001$).
IL-1α in GCFs from all horizontal and angular sites (Fig. 2b). The decrease was significant \((p<0.001)\) in both types of diseased sites. After treatment, there was no significant difference in IL-1α from healthy and diseased sites (Fig. 2b–d).

The amount of IL-1β in GCFs before treatment from sites with horizontal and angular bone loss was significantly \((p<0.001)\) higher compared with healthy sites (Fig. 3a). Treatment of sites with horizontal breakdown resulted in a reduction of IL-1β levels in 15 sites, no change in two (Fig. 3b). In sites with angular bone loss, 13 sites showed reduction in IL-1β levels, one site an increase and no change was seen in two sites (Fig. 3c). The decrease in both types of diseased sites was significantly \((p<0.001\) and \(p<0.01\), respectively) reduced after treatment. After treatment, however, the levels of IL-1β were still significantly \((p<0.01)\) higher in diseased sites compared with healthy ones (Fig. 3b–d).

Before treatment, levels of IL-1ra were also significantly higher \((p<0.001)\) in diseased sites compared with healthy ones (Fig. 4a). Treatment of
sites with horizontal bone loss resulted in reduction of the IL-1ra levels in 14 sites and an increase in two sites (Fig. 4b). In sites with angular bone loss, reduction was seen in 12 sites, an increase in two sites and no changes was noted in two sites (Fig. 4c).

Treatment resulted in statistically significant reduction in diseased sites showing horizontal bone loss ($p<0.01$). After treatment, the differences between healthy and diseased sites persisted only for sites with angular bone loss ($p<0.002$; Fig. 4b–d).

Initially, mean probing pockets depth was significantly deeper in pockets presenting angular bone loss compared with sites with horizontal loss, 8.1 ± 0.6 and 6.7 ± 0.3 mm, respectively. Treatment resulted in a significant decrease of 3.5 ± 0.5 mm ($p<0.001$) in sites showing angular bone loss and 2.8 ± 0.3 mm ($p<0.001$) in sites with horizontal bone loss (Table 1). The BRA in pre-treatment GCFs from sites with angular breakdown was significantly correlated to the pocket depth before treatment ($p<0.01$, $r_s=0.52$). In contrast, no such correlation was seen between BRA and pocket depth in sites with horizontal breakdown. After treatment no correlation between BRA and pocket depths were seen.

There were significant correlations between BRA and IL-1α, IL-1β in the diseased sites with angular bone loss before ($p<0.014$; $r_s=0.58$ and $p<0.0013$; $r_s=0.76$, respectively) and after treatment ($p<0.016$; $r_s=0.6$ and $p<0.008$; $r_s=0.66$, respectively). However, there were no significant correlations between the individual changes in each site of BRA and the corresponding changes of IL-1α, IL-1β and IL-1ra.

Discussion

Periodontal disease is associated with increased levels in GCFs of a variety of molecules involved in inflammatory and immune responses, as well as markers reflecting tissue destruction. In an attempt to elucidate which factors in inflammatory processes are responsible for stimulation of osteoclastic bone resorption we have studied the presence of BRA in different inflammatory exudates. Recently, we have found that GCFs collected from patients with adult periodontitis, when added to neonatal mouse calvarial bones in vitro, stimulate osteoclastic dependent bone resorption (Lerner et al. 1998, Rasmussen et al. 2000). Similarly, we have found that synovial fluids from patients with osteoarthritis, rheumatoid arthritis or loosen hip or knee joint prosthesis stimulate bone resorption in mouse calvariae (Lerner et al., in preparation).

In agreement with our previous studies, we found in the present investigation that GCF collected from sites with periodontal disease stimulates bone resorption. Since it has been claimed that the progression of bone loss is more severe in sites with an angular type of destruction compared with those with the horizontal breakdown (Papapanou & Wennström 1991), we compared intra-individually the amount of BRA in GCFs from these two types of sites. However, we were unable to reveal any significant differences in BRA from these sites.

Also in agreement with previous observations from our laboratory as well as others (Lerner et al. 1998, Engebretson et al. 2002), we found significantly increased levels of IL-1α and IL-1β in GCFs from sites with periodontal disease. Similar to BRA, no differences in IL-1α and IL-1β levels in GCFs from sites with horizontal and angular breakdown could be found, suggesting that there are no differences in the proinflammatory molecules present in granulation tissues in pockets with horizontal or angular bone loss. We have previously reported that IL-1ra, as well as antisera neutralising IL-1α, strongly inhibit the BRA in GCFs collected from patients with periodontal disease, indicating that IL-1 may be an important molecule in the GCFs responsible for activation of bone resorption (Rasmussen et al. 2000). In the present study, we noted significant correlations between BRA and levels of IL-1α and IL-1β for sites showing angular bone loss, both before and after treatment. In sites with horizontal bone loss, there was a significant correlation only between BRA and IL-1β levels after treatment, indicating that IL-1β are not the sole stimulator of bone resorption present in GCFs. The fact that pocket depth was significantly deeper in sites with angular bone loss might have contributed to the observed differences in correlations between BRA and IL-1α.

In the present study, changes of BRA after treatment in the individual site did not correspond to the changes of IL-1 levels, further indicating that molecules other than IL-1 are involved in the stimulation of BRA, in line with previous observations from our group (Rasmussen et al. 2000). The observation demonstrating that IL-1ra and antisera neutralising IL-1α inhibit the BRA in GCFs was based upon observations with GCFs pooled from several patients. In on-going studies, we have observed, using GCFs from individual sites, large variabilities in the susceptibility to inhibition by antisera neutralising IL-1α, IL-1β, or in the susceptibility to inhibition by IL-1ra, well in line with findings in the present study. These findings together suggest that factors besides IL-1α and IL-1β are also important for the capacity of GCFs to stimulate bone resorption in neonatal mouse calvariae. Further studies are required to elucidate the nature of such factor(s).

Interestingly, we have found that synovial fluids from patients with loosened hip prosthesis, another example of an inflammatory exudate, also stimulate bone resorption in neonatal mouse calvariae. The BRA present in the synovial fluids cannot be inhibited by antisera neutralising IL-1α or IL-1β (Andersson et al., in preparation).

Studies performed so far indicate an interindividual heterogeneity in the factors responsible for BRA in synovial fluids. It might very well be that different molecules are responsible for the BRA also in GCFs from different patients/sites.

In line with the increased levels of IL-1ra observed in synovial fluids and in serum from patients with rheumatoid arthritis (Malyak et al. 1993, Seitz et al. 1996), we found a 26–28-fold enhancement of IL-1ra in GCFs from patients with periodontal disease, with no differences observed for sites with horizontal and angular breakdown of bone. Thus, it seems that the IL-1 receptor agonists, as well as the IL-1 receptor antagonist, are increased in both periodontal disease and rheumatoid arthritis. It should, however, be noted that the concentration of the receptor antagonist is considerably (at least $\times 50$) higher than the receptor ligands. The fact that IL-1 bioactivity still is present is explained
Bone resorbing activity in gingival crevicular fluids

by the lower affinity of the antagonist to the receptors as compared with the agonists. Surprisingly, it has recently been reported that IL-1ra in GCFs is decreased in sites with periodontal disease (Rawlinson et al. 2000). However, our data showing increased IL-1ra are in line with those of Ishihara et al. (Ishihara et al. 1997). We have no explanation at present to the divergent observations.

Although we do not know, yet, whether or not the BRA that we can detect in GCFs is identical to the activity that is responsible for osteoclast activation in vivo, it is interesting to note that, 12 months after treatment, the amount of BRA was decreased in 28 out of 35 sites. The reductions of BRA were paralleled by reductions of the pocket depths. As regards BRA in GCFs from sites with angular breakdown, there was a significant correlation with the pre-treatment pocket depths. This correlation was not present 12 months after treatment, which might be due to the large reductions in pocket depths as compared with the decreases in BRA. It will be interesting to follow pocket depths and bone loss in the future in relation to BRA pre- and post-treatment.

The post-treatment samples were harvested 12 months after operation and the fact that the amounts of BRA were not decreased to baseline values indicates that some of the patients might not be in remission, which in turn might influence the relationship between BRA and pocket depth. Presently, we are measuring pre- and post-treatment levels of BRA at shorter periods of time after operation.

Treatment resulted in not only decreased amounts of BRA but also of IL-1β, IL-1α and IL-1ra, although the reductions did not reach statistical significance for IL-1ra in diseased sites with angular bone loss due to the large variabilities. The levels of IL-1α and IL-1β were decreased almost to those seen in healthy patients, whereas the amount of BRA was only partially decreased, further supporting the view that IL-1s are not the sole factors responsible for BRA. This is the first report demonstrating reductions of GCF cytokines after surgical treatment. In contrast, a recent study indicates that non-surgical mechanical therapy does not seem to reduce IL-1β in GCFs over a period of 3 month, unless smokers were excluded (Al-Shammari et al. 2001). In the present study, significant reduction of BRA after treatment was only seen in diseased sites with horizontal bone loss. Similarly, Al-Shammari et al. were unable to obtain any reduction of ICTP (a biochemical marker of bone resorption), although treatment resulted in reduction of clinical parameters in both studies.

In summary, we have demonstrated increased levels of activity(ies) stimulating bone resorption in vitro as well as of IL-1α, IL-1β and IL-1ra in GCFs from patients with periodontal disease, with no differences in sites with horizontal or angular breakdown of bone. The levels of the cytokines were decreased 12 months after treatment.

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