

MAST CELLS AS KEY PLAYERS IN PERIODONTAL DISEASE

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Abstract - Mast cell (MC) active mediators promote inflammation through changes induced in the connective tissue components of human gingiva. The aim of this study was to evaluate the distribution, mast cell density and their relationship with the degree of inflammatory infiltrate in gingiva from patients with periodontal disease. Thirty-nine cases with periodontal disease and 12 cases without significant changes to the gingival mucosa were investigated. MCs were identified on paraffin-embedded specimens by immunohistochemistry using anti-mast cell tryptase. The inflammatory infiltrate was scored from 0 to 3, and the MCs were counted using the hotspot method. Intraepithelial MCs were scored from 0 to 2. We found a significant increase of mast cell density in cases with mild and moderate inflammatory changes, and a slight decrease in patients with severe periodontal disease. We noticed a higher degranulation rate in patients with periodontal disease compared to those with healthy mucosa. Intraepithelial MCs were found in cases with periodontal disease only and were correlated with the severity of the inflammatory lesion. MCs are important cellular components of the early stages of periodontal disease. Contrary to other studies, we found that MC density and activation increases with moderate inflammation but decreases in severe inflammatory lesions. Our data suggest that MCs are key players in the progression of inflammatory lesions of the gingiva. In advanced-stage periodontal disease, intraepithelial MCs apparently play an important role, although their biological significance remains to be fully understood.

Key words: Mast cell, periodontal disease, gingiva, tryptase

INTRODUCTION

Mast cells (MCs) are connective tissue resident cells found in almost all human organs. In normal conditions, MCs release specific mediators that act on several connective tissue components as ground substance, blood vessels or nerve endings. In addition, the MC membrane has a relatively large spectrum of receptors capable of mediating the interaction with components of the immune system and thus they are considered to play an important

role in human immunity. It has been demonstrated that MCs are a powerful source of growth factors, such as vascular endothelial growth factor or nerve growth factor. Dramatic changes in the number, structure and active mediator content of specific granules were reported in several pathologic conditions. MC involvement in allergic diseases pathogenesis is well known, their behavior and clinical consequences being almost similar with regard to the stimulation of cytoplasmic granule release. The presence of MCs in tumor-associated stroma

was shown many years ago, but their role in tumor progression and metastasis is still controversial. A significant increase in their number was detected in the early stages of oral cavity squamous cell carcinoma and a dramatic decrease in advanced-stages of carcinoma, as well as in less differentiated tumors (Cheema et al., 2012). However, in many human tumors an MC-induced angiogenesis was clearly demonstrated, which finally contributes to tumor progression (Michailidou et al., 2012; Jahanshahi and Sabaghian, 2012). Therefore, at present it is difficult to conclude if mast cell density (MCD) reflects a good or poor prognosis; it most probably suggest a particular phenotype in terms of biological mediators. These findings lead to the concept of MC heterogeneity that recognizes many subtypes, based particularly on the content in mast cell tryptase and mast cell chymase.

Chronic inflammation is a good model to investigate the MC reaction as a response to minimal stimuli. Local activation of MCs is usually associated with an accumulation of lymphocytes, macrophages and plasma cells. Frequently this heterogeneous infiltrate is concentrated around blood vessels. Recently, the mast cell activation syndrome was described, characterized by systemic symptoms that involve a minimum of two organs, but most frequently the mucosa of the digestive tract and the skin (Frieri et al., 2013; Valent, 2013). Mast cell activation syndrome is a rare disorder (Picard et al., 2013), but local activation of MCs is a frequent event and even a condition of inflammation.

MCs were described some time ago in the mucosa of the oral cavity and particularly in human gingiva (Zachrisson, 1967) and experimental gingivitis (Zachrisson, 1969). As in many other locations in the human body, their intrinsic role in the gingiva is still elusive in both normal and pathological conditions. In periodontal disease, and particularly gingivitis, the mast cell density significantly increases, but without an explanation regarding their involvement in the maintaining or progression of inflammation (Lagdive et al., 2013). Moreover, there are no data on the relationship between MCs and the density of the

inflammatory infiltrate. A recent study has shown a correlation between MC degranulation and the severity of periodontitis (Huang et al., 2013). It was found that the density of degranulated tryptase-positive MCs is significantly higher in severe periodontitis compared to moderate periodontitis and normal tissue. However, it was not possible to conclude if degranulation was induced by the inflammatory infiltrate or a degranulation-induced accumulation of inflammatory cells. Moreover, increased mast cell density was reported by many authors in periodontal disease, but results are conflicting probably in part due to different counting methods and the relatively low number of cases (Steinsvoll et al., 2004; Batista et al., 2005).

In the present paper, we describe our investigation of MC distribution, density and relationships with other cells of the inflammatory tissue of the gingiva, including the particular aspect of intraepithelial MCs presence.

MATERIALS AND METHODS

Patients

Twelve patients without significant changes to the oral mucosa, 15 patients with mild, 16 with moderate and 8 with severe inflammatory lesions of the gingiva as found by the clinical examination were investigated. A biopsy was taken from each patient and washed with buffer saline. The local research ethics committee approved the protocol of the study and informed consent was obtained from all subjects according to the World Medical Association Declaration of Helsinki.

Primary processing

Gingival biopsies were fixed in buffered formalin and embedded in paraffin, using the standard histological procedure. Five-micrometer thick sections were obtained from each case and they were stained with the routine hematoxylin-eosin method. These slides were used to analyze the morphological changes of the epithelium and to evaluate the density of the

inflammatory infiltrate. Additional slides were prepared for the immunohistochemical study.

Immunohistochemistry

MCs were detected with anti-mast cell tryptase antibody (clone AA1, Dako Glostrup, Denmark). Briefly, we performed heat-induced epitope retrieval with citrate buffer (pH 6.0) (Novocastra, Newcastle upon Tyne, UK) for 30 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide and followed by incubation with the primary antibody for 30 min. The Bond Polymer Refine Detection System (Leica Biosystems, Newcastle upon Tyne, UK) was used to develop the immunohistochemical reaction and the final product was visualized with 3,3' diaminobenzidine dihydrochloride. Nuclei were stained with hematoxylin. The full immunohistochemical procedure was performed with Leica Bond-Max (Leica Biosystems, Newcastle upon Tyne, UK) autostainer.

Scoring

The inflammatory infiltrate was scored as 0 (absent), +1 (isolated inflammatory cells, less than 10, +2 (aggregates of inflammatory cells in the lamina propria only), and +3 (aggregates of inflammatory cells in the lamina propria associated with intraepithelial lymphocytes). Counting of mast cell in the lamina propria was based on the hotspot method. Three hotspot areas with high density of MCs were chosen at low power magnification. The MCs were counted at 400x magnification. The arithmetical mean of the three hotspots was the final result. Tryptase-positive granules scattered in the intercellular space were not taken into account to evaluate mast cell density. Degranulated MCs were expressed as a percentage from the total number of MCs. Intraepithelial mast cells were scored at 400x magnification as follows: absent scored with 0, rare scored with 1 (1-2/field) and numerous scored with 2 (3 or more/field).

Statistical analysis

Statistical analysis was performed with the commer-

cially available SPSS13.0 soft and Microsoft Excel 2010 soft. The relationships between the density of the inflammatory infiltrate, mast cell density in the lamina propria and intraepithelial MCs were evaluated, applying Spearman's test, and values of $p < 0.05$ were considered statistically significant.

RESULTS

Microscopically, in normal gingiva ($n=12$) we found no inflammatory changes except in one case, and the covering epithelium showed parakeratosis. MCs were found in the lamina propria, usually close to blood vessels and rarely in the vicinity of the epithelium. The mast cell density ranged between 12 and 31, with an average of 20.12. Degranulation was noticed in all of these cases. No intraepithelial MCs were found in cases without inflammatory infiltrate. We found inflammatory changes in 39 out of 51 patients. The inflammatory infiltrate consisted mainly of lymphocytes, neutrophilic granulocytes and macrophages, and more rarely contained eosinophilic granulocytes and plasma cells. Small islands of epithelial cells sequestered in the lamina propria were noticed, particularly in the cases with severe inflammatory lesions. We observed focal necrosis only in cases with high density of neutrophilic granulocytes. Based on the scoring system for the inflammatory infiltrate, we found 12 cases scored 0; 17 cases +1; 9 cases +2; 13 cases +3. The microscopic features of the normal gingiva, mild, moderate and severe periodontal disease are shown in Fig. 1.

Tryptase-positive MCs were identified in all of the cases included in the present study. They were medium-sized, with or without scattered granules in the pericellular space, signifying degranulation. In the gingiva without inflammatory changes we found a mast cell density of 20.12/high power field with a low rate of degranulation. MCs were preferentially located in the perivascular space and no intraepithelial MCs were noticed.

In cases with mild inflammation of the gingiva, we found a significant increase of mast cell density. MCs were diffusely distributed in the lamina propria,

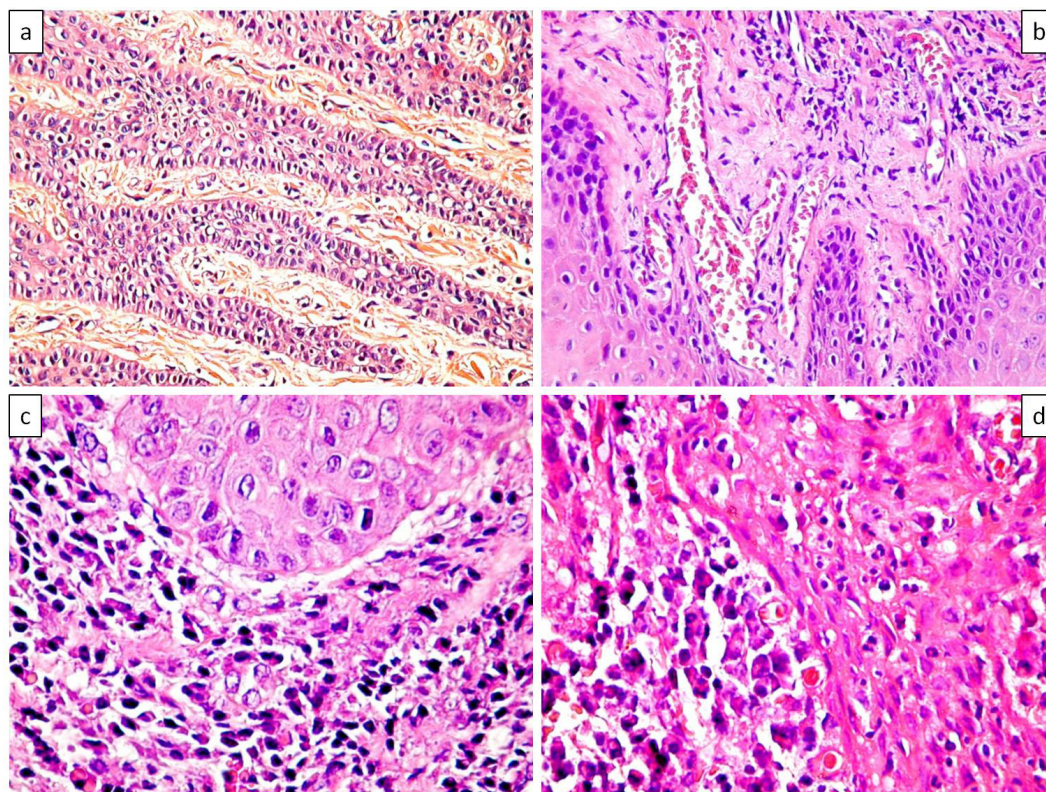


Fig. 1. Gingiva without inflammatory changes (a – at 200x magnification). Scattered inflammatory cells in the lamina propria (b – 200x); aggregates of inflammatory cells restricted to the lamina propria (c – 400x). Inflammatory cells in the lamina propria and within the epithelium (d – 400x). Hematoxylin and eosin staining.

they were not restricted to the perivascular space and showed a high rate of degranulation. Small aggregates of MCs were observed in close to the epithelium and intraepithelial MCs were found in 6 out of 17 cases. The assessed mast cell density in this group had an average of 44.08 MCs/high power field.

In the group with moderate inflammation, the average value for mast cell density was 63.87, and ranged between 42 and 97 per high power field. Numerous MCs were observed in the immediate vicinity of the epithelium and concentrated in the areas with dense inflammatory infiltrate. Strong degranulation was detected in almost all of the cases of this group. Intraepithelial MCs were found in 5 out of 9 cases, and we observed tryptase-positive cells even in the intermediate and superficial layers of the epithelium.

In cases with severe inflammation, we found a significant decrease in the value of mast cell density in comparison with moderate ($p < 0.0001$) and mild inflammation of the gingiva ($p < 0.023$). In these cases, MCs were located predominantly in the connective extensions of the lamina propria into the epithelium. We noticed an increased number of intraepithelial MCs that were identified in 12 of 13 cases, and half of the cases were scored as +2, with more than 3 intraepithelial MCs per high power field.

We found the highest value of mast cell density in periodontal disease with moderate inflammation and the lowest in cases with severe inflammation. Overall, the mast cell density in the gingiva of patients was 53.96, and compared with the gingiva without inflammatory infiltrate, the increase was significant ($p < 0.0001$). The relationship between the

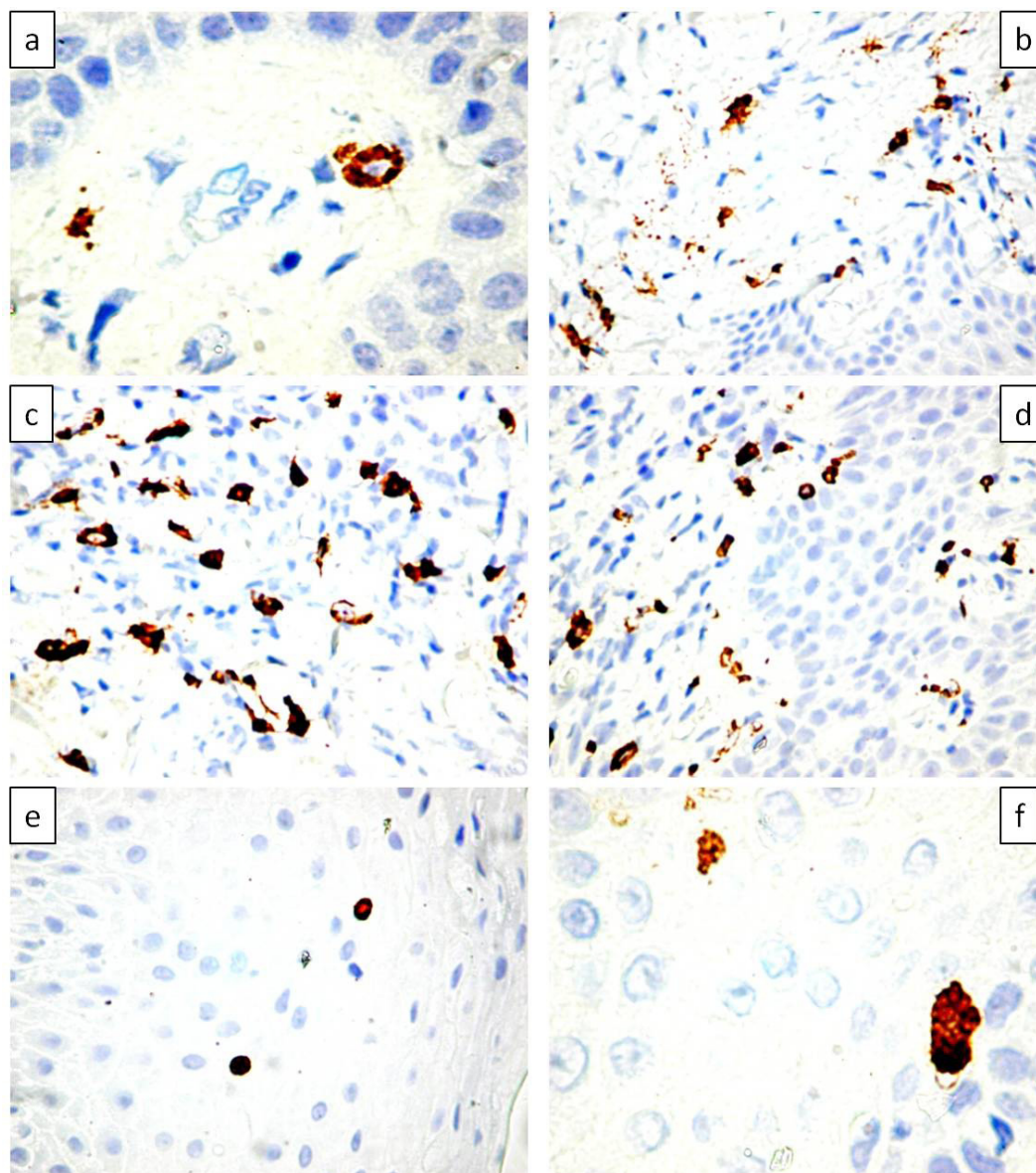


Fig. 2. Mast cells in the normal gingiva (a – at 900x magnification). Massive degranulation of mast cells of the lamina propria in a patient with mild inflammation (b – 200x); massive accumulation of mast cells in severe gingivitis (c – 400x); concentration of mast cells close to the epithelium (d – 400x); intraepithelial mast cells. Note their location in the intermediate and superficial layers (e – 400x). Intraepithelial mast cells, detail (f – 900x). Anti-mast cell tryptase, DAB.

number of MCs and the density of the inflammatory infiltrate was particularly significant for mild and moderate inflammation of the gingiva ($p < 0.002$), followed by severe inflammatory changes ($p < 0.027$). On the other hand, intraepithelial MCs correlated with the severity of the inflammatory changes in all

cases with periodontal disease. Data on the microscopic evaluation of the inflammatory infiltrate and mast cell density are shown in Table 1. Degranulation of MCs was a common finding in all of the cases and we noticed a higher rate of degranulated MCs in cases with periodontal disease, although we did not

Table 1. Distribution of cases based on the scoring of the inflammatory infiltrate and mast cell density

Score	Inflammatory infiltrate	Mast cell density	Minimum	Maximum
No inflammation (n=12)	0	20.12	12	31
Mild (n=17)	+1	44.08	19.5	65
Moderate (n=9)	+2	63.87	42	97
Severe (n=13)	+3	33.81	26	53

Table 2. Mast cell density versus degranulated MCs and intraepithelial MCs

Lesion/Degranulation rate	MCD/D-MCs	Intraepithelial MCs
No inflammation (n=12)	20.12/6.5 (3.09)	0
Mild (n=17)	44.08/18.08 (2.43)	6/17 (35.29%)
Moderate (n=9)	63.87/23.25 (2.74)	5/9 (55.55%)
Severe (n=13)	33.81/13.76 (2.45)	12/13 (92.30%)

Legend: MCD, mast cell density; D-MCs, degranulated mast cells

find a significant relationship to the severity of the inflammatory infiltrate (Table 2). The location, types and density of MCs in the normal gingiva and periodontal disease are shown in Fig. 2.

DISCUSSION

MCs develop from myeloid progenitors under the influence of stem cell factor and they are widely distributed in the connective tissue and mucosal surfaces. MCs play a crucial role in host defense and homeostasis, and are involved in many pathological conditions, such as inflammation and tumor progression. Degranulation induced by MC activation releases pro-inflammatory substances, like proteases, histamine, proteoglycans, arachidonic acid metabolites, chemokines and growth factors. From cytokines secreted by the mast cell, tumor necrosis factor alpha is of particular interest, being related to inflammation of the oral cavity (Walsh et al., 1995). MCs form a heterogeneous system and they are stratified on the basis of their content in proteases (Irani et al., 1986; Fonzi et al., 1995). Mast cell tryptase is expressed virtually by all mast cell subtypes and this is why we have chosen the specific immunohistochemical method to detect this highly specific MC marker.

Gingiva is the site in the oral cavity that contains the highest number of MCs and they were investi-

gated in both normal and pathological conditions by many authors (Fonzi et al., 1996; Kennett et al., 1993; Allam et al., 2008). Their density does not seem to be influenced by systemic therapy, such as antiretroviral drugs or immunosuppressive therapy (Segundo et al., 2012; Nurmenniemi et al., 2004). MCs of the gingiva are sensitive not only to chemical substances, but also to physical agents, like metal ions or low-intensity laser irradiation that induces massive degranulation (Schedle et al., 1998; Silveira et al., 2008).

Mast cell reaction has been investigated in periodontal disease by many authors and conflicting results have been reported (Lagdive et al., 2013; Steinsvoll et al., 2004; Batista et al., 2005; Gemmell et al., 2004). Some authors found a decreased mast cell density, while others noticed a significant increase. In none of these studies was the MC number correlated with the density and distribution of the inflammatory infiltrate. We found a significant increase of mast cell density in patients with periodontal disease in comparison to healthy gingiva. This was particularly evident in cases with mild and moderate inflammation, followed by a significant decrease in cases with severe periodontal disease. This finding might be explained on the one hand by the higher rate of degranulation, and on the other by the migration of MCs into the epithelium. Recently, Huang

et al. (2013) found a gradual increase in the number of MCs from healthy gingiva to severe gingivitis. We cannot confirm this finding as we found a significant decrease of MCD in cases with severe inflammation. The difference might be explained in part by the different counting method, and only moderate and severe periodontitis were included in the study.

Another possible explanation for the decrease in MCD in cases with severe inflammation could be related to the multiple functions of these cells. Most probably, MCs in periodontal disease govern not only recruitment of inflammatory cells, but also the formation of new blood vessels. The involvement of MCs in the induction and maintaining of angiogenesis and lymphangiogenesis was already demonstrated in both clinical and experimental conditions. This is supported by the expression of vascular endothelial growth factor by epithelial cells of the oral mucosa and the increased microvessel density in pathological conditions (Michailidou et al., 2012). Based on these data, we can suggest that the increase in the MC number is an early event during the progression of inflammatory changes in the gingiva.

Intraepithelial MCs were first reported in the lichen planus of the gingiva by ultrastructural examination, and at that time it was suggested that they might be in an early stage of differentiation (Barnett, 1975). Intraepithelial MCs have been also reported in other inflammatory diseases, like active *Helicobacter pylori* gastritis (Caruso et al., 2011) or in patients with asthma (Laitinen et al., 1993; Gibson et al., 1993). They were also found in intraepithelial neoplasia, recognized as a diagnostic tool and seem to promote progression to invasive carcinoma (Van de Nieuwenhof et al., 2010). In the present study, we found intraepithelial MCs only in cases with periodontal disease with a gradual increase in number with the severity of inflammation (from 35.29% in cases with mild to 92.3% in cases with severe inflammatory changes). Our results suggest that intraepithelial MCs can be useful to evaluate the severity of periodontal disease because we found a linear relationship with the density of the inflammatory infiltrate.

The migratory potential of MCs has been already demonstrated and seems to be stimulated by the mast cell growth factor. Mast cell growth factor is secreted by endothelial cells and cells of the covering epithelia and stimulates the homing of MC precursors into the epithelia (Walsh, 2003). The expression of mast cell growth factor is not modified by degranulation and this explains the accumulation of numerous MCs close to or within the epithelium in inflammatory conditions.

The presence and role of MCs in the epithelium of the gingiva is difficult to explain, despite the presence of scattered MCs with degranulation. Although the significance of intraepithelial MCs remains to be clarified, in an experimental model of MCs and epithelial cells from the corneas of patients with keratoconjunctivitis, a particular relationship was found. CCL2 protein/mRNA expression was induced by co-culture, with upregulation in MCs and CCL2 expression induced in the epithelial cells, with subsequent degranulation of MCs (Iwamoto et al., 2013). An increased number of endobronchial intraepithelial MCs was found in a clinical study performed on patients with asthma and a particular protease phenotype (tryptase and carboxypeptidase A3 high and chymase low) has been demonstrated by gene analysis (Dougherty et al., 2010). The protease spectrum of these intraepithelial MCs was found predictive for the response to corticoid therapy, and Il-13-stimulated production of stem cell factor by epithelial cells might explain MCs accumulation within the epithelium (Günhan et al., 1991).

CONCLUSION

Our results demonstrate a significant increase in mast cell density in patients with periodontal disease compared with healthy controls. This is associated with a significant increase of the degranulation rate. Accumulation of mast cells in the epithelium was found only in patients with periodontal disease and their density strongly correlated with the degree of inflammation. Morphological and immunohistochemical data concerning the correlation of MCs with inflammation in human periodontal disease

point to the necessity of a critical reassessment of periodontal disease treatment which is followed by the use of mast cell stabilizing agents as an adjuvant therapy for gingival pathology.

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