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ORIGINAL ARTICLE

Nafamostat mesilate, a potent tryptase inhibitor, modulates periodontitis in rats

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Abstract Previous reports have demonstrated increased tryptase-like proteolytic activity in the crevicular fluid of patients with periodontal disease. In the present study, we have investigated the effect of tryptase inhibition with nafamostat mesilate (NM, 6-amino-2-naphtlyl *p*-guanidinobenzoate dimethansulfonate) on the development of experimental periodontitis in rats. Eighty (80) male Wistar rats were randomly separated into four groups: Control group, NM group (daily 0.1 mg/kg body weight of NM, i.p.), Ligature group (ligature placed at lower right first molars), and NM+Ligature group. The amount of alveolar bone loss (ABL) around the mesial root surface of the first mandibulary molar, as well as the myeloperoxidase (MPO) activity, and total proteolytic activity [*N*-benzoyl-L-arginine-*p*-nitroanilide (BApNA) substrate] were determined at 7 and 14 days. NM led to significantly ($p<0.05$) decreased ABL in animals subjected to ligature-induced periodontitis. Tryptase inhibition prevented the onset of significant ABL at 7 days of experiment (0.44±0.16 and 0.60±0.22, $p>0.05$, NM+Ligature and Control, respectively) and significantly decreased the ABL at 14 days (0.97±0.17 versus 1.82±0.26, $p<0.001$, NM+Ligature versus Ligature, respectively). In addition, NM significantly decreased MPO and total proteolytic activity at 14 days ($p<0.05$). These data provided evidence that tryptase inhibition with NM attenuates gingival granulocyte infiltration and ABL in an experimental model of periodontitis in rats.

Keywords Periodontal diseases · Tryptases · Alveolar bone loss · Rats

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Introduction

Periodontal disease is the most important cause of tooth loss in adult population [1]. Although multifactorial, the pathogenesis of periodontitis involves the presence of a bacterial biofilm, which initiates a local inflammatory reaction in a predisposed host, therefore resulting in tissue destruction and alveolar bone loss (ABL) [2]. A number of cells and their mediators orchestrate the organized and complex periodontal immune system during the inflammatory process [3].

In all the inflammatory cascades of periodontal disease, there are some steps which are mediated by proteolytic enzymes. In fact, increased levels of proteolytic activity have been found at the gingival crevicular fluid, where a mixture of host endogenous enzymes and bacterial proteases combine each other in order to mediate connective tissue breakdown [4]. Host enzymes comprise the serine proteases like trypsin, elastase, plasmine, complement enzymes, and tryptase [5, 6].

Tryptase is a serine protease which is mainly stored at the mast cell granules found in the vast majority of the tissues, but especially at the mucosal tissue and at the subcutaneous connective tissue [7]. Mast cells are found in the connective and epithelial gingival tissues [8, 9]. Increased levels of mast cells have been found at the inflamed gingival tissue in patients with periodontitis in comparison with healthy periodontal patients [9–11]. It is well known that when activated by cytokines or bacterial products, mast cells undergo degranulation and release tryptase and other inflammatory mediators [7]. In fact, an increased trypsin-like proteolytic activity at the crevicular fluid of patients with chronic periodontitis correlates positively with the severity of clinical attachment loss and bone destruction [12, 13]. In addition, an improvement of the clinical parameters after periodontal therapy leads to a significant reduction of the tryptase-like activity present at the crevicular fluid [14, 15].

Tryptase involvement in the innate immune response has been shown in a number of chronic inflammatory diseases, such as rheumatoid arthritis [16], and inflammatory intestinal diseases [17]. Tryptase induces vascular changes and exudation of fluid and neutrophils [18], inflammatory cells chemotaxis [19], increased cyclooxygenase-2 expression [20], increased production of pro-inflammatory mediators, such as prostaglandins and interleukin-8 [7], and matrix metalloproteinase-2 (MMP2) activation [21], all known mediators of periodontal tissue destruction. In addition, anti-tryptase therapy, through nafamostat mesilate (NM, 6-amino-2-naphtlyl *p*-guanidinobenzoate dimethansulfonate) administration, the most potent tryptase inhibitor, has been considered efficient in the therapeutic management of inflammatory diseases [22, 23].

The importance of tryptase as an essential effector molecule on the immune response associated with several inflammatory diseases, besides its presence at the crevicular fluid and at the inflamed gingival tissues, may suggest a possible role for this enzyme on the pathogenesis of chronic periodontal disease. However, the biological significance of this host protease on periodontal tissue breakdown is still unknown. Therefore, the main objective of the present investigation was to evaluate the effect of serine protease inhibition with NM on the development of experimental periodontitis in rats.

Materials and methods

Experimental protocol

Male Wistar rats (12–14 weeks old, 250–300 g) were housed in temperature-controlled rooms and received water and food ad libitum. The Animal Care and Ethic Committees of the University of Taubaté approved all experimental protocols.

Induction of experimental periodontitis was accomplished through ligature placement around the right mandibular first molars. First, rats were anesthetized by intramuscular administration of ketamine (0.08 ml/100 g bodyweight) and xylazine (0.04 ml/100 g bodyweight), and then a 3.0 silk ligature was placed around the tooth as previously described [24]. Sham-operated rats were anesthetized and treated as ligatured rats with the exception of ligature.

The animals were randomly separated in four experimental groups: (i) control group (n=20) received daily intraperitoneal (i.p.) administration of saline (NaCl 0.9%); (ii) ligature group (n = 20) was subjected to ligature placement and received daily i.p. administration of saline (NaCl 0.9%); (iii) NM group (n = 20) was treated with NM[1] (0.1 mg/kg/day, i.p.), a potent tryptase inhibitor; and (iv) NM+Ligature group (n=20) was subjected to ligature placement and received daily i.p. administration of NM (0.1 mg/kg/day). Ten animals per group were killed at 7 and 14 days after ligature placement.

Myeloperoxydase (MPO) activity measurement

The gingivomucosal tissues encircling the right first mandibular molars were removed, homogenized and processed for MPO activity, an index of tissue granulocyte infiltration, as previously described [25].

Total proteolytic activity measurement

Trypsin-like activity was measured at the gingival tissues by using a specific substrate, *N*-benzoyl-L-arginine-*p*-nitroanilide (BApNA). Briefly, 100 µl of BApNa (1.8 mM) was added to 100 µl of the supernatant of the gingival tissue homogenate sample in the wells of a microplate. The mixtures were incubated at 37°C for 4 h, and the color was developed by additions of 10 µl of sodium dodecyl sulfate 10% w/v in 2 M Tris–HCl, pH 8.0. The absorbance at 415 nm was measured with a microplate reader. Trypsin-like activity was calculated in trypsin units per ml, using a standard reaction mixture with a serial dilution of trypsin (0.05–2 U/ml). All assays were performed in triplicate.

PAR_2 expression at the gingival tissues

Total ribonucleic acid (RNA) was isolated from the gingival tissues by the single-step method, using phenol and chloroform/isoamylalcohol. 20 µl of the reaction mixture containing 1.5 µl of total RNA sample, polymerase chain

[1] FUT-175, Biomol International, Plymouth Meeting, PA, USA

reaction (PCR) buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 5 mM MgCl2, 1 mM deoxyribonucleotide triphosphate (dNTPs), 20 U of RNase inhibitor, 2.5 mM of oligo (dT), and 100 U of Moloney murine leukemia virus reverse transcriptase were incubated at 42°C for 30 min and then heated to 95°C for 5 min to inactivate the enzyme activity and to denature RNA–complementary deoxyribonucleic acid (cDNA) hybrids. PCR was performed with two separate sets of oligonucleotide primers specific for rat protease-activated receptor 2 (PAR_2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. For PAR_2, upstream: 5′-ATGCGAAGTCTCAGCCTG-3′ and downstream: 5′-TCAGTAGGAGGTTTTCCG-3′. For GAPDH, upstream: 5′-ACCACAGTCCATGCCATCAC-3′ and downstream: 5′-TCCACCACCCTGTTGCTGTA-3′. PAR_2 and GAPDH cDNA amplification was performed in separate sets of reactions at a final concentration of 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.15 μM of specific primers, and 1.25 U of AmpliTaq DNA polymerase,[2] in a total volume of 50 μl. The amplification profile involved denaturation at 95°C for 1 min, primer annealing at 61°C for PAR_2 for 1 min or at 55°C for GAPDH for 1 min, and extension at 72°C for 1 min. PCR products were loaded in 1.6% agarose gel in Tris–borate/ethylenediaminetetraacetic acid buffer, loading 10 μl of either PAR_2 or GAPDH PCR products for each sample. Amplified samples were visualized under ultraviolet light after being stained with ethidium bromide. Results are expressed as PAR_2 to GAPDH ratios.

ABL evaluation

Mandibles were removed, hemisected, exposed to NaOH (2 N), and then mechanically defleshed. The distance between the cemento-enamel junction and the height of alveolar bone was determined for mesial root surfaces of lower right first molars with the aid of a dissecting microscope (×40) and the software ImageJ.[3] Millimeters of bone loss were measured three times in a random and blinded protocol by one evaluator.

Data analysis

One-way analysis of variance (ANOVA) was used to compare means among groups. In cases of significant differences among the groups, post hoc two group comparisons were assessed with Tukey–Kramer test. A p value<0.05 was considered statistically significant. Data are expressed as mean + SEM.

[2] Perkin-Elmer Cetus, Boston, MA

[3] ImageJ, National Institute of Mental Health, Bethesda, MD, USA

Results

Tryptase plays a role on ABL in ligature-induced periodontitis

The satisfactory outcome of the experimental periodontitis model was confirmed, as increasing bone loss over the 14-day period after ligature was observed, with significant increases in mean bone loss from day 7 to day 14 (Table 1). So there was a statistical significant difference ($p<0.001$) between Control and Ligature groups at 7 and 14 days (Fig. 1a and b). In addition, a progressive ABL was observed after ligature placement, with significative increases of mean ABL from 7 to 14 days for both Ligature and NM groups.

Tryptase inhibition, with NM, led to a decreased ABL in animals subjected to ligature placement. At day 7, the mean alveolar bone level of the animals subjected to ligature-induced periodontitis and treated with NM (NM+Ligature group) was statistically similar to the ABL observed in Control and NM groups and was statistically lower than the ABL observed at the Ligature group ($p<0.001$). At 14 days after ligature placement, a significative increased ABL was observed at NM + Ligature group (Fig. 1c) compared to Control ($p<0.001$) and NM ($p < 0.001$) groups. However, the mean ABL was statistically lower compared to the Ligature group ($p < 0.001$).

Tryptase inhibition decreases gingival inflammation in ligature-induced periodontitis

Ligature placement led to increased gingival granulocytic infiltration at 7 and 14 days after ligature placement (Table 2). There was a statistically significant difference with regard to the mean MPO activity between Control and Ligature groups at 7 ($p<0.001$) and 14 days ($p < 0.001$). In

Table 1 Mean ABL (mm) for Control, Ligature, NM and NM+Ligature groups at 7 and 14 days of experiment at 7, and 14 days after beginning the treatment

	7 days	14 days
Control	0.60±0.22	0.60±0.26
Ligature	1.20±0.38*	1.82±0.26*,***
NM	0.42±0.16	0.39±0.23
NM + Ligature	0.44±0.16**	0.97±0.17**,***

$n=10$ animals/group/period; data expressed as mean ± SEM

* Significant difference ($p<0.05$) compared with Control group at the same time period

** Significant difference ($p<0.05$) compared with Ligature group at the same time period

*** Significant difference ($p<0.05$) compared with the same experimental group at 7 days (ANOVA)

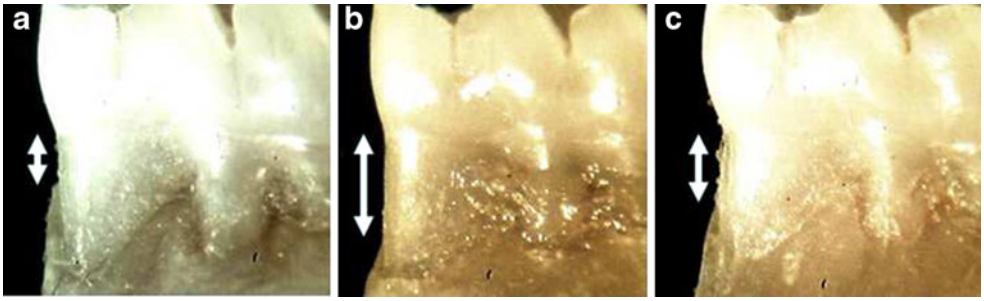

Fig. 1 The lower right first molar images of Control group (**a**), Ligature group (**b**) and NM+Ligature group (**c**) at 14 days of experiment. The *light-blue arrow* indicates the distance between the cemento-enamel junction and the height of alveolar bone

addition, MPO activity was statistically increased ($p < 0.05$) in Ligature group at 14 days compared to 7 days.

Treatment with tryptase inhibitor NM led to a decreased MPO activity in animals subjected to ligature placement (NM+Ligature group) compared to Ligature group at 14 days. No statistical differences ($p<0.05$) with regard to the MPO activity were found between Ligature and NM+Ligature groups at 7 days of experiment.

Tryptase inhibition decreases gingival proteolytic activity and downregulates PAR_2

Table 3 shows the mean total proteolytic activity for Control, Ligature, NM, and NM+Ligature groups at 7 and 14 days of experiment. Ligature placement led to a significant increase ($p<0.001$) with regard to the trypsin-like activity in the gingival tissue at 7 and 14 days when compared to healthy controls. NM statistically decreased ($p < 0.001$) trypsin-like activity in the gingival tissue at 14 days of ligature placement. No statistical differences ($p < 0.05$) with regard to the trypsin-like activity were found between Ligature and NM+Ligature groups at 7 days of experiment.

PAR_2 expression was determined at 14 days of experiment for Ligature and NM+Ligature groups (Fig. 2). Compared to the level of expression of PAR_2 in Ligature group (0.63±0.07), NM+Ligature group presented a significant decrease ($p<0.0001$) in PAR_2 messenger RNA (mRNA) expression relative to the GAPDH RT-PCR signal (0.38±0.05). Therefore, tryptase inhibition led to a 1.6-fold decrease in gingival PAR_2 expression.

Discussion

Traditionally, mast cells have been considered as effector cells in the allergic reaction. However, in the last decade, a number of studies have demonstrated that mast cells are also involved in the innate and acquired immunity [26]. Through activation by degranulation, mast cells may lead to the secretion of several soluble mediators, which can interact with other cells like endothelial cell, keratinocytes, sensorial nerves, neutrophils, T cells, and antigen-presenting cells, which participate in the inflammatory process [27].

Tryptase is a preformed mediator stored in the granules of mast cells, whose most important functional activity is to facilitate granulocyte recruitment through induction of interleukin-8 secretion [28, 29] that acts in vasodilatation and in inflammatory cells infiltration [30].

Table 2 Mean MPO activity (U/g) for Control, Ligature, NM and NM+Ligature groups at 7, and 14 days after beginning the treatment

	7 days	14 days
Control	6.38±1.58	5.10±0.55
Ligature	24.04±2.92*	31.47±1.65*,**
NM	5.23±0.72	6.77±0.93
NM + Ligature	26.33±1.72*	8.38±0.72**,***

$n=10$ animals/group/period; data expressed as mean ± SEM

* Significant difference ($p<0.05$) compared with Control group at the same time period

** Significant difference ($p<0.05$) compared with the same experimental group at 7 days (ANOVA)

*** Significant difference ($p<0.05$) compared with Ligature group at the same time period

Table 3 Mean total proteolytic activity (U of trypsin/g) for Control, Ligature, NM, and NM+Ligature groups at 7 and 14 days of experiment

	7 days	14 days
Control	1.58±0.14	1.51±0.35
Ligature	2.23±0.78*	2.44±0.81*
NM	1.42±0.28	0.23±0.293*
NM + Ligature	1.42±0.32	0.85±0.66**,***

$n=10$ animals/group/period; data expressed as mean ± SEM

* Significant difference ($p<0.05$) compared with Control group at the same time period

** Significant difference ($p<0.05$) compared with Ligature group at the same time period

*** Significant difference ($p<0.05$) compared with the same experimental group at 7 days (ANOVA)

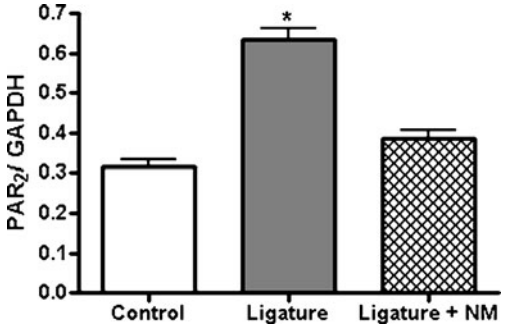

Fig. 2 Detection of PAR_2 in gingival tissue by RT-PCR. Mean ± SEM mRNA ratio PAR_2/GAPDH for Ligature and NM+Ligature groups at 14 days of experiment. * Significant difference ($p<0.0001$) versus Ligature group (unpaired t test); $n=10$ animals/group

Accordingly, the present study showed that tryptase inhibition with 0.1 mg/kg/day of NM not only led to reduced levels of gingival trypsin-like serine activity, therefore showing the effectiveness of the dosage, but also led to a significative reduction of the myeloperoxidase (MPO) activity, a tissue granulocyte infiltration marker, in gingival tissues of rats with ligature-induced periodontitis.

The association between tryptase and bone resorption has been studied for almost 50 years. Tryptase has been associated with an exacerbated bone loss, with inhibition of osteoblasts and increased expression and activity of osteoclasts [31, 32]. In the present study, it was observed that tryptase inhibition led to a significant reduction of periodontal destruction in rats with ligature-induced periodontal disease. Tryptase inhibition prevented the onset of bone resorption at 7 days of experiment, and at this period, no significant differences were observed between the mean alveolar bone level of the group of animals treated with ligature and control group. In addition, at 14 days after ligature placement, although a significant alveolar bone level alteration had occurred in comparison to the previous experimental period, it was observed that tryptase inhibition led to a statistically significant reduction of the ABL compared to the nontreated group. Thus, the findings of the present study highlighted the important role of tryptase as a mediator in the progression of periodontal tissue destruction and may explain a possible association between mast cells and bone metabolism.

The mechanisms by which tryptase exert its effects on tissue degradation are not completely known, although some recent scientific evidences had suggested that tryptase could lead to connective tissue destruction by the direct activation of pro-collagenases and through the initiation of matrix metalloproteases cascades [27]. In addition, it is believed that some of the physiological actions of tryptase are mediated by PAR_2 activation [33, 34]. Tryptase, as well as trypsin and coagulation factors (VIIa/Xa), have the ability to cleave the N-terminal domain of PAR_2 therefore generating a new N-terminal sequence which binds to the receptor itself, activating it, and resulting in pro-inflammatory responses [35] such as vascular dilation, hypotension, increased vascular permeability, increased leukocytes adhesion and margination, granulocyte infiltration, and pain [36–38]. There are substantial evidences showing that PAR_2 is involved in periodontal inflammation through its activation by endogenous proteases and gingipain, a bacterial protease produced by *Porphyromonas gingivalis* [39–44], a major periodontal pathogen. Interestingly, the present study showed that at 14 days of experiment, NM therapy decreased the gingival expression of PAR_2 mRNA in rats subjected to ligature-induced periodontitis, therefore reinforcing its role as a potential modulator of inflammation.

Although been considered infectious in nature, the pathogenesis of periodontal disease is similar to other inflammatory diseases with regard to the pathways of progression. The presence of specific bacteria stimulates immune responses which are responsible for the periodontal breakdown. The attempt to manipulate the immune response is defined as host response modulation and aims to modify the host response by changing the inflammatory process, therefore suppressing the degradation of connective tissue and alveolar bone. Attempts to eliminate infectious agents not always represent a definitive therapy in periodontal disease. There are, for instance, some nonmicrobial risk factors that are difficult to be diminished or eliminated (smoking, diabetes) or that are beyond the ability of the clinician to be controlled (genetic predisposition). In these cases and in groups of individuals that are more susceptible to periodontal disease, the combination of host modulation therapies and the antibacterial treatments may be advantageous.

In conclusion, within the limits of the present study, the data clearly demonstrated that tryptase actively participates to the inflammatory destructive process that takes place in the periodontal tissues of rats subjected to experimental periodontal disease. Therefore, it is believed that future studies could evaluate the role of pharmacological modulation of tryptase on preventing human inflammatory periodontal disease.

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Conflicts of Interest None

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