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Purine nucleoside phosphorylase activity and expression are upregulated in sites affected by periodontal disease

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Background and Objective: Purine nucleoside phosphorylase (PNP) is an enzyme that catalyzes the reversible phosphorolysis of purine nucleosides, playing a key role in the purine salvage pathway. Activated T cells seem to rely heavily on PNP to remain functionally active and are particularly sensitive to PNP deficiency. The role of PNP in periodontal tissues has not been characterized thus far. The aim of this study therefore was to assess the activity and expression of PNP in the gingival tissues of periodontitis patients.

Material and Methods: Ten patients consecutively admitted for treatment had their periodontal clinical variables recorded and their gingival crevicular fluid collected. After periodontal treatment the patients were seen once a month for plaque and bleeding control, and had their periodontal variables recorded and gingival crevicular fluid collected at 90 and 180 d. Purine nucleoside phosphorylase-specific activity was assessed using a spectrophotometer through the addition of the PNP substrate analog 2-amino-6mercapto-7-methyl purine riboside to the gingival crevicular fluid. In parallel, PNP expression was assessed by immunohistochemistry and real-time PCR in gingival biopsies and cell culture.

Results: Purine nucleoside phosphorylase activity was higher in the gingival crevicular fluid of periodontally diseased sites, which was positively correlated with improvements of the clinical variables. Treatment of periodontal disease induced a striking decrease of PNP activity in periodontally diseased sites. Expression of PNP was more pronounced in mononuclear cells and endothelial cells of the gingiva, and the mRNA levels were 5.7-fold higher in inflamed tissues compared with control samples.

Conclusion: Purine nucleoside phosphorylase activity and expression are upregulated in periodontally diseased sites and can be detected in the gingival crevicular fluid.

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Periodontitis is a complex disease that involves the activation of cells from the innate and acquired immune systems leading to destruction of the supporting tissues (1). The role of monocytes and lymphocytes has been extensively studied in periodontal lesions, pointing out important functions for these cell subsets in the modulation of tissue destruction (2). A series of findings indicates that activation of the immune response is key in triggering mecha-

nisms that will ultimately lead to bone loss in response to the accumulation of microorganisms around teeth (3,4). In this scenario, T lymphocytes and the corresponding array of cytokines secreted in response to bacteria and their products lead to bone loss through the expression of RANKL and the modulation of osteoclastogenesis that causes bone loss (5).

Purine nucleoside phosphorylase (PNP) is a purine-metabolizing enzyme that catalyzes the reversible phosphorolysis of purine nucleosides such as deoxyinosine and deoxyguanosine to their respective bases and deoxyribose-1-phosphate (6). Purine nucleoside phosphorylase is a key enzyme in the purine salvage pathway of mammalian cells, where inosine and guanosine derived mainly from ribonucleotide hydrolysis, and 2′-deoxyguanosine derived from DNA degradation, are its main substrates (7). Activated T cells seem to rely heavily on PNP activity to maintain their functions and are particularly sensitive to PNP deficiency (8), which is attributed to a relatively high level of kinase and a low level of nucleotidase activity compared with other cells (6,9). Inherited genetic deficiency of PNP leads to a selective depletion of T cells with normal to elevated B-cell function (10).

Cytokines, lymphokines and enzymes have been assayed in the periodontium using different techniques and study models, in an attempt to characterize, in greater detail, the events that take place in the periodontium during attachment loss (11–15). As bone destruction in periodontal disease is critically affected by cells of the immune system, notably $CD4^+$ T-cell subsets, which seem to rely heavily on the purine salvage pathway in the activated state, we sought to assess the activity of PNP in the gingival crevicular fluid of patients suffering from periodontal disease.

Material and methods

Examination of patients and gingival crevicular fluid sampling

Patients consecutively admitted to the clinic of Periodontology of the Dental School at the Pontificia Universidade Catolica do Rio Grande do Sul (PUCRS) for periodontal treatment were enrolled in this trial. All subjects received a detailed description of the study and signed an informed consent form before being allowed to participate. The outline of this study followed the ethical principles adopted by the World Medical Association Declaration of Helsinki and was approved by the Ethics Committee of the Dental School, the University General Ethics Council and the National Council of Ethics (protocol #07/04023). Six patients with aggressive periodontitis (28.6 ± 3.78 years of age) and four patients with chronic periodontitis (65.3 ± 4.31 years of age), who were consecutively admitted, had no signs or history of compromised health, no evidence of diabetes and were non-smokers, and who presented at least six sites with attachment loss in the form of pockets ≥ 5 mm, were enrolled in this trial. During baseline consultation the patients underwent a through periodontal examination; at this time the probing pocket depth, relative clinical attachment level, visible plaque and bleeding on probing were electronically recorded using a constant force probe (Pocket Probe and Disc Probe; Florida Probe®, Gainsville, FL, USA). The target teeth were washed with a spray of water, air-dried and isolated with cotton rolls; when present, visible plaque was removed with a sterilized curette, and an 8 mm × 2 mm strip of sterile Whatman chromatography paper was inserted approximately 4 mm deep into the crevice or until resistance was encountered; the paper strips were held in position for 30 s and were then transferred to 1.5-mL microcentrifuge tubes containing 80 μL of cold sterile 10 mM NaCl. Enrichment of the gingival crevicular fluid mixture was further increased, as previously described (16), with modifications. Briefly, filter papers containing the gingival crevicular fluid were transferred to 600-μL tubes pierced with a sterilized needle, which was positioned at the rim of the 1.5 mL microcentrifuge tube and centrifuged at 8000 *g* in a tabletop centrifuge for 10 min at 4°C. The eluate was subjected to a further two to five centrifugations until no signs of cells and/or debris were visible at the bottom of the tube as pellets. Paper strips containing blood and the respective sites were excluded from the analysis. The gingival crevicular fluid eluates were kept on ice, aliquoted and stored at −80°C until further analysis. The total protein concentration present in the gingival crevicular fluid was determined spectrophotometrically, at 595 nm, on a plate reader using the Bradford assay, against serial dilutions of bovine serum albumin.

Periodontal treatment

Aggressive periodontitis was diagnosed according to established criteria (17) and patients received full-mouth scaling and root planing treatment with hand and ultrasonic instruments, and systemic antimicrobial therapy was prescribed in select cases (amoxycillin 500 mg, every 8 h; metronidazole 400 mg, every 12 h) for 10 d according to the protocol of the Department of the Clinic. Patients with chronic periodontitis were also treated with hand and ultrasonic instruments at single appointments, but no systemic antimicrobials were prescribed. During the post-treatment phase, patients rinsed with chlorhexidine gluconate 0.12% (Periogard, Colgate, Sao Paulo, SP, Brazil), twice daily for 10 d and then mechanical plaque control measures were re-established. Patients received instruction on the correct toothbrushing techniques and flossing and were seen once a week in the first month and every 15 d for 6 months after treatment. During maintenance visits the presence of visible plaque and bleeding were assessed and the teeth were subjected to crown polishing. Probing pocket depth, relative clinical attachment level, visible plaque and bleeding on probing were re-assessed at 90 and 180 d after treatment, and gingival crevicular fluid was collected as described above.

Specific activity of PNP in the gingival crevicular fluid

Specific activity of PNP was assessed using a modification of the phosphate assay as previously described (18). In

the presence of inorganic phosphate the synthetic 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) substrate (Enzcheck phosphate assay kit®; Molecular Probes, Carlsbad, CA, USA) is enzymatically converted by PNP to 2-amino-6-mercapto-7-methylpurine, which leads to a shift in the maximum absorbance from 330 to 360 nm over the pH range 6.5–8.5. For each sample collected, a reaction mix containing 50 mM Tris–HCl, 1 mM $MgCl_2$ (pH 7.5), 200 μM KH_2PO_4 and 200 μM MESG was assembled in a 0.5 mL quartz cuvette; after 1 min of incubation at 25°C, 20 μL of gingival crevicular fluid was added. The enzymatic conversion of the MESG substrate was immediately monitored at 360 nm for 60 s, at 5 s intervals in a spectrophotometer (Shimadzu UV-2550, Columbia, MD, USA). The substrate conversion was established within 1 min under initial rate conditions and PNP activity (units/mL) was calculated (using the Beer equation):

$$\Delta A = \varepsilon(M^{-1}cm^{-1}) \times C(Molar) \times b(cm),$$

Where: where ΔA = absorbance (Abs/min); ϵ = coefficient of molar absorbance at 360 nm; C = Molar concentration; and b = optical path (1 cm).

The number of units/mL was then divided by the total protein concentration (mg/mL) of the gingival crevicular fluid sample to adjust for variations in the total protein content, and the shift in absorbance was expressed as purine nucleoside phosphorylase specific activity (U/mg) (i.e. the amount of enzyme necessary to generate 1 μmole of product per minute in an optical path of 1 cm). In order to access the specificity of the purine nucleoside phosphorylase-catalyzed phosphorolysis, two randomly selected gingival crevicular fluid samples were co-incubated with a selective purine nucleoside phosphorylase inhibitor (Immucillin-H; BioCryst Pharmaceuticals, Birmingham, AL, USA) to a final concentration of 1 μM, as previously reported (19).

Histology and immunolocalization of PNP in gingival tissues

Tissue biopsies were harvested during routine periodontal surgery carried out for pocket elimination or crown lengthening. Immediately after harvesting, the tissues were washed in sterile phosphate-buffered saline (PBS) and transferred to microfuges containing 10% buffered formalin. Samples were fixed at room temperature for 48 h and processed for paraffin embedding. Five-micrometer serial sections of paraffin-embedded samples were cut and mounted over silanized glass slides for hematoxylin and eosin staining. For immunohistochemistry, endogenous peroxidase was blocked with H_2O_2 and further incubated overnight with diluted (1 : 100) goat anti-hPNP polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing unbound antibodies with PBS, the sections were incubated with horseradish peroxidase-conjugated donkey anti-goat secondary antibody for 2 h at room temperature. Diaminobenzidine substrate was added to the reaction and the sections were counterstained with eosin. Negative controls included sections not treated with secondary antibodies and sections treated with nonimmunized goat sera. The slides were analyzed under a light microscope and the images of interest were captured through a digital camera. The whole tissue was screened for qualitative detection of PNP expression at microfields extending from the base of the sulcus towards the oral epithelium. Only representative fields were presented.

PNP mRNA levels in periodontal tissues

RNA purification For RNA purification, six samples obtained during pocket elimination surgical procedures and six gingival samples obtained during esthetic surgical crown-lengthening procedures, with no signs of clinical inflammation, were used. The specimens were immediately stored in RNA Stabilization Reagent (RNA later™, RNA Stabilization Reagent; Ambion, Austin, TX, USA) and stored at −20°C. Total RNA was isolated using affinity chromatography and on-column DNase I treatment according to the manufacturer's protocol (RNasy® Protect; Qiagen, Valencia, CA, USA). RNA concentrations were spectrophotometrically determined at 260 nm (1 OD = 40 μg/mL), and sample purity was determined by the 260/280 ratio. Only samples with a 260/280 ratio of >1.7 were included. The purified RNA was stored at −80°C. The quality of RNA was assessed by separation in 1% formaldehyde-denaturing gels stained with SYBRGold™ (Invitrogen™; Molecular Probes). Sharp 28S and 18S ribosomal RNA bands without smearing were obtained for all samples, enabling their use for reverse transcription.

Differential expression of the PNP gene Complementary DNA (cDNA) was synthesized from 100 ng of total RNA with random hexamers in a reaction mixture containing 10 mM of each dNTP, 2 mM $MgCl_2$ and one unit of the Murine Leukemia Virus Reverse Transcriptase (MultiScribe™ Reverse Transcriptase, TaqMan® Reverse Transcription Reagents; Roche Molecular, Indianapolis, IN, USA) in 20-μL reaction mixtures (giving a final concentration of approximately 25 ng/μL of cDNA). The reaction mixtures were subjected to cycles of 25°C for 10 min, 48°C for 30 min and 95°C for 5 min in a thermal cycler (Techne™ TC-412; Duxford, Cambridge, UK). Real-time RT-PCR was performed using primers and TaqMan probes labeled with FAM-MGB dye (Assays-on-Demand; Applied Biosystems, Foster City, CA, USA) for human *PNP*. Amplification was carried out in 25-μL duplicates, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Human Endogenous Controls; Applied Biosystems) was amplified in parallel with the gene of interest and used for normalization of the reverse transcription reactions. Before starting the amplification assays, the efficiency of the system was checked for every gene of interest according to previously established guidelines (20). Briefly, approximately 500 ng of a randomly selected gingival cDNA was subjected to a 10-fold serial dilution (500–0.5 ng and nontemplate control) and tested with *PNP* and *GAPDH* primers/

probes. R^2 values were 0.9789 and 0.9976 for *PNP* and *GADPH*, respectively. Relative expression was calculated according to a previously described method ($2^{\Delta\Delta Ct}$) having the control tissue as the calibrator or reference variable.

PNP activity and expression by CD4+ T-memory cells

$CD4^+$ T-memory cells were isolated from the peripheral blood by venipuncture from three male healthy donors using negative selection with magnetic beads as described by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, monocytes were isolated through gradient sedimentation using Histopaque 1777 then washed in cold PBS and treated with a biotin-labeled antibody cocktail that recognized a series of markers found in nonmemory $CD4^+$ T cells. A monoclonal, microbead-conjugated antibody against biotin was then added, which magnetically retained nonmemory $CD4^+$ T cells while the target cells passed freely through the column. Cell purity was assessed by flow cytometry using fluorescein isothiocyanate-labeled anti-CD4 Ig1 (93%; data not shown). Aliquots containing 2×10^6 cells were plated in triplicate and transferred to 24-well plates containing RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum. Cells were incubated at 37°C and 5% CO_2 in a humidified chamber, and, after 24 h, were treated with either the mitogen concanavalin A (10 mg/mL) or PBS, and incubated for 4 h. Cells were then harvested, counted and 5×10^5 cells from each group were used to determine PNP activity. Cell aliquots were sonicated in cold Tris buffer (pH 7.6) for 3 min, maintained on ice and processed for PNP activity as described above. Total protein concentration was assessed using fluorimetry (Qubit; Invitrogen, Carlsbad, CA, USA). In parallel, aliquots containing 2×10^6 cells were incubated either with concanavalin A or vehicle for 0, 8, 12 and 24 h. Total RNA isolation, concentration, purity and quality were evaluated as described above. For PNP quantitative analysis, approximately 10 ng of cDNA was amplified in duplicate ($n = 3 \times 2$), as described above.

Statistical analysis

Descriptive statistics were calculated for all variables. The Shapiro-Wilk test was used to determine the distribution patterns of the variables considered herein. The Friedman nonparametric test for repeated measures was chosen, along with the Dunn's post-hoc test, to assess PNP activity in gingival crevicular fluid. Changes in the categorical variables assessed herein (i.e. bleeding on probing and visible plaque) were analysed using the nonparametric Chi-square test. The Student's *t*-test for independent groups was used to assess PNP activity in $CD4^+$ T cells and PNP gene expression in healthy and inflamed gingival tissues. Analysis of variance (ANOVA), followed by Bonferroni's post-hoc test, was used to analyse *PNP* gene expression changes in $CD4^+$ T cells. Tests were carried out using a statistical package (Prism 5 for Mac, GraphPad Inc., La Jolla, CA, USA) and differences were regarded as significant if the *p*-value was < 0.05.

Results

Clinical variables

An overall significant improvement was observed in all 83 periodontal sites evaluated as well as in patient compliance throughout treatment. As a consequence, a marked reduction in bleeding on probing, suppuration and visible plaque was observed for all 10 patients. The observed mean probing pocket depth at baseline was progressively reduced from a mean of 6.39 ± 1.06 mm at baseline to 4.22 ± 0.80 and 3.5 ± 0.67 mm at 90 and 180 d, respectively ($p < 0.01$, Fig. 1A). Likewise, a mean clinical attachment level gain of 2.75 ± 0.752 mm at 180 d was observed, which was significantly different from the 90-d clinical attachment level gain (Fig. 1B). There was also a striking improvement in the overall gingival status, as depicted by a reduction in the number of bleeding sites and a reduction in visible plaque (data not shown).

PNP specific activity in the gingival crevicular fluid

Purine nucleoside phosphorylase specific activity in the gingival crevicular fluid underwent a significant decrease at our first post-therapy evaluation at 90 d, which continued until the 180-d period after periodontal treatment (Fig. 1C; $p < 0.01$). Between 90 and 180 d, PNP activity continued to decrease ($p < 0.05$), which reflected the overall continuous improvement observed in the clinical variables assessed. There was a positive correlation between PNP specific activity and probing pocket depth reduction ($r = 0.561$, $p < 0.01$), as well as between PNP specific activity and the explanatory variable 'time after treatment' ($r = 0.636$; $p < 0.001$). Clinical attachment level gain was inversely correlated to PNP specific activity ($r = -0.516$; $p = 0.005$), corroborating the association between periodontal outcome after treatment and PNP activity. No differences were observed for localized aggressive periodontitis and patients with chronic periodontitis with respect to PNP activity ($p > 0.05$). The addition of a specific pharmacological inhibitor of PNP to two randomly selected gingival crevicular fluid samples showed complete absence of shift in the absorbance, confirming the specificity of phosphorolysis mediated by the PNP present in the gingival crevicular fluid (data not shown).

Distribution of PNP in gingival tissues

Purine nucleoside phosphorylase was detected in the periodontal tissues in the three samples analyzed. In general, the tissue samples had a rather diminished number of inflammatory cells because the tissue was harvested from patients who had undergone basic therapy before to pocket-reduction surgery. The samples comprised only keratinized soft tissue, and the major areas screened were those bordering

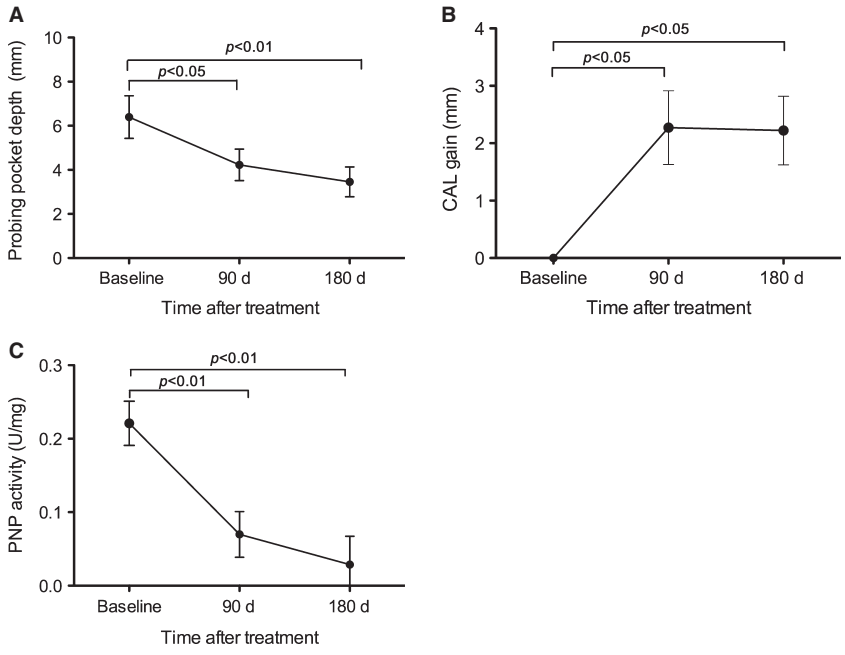

Fig. 1. Changes in clinical variables and in purine nucleoside phosphorylase (PNP) activity. Treatment led to a decrease in probing pocket depth (A) and to an increase in clinical attachment gain (B) at 90 and 180 d. The specific activity of PNP decreased after therapy, which accompanied the clinical improvements observed (C). Means and standard deviations of 83 periodontal sites from 10 individuals are shown.

the anatomical sulcus and connective tissue. Purine nucleoside phosphorylase expression was detected mainly in monocytes, lymphocytes, endothelial cells and cells of the basement membrane (Fig. 2).

mRNA levels of PNP in gingival tissues

The results of the fluorescence-assisted relative expression analyses of PNP revealed that gingival samples harvested from pocket-elimination procedures showed higher PNP mRNA levels than calibrator samples (i.e. those harvested from crown-lengthening procedures carried out for esthetic reasons). In fact, the expression of purine nucleoside phosphorylase was generally 5.7-fold higher in the gingiva of sites subjected to pocket elimination, mostly distal wedges (Fig. 3).

PNP activity and expression in stimulated CD4+ Tlymphocytes

The results showed that when the same amount of total protein and RNA from a strictly controlled number of human memory $CD4^+$ T cells were stimulated with a mitogen, there was a rapid and significant increase in the PNP enzyme activity (Fig. 4) and in the expression of PNP mRNA (Fig. 5). RNA levels took longer to be upregulated after stimulation with the mitogen (24–48 h), while no changes were observed in cells incubated with vehicle alone up to 48 h (data not shown).

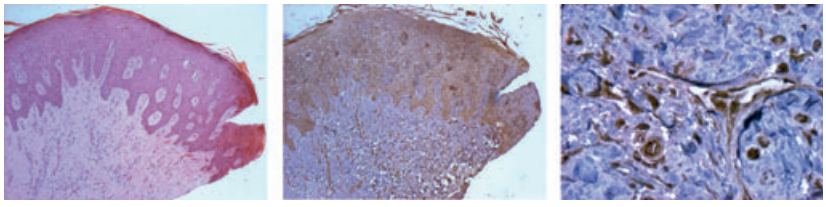

Fig. 2. Purine nucleoside phosphorylase (PNP) immunolocalization in gingival tissues. Gingival tissues were harvested and stained with hematoxylin and eosin (left panel, 50X) and further incubated with antibodies against human PNP; PNP expression was broadly observed in the gingiva (central panel, 50X). Right panel is a higher magnification (400X) of the connective tissue area of the central panel, showing that PNP is expressed mainly in monocytes and endothelial cells. The figure shows representative images of three tissue samples harvested.

Discussion

In the present investigation we sought to assess the presence of purine nucleoside phosphorylase activity in the gingival crevicular fluid of periodontally diseased individuals. Purine nucleoside phosphorylase catalyzes the reversible phosphorylation of purine nucleosides, such as deoxyinosine and deoxyguanosine, to their respective bases and deoxyribose-1-phosphate (6,7). Purine nucleoside phosphorylase uses inosine and guanosine derived from the hydrolysis of ribonucleotides and 2-deoxyguanosine, the latter directly derived from the degradation of DNA. As a result of this catalytic reaction, purines are salvaged as opposed to being synthesized *de novo*, which is more demanding for the cell from an energy consumption standpoint.

In this context, purine metabolizing enzymes, notably PNP, seem to have a key role in the modulation of cell-based immune responses. The importance of PNP in the immune response is highlighted by the fact that genetic deficiencies of PNP have been linked to impairment of lymphocyte proliferation without concomitant compromise of B lymphocytes (10,21). Activated lymphocytes seem to rely heavily on the action of PNP for the activation of the purine salvage pathway, rather than the *de novo* synthesis of purines (9). Among the different enzymes involved in the metabolism of purines, there seems to be some specificity within the T-cell lineage; T-helper cells seem to have higher PNP activity compared with other enzymes of the same family and slightly higher activity than T-suppressor cells (22). Indeed, it has been noted that cells exhibiting high levels of PNP activity are partic-

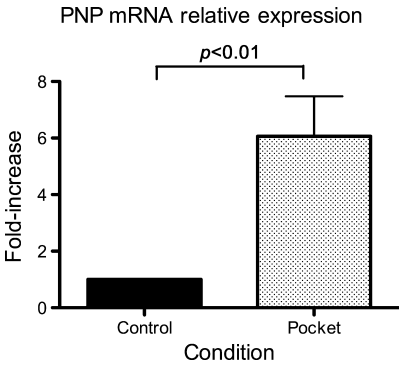

Fig. 3. Quantitative assessment of purine nucleoside phosphorylase (PNP) mRNA expression in gingival tissues. Purine nucleoside phosphorylase was upregulated in gingival tissues harvested for pocket elimination (test) compared with clinically non-inflamed tissues (controls). The means and standard deviations of three pairs of samples (three controls and three tests), run in triplicate, are presented. Control tissue is the calibrator and therefore is assigned a value of '1' in relative expression.

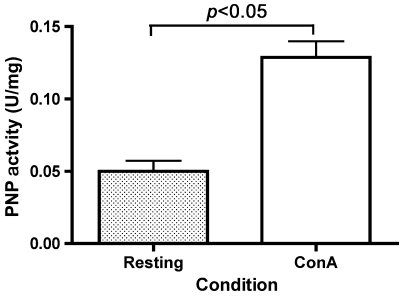

Fig. 4. Specific activity of purine nucleoside phosphorylase (PNP) in $CD4^+$ T cells. Incubation of memory cells, isolated from peripheral blood, with the mitogen concanavalin A (ConA) resulted in the upregulation of PNP specific activity (results are shown from cells isolated from three individuals and measured in duplicate).

ularly dependent on this enzyme for survival (23). Functionally, decrease/abrogation of PNP activity in T cells leads to an accumulation of intracellular deoxyguanosine; this imbalance causes activation of deoxycytidine kinase and phosphorylation of deoxyguanosine to deoxyguanosine monophosphate, which is, in turn, converted by cellular kinases into deoxyguanosine triphosphate. Accumulation of the latter in the cell causes a major imbalance, which inhibits ribonucleotide reductase activity and thus prevents the production of deoxyribonucleoside diphosphates. This process translates into the inhibition of DNA synthesis, impairing cell proliferation and activation (24,25). Functional studies of human peripheral blood lymphocytes *in vitro* have shown that the generation of antigen-specific T lymphocytes is selectively affected by deoxyguanosine levels, corroborating the importance of the purine salvage pathway in the effector function of these cells (6). Previous evidence related to the physiology of lymphocytes also supports the fact that different T-cell subsets vary in their sensitivity to the accumulation of deoxyguanosine (8).

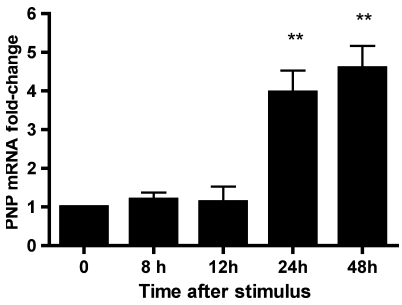

Fig. 5. Purine nucleoside phosphorylase (PNP) mRNA expression in $CD4^+$ T cells. Memory cells were isolated from the peripheral blood of three individuals and incubated with a mitogen for 8, 12, 24 and 48 h. Increase of PNP mRNA levels was observed only after 24–48 h and reached approximately fourfold. Basal expression levels are represented by a value of '0', and cells not incubated with mitogen served as the calibrator and are therefore assigned a value of '1' for relative expression ($^{**}P < 0.01$).

As purine nucleoside phosphorylase has been claimed to be an important enzyme involved in T-lymphocyte activation, it seemed reasonable to assume that it might also have some role in the processes that take place in the periodontium. Our results showed that PNP activity is high in sites suffering from untreated periodontitis and undergoes a progressive decrease once treatment is implemented. Periodontal disease has been regarded as a complex malady modulated by different cell subsets in response to a myriad of virulence factors imposed by a highly organized biofilm. Several factors pinpoint the multiplicity of mechanisms triggered during the host response, reflecting the activation of distinct pathways that may overlap with regards to the inflammatory/immune outcome. The outcome of such a highly orchestrated scenario is destruction of the periodontal supporting tissues in many instances. Although polymorphonuclear cells have a key role in the orchestration of the host response as a link between innate and adaptive immunity (26), evidence highlights the involvement of lymphocytes in the destruction of alveolar bone (1,27). Our findings could be explained by the nature of the inflammatory response that is present in the periodontium and the importance that lymphocytes have in this process, although the role of other cell types cannot be ruled out. In order to allow the healing process to occur undisturbed, no attempts were made to assess the target sites for 30 d after treatment using additional probing and sampling procedures, so it would be difficult to reach any conclusions regarding PNP activity in the early phases of healing. Nevertheless, considering the progressive improvement observed, this premise should not be ruled out. In fact, the progressive decrease in PNP activity over 6 mo of evaluation in the presence of a strict plaque control accompanied the improvements observed for the clinical variables over the same period of time. It is noteworthy that the activity of PNP in the periodontium of individuals who have not been affected by periodontal disease was not assessed here; in fact, analysis of the gingival crevicular fluid of healthy individuals would be very helpful in determining possible thresholds between health and disease, so that PNP could potentially serve as a marker of disease status. Therefore, currently, no assumptions can be made about PNP as a periodontal disease marker.

Purine nucleoside phosphorylase is an intracellular enzyme, and its presence in the gingival crevicular fluid shows that it is able to access the

extracellular environment and drain into the gingival crevicular fluid. This could be a result of host cell damage, release of the intracellular component during the inflammatory process, aspects related to the collection and processing of gingival crevicular fluid, and even the existence of a soluble form of the PNP enzyme. Interestingly, PNP activity has been found in the rat cerebrospinal fluid (19); PNP could have a neuroprotective function in the cerebrospinal fluid, as a regulator of inosine and guanosine released by neural cells. Despite the obvious differences between the nervous system and the periodontium, and the states where those have been assayed (i.e. physiological vs. inflammatory, respectively), it must be recognized that the source of PNP in the gingival crevicular fluid might not be restricted only to lymphocyte subsets as PNP may be upregulated in many other cell types during inflammation.

The gene expression results seemed to corroborate the gingival crevicular fluid findings at a different level, as *PNP* mRNA was directly measured from tissue samples and from $CD4^+$ T cells. In our approach we sought to obtain tissue samples routinely harvested from pocket elimination procedures distal to upper molars (distal wedges) 30 d after basic therapy and after routine crown lengthening for esthetic purposes. The latter was chosen as a control because the buccal anterior areas are usually less prone to inflammation, and indeed none of the harvested tissues presented bleeding on probing just before tissue harvesting. Our findings showed that *PNP* expression was higher in the gingival tissues of the distal wedges probably because there was still some residual activity of the inflammatory/immune response in deeper aspects of the pocket. However, this approach does not discriminate between different cells expressing *PNP* because a pool of tissue RNA was analyzed. As a matter of fact, immunohistochemistry showed that, along with mononuclear cells, cells from the basement membrane and endothelial cells also expressed the enzyme that can also lead to higher drainage of PNP to the gingival crevicular fluid. Regardless of the predominant cell subsets contributing to the increase of PNP activity detected in the gingival crevicular fluid, treatment has been very effective in reducing it.

If we consider the aforementioned evidence only in the context of the immune response that occurs in the periodontium, it can be assumed that the higher activity of PNP observed before treatment is, at least in part, related to the increase in the activation of lymphocytes, among other cells, that ultimately rely on the purine salvage pathway to efficiently respond to antigens (6). Nevertheless, the findings could simply reflect the increased number of cells recruited to the tissues, rather than the actual upregulation of gene expression and enzyme activity. Importantly, an increased number of cells at sites of injury, and total crude enzyme levels, do not necessarily reflect changes in enzyme activity. Therefore, *ex vivo* assays using memory $CD4^+$ T lymphocytes that enabled a strict control of cell numbers, as well as the total RNA and total protein amounts, were employed. The findings showed that the activation of $CD4^+$ T cells with a mitogen led to increased activity and expression of PNP that was not observed in cells which received vehicle alone. These findings substantiate the upregulation of PNP activity and expression as being the result of a response of cells to biological agonists rather than just an increased cell number. Purine nucleoside phosphorylase activity is increased in the event of a signal that induces cell proliferation, but the events that take part in this process, such as upstream activators and the fate of the protein after acting upon the substrate, are poorly known.

We have shown, for the first time, the specific activity of PNP in the gingival crevicular fluid obtained from periodontal sites presenting attachment loss and bleeding on probing. Noteworthy, periodontal treatment did lead to a reduction of PNP activity in the gingival crevicular fluid, which probably reflects the shift in the state of health of the periodontal site. This study paves the way for ongoing and future investigations aiming at evaluating PNP as a potential marker and modulator of periodontal disease activity, and as a target for disease progression arrest.

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