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The Effect of Amino-Bisphosphonate Therapy on Gingival Crevicular Fluid Alkaline Phosphatase Activity in Cancer Patients

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Abstract

The enzyme Alkaline Phosphatase (ALP) is considered a biomarker of bone formation and it is used for monitoring intravenous Bisphosphonate (BP) therapy. It can be measured in serum and urine, but also in Gingival Crevicular Fluid (GCF). The aim of the present study was to monitor the GCF-ALP activity fluctuations in cancer patients before and during the first six months of BP therapy, comparing them with systemic serum ALP concentrations. 33 patients and 25 controls were enrolled according to the eligibility criteria. The GCF was collected at baseline, and after every BP administration for 6 times. The total ALP activities were determined spectrophotometrically. The results show how the differences in GCF-ALP activity over time appear to be significant in the BP-taking group ($p=0.003$), while the differences in serum ALP in the BP-taking group and in GCF-ALP activity in the control group were not significant ($p>0.05$). GCF-ALP activity may thus represent a possible new biomarker for bone turnover rate evaluation in patients taking intravenous BP therapy. Up to date, this is the first study that investigates on variations of GCF-ALP activity due to changes in bone metabolism caused by intravenous nitrogen-containing BP therapy in cancer patients.

Keywords: Bisphosphonates; Alkaline phosphatase; Gingival crevicular fluid

Introduction

In the last twenty years, Bisphosphonate (BP) therapy has become the standard treatment for several disorders of bone metabolism: bone metastases, osteoporosis, Paget's disease, osteogenesis imperfecta, primary hyperparathyroidism and fibrous dysplasia [1-3]. The mechanisms of action of BPs have been thoroughly studied and explained: they act causing the suppression of bone resorption inhibiting the osteoclast activity and inducing their apoptosis. As a consequence, also bone formation is slowly reduced affecting the whole bone turnover rate. Especially the more potent nitrogen-containing BPs with intravenous administration (eg. Zoledronic acid and Pamidronate) are highly active in terms of antiresorptive effect [4]. Biomarkers of bone formation and resorption are molecules that reflect the overall osteoblastic and osteoclastic activity in the skeleton. A number of assays have been developed which can provide clinically useful information about bone cell activity in skeletal disorders and during antiresorptive therapy. Bone metabolism markers have been detected in several body fluids such as serum, urine, saliva, Gingival Crevicular Fluid (GCF). Up to date, only bone metabolism markers derived from serum and urine have been investigated and used to monitor bone metabolism in bone metabolism disorders, especially in cancer patients. Among them, serum Alkaline Phosphatase (ALP) is considered a biomarker of bone formation and it is already used for monitoring intravenous BP therapy. Although the use of biochemical markers of bone turnover is increasing, there are still some concerns on their reliability because of their high variability [5-7].

GCF is considered either a serum exudate or a transudate, depending on the inflammatory conditions of the gingival crevice. It contains components of serum, inflammatory cells, connective tissue, epithelial cells, microbial flora and host-derived products including enzymes such as ALP [8,9]. GCF-ALP is sensitive to bone remodeling when released from osteoblasts [10], to inflammation when released from polymorphonuclear cells [11] and to periodontal regeneration when released from periodontal ligament fibroblasts [12]. GCF-ALP activity has been shown to have a predictive value in chronic periodontitis in terms of attachment loss and recurrent inflammation after sessions of scaling and root planing [13]. Under healthy periodontal conditions, total GCF-ALP activity has been also shown to be a reliable marker of bone turnover during orthodontic tooth movement [14,15]. It has also been proposed as a noninvasive diagnostic aid for the determination of optimal treatment timing in functional jaw orthopaedics, as it significantly increases during periods of increased bone deposition due to pubertal growth spurt [16]. Up to date, no studies have investigated yet on variations of GCF-ALP activity due to changes in bone metabolism caused by intravenous nitrogen-containing BP therapy in cancer patients.

Objective of the study

The aim of this study is to evaluate the possible role of GCF-ALP activity as a biomarker of bone turnover in patients undergoing intravenous nitrogen-containing BP therapy for the treatment of solid tumor bone metastases or multiple myeloma. The objective of the present study was to monitor the GCF-ALP activity fluctuations in cancer patients before and during the first six months of BP therapy, comparing them with systemic serum ALP activity and GCF-ALP activity in non treated controls.

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Materials and Methods

Sample subjects selection and study design

From December 2010 to April 2012, 50 cancer patients were visited at the Division of Oral Medicine, Dental Science Department, University of Trieste, Italy for an oral health evaluation before starting intravenous nitrogen-containing BP therapy. 33 of them were selected to participate to the study according to the eligibility criteria listed in Table 1.

Also 25 control subjects were selected among patients visited in the department of Oral Medicine and Pathology, that followed the same eligibility criteria but didn't undergo BP therapy either before or throughout the study. Both BP-taking and control subjects underwent professional supragingival and subgingival scaling at least 10 days before the first sampling, and all subjects also received oral hygiene instructions to maintain a healthy periodontal status during the study period. In the BP group, GCF samples were collected before starting BP therapy (baseline) and then after each intravenous BP administration (every 28 days) for 6 administrations (T0 to T6, total of 7 sample collections). GCF samples were collected for each patient in a period ranging from one week after BP administration and one week before the next one. In the controls group, GCF samples were collected 7 times at monthly intervals (T0 to T6). Serum ALP activity values measured before each intravenous BP administration were obtained from the Ospedali Riuniti di Trieste clinical recording data-base for each patient. Signed informed consent was obtained from the subjects, and the protocol was reviewed and approved by the local Ethics Committee.

GCF collection method

GCF collection and processing method are modified from a previously published protocol [13]. GCF was collected for evaluation as follows: the sites were first isolated with cotton rolls and after gentle removal of supragingival plaque when present with a sterile curette, the gingival surface was dried with a gentle air flow. GCF samples were then obtained by insertion of four standardized #25 sterile paper points (Krugg, Buccinasco, Milan, Italy) into the deepest part of each gingival site, and left in situ for 60s to collect the resting GCF. Four samples were obtained from the upper jaw central incisors and four from the lower jaw central incisors. The GCF samples were then transferred to plastic Eppendorf tubes and immediately stored at -80°C until analyzed.

Enzymatic activity determination

The biochemical assays were performed by a single operator. The four GCF samples from the four collection sites were resuspended in 200 µL of buffer containing 200 mMTris, 20 mM $MgCl_2$ (pH 9.8 ± 0.1) and 1 mg/mL of p-nitrophenyl phosphate (N2770-5SET; Sigma FastTM; Sigma-Aldrich, St Louis, MO,USA). The samples were then incubated in dark at 37°C (± fluctuations of <0.1°C) for 3 h. During this incubation, the ALP in the samples hydrolyses the p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate. The relevant control for each analysis consisted of the substrate and the Tris buffer without the sample. The reactions were then stopped by the addition of 5 µL of 3 M NaOH, and 180 µL of the resulting solution from each sample tube were transferred to a well in a plastic plate for spectrophotometral reading. The absorbances were read using a spectrophotometer at 405 nm wave length (Tecan Group Ltd., Männedorf, Switzerland). The samples were analyzed in three different sessions. Using 18.45 as the p-nitrophenol mM absorptivity, the absorbance was converted into enzyme activity units (1 unit=1 µmol of p-nitrophenol released per minute at 37°C) and expressed as total activity in mU/sample.

Eligibility criteria
Presence of at least two teeth in both 2nd and 5th sextant
PSR 2 or less in the 2nd and 5th sextant
No active periodontal disease in the considered sites, evaluated considering the absence of bleed on probing and probing depth <3mm
Non-smokers

Table 1: Eligibility criteria for patient selection for the present study.

Data processing

The SPSS software, version 13.0 (SPSS® Inc., Chicago, Illinois, USA) was used to perform the statistical analyses. A Wilcoxon paired sign rank test assessed the significance of the differences in the GCF-ALP activity between the maxillary and mandibular sites within each group and time point. All of the subsequent analyses have been performed by using the merged maxillary and mandibular GCF-ALP activities, i.e. the patient was the statistical unit. The Friedman test was used to assess the significance of the difference in the GCF-ALP activity over time within each group. Moreover, the Friedman test was also employed to assess the significance of the differences in the serum ALP activity over time for the treated group. Finally, for the BP group, the significance of the correlation between the merged GCF-ALP activity and serum ALP activity within each time point was assessed by a rho Spearman correlation coefficient. A $p<0.05$ was used for rejection of the null hypothesis.

Results

Study population

A total of 221 GCF samples were collected and analyzed throughout the present study. The characteristics of the subjects are listed in Tables 2 and 3. Among the 33 patients group, 14 were male and 19 female. The age range was 54-84, with a mean age of 71 ± 6. 13 patients suffered from breast cancer, 8 prostate cancer, 5 multiple myeloma, 5 lung cancer and 2 colon cancer. All patients underwent BP therapy due to the development of bone metastases. The most prescribed type of BP was zolendronic acid (30 patients), while 3 patients took pamidronate. The duration of BP therapy ranged from 1 to 31 months, with a mean duration of 12 months. Among the 25 controls enrolled, 15 underwent all 7 sample collections. 8 of the controls were male and 17 were female. The age range was 40-84 with a mean age of 64 ± 12 years. Both BP-taking patients' and controls' periodontal conditions remained stable throughout the study.

GCF and serum ALP activity

ALP activity is expressed in mU/sample where 1 Unit=1 mmol of p-nitrophenol released per minute at 37°C. Serum ALP activity is expressed in U/L. The merged upper and lower jaw GCF-ALP activities for each time point in the treated and control group are reported in Figure 1. The serum ALP activity values are reported in Figure 2.

Considering all the GCF-ALP activity values measured in the present study, the patterns of fluctuations over time are very similar comparing the upper and lower jaw in both BP and control groups, and a Wilcoxon paired sign rank test assessed significant difference only in T2 time point in the BP group ($p<0.02$). Comparing the merged upper and lower jaw GCF-ALP fluctuations in the BP and control group, the patterns are different as shown in Figure 1. The GCF-ALP analysis made in the BP group shows a gradual decrease after the first three intravenous nitrogen-containing BP administration, followed by a significant peak after the fourth one. Afterwards, GCF-ALP activity

N pts	Gender		Age range	Mean age ± SD	Underlying disease	BP type
	M	F				
33	14 (42%)	19 (58%)	54-84	71±6	Breast 13(39%) Prost 8 (24%) MM 5 (15%) Lung 5 (15%) Colon 2 (6%)	ZA 30 (91%) PA 3 (9%)

Table 2: Characteristics of the patients group enrolled in the present study. N pts number of patients, M male, F female, SD standard deviation, BP bisphosphonate, Breast breast cancer, Prost prostate cancer, MM multiple myeloma, Lung lung cancer, Colon colon cancer.

N controls	Gender		Age range	Mean age ± SD
	M	F		
25	8 (32%)	17 (68%)	40-84	64±12

Table 3: Characteristics of the control group enrolled in the present study. N controls number of controls, SD standard deviation.

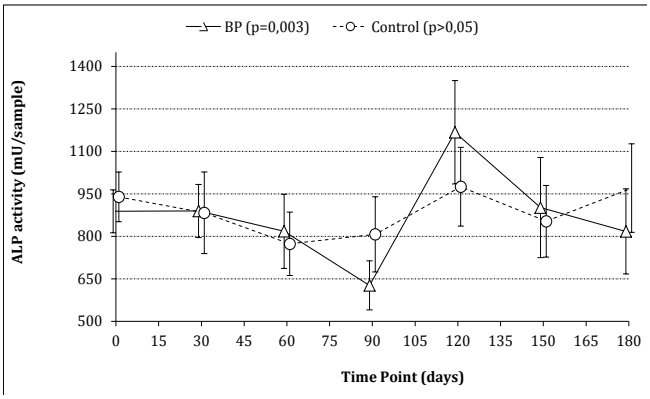

Figure 1: Total GCF-ALP activities (mU/sample) in the BP and control groups. Data presented as mean values (bars represent the standard error.

decreases again. The Friedman test assessed the significance of the differences in GCF-ALP activity over time with p=0.003. The GCF-ALP activity fluctuation in the control group doesn't show a recognizable pattern. According to the Friedman test, this fluctuation results are not significant (p=0.145).

Serum ALP activity differences over time appear to be not significant (p=0.429) according to the Friedman test. The Spearman rho correlation coefficient was used to assess the significances of correlations between GCF-ALP activity and serum ALP activity for each sampling time in the BP group, finding a significant correlation only for the combination of T4 in the GCF-ALP activity and T4 in the serum ALP activity (p<0.05).

Discussion

ALP is an enzyme particularly concentrated in bone, where it is tipically secreted to extracellular matrix by osteoblasts in bone-forming periods for hydrolyzation of inorganic pyrophosphate, which is a strong inhibitor of bone deposition [17]. It is already known and used as a serum biomarker for bone formation in several diseases. As physiologically bone formation and resorption take place simultaneously during bone remodeling, ALP can be considered a biomarker for bone turnover rate [5]. Serum ALP activity is normally used for monitoring intravenous BP therapy, especially in cancer patients. Since GCF is a serum transudate/exudate, it also contains ALP, which is detectable with the colorimetric essay used in the present study [13]. GCF-ALP is sensitive to bone remodeling when released from osteoblasts [10], to inflammation when released from polymorphonuclear cells [11] and to periodontal regeneration when released from periodontal ligament fibroblasts [12]. In case of periodontal health or stability, as maintained throughout the present study, GCF-ALP fluctuations are most likely dependent on changes in bone turnover rates. To our knowledge, this is the first study that investigates on variations of GCF-ALP activity due to changes in bone metabolism caused by intravenous nitrogen-containing BP therapy in cancer patients.

The present study results show how the GCF-ALP activity patterns are very similar both in the upper and lower jaw, in both BP and control groups. Only one significant difference has been found in the BP group time point T2. This minimizes the bias that the ALP activity could be influenced by periodontal inflammation. During BP therapy, the total ALP levels are expected to gradually decrease, as observed in previously published trials [18]. Statistical analysis show how in our study merged upper and lower jaw GCF-ALP activity in BP-taking cancer patients has significant variations over time (p=0.003) in the first six months after the beginning of the therapy. The pattern is characterized by a gradual decrease until the third month as expected, followed by a pronounced increase in the fourth month. Afterwards, GCF-ALP activity decreases again. A similar pattern was found also in serum ALP fluctuations, but the differences in activity over time resulted to be non significant. Comparing GCF and serum ALP activity over time, a significant correlation was found only for the combination of T4 in the GCF-ALP activity and T4 in the serum ALP activity (p<0.05), when ALP activity suddenly increases.

These results show how after an initial decrease in bone turnover as expected, due to the inhibition of osteoclast activity, there is probably a rebound reaction with a sudden and pronounced but temporarily short period of increase in bone turnover rate which is significantly evident in GCF and not significantly in serum. BPs appear not only to slow down bone resorption, but also to enhance bone formation increasing osteoblast proliferation, collagen and osteocalcin production and the formation of mineralization nodules *in vitro* [19,20]. This peak in ALP activity may mean that in bones affected by bone metastases where bone structure is impaired due to the increase in bone resorption, after the inhibition of osteoclast function achieved in the first months of treatment, there can be a sudden and pronounced increase in bone formation caused by the BP promoting action on osteoblasts to refill the lacunae, until a new equilibrium between bone resorption and formation is gained.

The results of our study show how there are significant variations

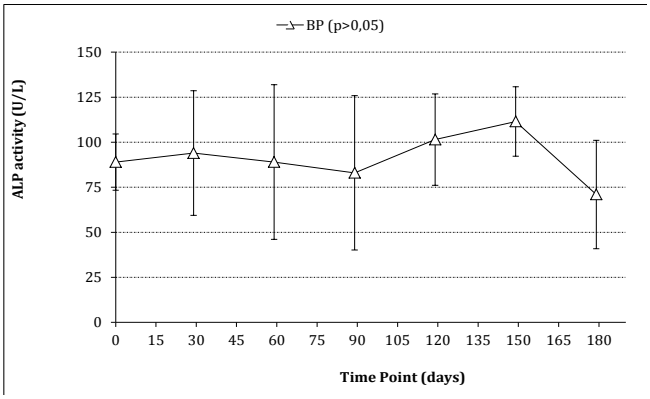

Figure 2: Serum ALP activities in the BP group expressed in U/L. Data presented as mean values (bars represent the standard error).

in GCF-ALP activity in cancer patients taking intravenous BPs over time, compared to serum ALP and a control group. Further studies are needed to evaluate whether GCF-ALP variations follow the variations of other bone metabolism markers present in GCF, such as osteocalcin, Ntx or Ctx [21,22].

In conclusion, GCF-ALP may be considered as a possible new biomarker of bone turnover rate in patients taking intravenous BP therapy. Future studies are warranted to assess whether it can be clinically applied to cancer patients, and possibly also to patients taking oral BPs to monitor the antiresorptive therapy in a non-invasive and less expensive way compared to serum analysis.

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