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ORIGINAL ARTICLE

Quantification of mast cells in different stages of human periodontal disease

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OBJECTIVE AND METHODS: Among the cells involved in immune and inflammatory responses in periodontal disease, mast cells have been shown to be capable of generating a large number of biologically active substances. The present study was undertaken to identify and quantify the presence of mast cells in different stages of human periodontal disease using histochemical (toluidine blue) and immunohistochemical (tryptase-positive mast cells) techniques.

RESULTS: Mast cell densities (cells per mm^2) were significantly increased in chronic periodontitis/gingivitis lesions compared with clinically healthy gingival tissues (Health) uniquely by immunohistochemical technique. Interestingly, mast cells were distributed specially in close apposition to mononuclear cells.

CONCLUSIONS: In human periodontal disease there is an increase in the number of mast cells that may be participating either in the destructive events or in the defense mechanism of periodontal disease via secretion of cytokines, including perpetuation of the Th2 response, and cellular migration and healing processes.

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Introduction

Bacterial plaque has been implicated as the primary etiologic factor in inflammatory periodontal disease, but recently several studies have focused on the role of the immune system in the evolution of periodontal disease, indicating that bacterial antigens trigger an immunopathologic reaction and that the susceptibility of the patient determines the ultimate outcome of the disease process (Asaro *et al*, 1983; Jeffcoat *et al*, 1985; Page, 1991, 1998; Wilson *et al*, 1996; Gemmell *et al*, 2002). Among the cells found in periodontal tissues, mast cells have been detected in both healthy and inflamed gingiva, in different numbers at various sites (Carranza and Cabrini, 1955; Shapiro *et al*, 1969; Robinson and De Marco, 1972; Asaro *et al*, 1983; Jeffcoat *et al*, 1985; Günhan *et al*, 1991; Walsh *et al*, 1995). However, comparisons of cell counts in normal and inflamed tissues are contradictory, as some investigations report an increase in mast cell density at inflamed sites (Dewar, 1955; Shapiro *et al*, 1969; Zachrisson, 1969; Kennett *et al*, 1993), others show a decrease (Carranza and Cabrini, 1955; Zachrisson, 1967; Shelton and Hall, 1968; Robinson and De Marco, 1972; Gemmell *et al*, 2004). In fact, it is not clear whether there is a real change in the population of mast cells in gingival tissue during inflammation. Mediators derived from mast cells are stored in secretory granules and released by degranulation, or can be newly generated when mast cells are properly activated (Metcalfe *et al*, 1997; Mekori and Metcalfe, 1999; Walsh, 2003). Preformed mediators include histamine, proteoglycans and proteinases, while newly generated mediators include the arachidonic acid metabolites, platelet activating factor and a variety of cytokines, including basic fibroblast growth factor (bFGF) and tumor necrosis factor-alpha (TNF-α), as well as nitric oxide (NO) (Harvima and Schwartz, 1993; Qu *et al*, 1995; Bidri *et al*, 1997; Metcalfe *et al*, 1997).

Mast cells are involved in numerous activities ranging from control of the vasculature to tissue injury and repair, allergic inflammation, and host defense. The significant contribution of mast cell mediators to tissue damage and the propagation of inflammatory responses make the control of mast cell activity vital to the management of many inflammatory diseases (Forsythe and Befus, 2003). Nowadays, there is an increased awareness of the potential interactions between mast cells and other components of the immune response,

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contributing to the modulation of humoral and cellular events in host defense mechanisms against bacterial infections (Welle, 1997; Villa *et al*, 2001), and probably participating in the pathogenesis of inflammatory conditions such as periodontal disease.

Some researchers (Neiders *et al*, 1979; Asaro *et al*, 1983) believe that immediate and/or delayed hypersensitivity may be one of the immunologic mechanisms active in human periodontal disease, because all the necessary components for these reactions have been identified in the gingival tissues. However, mast cells may significantly influence IgE-independent responses, extending their potential from pro-inflammatory effector cells to regulatory components of the immune system, contributing to the development of non-specific as well as specific inflammatory responses (Gordon *et al*, 1990; Galli, 1993; Skokos *et al*, 2001). Thus, the contribution of mast cell mediators to periodontal disease progression is not yet established. The administration of locally or systemically acting pharmacological agents that inhibit mast cell degranulation may be a useful method to probe the role of mast cells in disease. Jeffcoat *et al* (1985) examined the effect of daily application of lodoxamide ethyl on the progression of naturally occurring chronic destructive periodontal disease in beagles and observed a significant decrease in the rate of bone loss. Therefore, the aim of this study was to analyze the variations and alterations in mast cell densities in human samples of dental plaque-induced gingivitis (DPIG) and localized chronic periodontitis (LCP), via toluidine blue (TB) staining and immunoperoxidase immunohistochemical (IH) technique.

Material and methods

Tissue

A total of 20 cases of LCP characterized by advanced loss of periodontal support, 20 cases of DPIG with no attachment loss and 20 cases of clinically healthy gingival tissues (Health) were used in this study. The healthy samples were obtained from tooth extractions, generally of premolars, for orthodontic reasons. The samples of groups LCP and DPIG were obtained from patients undergoing periodontal surgery. Diagnosis of periodontal status was previously made on the basis of radiographic analysis, physical examination, medical and dental history and assessment of attachment loss. The American Academy of Periodontology guidelines were followed for classification of the periodontal disease and conditions (Armitage, 1999). The patients included in the present work had no history of systemic disease. The samples were fixed in 10% buffered formalin and were selected from a previous examination of slides stained with hematoxylin and eosin. Samples of clinically healthy gingival tissues presenting mild to intense inflammatory infiltrate were excluded. The most recent cases and most representative lesions, presenting integrity of tissue arrangement were selected to ensure better preservation of the antigens under investigation. This study was approved by the institutional ethics committee for human subjects.

Histochemistry

Sections measuring 5 μm in thickness were deparaffinized and hydrated with water. TB staining was performed with a 1% TB solution (A1111.01.AD, Synth, São Paulo, Brazil) diluted in phosphate buffer (pH = 4–6) for 45 s. After rinsing in phosphate buffer for 1 min, sections were quickly dehydrated through 70, 96% ethanol and acetone p.a. (or absolute ethanol) to xylene and mounted in synthetic resin. TB method was modified from Heaney *et al* (1997).

Immunohistochemistry

From the tissues embedded in paraffin wax, 3-μm thick sections were obtained (RM 2245, Leica, Wetzlar, Germany) and collected on silane-coated glass slides (S3003, Dako, Glostrup, Denmark) for processing by standard IH technique (immunoperoxidase: avidin-biotin-peroxidase). The samples collected for immunohistochemistry were deparaffinized through immersion in xylene and ethanol followed by incubation with 3% hydrogen peroxide diluted in phosphate-buffered saline (PBS; pH = 7.2) for 40 min. Thereafter, the sections were incubated in 3% normal serum diluted in distilled water at room temperature for 20 min and were sequentially incubated with murine anti-human mast cell tryptase (Dako, M7052, clone AA1) monoclonal antibodies, diluted in 1% PBS-bovine serum albumin (BSA) at 0.035 μg ml^{-1}, at 4°C overnight. Following the incubation period, the sections were washed in PBS with Triton X-100 p.a. (H282, Mallinckrodt, Phillipsburg, NJ, USA) and incubated with biotinylated mouse anti-IgG (Dako, K0492) antibodies in 1% PBS-BSA for 60 min at room temperature. Sections were then washed in PBS with Triton X-100 p.a. and incubated with avidin-biotin complex (Dako, K0492) for 45 min at room temperature. After further washing, sections were incubated in a solution with 5 mg 3,3′-diaminobenzidine (D4293, Sigma, St Louis, MO, USA) dissolved in 10 ml of PBS containing 180 μl of H_2O_2 (20 vol), for 5 min at room temperature. After washing with distilled water, the slides were counterstained with Mayer's hematoxylin for 5 min. Negative controls consisted of sections in which primary antibodies were omitted and replaced with non-immune murine serum (Dako, X 0910) and 1% PBS-BSA.

Quantitative and statistical analysis

Assessment and quantification of immunostaining was carried out by two investigators. The number of positively stained cells in the periodontal tissues was determined in 25 consecutive microscopic high-power (×800: objective ×64; eyepiece ×12.5; tube factor ×1) fields [each field has an area of 0.0176625 mm^2, obtained from the mathematical expression $A = (\pi d^2)/4$, where $\pi = 3.14$, $d = 0.15$ mm] in a representative section of each specimen. Mast cell counts in inflammatory cell-rich tissue areas and in sites adjacent to the inflammatory cell infiltrates of diseased tissues have been performed separately. The results of mast cell immunostaining as well as TB-stained cells were expressed as mean ± s.d. of n observations per mm^2. Comparative

analysis of the number of mast cells per mm^2 between TB staining and IH technique was also conducted. The statistical study was performed using ANOVA followed by Tukey test or paired, two-tailed Student's *t*-test. A P value of less than 0.05 was considered statistically significant.

Results

The samples of periodontal disease presented an intense inflammatory infiltrate, predominantly mononuclear and focally distributed, with large presence of lymphocytes, plasma cells and mast cells, as well as a discrete presence of macrophage-like cells and foci of polymorphonuclear cells.

Quantitative analysis of mast cells in healthy tissues and in inflammatory cell-rich areas of diseased tissues

Quantitative analysis of mast cells per mm^2 with TB staining revealed no statistically significant difference (ANOVA: $F = 2.21$; $P = 0.0801$) among the three groups examined; clinically healthy gingival tissues (Health) (35.73 ± 37.77 mast cells per mm^2), LCP (71.38 ± 59.15 mm^2) and DPIG (44.54 ± 30.31 mm^2) (Figure 1). Nevertheless, IH analysis revealed a statistically significant difference (ANOVA: $F = 3.77$; $P = 0.0019$) in the number of mast cells per mm^2 among the three groups examined. To specify between which groups the difference was significant, we performed the Tukey test that revealed a statistically significant difference between LCP (112.9 ± 45.41 mm^2) and Health (58.3 ± 25.7 mm^2) ($P = 0.039$), and between DPIG (101.3 ± 53.6 mm^2) and Health ($P = 0.002$), with no statistically significant difference between DPIG and LCP ($P = 0.930$) (Figure 1). Then, a significant difference in the number of tryptase-positive cells (mast cells) was observed only between the periodontal disease and Health groups, yet with no significant differences between the two stages of periodontal disease.

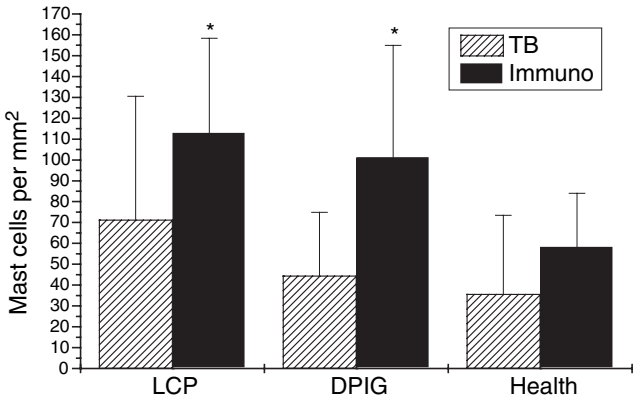

Figure 1 Comparative results of toluidine blue (TB) and immunohistochemical (IH) staining in the three groups studied: localized chronic periodontitis (LCP), dental plaque-induced gingivitis (DPIG) and clinically healthy gingival tissues (Health). *Statistically significant different from Health (Tukey test: $P < 0.05$)

Additionally, Student's *t*-test revealed that the number of tryptase-positive mast cells was significantly higher than the number of TB-stained cells within any groups examined (LCP, $P = 0.027$; DPIG, $P = 0.003$; Health, $P = 0.028$). The negative controls did not show any immunostaining.

Quantitative analysis of mast cells in healthy tissues and in sites adjacent to the inflammatory cell infiltrates of diseased tissues

Based on better identification of mast cells by immunohistochemistry, only tryptase-positive mast cell counts have been performed in sites adjacent to the inflammatory cell infiltrates of diseased tissues. Again, a statistically significant difference (ANOVA: $F = 13.26$; $P = 0.00014$) in the number of mast cells per mm^2 was observed among the three groups examined. Tukey test performance specified the statistically significant difference only between the DPIG (98.39 ± 46.22 mm^2) and Health group (58.30 ± 25.70 mm^2) ($P = 0.0001$), yet with no statistically significant difference between DPIG and LCP (81.28 ± 47.30 mm^2) ($P = 0.55$) and or LCP and Health ($P = 0.82$). By comparison of mast cells number in areas containing inflammatory cells and its adjacencies, there was an increase of approximately 28% and 3% of mast cells from adjacent sites to inflammatory cell-rich area, in DPIG and LCP respectively; although with no statistically significant difference (Student's *t*-test: DPIG, $P = 0.916$; LCP, $P = 0.4111$).

Subjective analysis of tryptase-positive mast cells in DPIG and LCP

Based on subjective microscopic analysis, mast cells in DPIG were mostly found in active inflammation sites mainly adjacent to the epithelial pocket and near the blood vessels. In LCP, mast cells were diffusely distributed both in close apposition to a dense mononuclear inflammatory infiltrate and among collagen fibers and fibroblast-like cells.

Discussion

In relation to staining techniques for mast cells, both TB and IH identification techniques reliably identify mast cell granules. However, the present study observed that the IH method is more specific than metachromatic staining through TB (Figure 1). The present results showed that the number of tryptase-positive mast cells was significantly higher than the number of TB-stained cells within any groups examined. In addition, only with the IH method was there a significant difference between groups, yet with no significant differences between the two stages of periodontal disease. In accordance with these findings, Walls *et al* (1990) observed that the immunoperoxidase staining procedure with monoclonal antibody AA1 is a highly specific method that allowed identification of significantly more mast cells than a standard procedure with TB in routinely processed tissues. In addition, Heaney *et al* (1997) reported mast cell counts in endobronchial biopsies from asthmatic subjects using different methodologies and recommen-

ded the IH method (tryptase), as the resultant tissue sections facilitated clear, accurate and rapid counts.

In all three groups, the mast cells showed intense immunostaining and were diffusely distributed in close physical proximity to plasma cells and lymphocyte-like cells. In light of these results, it may be concluded that there is an increased number of mast cells in human periodontal disease compared with healthy tissues, considering inflammatory cell-rich areas of diseased tissues. If mast cells do not proliferate locally, it can be concluded that there must be some process of directed migration of mast cells into such lesions (Walsh *et al*, 1995). But, we also observed numerous mast cells in sites adjacent to the inflammatory cell infiltrates of diseased tissues, with statistically significant difference only comparing DPIG and Health. Accordingly, in LCP there was an increase of approximately 28% in the number of mast cells from adjacent sites to the inflammatory cell-rich area, suggesting important dynamic alterations in the migration and localization of mast cells in the evolution of periodontal disease, which need to be more precisely speculated.

Conversely, Gemmell *et al* (2004), using the same IH marker, observed that the numbers of tryptase-positive mast cells were decreased in chronic periodontitis tissue sections compared with healthy/gingivitis samples. One possible explanation for this discrepancy could be that the clinically healthy and marginal gingivitis samples were grouped together (healthy/gingivitis) in Gemmell's study, while we used separate samples of DPIG and clinically healthy gingival tissues (Health).

Based on our results, the increase of mast cells *in vivo* has called attention with respect to the possible participation of mast cells in the defense mechanism and destructive events both as effector and responsive cells in chronic inflammation, as well as the possible functional relationship between mast cell and immunocompetent cell populations in periodontal lesions.

Chronic synthesis and release of bFGF (Qu *et al*, 1995; Takayama *et al*, 1997; Murakami *et al*, 1999) and TNF-α from mast cells within oral lesions such as gingivitis (Moughal *et al*, 1992; Verdickt, 1992) and periodontitis (Gemmell *et al*, 1993) may be responsible for maintaining chronicity and healing (Walsh *et al*, 1990; Verdickt, 1992; Walsh and Murphy, 1992) and leukocyte migration and proliferation. TNF-α has been shown to induce endothelial expression of E-selectin (ELAM-1) (Walsh *et al*, 1991), which is critically required for the rapid adhesion of neutrophils, T lymphocytes, monocytes, and other leukocytes to endothelial cells (Walsh and Murphy, 1992; Bevilacqua, 1993). Besides, TNF-α participates in the differentiation and proliferation of osteoclasts (Birkedal-Hansen, 1993; Manolagas, 1995; Assuma *et al*, 1998) and induces the expression of metalloproteinases, which are capable of cleaving several components of the extracellular matrix, leading to tissue destruction (Birkedal-Hansen, 1993). In fact, high levels of TNF-α have already been detected in gingival crevicular fluid and in periodontal tissues from periodontitis (Rossamando *et al*, 1990), as well as in the serum of patients presenting with periodontal disease (Meyle, 1993).

A change from gingivitis to periodontitis involves a shift from a predominantly T-cell lesion to a B-cell/plasma cell lesion (Seymour *et al*, 1979). Still, there is a hypothesis that Th1 cells are associated with the stable lesion – gingivitis – while a Th2 response is linked to disease progression – periodontitis (Gemmell *et al*, 2002). The notion of a functional relationship between mast cells and T lymphocytes has been strengthened as activated mast cells reside in close apposition to T cells in some inflammatory processes (Bhattacharyya *et al*, 1998; Mekori and Metcalfe, 1999) and mast cells can secrete both Th2-type (De Pater-Huijsen *et al*, 1997; Hültner *et al*, 2000; Coulombe *et al*, 2002; De Pater-Huijsen *et al*, 2002) and Th1-type (Metcalfe *et al*, 1997; Tkaczyk *et al*, 2000) cytokines, influencing the differentiation of T cells (Robinson *et al*, 1993; Huels *et al*, 1995). Nevertheless, mast cells seem to be able to present antigens to T cells *in vitro* (Fox *et al*, 1994; Love *et al*, 1996; Malaviya *et al*, 1996; Villa *et al*, 2001). The resultant T cell activation would activate mast cells, leading to both degranulation and cytokine release. In addition, Th2 responses can be perpetuated through activation of FcR^+ mast cells (Aoki *et al*, 1995, 1996). Gemmell *et al* (2004) suggested a predominance of Th2 response in periodontitis, although they have not observed an increased number of mast cells in periodontal disease lesions. Conversely, we believe that as mast cells are widely distributed in periodontal tissues and their activation results in the release of a great number of mediators, these cells may contribute to the magnitude and perpetuation of a Th2-biased response, as postulated by Aoki *et al* (1999), by examination of the response of mast cell-deficient mice after ovalbumin-induced immunization. In fact, we noted mast cells diffusely distributed in close apposition to immune cells including plasma cells and lymphocytes. In summary, periodontitis is not unidirectional, but rather it is interactive; the same cells that produce the destructive proinflammatory cytokines can also produce mediators that activate the healing process (Page, 1998). The mast cells and mediators released probably contribute to this event. Heparin, histamine, Th2-type cytokines, TNF-α and NO possibly contribute to tissue destruction and bone loss in an attempt to eliminate bacterial products, while bFGF may influence tissue repair.

Our findings on the participation of mast cells in inflammatory diseases seem to be not a unique feature of periodontal disease, as mast cells have been observed in chronic periapical lesions (Teronen *et al*, 1996; Rodini *et al*, 2004) and in oral lichen planus (Zhao *et al*, 2002), with emphasis in the mast cell–T cell interaction. However, these findings have not been compared with healthy gingival tissues. However, Walsh *et al* (1995), have shown that mast cell numbers were dramatically increased in inflamed sites of periapical granulomas and lichen planus when compared with non-lesional sites or normal buccal mucosa, indicating higher activity of mast cells in that area. Interestingly, some inflammatory oral diseases such as paracoccidioidomycosis present rare mast cells inside the granulomas, probably restricting its participation in

immunopathological mechanisms of these oral manifestations (Batista, 2004).

The therapeutic implications of the findings and suggestions herein presented include strategies directed toward the possible use of drugs to influence mast cell secretion and thereby prevent inflammation and maintenance of chronicity, or even with the aim of improving periodontal regeneration.

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