Original Article

Detection of Ostecalcin in gingival Crevicular fluid in a Group of Orthodontic Patients

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Abstract

Aim: To detect osteocalcin (OC) in gingival crevicular fluid (GCF) and to monitor the concentration of OC at what stage inflammation and bone resorption reaches their maximum following orthodontic activation.

Materials and Methods: GCF samples were collected from six adult orthodontic patients (mean age = 22.3, range 20–24 years) on 3, 7, 10, 14, 21, 28, and 35 days after activation of orthodontic appliance, from the tooth surface where bone resorption was expected to occur. A total of 330 GCF sample were collected using filter paper strip, the volume measured by weighing. OC was analyzed using Enzyme-Linked immunoassay technique. Data were analyzed using the Statistical Package for Social Sciences software, SPSS (SPSS Inc., Chicago, IL, USA) version 15.

Results: An increase in GCF volume and flow rate was noted in the 10th day after activation of the orthodontic appliance activation; however, due to high-standard deviation, the result was not significant. OC was detected in all GCF samples. The amount and concentration were quite variable. Increase in the amount of OC was observed between days 7 and 14.

Conclusion: OC was detected in all samples. The quantity of OC increased at day 10 in a number of samples. There was no obvious association between OC concentration and time of collection.

Keywords: *Flow rate, gingival crevicular fluid, orthodontic appliance activation, osteocalcin, volume*

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Introduction

Detection of an indicator of active phases of tissue destruction, particularly, that of alveolar bone during periodontal disease is the dream of dental investigators. The shortcoming of current clinical indices, that assess periodontal disease has led to the development of more precise,[1] noninvasive means of determining active disease, prediction of sites of future deterioration, and response to treatment.[2-7] The composition of gingival crevicular fluid (GCF) is the result of interplay between bacterial biofilm adherent to the teeth surfaces and the cells of the periodontal tissues. Analysis of specific constituents in GCF provides quantitive biochemical indicators for the evaluation of the local cellular metabolism that reflects a person's periodontal health status,[8] around implant in health and disease.[9]

Osteocalcin (OC is a noncollagenous calcium-ion binding protein also known as γ-carboxy-glutamic acid protein (bone-Gla-protein).[10,11] It is produced by both the osteoblast and odontoblast and can be measured by immunoassay.[12] OC has been described as one of the important markers of bone metabolism and is considered as one of the markers that reflect the rate of bone turn over in both health and disease states when bone resorption and formation are coupled.[13] Serum OC is considered a specific marker of metabolic bone diseases such as hyper- and hypo-parathyroidism, hyperthyroidism, osteoporosis, and acromegaly.[8]

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OC plays a role in systemic bone remodeling that encouraged dental investigators to study its role in the dental field. Many studies have shown that OC is detectable in GCF in a variety of clinical situations.[14-16] In a cross-sectional study performed by Kunimatsu *et al*.,[15] GCF was collected from five patients with gingivitis and 14 patients with adult periodontitis. In individuals with gingivitis, no significant amounts of OC were detected whereas in the subjects with adult periodontitis varying amounts of OC were detected, and these were positively correlated with clinical variables. In a similar study, Nakashima *et al*.[16] examined the GCF levels of OC, prostaglandin E2 and alkaline phosphatase at 224 sites from 17 individuals. Thirty-four of the sites were diagnosed as healthy, 72 gingivitis, and 118 exhibited periodontitis. In contrast to the findings of Kunimatsu *et al*.,[15] Nakashima *et al*.[16] found OC in the GCF collected from both gingivitis and periodontitis sites. Total OC significantly correlated with the gingival index and probing index. The concentration of OC was more than 10 times that of serum, which suggesting a significant amount of OC in GCF is produced locally. In addition, OC analyzed in peri-implant crevicular fluid. OC was detected around dental implant between 7 and 30 days after placement.[17] In addition, it was detected in peri-implant mucositis, peri-implantitis that detection may reflect increased local bone turnover around implant,[18] and decreased after treatment[9] OC was only decreased in the GCF of patients treated with cyclosporine either with gingival overgrowth or without.[19]

Successful orthodontic tooth movement requires the remodeling of the periodontium, particularly the alveolar bone. When small orthodontic forces applied for a prolonged period, inflammatory events occur within the periodontium, resulting in bone resorption and so release of inflammatory mediators.[20] Last *et al*.[21] were the first who used orthodontic model to study changes in GCF. After that, many investigators[14,22-24] employed a similar method to study different indicators in GCF. Griffiths *et al*.[14] investigated local production of OC in GCF by studying the orthodontic tooth movement model. OC was detected in all samples collected without any relation with the stages of orthodontic treatment.

Isik *et al*.[22] proposed if orthodontic treatment could be possible to biologically monitor and predict the outcome of orthodontic forces then the appliance management could be based on individual tissue response and effectiveness of the treatment.

Hence, the aims of this study are to establish if OC can be detected in GCF and to monitor the concentration of this marker to determine at what stage, inflammation, and bone resorption reach its maximum following orthodontic activation.

Materials and Methods

Study design

Each experimental period was run from appliance activation to the next period of activation (4–8 weeks). To examine the effect of the appliance activation on the GCF levels, six individuals were examined, and samples were collected on 7 occasions over 6 weeks. Samples were collected 3, 7, 10, 14, 21, 28, and 35 days after activation of orthodontic appliance. Then, if treatment was continuing and the patients were willing, the individuals were resampled.

Subjects selection

The participants who participated in this investigation, from the staff of the Eastman Dental Institute and London Hospital, those who were undergoing orthodontic treatment. The inclusion criteria were the presence of fixed orthodontic appliance without any kind of extractions, no signs of gingivitis or periodontitis, probing depths should not more than 3 mm in the whole dentition, plaque score ≤30, and bleeding score ≤10. Systemically, participants should have good general health, no use of anti-inflammatory or antibiotic drugs, no systemic diseases, and nonsmoker. Ethical approval for the study had been obtained according Eastman Dental Institute and London Hospital. All suitable participants were given a verbal and written explanation of the study, and a written consent was obtained. Six participants (4 females and 2 males) with mean age 22.30 years (range 20–24 year) were available for sampling. The small number of the participants was due to the frequency of sampling, and it is compensated by the number of samples were collected.

Samples were collected from the teeth undergoing orthodontic movement likely to be inducing bone resorption. The collection was from the tooth surfaces where bone resorption was expected to be most pronounced, for example, distal surface of canine during retraction of canines. The filter paper strip, 2x10 mm filter f.p.s of Whatman 3 MM chromatography paper down the centrifuge tube (a hole was created in the cup with a needle, before it was positioned in the microcentrifuge tube). One fps was placed inside a microcentrifuge tube and weighed before sampling with a Cahn 500 microbalance accurate to the nearest 0.01 mg.

Sites of collection were isolated with cotton rolls and a saliva ejector. High-volume aspiration was used close to the area of collection to prevent any salivary contamination. Using a periodontal probe, the tooth surface was cleaned of any plaque or debris, without disturbing the gingiva.

Blood

A single 10 ml sample of blood from each subject was collected to provide a reference for serum concentrations of OC. In addition, it was used as a positive control in the assays and was analyzed in conjunction with the GCF samples. Serum was separated from the blood clot by centrifuging at 150 g for 5 min. The supernatant was removed and centrifuged at 350 g for 15 min. To destroy the heat labile components of complement, the serum was heated in water bath at 56°C for 30 min. The serum was then transferred to containers and stored at −70°C.

Collection of gingival crevicular fluid and sampling protocol

The fps was placed at the entrance of the gingival crevice. The first fps was maintained in the position for 5 s, followed by an interval of one min. The second fps was similarly maintained in the same position for a 5 s collection. Following an interval of 30 s, a final fps. was placed in the position for a 30 s collection period. The first strip sample represents the pooled volume of GCF, and the second strip represents the GCF flow rate, and to increase the volume of GCF available for subsequent laboratory analysis, the third fps was used. Following GCF collection, strips were replaced and sealed in the microcentrifuge tube and reweighed. Differences in weights were calculated for each strip and represent the amount of GCF collected. GCF samples were stored inside the microcentrifuge tubes at −70°C.

Sample analysis and laboratory procedures

The total number of GCF samples was 330 was collected from various tooth surfaces. Initial aim of the laboratory investigations was to determine whether (OC) could be consistently detected in the GCF and to establish the level of sensitivity. This required samples to be pooled to ensure that detection is not limited by the paucity of material collected.

An enzyme-linked immunoassay kit (Gla-type OC EIA KIT, code No. MK011, TAKARA SHUZO CO. LTD, JAPAN) was utilized for detection of gla-type OC in GCF. The Gla-OC EIA Kit is a solid phase based on a sandwich method that utilized two mouse monoclonal anti-Gla-OC by two-step procedure. Data were analyzed using the Statistical Package for Social Sciences software, SPSS (SPSS Inc., Chicago, IL, USA) version 15.

Results

A total number of 330 GCF samples was harvested from various tooth surface and then analyzed for the purpose of the study. Table 1 shows the mean and standard deviation (SD) of GCF collected on the second strips, at different days following activation of the orthodontic appliance, which represents the flow rate of GCF. The mean values of GCF flow rate show some variation between the days of collection. Although 14 and 21 days represent high values (0.37 µl/min), still the 10 days represents the maximum value (0.43 µl/min), and 35 days represents the minimum value (0.10 µl/min). Statistical analysis using analysis of variance showed no statistically significant effect (P = 0.144) for the time of sample collection. Further paired t-test between days 10 and 35 showed no statistically significant difference (P = 0.77).

Table 2 shows the mean and SD of the total volume of GCF collected on the three strips at different days following activation of the orthodontic appliance. The maximum mean value (1.50 µl) occurred at 10 days, and it was almost four times the minimum mean value (0.40 µl) which occurred at 35 days. The mean values at 14 and 21 days were also slightly higher than the means on the remaining days. Analysis of variance revealed no statistically significant differences between the total volume collected on any of the days. Similarly, paired t-test of day 10 and day 35 (P = 0.10) revealed no statistically significant differences.

An enzyme immunoassay kit was used for the analysis OC in six participants whom GCF samples were collected at 3, 7, 10, 14, and 21 days after activation of an orthodontic appliance. As a pilot study, the samples from subject[1] were analyzed alone. The concentration of gla-OC was determined for the GCF samples by reference to a standard curve obtained by plotting absorbance versus gla-OC concentration of the standard solution. Each sample was analyzed, and OC was detected by reference to the standard curve and presented in Figure 1.

The absolute level of OC was low and ranged from 0.060 pg/strip, which barely above the threshold of detection to a maximum value of 5.012 pg/strip in tooth

Table 1: Mean (µl/min) (+ standard deviation) of gingival crevicular fluid flow (2nd strip) following adjustment of appliance

Days	3	7	10	14	21	28	35
Mean (µl/min)	0.24	0.19	0.43	0.37	0.37	0.23	0.10
SD	0.24	0.24	0.62	0.72	0.48	0.23	0.11

P: 0.144. SD=Standard deviation

Table 2: Mean and (+ standard deviation) of the total volume of gingival crevicular fluid (three strips) following orthodontic appliance adjustment

Days	3	7	10	14	21	28	35
n	51	48	49	50	45	43	44
Mean	0.92	0.76	1.50	1.12	1.11	0.92	0.40
SD	0.96	1.00	2.13	1.60	1.10	0.79	0.34

P: 0.77. SD=Standard deviation

24 10 days after activation of the appliance. By reference to the volume of GCF collected, the absolute amount of GCF can be converted to the concentration of OC [Figure 2]. The range of concentration was 97.8–3200.00 pg/µl. Increase in OC concentration was observed at day 7 and 21 after activation of orthodontic appliance. Calculation of the serum OC concentration from the single blood sample collected from the same subject and analyzed in the same kit gives a concentration of 67 pg/µl.

Further analysis was carried out for samples collected from the rest of the participants. OC was detected in all samples; absolute level of OC was 0.57–4.79 pg/strip, [Figure 3]. An increase in OC level was noted in most of the participants. All participants showed decrease in OC level of OC at day 21. The volume of the samples ranged from 0.37 to 2.60 µl. The absolute concentration of OC in GCF samples was calculated and plotted in [Figure 4]. It was ranged 176–3984.38 pg/µl. There was a little variation in the majority of samples; however, a consistent trend to increase toward the late stages of collection which is after day 21.

Analysis of the serum samples revealed a concentration of 63.0–97.8 pg/µl. This was substantially lower than the figures obtained for GCF samples, which suggests that OC is reflecting local activity of bone turnover and tooth movement rather than the systemic activity.

Discussion

Knowledge of all the biomarkers present in the GCF that can be used to mark the changes in tooth that is undergoing orthodontic treatment may be of clinical usefulness leading to proper choice of mechanical loading, to improve and to shorten the period of treatment, avoiding adverse consequences.[24]

During active periodontal disease, there is an increased alveolar bone turnover. Although there may be a dominance of bone resorption that represented as alveolar bone loss. Some studies[25,26] reported that OC level in GCF in periodontally diseased sites is higher than that in healthy sites. Orthodontic treatment represents a model a periodontaly diseased sites and when small orthodontic forces applied inflammatory mediators release that similar to what occurs during during periodontal diseases.

Analysis the results of the current study, noted a large variation in the absolute level of OC in GCF samples collected from all participants, although most of the samples showed an increase in OC level after day 10

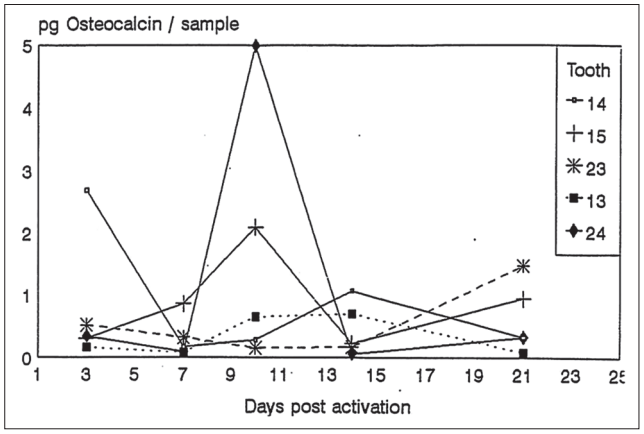

Figure 1: Absolute level of osteocalcin (subject 1)

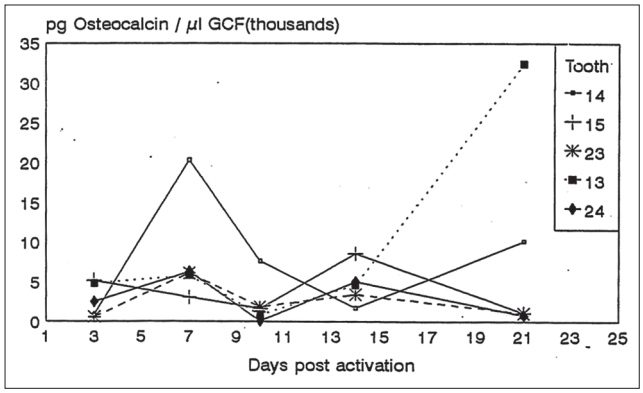

Figure 2: Absolute concentration of osteocalcin (subject 1)

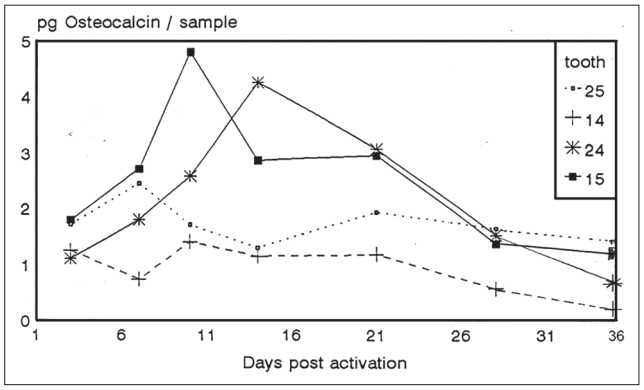

Figure 3: Absolute level of osteocalcin (rest of participants)

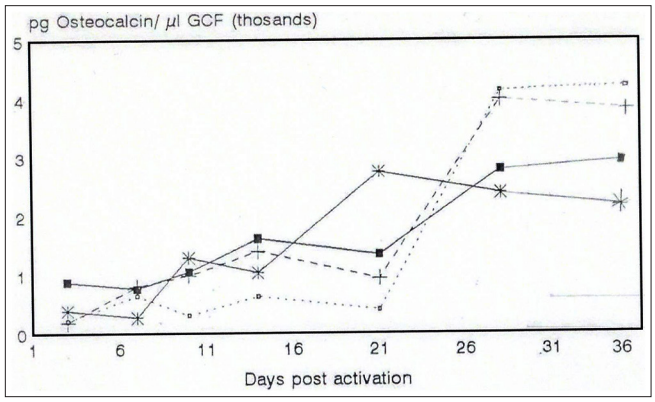

Figure 4: Absolute concentration of osteocalcin (rest of participants)

and 14. The maximum amounts of OC detected at day 10 represented approximately 2–12 pg/sample, which comes with results of Alfaqeeh and Anil[27] where they reported a significant changes in the level of OC on days 7, 14, 21, and the peak occurred on day 14 after canine retraction. In addition, it was reported in the elevation of alkaline phosphatase at days 14 and 28 after activation of orthodontic appliance.[28]

Further, the values of our results are lower than Kunimatsu *et al*.,[15] who presented values of 0–540 pg/sample. The increase in OC at days 10 is of interest as Lynch *et al*.[29] reported that it is the time, when bone resorption and deposition are ongoing osteoclast and monocleated cells in the resorption sites stained distinctly for IL-β. In contrary to the result of Isik *et al*.,[22] who reported an increase in OC on the 7th day after the first activation of orthodontic appliance. The 1st day after the second activation also shows a dramatic decrease following with a slight recovery at 28th day.

In this study, a large variation in OC concentration was observed, and it did not follow any particular pattern during the period after activation of orthodontic treatment and collection of GCF samples. The range of OC concentration 176–3984.38 pg/μl was comparable to some of the highest values presented by Kunimatsu *et al*.[15] 1.3750 and 2723 pg/μl. However, they are higher than the values reported by Griffiths *et al*.[14] 250–860 pg/μl and substantially higher than Nakashima *et al*.[16] 56.9–155.9 pd/μl.

Variation of OC concentration between participants may be explained by variation in time of treatment and type of appliances used during treatment. Variation in treatment between subject may be indicated variation in the amount of force and the time of treatment and that result in variation in the amount of bone remodeling. Rönnerman *et al*.[30] reported that the occurrence of bone resorption or bone remodeling during orthodontic treatment may be related to differences in the type of appliances, treatment time and forces, or site of collection.[31]

Bullon *et al*. (2005)[18] analyzed OC level in serum, saliva, and GCF and correlated them with periodontitis and osteoperosis. OC concentration was higher in GCF, and OC level in GCF correlates with periodontal condition. Analysis of serum blood samples in this study revealed a concentration of 63.0 pg/μl and 97.8 pg/μl. This was substantially lower than the figure obtained for GCF samples which suggested that OC is reflecting local activity of bone turn over and tooth movement rather than systemic activity. If this is true for OC, continued sampling would produce a more serum like sample which will dilute the level of OC detected in GCF.

Conclusion

Using of OC agent as indicator of bone remodeling during orthodontic treatment and therefore, as potential indicators of periodontal disease activity, still need more investigation. Ideally, there should be more control to the participants and sites involved in this study; however, as it is difficult to recruit an appropriate number of suitable adult patients with this frequency of GCF collection. As possible solution is to use animals and apply exactly the same treatment using constant orthodontic forces with the frequent of collection.

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Conflicts of interest

There are no conflicts of interest.

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