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PROPERTIES OF ALKALINE PHOSPHATASE IN THE GINGIVAL CREVICULAR FLUID

BY

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ABSTRACT

The isoenzymic properties of the alkaline phosphatase (ALP) of the gingival crevicular fluid (GCF) were investigated and compared with those in other cells, such as human polymorphonuclear leukocytes (PMNs), and human periodontal ligament cells (PDLs), and with those of three species of periodontopathic bacteria: *Porphyromonas gingivalis* 381 (*P. gingivalis*), *Prevotella intermedia* ATCC25611 (*P. intermedia*), and *Capnocytophaga sputigena* ATCC33123 (*C. sputigena*). The biochemical properties of the isoenzymes were analyzed by the following methods: enzyme assays, inhibition pattern using three chemical inhibitors, 4 to 20% gradient polyacrylamide gel electrophoresis, thermostability, immunological specificity, and phosphatidylinositol-specific phospholipase C (PI-PLC) treatment. The inhibition experiment showed that ALP of the PMNs and PDLs possessed almost the same enzymatic properties of tissue-nonspecific ALP (bone/liver/kidney; TNSALP), and the ALP of the three species of periodontopathic bacteria possessed specific properties that were different from those of TNSALP, intestinal, or placental ALP. The ALP of the GCF was only slightly susceptible to levamisole (1 mM), L-phenylalanine (20 mM), and SDS (1%). An electrophoresis thermostability test demonstrated that the enzyme activity of the GCF was separated into one or two bands. The main heat-labile slow band contained the phosphatidylinositol (PI)-moiety-anchored ALP and possessed immunological specificity against anti-bone type ALP. The minor fast band was heat stable and showed mobility similar to that in *P. gingivalis*. These results indicated that the ALP of the GCF consisted of several ALP isoenzyme types whose possible origins are considered to be derived from phosphatidylinositol (PI) anchored ALP and periodontopathic bacterial ALP. The quantitative isoenzyme analysis of ALP in GCF may elucidate the mechanism of the disease activity of periodontitis.

Key words: Alkaline phosphatase; gingival crevicular fluid; isoenzymes.

INTRODUCTION

There have been various efforts to develop a diagnostic test to distinguish between disease-active and inactive sites for the progression of periodontitis (Fine and Mandel [1]; Page [2]). One of the efforts was based on the analysis of gingival crevicular fluid (GCF), which offers a potential source of factors associated with active tissue destruction (Lamster et al. [3]). Alkaline phosphatase (ALP) is an enzyme known to be associated with bone metabolism (Fleish and Neuman [4]) and periodontal disease (Ishikawa and Cimasoni [5]; Binder, Goodson and Socransky [6]). It may be localized in several cells in the periodontium, such as osteoblasts (Takimoto, Deguchi and Mori [7]), fibroblasts (Abe et al. [8]), and neutrophils

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(Vergnes, Grozdea and Corberand [9]; Nakashima, Demeurisse and Cimasoni [10]), and is also produced by some periodontopathic bacteria found in subgingival plaque (Poirier and Holt [11]; Yamashita et al. [12]; Lo Storto, Silvestrini and Bonucci [13]; Shibata et al. [14]). ALP could break down connective tissue ground substance (Ten Cate and Syrbu [15]) and could adversely affect the phosphoproteins of mineralized tissue (Kreitzman, Fritz and Saffir [16]; Poirier and Holt [17]; Yamashita et al. [12]). This enzyme is also known to be released during periodontal disease by way of microbial challenge and host response mediated by neutrophils (Van Dyke, Levine and Genco [18]). On the other hand, as an enzyme of osteoblasts, ALP activity is often elevated when bone formation is increased (Van Lente [19]). This phenomenon has been reported to occur in the periodontium after orthodontic tooth movement (Takimoto, Deguchi and Mori [7]). This paradoxical behavior of ALP may be cited as evidence that there are four main types of human ALPs: tissue-nonspecific ALP (bone/liver/kidney; TNSALP), intestinal, placental, and placental-like types (Seargeant and Stinson [20]; Vergnes, Grozda and Corberand [9]). Though they are products of homologous genes (Henthorn et al. [21]), the four isoenzymes are distinguished by their enzymatic properties. To characterize the isoenzymatic properties of ALP of the GCF from periodontal patients, we have compared the biochemical characteristics of ALP of the GCF with those in other cells, such as human polymorphonuclear leukocytes (PMNs), and cultured human periodontal ligament cells (PDLs), and with those from three species of periodontopathic bacteria, *Porphyromonas gingivalis* 381 (*P. gingivalis*), *Prevotella intermedia* ATCC25611 (*P. intermedia*), and *Capnocytophaga sputigena* ATCC33123 (*C. sputigena*).

Materials and Methods

I. *Collection of GCF samples.*

Eight patients (4 females and 4 males) ranging in age from 46 to 72 years (means 61.5) with chronic adult periodontitis were selected for this study. GCF was collected from all periodontally diseased sites of each patient following a standardized collection and processing procedure. Each crevicular area was gently air-dried and supragingival plaque was removed. A precut filter-paper strip (Toyo Roshi Co., Ltd.; Tokyo, Japan) was inserted into the pocket until mild resistance was felt, left in place for 30s, and transferred to the Periotron 6000 (Harco Electronics; Winnepeg, Canada) for GCF volume determination. All strips collected from each patient were eluted for 1 h at room temperature in 200 μl of 10 mM Tris-HCl buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.7) in a microcentrifuge tube. In case of visible contamination with blood, the strips were discarded. After 1 h, the strips were shaken vigorously for 1 min, and then the tubes were centrifuged at 10,000 rpm for 1 min in order to completely elute the GCF components. Following removal of the strips, each supernatant sample was recorded with a coded number and then stored at -20°C until analysis.

II. *Isolation of PMNs.*

Peripheral blood (30 ml) from systemically healthy adults was obtained and placed in heparinized tubes. The heparinized blood was separated on a dextran gradient at room temperature for 90 min. The PMNs-rich fraction was collected, resuspended in 18 ml PBS containing anticoagulant, and the cells were pelleted by centrifugation at 1,500 rpm for 10 min. The cell pellet was resuspended in 0.83% NH_4Cl(4°C). The PMNs suspension was

subjected to cooling at 4°C for 40 min and centrifuged at 800 rpm for 10 min at 4°C. The cell pellet was resuspended in 10 mM Tris-HCl buffer containing 1 mM PMSF (pH 7.7) and stored at −20°C until analysis. PMNs were homogenized using a Polytron homogenizer prior to analysis.

III. *Culture of PDLs.*

PDLs were cultured by the method of Mariotti and Cochran [22] with minor modification. PDLs were isolated from freshly extracted premolars obtained by consent from patients undergoing orthodontics treatment. To avoid contamination from neighboring tissues, the PDLs were scrapped from the middle one-third of the root. The pieces of periodontal ligament were separately rinsed with Dulbecco modified Eagle medium (DMEM) containing antibiotics (200 U/ml penicillin G, 200 μg/ml streptomycin, and 0.5 μg/ml amphotericin B). The tissue explants were separately placed on the bottom of 60 mm Petri dishes and were incubated in humidified 5% CO_2–95% air atmosphere at 37°C in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) containing antibiotics at concentrations half those in the medium.

After 3 to 5 weeks, the cells were trypsinized and then grown to confluence under the same conditions. After three to five passages, the cells were washed with PBS and then scraped and harvested in TBS-PMSF containing 1% Triton X-100. After that, the cells were homogenized using a Polytron homogenizer, and the supernatant was collected by centrifugation at 12,000 rpm for 1 min and stored at −20°C until analysis.

IV. *Bacterial suspension.*

The bacterial strains *P. gingivalis, P. intermedia*, and *C. sputigena* were cultured by the method of Naito, Okuda and Takazoe [23]. Bacterial cells were cultured in 2 liters of the broth for 48 to 72 h in an anaerobic glove box. Cells were harvested by cold centrifugation at 12,000 rpm for 20 min at 4°C and washed three times with sterile phosphate-buffered saline (PBS, pH 7.2). Washed cells were suspended in PBS to give a concentration of 200 mg (wet weight) per ml and were subjected to ultrasonic disruption (model 5213, 300W; Ohtake; Tokyo, Japan) in an ice bath. The cell disruption was monitored by phase-contrast microscopy. The sonicates were centrifuged at 12,000 rpm for 20 min at 4°C. The supernatants were dialyzed against distilled water at 4°C, lyophilized, and stored at −20°C. Prior to analysis, lyophilized supernatants were dissolved in 10 mM Tris-HCl buffer containing 1 mM PMSF (pH 7.7) at a concentration of 1 mg/ml.

V. *Enzyme-activity assay.*

ALP activity was determined using 96-well microtiter plates (Falcon, Becton Dickinson Labware; Lincon Park, NJ). The samples were incubated at 37°C with 10 mM p-nitro-phenylphosphate as substrate in 100 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer, pH 10.0, containing 5 mM $MgCl_2$. The enzyme activity was also assayed in the presence of inhibitors, such as levamisole (1 mM), L-phenylalanine (20 mM), and SDS (1%) (Goseki et al. [24]; Shibata et al. [14]).

VI. *Protein determination.*

Protein concentration was determined using the BCA protein assay kit from Pierce (Rockford, IL; U.S.A.)

VII. *Electrophoresis.*

Four to twenty percent gradient polyacrylamide gel electrophoresis was carried out in 0.1 M Tris-borate buffer (pH 9.5) at 125 volts at 4°C for 2–3 hrs. ALP isoenzyme activity in the gel was stained by the β-naphthyl phosphate method. All samples were electrophoresed in the presence of 1% (v/v) Triton X-100.

1. *Thermostability.*

Prior to electrophoresis, the samples were incubated at 60°C for 30 min.

2. *Production of antibodies.*

Polyclonal antibodies against purified bovine intestinal ALP (anti-intestinal type) and swine kidney ALP (anti-bone type) were raised in rabbits as described previously by Oida, Goseki and Sasaki [25] and Goseki et al. [26]. Both antibodies were purified by affinity chromatography with Protein A (Affi-Gel Protein A MAPS™ Kit, Bio-Rad Lab.) and stored at −80°C. IgG contents in the anti-intestinal and anti-bone type ALP preparations were 1.5 and 0.5 mg/ml, respectively.

3. *Immunological specificity.*

The enzyme preparations (10 μl each, 0.1 U/ml) were incubated with 10 μl of polyclonal antibody solution (anti-intestinal or anti-bone) or 10 mM Tris-HCl buffer (pH 7.7) overnight at 4°C prior to electrophoresis with 4 to 20% gradient polyacrylamide gel.

4. *Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment.*

The enzyme preparations were treated with PI-PLC (0.1 U/ml) overnight at room temperature prior to electrophoresis with 4 to 20% gradient polyacrylamide gel.

Results

I. *Inhibition Studies*

The enzyme activity in the samples was assayed in the presence of inhibitors such as levamisole (1 mM), L-phenylalanine (20 mM), and SDS (1%) (Goseki et al. [24] and Shibata et al. [14]). The results showed that the ALP of the PMNs and PDLs possessed enzymatic properties almost the same as that of TNSALP, and the ALP of the three species of periodontopathic bacteria possessed specific properties which were different from those of TNSALP, intestinal ALP, or placental ALP. The ALP of the GCF was not so susceptible to levamisole (1 mM), L-phenylalanine (20 mM), and SDS (1%). No correlation was found the ALP of the GCF and those of the PMNs, PDLs, intestine, placenta, and the three species of periodontopathic bacteria (Fig. 1).

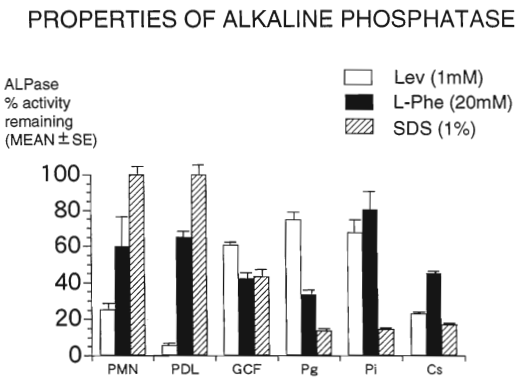

Fig. 1. Inhibition studies.
Inhibitory effects of levamisole (1 mM), L-phenylalanine (20 mM), and SDS (1%) on the ALP activities of PMNs, PDLs, GCF, *P. gingivalis, P. intermedia*, and *C. sputigena*. Percentage of remaining ALP activity.

II. *Electrophoresis thermostability test*

The samples were heated at 60°C for 30 min prior to electrophoresis, and their electrophoretic patterns were compared. The results demonstrated that the enzyme activity of the GCF preparation was separated into one or two bands. The main slow band was heat labile, and its inhibition profile indicated that the GCF contained a thermo-unstable isoenzyme. The minor fast band was heat stable and its electrophoretic pattern showed mobility similar to that in *P. gingivalis* (Fig. 2).

III. *Electrophoresis immunological specificity*

The samples were incubated with anti-bone type ALP or anti-intestinal type ALP prior to electrophoresis. In this respect, when a cross-reaction occurs between ALP and the corresponding antibodies, the ALP antibody complex shows some delay compared with the reaction time of the non-treated samples. Fig. 3 shows that a cross-reaction occurred between the anti-bone type ALP and the ALP of the GCF,

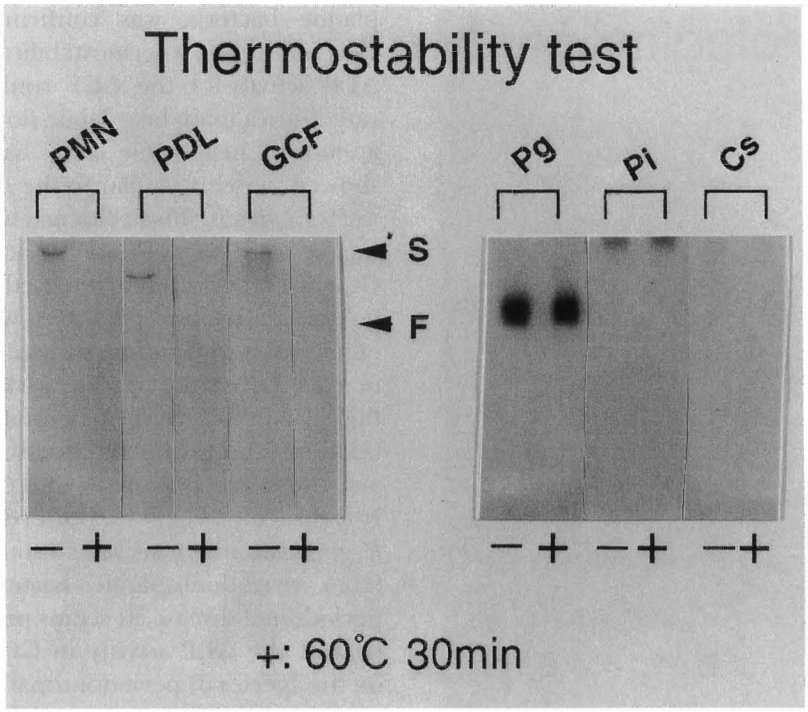

Fig. 2. Electrophoresis thermostability test.
Effect of heat treatment at 60 °C for 30 min on ALP activities of PMNs, PDLs, GCF, *P. gingivalis, P. intermedia,* and *C. sputigena.*
+: Heated at 60°C
−: Unheated
S: Heat labile slow band of the GCF
F: Heat stable fast band of the GCF

PMNs, and PDLs, because their ALP antibody complexes showed retarded electrophoretic mobility. The ALP of the *P. gingivalis, P. intermedia,* and *C. sputigena* did not cross-react with any anti-bone type ALP or anti-intestinal type ALP (data not shown).

IV. *Electrophoresis PI-PLC treatment*

ALP belongs to a growing family of known cell surface proteins that are covalently bound to phosphatidylinositol (PI) phospholipid complexes in the plasma membrane. Thus, such membrane-bound ALP can be released from cells by PI-specific phospholipase C (Noda et al. [27]). Fig. 4 shows that the main slow band of the GCF contained the phosphatidylinositol (PI)-moiety-anchored ALP. The ALP of the *P. gingivalis, P. intermedia,* and *C. sputigena* did not contain the phosphatidylinositol (PI)-anchored ALP.

Discussion

Qualitative evaluation of ALP in the GCF may open a possible way to detect periodontal disease activity, because ALPs in humans are classified into four main types of isoenzymes. In the present study, the biochemical characteristics of the ALP in the GCF from periodontal diseased sites were investigated to find its possible source and to identify that source, by means of isoenzyme identification procedures. The data obtained from the inhibition experiment showed that the ALP in the GCF was only slightly susceptible to levamisole (1

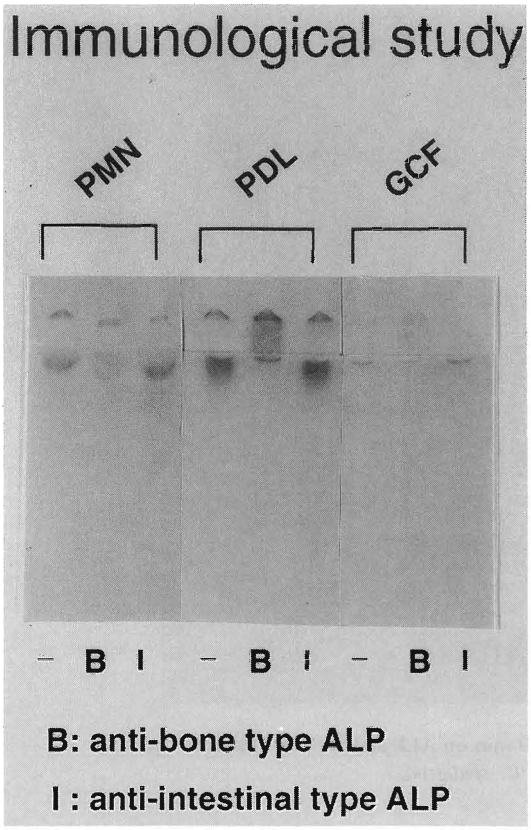

Fig. 3. Electrophoresis immunological specificity. 4 to 20%-polyacrylamide gel electrophoresis of ALP preparations of PMNs, PDLs, and GCF incubated with; –: 10 mM Tris-HCl buffer (pH 7.7), B: anti-bone type ALP, and I: anti-intestinal type ALP.

mM), L-phenylalanine (20 mM), and SDS (1%), and its properties were different from those ALPs in PMNs, PDLs, intestine, placenta, and three species of periodontopathic bacteria. Those bacteria seem to be associated with strong ALP activity (Poirier and Holt [11]; Yamashita et al. [12]; Shibata et al. [14]). The result of the ALP assay in GCF combined with SDS (1%) was similar to that reported by Shibata et al. [14] who distinguished oral bacterial ALP from human ALP by this method. The hypothesis that part of the ALP activity in GCF from periodontal diseased sites should be associated with subgingival plaque bacteria was confirmed by the electrophoresis thermostability test. The ALP activity of the GCF could be separated into a main heat-labile slow band and a minor heat-stable fast band, which showed mobility similar to the ALP activity in *P. gingivalis*. This evidence was interesting, because part of the ALP activity in the GCF from periodontal diseased sites might be derived from *P. gingivalis*, which is one of the most important pathogens involved in adult periodontitis (Socransky and Haffajee [28]). We could not establish a correlation among the ALP activity in GCF and those in *P. intermedia* and *C. sputigena*. It is difficult to believe, however, that only *P. gingivalis* might serve as a source of ALP from periodontopathic bacteria during periodontal disease. It seems probable that part of the ALP activity in GCF depends on the species of periodontopathic bacteria colonizing the periodontal pocket and thus promoting the periodontal disease. When we carried out an electrophoresis thermostability test using GCF from patients with disease-inactive periodontal pockets (data not shown), we could not easily identify any ALP isotypes. It was also impossible to detect the minor heat-stable band even by analyzing more than 10 μl of GCF. This evidence suggests that the ALP from periodontopathic bacteria may play an important role in periodontal pockets and could raise the level of ALP. The results of the electrophoresis thermostability test also showed that the ALP activity of the GCF contained a thermo-unstable isoenzyme which might be derived from host cells (TNSALP). The evidence that ALP activity of the GCF contains a TNSALP from host cells was also identified by an electrophoresis immunological specificity test. A cross-reaction occurred between the ALP of the GCF and anti-bone type ALP, which is a polyclonal antibody used to distinguish TNSALP from other ALP

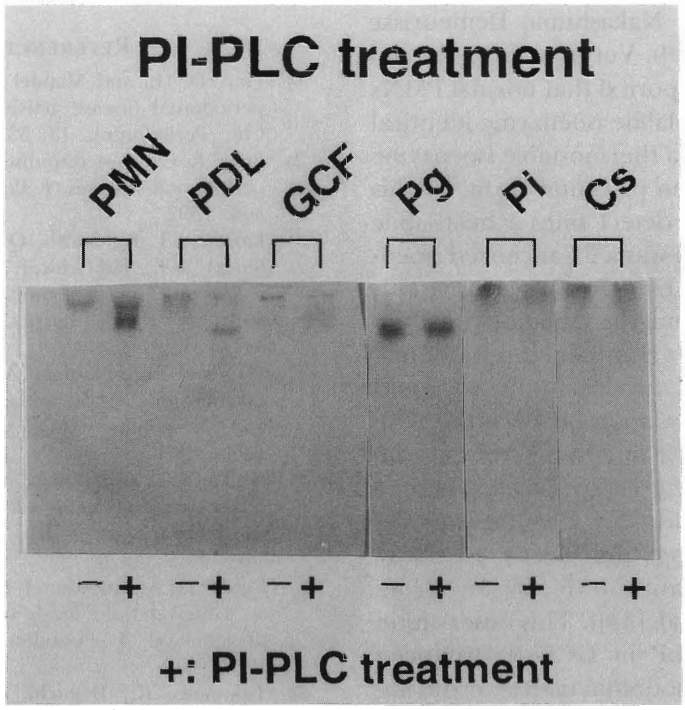

Fig. 4. Electrophoresis PI-PLC treatment.
4 to 20%-polyacrylamide gel electrophoresis of ALP preparations of PMNs, PDLs, GCF, *P. gingivalis, P. intermedia*, and *C. sputigena*. +: the enzyme preparations were treated with PI-PLC (0.1 U/ml), −: non-treated.

isotypes (Goseki, Oida and Sasaki [29]). Mammalian ALPs are present in two forms: an ALP-soluble form and an ALP-bound form covalently anchored to phosphatidylinositol (PI) phospholipid complexes located in the plasma membrane of cells. PI-PLC treatment releases mammalian ALP from the plasma membrane of cells (Noda et al. [27]) and changes its electrophoretic mobility. In contrast, bacterial ALPs are located in the periplasmic space at the cell surface (Poirier and Holt [30]; Lo Storto, Silvestrini and Bonucci [13]) and are not sensitive to PI-PLC treatment. Considering these different characteristics, we performed the electrophoresis PI-PLC treatment to distinguish between mammalian ALP and bacterial ALP. The results proved to be useful in that the GCF contained PI-anchored ALP only in the main heat-labile slow band, which was related to mammalian ALP. On the contrary, the minor heat-stable fast band, which was associated with *P. gingivalis*, did not appear to be sensitive to PI-PLC treatment. This result confirmed that the origin of the ALP activity in GCF can be distinguished by PI-PLC treatment. Despite the fact that PDLs and fibroblasts in the inflammatory connective tissue possess different levels of ALP activity (Oshima et al. [31]; Abe et al. [8]), these cells express the same TNSALP isotype (Yamashita, Sato and Noguchi [32]; Abe et al. [8]), which might contribute to an increase in the level of ALP of the GCF. The major source of ALP in the GCF is probably derived from PMNs (Fine

and Mandel [1]; Nakashima, Demeurisse and Cimasoni [10]). Vergnes, Grozdea and Corberand [9] reported that normal PMNs contain a thermolabile isoenzyme identical to TNSALP and a thermostable isoenzyme that resembles the placental form. In this study, we could detect only a heat-labile isoenzyme, which was a PI-anchored isoenzyme. GCF is an exudate from the vessels of the gingival microcirculation into the sulcus or pocket via inflamed periodontal tissue (Page [2]). In this study, we could not find any correlation between the ALP activities from GCF and those from serum, even by 4 to 20% gradient polyacrylamide gel electrophoresis. This method is useful for distinguishing the liver-type ALP, which is dominant in the serum from adults (Goseki et al. [33]). This observation suggests that ALP in GCF is produced locally in the periodontal tissues or during periodontal disease progression and consists of several types of ALP. Using the isoenzyme method, we could distinguish that these ALPs originated from bacterial and host cells. The present findings suggest that their origins might be host response PI-anchored ALP and periodontopathic bacterial ALP. More qualitative and quantitative evaluations of ALP in the GCF will be necessary, however, to fully understand the significance of the different ratios of activity of the ALP isotypes in the GCF during disease activity.

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