

## Mast Cells Stimulate Lymphangiogenesis in the Gingiva of Patients with Periodontal Disease

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**Abstract.** *The presence and distribution of lymphatic vessels and mast cells in the gingiva under normal and pathological conditions have been reported by several studies, but the relationship between them during inflammatory lymphangiogenesis is virtually unknown. The aim of the present study was to investigate the lymphatic microvessel density (LMVD) and mast cell density (MCD) in the gingiva of patients with periodontal disease compared to normal-like gingiva. Gingival punch biopsies from 51 patients with periodontal disease were investigated. MCs and LVs were detected by double-immunohistochemistry, using primary antibodies against mast cell tryptase and D2-40. The inflammatory infiltrate was evaluated on a scale from 0 (absent) to +3 (severe inflammation). MCs and LVs were counted in the same microscopic field for each case at ×200 magnification. We found a significant increase in the number of both MCs and LVs in cases with mild and moderate inflammatory changes, followed by a slight decrease in cases with severe inflammation. We have shown a particular association between MCs and LVs that may support the contribution of MCs to the development of the lymphatic vasculature in inflammatory conditions. MCD correlated with LMVD in all cases with mild and moderate inflammatory changes, but not in cases with severe inflammation. No correlation was found between MCD/LMVD and the density of the inflammatory infiltrate. Our results suggest the potential involvement of MCs in the*

*induction and maintenance of lymphangiogenesis in the gingiva of patients with periodontal disease in the early steps of evolution.*

Lymphangiogenesis defines the process of new lymphatic vessel (LVs) formation from post capillary venules or from pre-existing lymphatics. In the past three decades, LVs have been less investigated in comparison to blood vessels, especially for benign conditions. These studies were restricted particularly by the lack of highly-specific markers of the lymphatic endothelium. Such markers were introduced in practice only in the last 15 years, and nowadays podoplanin, prospero-related homeobox 1 gene (PROX1) or lymphatic vessel endothelial hyaluronan receptor (LYVE1) are largely used to discriminate between lymphatic and blood vessels (1). Podoplanin is expressed by developing and mature lymphatic endothelial cells and its formalin-insensitive epitope, called D2-40, is the most used antibody to identify LVs in normal and pathological conditions. Although highly specific for the lymphatic endothelium, podoplanin is also expressed by a variety of cells in both normal and pathological conditions, but not by the blood vessel endothelium (2).

The molecular mechanisms that initiate and maintain normal and pathological lymphangiogenesis are strongly related to the vascular endothelial growth factors (VEGF)-C and -D, which specifically bind two tyrosine kinase receptors, VEGF receptor (VEGFR)-3 and VEGFR2. The VEGFC/D-VEGFR3 axis increases vascular permeability, and stimulates proliferation, migration and survival of lymphatic endothelial cells *in vivo* and *in vitro* (3, 4). Lymphangiogenesis has been extensively investigated in malignant tumors, based on the prognostic relevance of lymphovascular invasion and the predictive role of lymph node metastasis (5-7). Despite the first early description and characterization of LV architecture and histology in various animal models of normal and inflamed gingiva (8-10), few data are available about lymphangiogenesis in inflammatory conditions, and fewer than 10 articles have

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been published on the topic of periodontal disease, most of them being in the past three years (11-14). Previous data particularly analyzed the number and structure of LVs, but the relationship between LVs and the microenvironment of gingival *lamina propria* has been less investigated.

Heterogeneity of inflammatory cells involved in periodontal disease and, moreover, their different mechanism of action during the development of inflammation hampered the full characterization of each type of inflammatory cell during periodontal disease progression. Currently, at least two resident cells of the *lamina propria* of the gingiva may be involved in lymphangiogenesis, based on their ability to secrete growth factors in normal and pathological lesions. From these, macrophages have been reported as active players in tumor-associated lymphangiogenesis (15, 16), but have been less studied concerning their involvement in lymphangiogenesis in periodontal disease (17). Mast cells are inflammatory cells commonly involved in angiogenesis and it has been demonstrated that their activation leads to VEGFA secretion, which stimulates proliferation of blood vessel endothelium (18-20). Mast cells are bone marrow-borne granulated cells normally found in the connective tissue and found in large numbers in the gingiva. Previous studies have demonstrated the increase of mast cell density (MCD) in inflammatory conditions in both connective and epithelial compartments of the gingiva of patients with periodontal disease. Although the MC reaction in the gingiva of patients with periodontal disease has been noticed by several authors (21-23), the basic significance of this change is still elusive (24).

MCs are very complex cells that contain many biologically-active substances in cytoplasmic granules, including biogenic amines, glycosaminoglycans, proteolytic enzymes and growth factors. The release of these substances by degranulation induces many changes in the connective tissue that also includes the vasculature (25, 26).

Currently, as far as we are aware of, there are no data in the literature on the relationships between MCs and LVs, and we hypothesized that MCs activation stimulates changes in the structure of LVs and an increase of lymphatic microvessel density (LMVD). In the present study, we investigated the distribution and number of LVs and MCs in the gingiva of patients with periodontal disease using a double immunostaining method. To the best of our knowledge, it is the first report to suggest the involvement of MCs in inflammatory lymphangiogenesis of the gingiva.

## Materials and Methods

**Patient data.** We investigated 12 patients without significant changes of the oral mucosa, and 15 patients with mild, 16 with moderate and eight with severe inflammatory lesions of the gingiva as found on clinical examination. Punch biopsies were taken from each patient and washed with buffered saline. The local Research Ethics

Committee approved of the protocol of the study and informed consent was obtained from all study participants, according to the World Medical Association Declaration of Helsinki.

**Primary processing.** Biopsies were fixed in 10% buffered formalin and paraffin embedded, using the standard histological technique. Five micrometer-thick sections were prepared for each case and were stained with the routine haematoxylin-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 and selected for the immunohistochemical study.

**Immunohistochemistry.** Immunohistochemical detection of LVs was performed by using monoclonal mouse antibody against human podoplanin (clone D2-40, dilution 1:200; Dako, Carpinteria, CA, USA). MCs were labeled with monoclonal mouse antibody to human MC tryptase (MCT, clone AA1, dilution 1:500; Dako).

Briefly, sections were de-waxed and then a heat-induced epitope retrieval method was applied by treating sections with citrate buffer (pH 6) for 30 min at 99°C following the scheduled PT Link automatic protocol (Dako, Carpinteria, USA). Endogenous peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide solution for 10 minutes.

Co-localization of D2-40 and MCT was obtained by performing a double immunostaining method. The first step of the method included 30 minutes' incubation with MCT, followed by the use of Novolink Polymer/HRP detection system (Novocastra, UK) and 3,3'-diaminobenzidine as chromogen, highlighting the MCT-containing granules stained in dark brown. The second step of the procedure started with endogenous peroxidase block step performed in the same manner as previously described. D2-40 antibody to podoplanin was applied to the slides for 30 min and the same detection system was used, followed by incubation with Vina Green chromogen kit (Biocare, Concorde, CA, USA) for 15 minutes, followed by nuclear counterstaining with modified Lillie Haematoxylin. Vina Green detected the cytoplasmic expression of podoplanin as a bright green signal observed in basal cells of gingival epithelium (used as internal control of the reaction) and also inside LV endothelium.

All steps of the immunohistochemical procedure were performed in a fully automated manner by using Dako Autostainer Plus System (Dako Cytomation, Carpinteria, CA, USA) and the slides were mounted with permanent mounting media (Leica Mount; Leica Microsystems, Nussloch, Germany).

**Scoring.** The inflammatory infiltrate was scored as 0 (absent), +1 (isolated inflammatory cells, fewer than 10 per ×400 field), +2 (aggregates of inflammatory cells in the *lamina propria* only), and +3 (aggregates of inflammatory cells in the *lamina propria* associated with intraepithelial lymphocytes). MCD and LMVD were calculated using the hot-spot method, at ×200 magnification (Figure 1A). Three hot spots with higher density of MCs and LVs were chosen for each case and the arithmetic mean of the three hot spots was calculated as the final result. Tryptase-positive granules scattered in the intercellular space were not included in evaluation of MCD.

**Statistical analysis.** Statistical analysis was performed with the commercially available SPSS20.0 software (IBM Corporation, Armonk, NY, USA) and Microsoft Excel 2010 software (Redmond,

WA, USA). The relationships between the density of the inflammatory infiltrate, MCD in the *lamina propria* and intraepithelial MCs were evaluated, applying Spearman test, and values of  $p < 0.05$  were considered statistically significant.

## Results

MCs and LVs were identified by double immunostaining in all cases included in the study and were quantified in close relationship in the same microscopic field.

Normal-like gingival tissues and gingiva with microscopic changes specific for periodontal disease contained both LVs and MCs but several significant morphological and numerical differences were observed for LVs and MCs between normal and pathological conditions of the studied gingival tissues.

Gingiva lacking microscopic inflammatory changes exhibited a D2-40-positive reaction, with a diffuse cytoplasmic pattern located in the lymphatic endothelium and basal epithelial cells of the gingival epithelium. Most of the superficial LVs were small, irregular in shape, with narrow lumen. LVs located deeply inside the *lamina propria* had a larger and regular lumen (Figure 1B). MCs were located at the some distance from LVs and no particular association was found between MCs and LVs in cases without inflammatory changes. Degranulation was rarely observed, and was restricted to MCs located in the superficial *lamina propria*. The MCD ranged between 17 and 49 (average=33.62) and the LMVD ranged between 4 and 27 (average=15). No significant correlation was found between MCs and LVs in the normal-like gingiva.

In cases with periodontal disease with mild inflammatory changes scored as +1, we found LVs with a lymphatic vessel endothelial hyaluronan receptor *lamina propria*, close to the epithelium. In some cases, we also noticed D2-40-positive structures without evident lumen associated with one or more MCs at the tip. We noticed a dramatic increase in the number of MCs in both superficial and deep *lamina propria*, but in association with a low rate of degranulation. More than half of the cases exhibited a significant number of intraepithelial MCs. The MCD ranged between 35 and 111 (average=73.16) and the LMVD ranged between 11 and 28 (average=20.66). A significant positive correlation was found between MCD and LMVD for  $p < 0.0001$ .

In cases with moderate inflammatory infiltrate, no significant changes in the number of LVs were found compared to the group of periodontal disease with mild inflammation, and values of MCD were similar to those found in cases with mild inflammatory infiltrate (Table I). The MCD ranged between 64 and 121, and LMVD ranged between 14 and 29. We found a significant positive correlation between MCD and LMVD ( $p < 0.0023$ ), but not between MCD/LMVD and the grade of inflammation.

For periodontal disease associated with mild or moderate inflammation, an increased number of both LVs and MCs was noted. Moreover, MCs were grouped around LVs, some of them being identified in close apposition to the outer surface of the LV wall. The morphology of LVs was also changed. For the specimens with no inflammation or mild inflammatory changes, LVs with large and well-defined lumen predominated; in contrast, in cases with moderate inflammatory infiltrate there was a predominance of small LVs with or without patent lumen. Morphological heterogeneity of the LVs included rarely observed vessels with a large lumen and several LVs with a slit-like lumen and cord-like structures lined by D2-40-positive endothelial cells but lacking a visible lumen (Figure 1C, red arrows). For periodontal disease with microscopically detected moderate inflammatory changes, all LV types had a tendency to be distributed in small groups associated with an increased number of both degranulated and non degranulated MCs (Figure 1C, arrowheads). Interestingly, the highest MCD was observed around D2-40-positive LVs without lumen, suggesting a strong involvement of MCs in the early stages of new LV development (Figure 1C and 1F).

In cases with severe inflammation, scored as +3, we found a marked decrease in the number of MCs and a high rate of degranulation (Table I). In this group, MCs were frequently associated with the inflammatory infiltrate. Additionally, most of the LVs were of mature type, having a fully developed and perfused lumen, as shown in Figure 1D. Occasionally, scattered LVs with cord-like morphology or isolated D2-40-positive stromal cells with endothelial-like morphology were detected, both being surrounded by a higher number of MCs compared to mature ones LVs. No correlation was found between MCD and LMVD in this group of patients.

## Discussion

The investigation of LVs in the normal gingiva and periodontal disease is not new. On the basis of classic morphological methods, LVs were reported in this location many years ago (8, 9). LVs of the gingiva are located in the *lamina propria* and travel close to the external surface of the alveolar bone (12). It is supposed that LVs drain fluids and regulate the interstitial fluid pressure and volume. Formation and maintenance of LVs is under the control of growth factors, such as VEGFC and -D, and most probably, Platelet Derived Growth Factor BB (PDGF BB), *via* their cognate receptors VEGFR3 and PDGFR $\beta$ , respectively (12, 27). The growth factors are secreted by cells of the covering epithelium and by some immune cells found in the *lamina propria*. VEGFC seems to be an important player in lymphangiogenesis associated with periodontal disease, but the knowledge in this field is limited as very few studies have been published on this

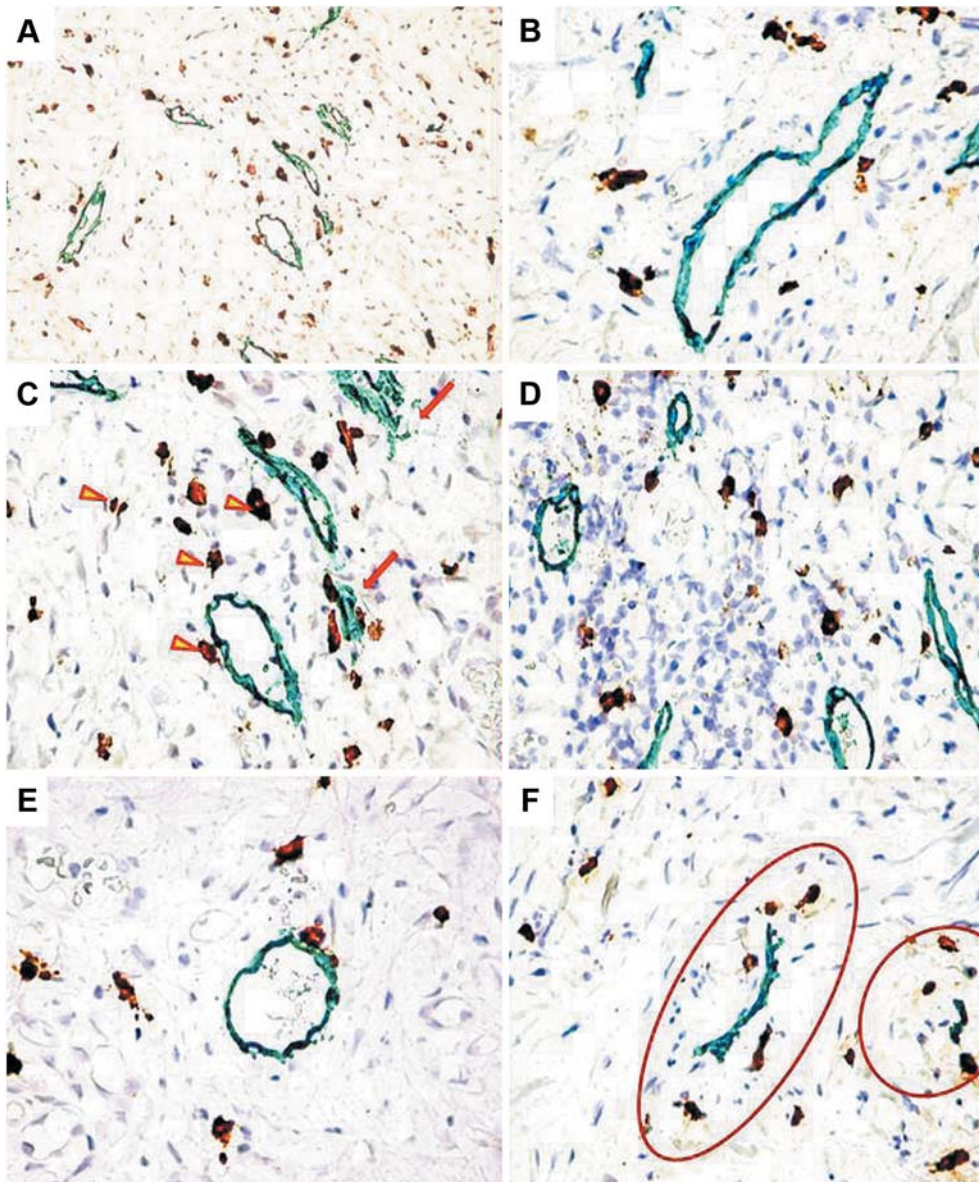


Figure 1. A hot-spot field used to count mast cells and lymphatic vessels (A,  $\times 200$ ). Lymphatic vessels (green) and mast cells (brown) in gingiva without inflammatory changes (B,  $\times 400$ ), with moderate inflammatory infiltrate (C,  $\times 400$ ) and with severe inflammatory changes (D,  $\times 400$ ). Mast cell apposition to the external wall of the lymphatic vessel (E,  $\times 400$ ). D2-40-positive tube-like structure surrounded by mast cells, without inflammatory infiltrate (F,  $\times 400$ ).

topic. Moreover, there is a lack of data concerning the role of MCs in periodontal disease-associated lymphangiogenesis as a potential source of lymphangiogenic factors.

Highly specific markers of the lymphatic endothelium were introduced in practice only in last 15 years. However, LV number and distribution in the gingiva were evaluated with less specific methods, such as 5'-nucleotidase or transmission electron microscopy, and it was noticed that a well-developed network can be detected (28, 29). In the

present study, we investigated LVs using the most specific marker, D2-40, which recognizes the formalin-insensitive epitope of podoplanin. D2-40 is highly specific for lymphatic endothelial cells and does not stain blood vessel endothelium (2). The double immunostaining for D2-40 and MCT allowed for counting of LVs and MCs in the same microscopic field. To the best of our knowledge, this is the first application of this method to quantify both LVs and MCs in the gingiva of patients with periodontal disease.

Table I. Average mast cell density and lymphatic microvascular density in normal gingiva and periodontal disease.

Inflammatory infiltrate	0 (n=12)	+1 (n=15)	+2 (n=16)	+3 (n=8)
MCD	33.62	76.16	87.75	40.7
LMVD	15	20.66	21.75	15.1

There are very few data concerning the relationships between MCs and lymphangiogenesis, and this aspect was evaluated mainly for malignant tumors (30). To the best of our knowledge, this is the first report on MCs and LVs in inflammatory conditions of the gingiva with periodontal disease. We found no correlation between MCD, or LMVD with the inflammatory infiltrate, but we report a strong positive correlation between MCD and LMVD, which suggests an important contribution of MCs to the development of the lymphatic vasculature. Variability of MCD around different types of LVs, with a high density around less mature LVs with cord-like morphology, together with MCs being found located in close contact with the outer wall of LVs or surrounding D2-40-positive isolated endothelial cells supports the involvement of MCs in the early steps of inflammatory-induced lymphangiogenesis from periodontal disease.

In inflammatory conditions, some immune cells produce lymphangiogenic factors that stimulate enlargement and proliferation of LVs (31, 32). In an experimental model of periodontal disease, Mkonyi *et al.* have shown that proliferation of lymphatic endothelial cells using the Ki67/LYVE1 double-immunostaining is an early event (13). Our findings support this, as we found a significant increase in the MCD in early stages of gingival inflammation. In addition, we found a slight decrease of MCD in advanced-stage inflammation, associated with severe degranulation.

Our findings suggest MC involvement in all stages of periodontal disease but acting through a different mechanism regarding development, morphology and function of LVs. While the first stages of lymphangiogenesis in periodontal disease seems to be governed by MCs as stimulators of proliferation, migration and organization of lymphatic endothelial cells in new LVs (most probably by MC secretion of lymphangiogenic growth factors), later stages characterized by increased inflammation exhibited high degranulation of MCs, most probably being responsible for increasing the permeability of newly-formed LVs for proper drainage of inflammation-induced tissue fluid.

Periodontal disease is associated with chronic inflammatory infiltrate and destruction of supporting tissues of the teeth. It has been shown that LVs of the gingiva regulate the normal tissue fluid balance in both normal and inflammatory conditions (14, 33). We found enlarged LVs in the gingiva of

patients with periodontal disease and this finding confirms previous studies in inflammatory conditions (34, 35). Based on our data, we can speculate that MCs are no longer necessary after the massive accumulation of other inflammatory cells, and this is supported by the slight decrease of MCD in patients with severe inflammatory changes, the lack of morphological association between MCs and LVs, and the lack of correlation in this stage between these two elements. The decrease of LMVD in this group is coincident with the marked decrease of MCD and massive accumulation of lymphocytes, although we found no correlation between them.

Although the immunohistochemical investigation, as performed in the present study, is only an indirect proof of the potential effect of MCs on lymphangiogenesis, it suggests further investigations in experimental models are required to demonstrate the effects of biologically-active substances on lymphatic endothelial cells. In addition, we believe that inflammation could represent an attractive model for evaluating lymphangiogenesis in both clinicopathological and experimental conditions.

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