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Mast cells and matrix metalloproteinase 9 expression in actinic cheilitis and lip squamous cell carcinoma

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Objectives. The aim of this study was to evaluate mast cell (MC) density and migration and their association with matrix metalloproteinase (MMP) 9 expression in squamous cell carcinoma (SCC) and actinic cheilitis (AC).
Study design. Tryptase, c-Kit, and MMP-9 expression was evaluated in 20 cases of SCC, 20 cases of AC, and 7 cases of normal lip (control samples) by immunohistochemistry techniques.
Results. Tryptase$^+$ and c-Kit$^+$ MC densities were significantly higher in SCCs than in ACs and control samples ($P < .001$). However, no significant difference was found when comparing tryptase$^+$ and c-Kit$^+$ MC densities between ACs and control samples (P values .185 and .516, respectively). MMP-9 was strongly expressed in SCCs and moderately expressed in ACs and control samples. A highly significant association was found between tryptase$^+$ MC density and the expression of MMP-9 ($P < .001$).
Conclusions. The increase in MC density associated with the strong expression of MMP-9 may favor SCC progression. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:342-348)**

Actinic cheilitis (AC) is a chronic inflammatory disorder that occurs mainly in the lower lip of middle-aged men. It is usually caused by chronic and excessive exposure of the lips to solar ultraviolet (UV) radiation.[1,2] The lesion is potentially malignant and may transform into squamous cell carcinoma (SCC).[3]

Mast cells (MCs) are multifunctional cells that play an important role in inflammation and have been associated with both resistance and greater susceptibility to tumor development.[4,5] These cells are present in a large number of tissues, including skin.[6,7] MC prevalence in human skin is modified by intrinsic (e.g., regulatory mechanisms of c-Kit expression) and extrinsic factors (e.g., chronic sun exposure).[8,9]

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent proteolytic enzymes that degrade the extracellular matrix (ECM) constituents and nonproteins.[10] More than 20 different members are currently known and was classified according to the domain organization: collagenases (MMP-1, -8, -13, and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and -10), matrilysins (MMP-7, -26, and -11), membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25), and other MMPs (MMP-12, -19, -20, -21, -23, -27, and -28). Among MMPs, gelatinase B (MMP-9) plays an important role in angiogenesis as well as in tumor invasion and metastasis, especially for its ability to cleave type IV collagen in the basement membrane.[10-12] This gelatinase also cleaves other collagens, such as types I, V, VII, and X, and substrates, such as gelatin, fibronectin, tenascin-C, fibrillin, osteonectin, decorin, α2-M, laminin-5, prointerleukin (IL) 1β, pro–tumor necrosis factor (TNF) α, pro–transforming growth

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Table I. Antibodies used

Antibody clones	*Specificity*	*Dilution*	*Antigen retrieval*	*Incubation*
AA1*	Tryptase	1:200	Citrate pH 6.0, steamer	60 min
A4502*	c-Kit	1:400	Tris/EDTA pH 9.0, steamer	Overnight (18 h)
15W2†	MMP-9	1:80	Citrate pH 6.0, steamer	Overnight (18 h)

*Dako, Glostrup, Denmark.
†NovoCastra, Fremont, CA.

Table II. Immunoreactive scores used for analysis of metalloproteinase 9 in lip squamous cell carcinoma, actinic cheilitis, and normal lip

Score	*Percentage of immunopositive cells*	*Staining intensity*
0	0	Negative
1	<10%	Weakly positive
2	10%-50%	Moderately positive
3	>50%	Strongly positive

factor (TGF) β, fibroblast growth factor receptor 1, α1-proteinase inhibitor and pro–MMP-1, -2, and -13.[13]

MMPs, including MMP-9, are generally synthesized and secreted as latent soluble enzymes that require activation in the extracellular space.[14] They are regulated at the transcription level, and their expression is modulated by a variety of stimuli produced by cytokines,[15,16] growth factors,[17] and cell-cell and cell-matrix interactions.[18-20] In addition, some studies have also identified MCs derived from tryptase and chymase as powerful MMP activators.[21,22] The literature agrees with the role of the stromal microenvironment in tumoral progression. Various experiments show evidence of cooperation or synergy between neoplasic and stromal cells in MMP production.[23,24]

The purpose of the present study was to evaluate MC density and migration and their association to MMP-9 expression in AC and lip SCC to better understand the role of MCs and MMP-9 in these lesions.

MATERIALS AND METHODS

We selected 20 cases of AC, 20 cases of SCC, and 7 cases of normal lip (used as control), all embedded in paraffin, from the files of the Pathologic Anatomy Service of the Oral Pathology Course, Dentistry Program, Federal University of Rio Grande do Norte (UFRN). For standardization purposes, selected cases were microscopically examined by 2 independent examiners through the review of histologic sections stained with hematoxylin and eosin. Histologic features for AC included epithelial changes (keratosis, hyperkeratosis, hyperplasia, atrophy, acanthosis, ulceration, and dysplasia) and connective tissue alterations (solar elastosis and inflammation).[1,3] Microscopic features for SCC were analyzed according to the World Health Organization tumor classification.[2] The study was approved by the Institutional Review Board at UFRN.

Immunohistochemical methods

Paraffin-embedded tissues were sectioned (3 μm) and extended in glass slides coated with 2% 3-aminopropyltriethoxy-silane (Sigma Chemical Co., St. Louis, MO, USA). Sections were deparaffinized by immersion in xylene, followed by immersion in alcohol with 3% hydrogen peroxide to block endogenous peroxidase activity, and then washed in Tris-buffered saline solution (TBS; pH 7.4). Antigen retrieval, incubation, dilution are shown in Table I. Sections were blocked by incubation with 3% normal goat serum diluted in distilled water at room temperature for 20 minutes. Slides were then incubated with the primary antibodies in a humidified chamber. After washing in TBS, sections were treated with labeled streptavidin-biotin kits (K0690; Dako, Glostrup, Denmark) for tryptase and MMP-9 and with the Envision system (K4001; system-labeled polymer–horseradis peroxidase; DakoCytomation, Carpinteria, CA, USA) for c-Kit. We used 0.03% diaminobenzidine (DAB; Sigma, Chemical Co.) as chromogen, and counterstaining was performed with Mayer hematoxylin. Positive control samples for tryptase, c-Kit, and MMP-9 were, respectively, sections of lung, gastrointestinal stromal tumor, and liver. As negative control subjects, samples were treated as above, except that the primary antibody was replaced by a solution of bovine serum albumin in phosphate-buffered saline solution.

Immunohistochemical expression of MC evaluation

Tryptase– and c-Kit–immunoreactive MC counting was performed with the aid of a graticule coupled to an optical microscope (CX31; Olympus, Tokyo, Japan). The number of immunomarked MCs for each antibody was separately determined for each group in histologic fields of higher density. Two previously calibrated and independent observers performed MC counting in 10 fields of control cases (5 fields at the epithelium–connective tissue junction and 5 in the reticular lamina propria), in 10 fields of ACs (5 at the epithelium–connective tissue junction and 5 in areas of solar elastosis), and in 15 fields of SCCs (5 at the epithelium–connective tissue junction, 5 in the tumor parenchyma, and 5 in the peritumoral stroma), at ×400 magnification (counting field area 0.20 mm^2).

Table III. Distribution of main histologic findings of the 20 specimens of actinic cheilitis

Histologic finding	*n*	*%*
Parakeratosis	4	20
Orthokeratosis	8	40
Hyperorthokeratosis	8	40
Acanthosis	8	40
Epithelial atrophy	13	65
Solar elastosis	20	100
Inflammatory infiltrate	16	80
Dysplasia		
Mild	5	25
Moderate	2	10

Table IV. Tryptase$^+$ and c-Kit$^+$ mast cell (MC) densities in lip squamous cell carcinoma (SCC), actinic cheilitis (AC), and normal lip (control)

Group	*n*	*Tryptase$^+$ MC density* (95% CI)*	*c-Kit$^+$ MC density* (95% CI)*	*P value†*
SCC	20	181.173-246.327	75.551-110.322)	<.001
AC	20	53.727-77.073	51.089-68.111	
Control	7	19.583-34.702	12.345-69.083	

CI, Confidence interval.
†Analysis of variance.
*Number of positive cells/mm^2.

Immunohistochemical expression of MMP-9 evaluation

MMP-9 immunohistochemical staining was evaluated through descriptive and semiquantitative analysis. In the latter, we used scores adjusted from Franchi et al.[25] for analysis of the epithelial tissue in ACs and control samples and of tumor cells in SCCs, based on the percentage of immunoreactive cells and their staining intensity (Table II). The analysis was performed using a light microscope by 2 previously calibrated and independent observers.

Statistical analyses

MC density descriptive analysis was expressed as confidence intervals (CIs) of the number of observations per mm^2. Comparative analysis of means between groups and between histologic fields was performed using parametric 1-way analysis of variance (ANOVA). Paired multiple comparisons were then performed by using the Tukey test. MC migration was expressed as the ratio between c-Kit$^+$ and tryptase$^+$ densities, and the comparative analysis between groups was performed with 1-way ANOVA and Tukey post hoc tests. Association between MC density and MMP-9 expression was assessed by using the Student *t* test. Differences were considered to be statistically significant when $P < .05$.

RESULTS

The main histologic findings regarding the 20 specimens of ACs are summarized in Table III. In the SCC analysis, 7 cases were classified as well differentiated, 3 as moderately differentiated, and 10 as undifferentiated.

Resident MCs were identified by the use of antitryptase antibody, and MC migration was evaluated with an anti–c-Kit antibody. Analysis of material submitted to immunohistochemistry showed that tryptase$^+$ MCs were more strongly expressed in SCCs than in ACs and control samples ($P < .001$ [Tukey post hoc test]; Table IV). In these tumors, a significantly high density of tryptase$^+$ MCs was found in the tumor stroma, surrounding the invasive epithelial nests and cords (Fig. 1, *a*). A high expression of these cells was also observed near the lining epithelium adjacent to the tumor, though less than in the stroma ($P = .007$ [Tukey]). Moreover, a sparse density of these cells was observed in the lesion parenchyma compared with the tumor stroma ($P < .001$ [Tukey]).

Regarding the immunostaining for c-Kit, we found a higher concentration of c-Kit$^+$ MCs in SCCs than in ACs and control samples ($P < .001$ [Tukey]; Table IV), similar to what was found for tryptase. A higher c-Kit$^+$ MC density was also found in the tumor stroma (Fig. 1, *b*) and at the epithelium–connective tissue junction compared with the tumor parenchyma ($P < .001$ [Tukey]).

In the analyzed ACs, tryptase$^+$ and c-Kit$^+$ MCs were present in areas of elastosis and near the epithelium/connective tissue junction (Fig. 1, *c* and *d*), but the difference was not significant in the expression of these markers between these regions (*P* values .195 and .496, respectively).

In specimens of normal lip, used as the control group, lower tryptase$^+$ and c-Kit$^+$ MC densities were observed. These cells were mainly located in the epithelium/connective tissue junction and in the reticular lamina propria (Fig. 1, *e* and *f*), but the difference was not significant in the expression of these markers between these regions (*P* values .165 and .626, respectively). Nonetheless, no significant difference was found when comparing tryptase$^+$ and c-Kit$^+$ MC densities between ACs and control samples (*P* values .185 and .516, respectively [Tukey]).

MC migration (c-Kit$^+$–tryptase$^+$ relationship) was 75% in SCCs, 103% in ACs, and 138% in control samples. When the MC migration was compared between lesions, the difference was significant only between SCCs and control samples ($P = .012$) and not between SCCs and ACs ($P = .166$) nor between ACs and control samples ($P = .231$).

All SCC specimens exhibited strong expression of MMP-9 in tumor nests (Fig. 2, *a*). Expression of this gelatinase was also observed in inflammatory and endothelial cells. All AC cases showed a moderate MMP-9 expression, which was heterogeneously evident in the epithelium. Staining was generally negative in the epithelial surface layers (Fig. 2, *b*). MMP-9 also showed mod-

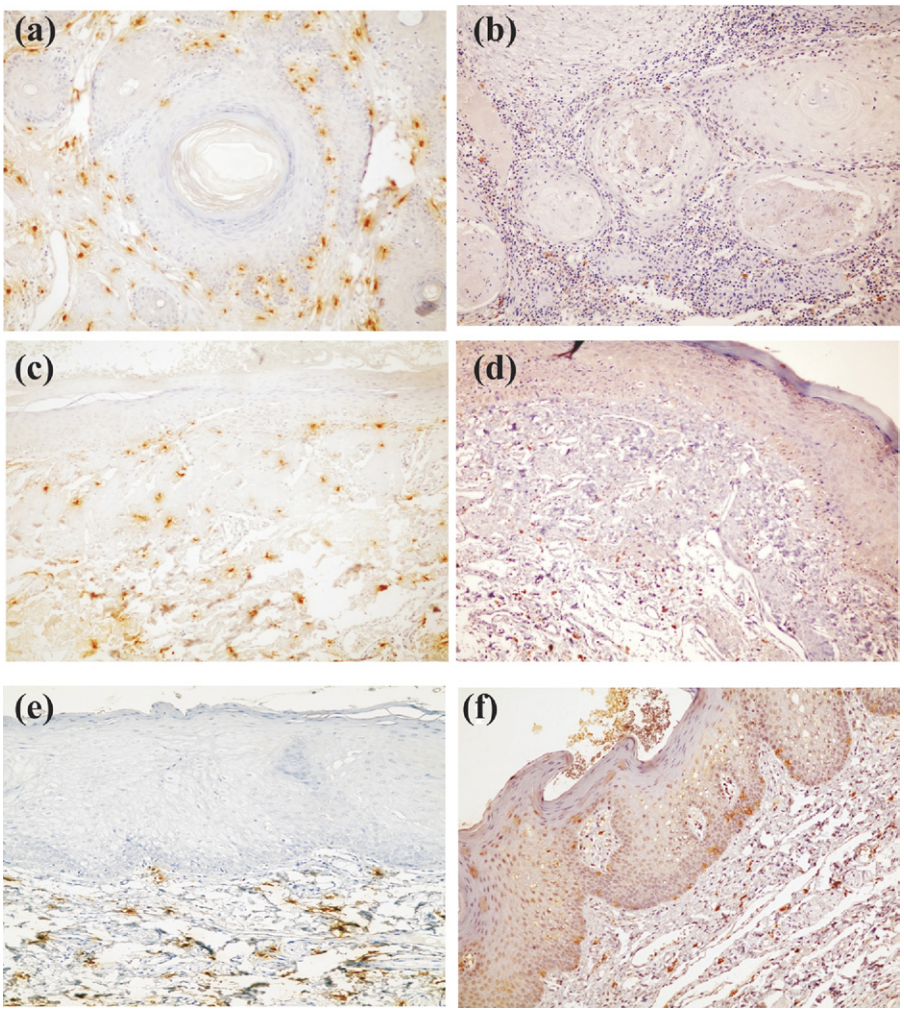

Fig. 1. Tryptase$^+$ and c-Kit$^+$ mast cell immunoexpression in lip squamous cell carcinoma (SCC), actinic cheilitis (AC), and normal lip (control). Tryptase$^+$ (**a**) and c-Kit$^+$ (**b**) mast cell immunoexpression in stromal region of SCC. Tryptase$^+$ (**c**) and c-Kit$^+$ (**d**) mast cell immunoexpression in solar elastosis region of AC. Tryptase$^+$ (**e**) and c-Kit$^+$ (**f**) mast cell immunoexpression in epithelium–connective tissue junction and lamina propria region of control (Strept avidin biotin complex [SABC] method, original magnification ×200).

erate expression in control samples, with positive staining in most of the epithelium, although it was occasionally negative in focal areas of keratinized, granular and prickle layers (Fig. 2, *c*). A highly significant association was found between the tryptase$^+$ MC density and the expression of MMP-9 ($P < .001$; Table V).

DISCUSSION

MCs are multipotent hematopoietic progenitor cells that circulate through blood vessels and subsequently migrate to peripheral tissues where they undergo terminal differentiation and participate in regulating the immune response. The migration process is influenced by the stem cell factor (SCF), also known as MC growth factor, and the local microenvironment.[9,26] These cells play a variety of roles. Besides acting in the innate and acquired immune response, they are also able to degrade the ECM. MC degranulation releases specific products, such as tryptase, chymase, MMPs, basic fibroblast growth factor, heparin, histamine, TNF-α, various interleukins (IL-3, -4, -5, -6, -8, -10, -13, and -16), chemokines (MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4 and RANTES/CCL5), and lipidic mediators.[5,27-30] Of these, tryptase is the most abundant serine proteinase stored in MC granules.[29] It promotes inflammation, ECM destruction, and tissue remodeling[31,32] and is considered to be an important angiogenic factor.[33]

The results of the present study showed a significant increase of tryptase$^+$ MCs in SCCs compared with normal lip. These findings agree with other results in

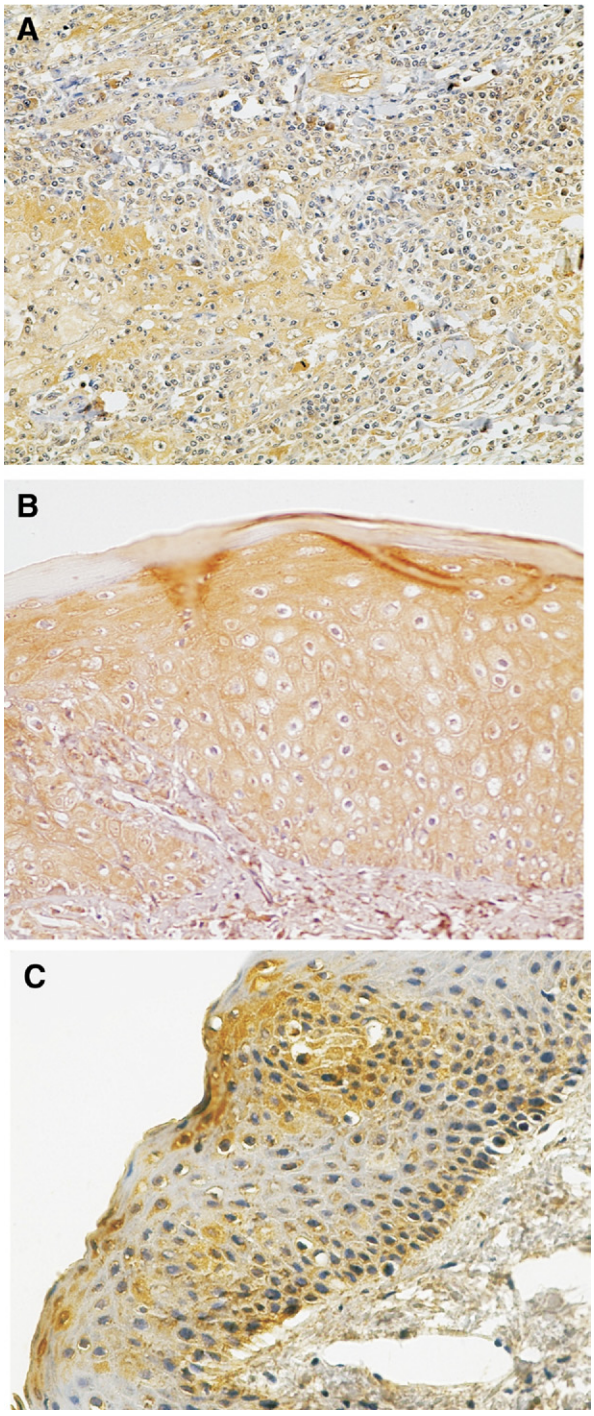

Fig. 2. MMP-9 immunoexpression in lip squamous cell carcinoma (SCC), actinic cheilitis (AC), and normal lip (control). **A,** Immunoexpression in tumor nests of SCC. **B,** Immunoexpression in epithelium of AC. **C,** Immunoexpression in epithelium of control (SABC method, original magnification ×400).

Table V. Tryptase$^+$ mast cell (MC) density and metalloproteinase (MMP) 9 immunoexpression

n	*MMP-9 immunoexpression*	*MC density* (mean ± SD)*	*P value†*
27	Moderate	55.48 ± 27.60	<0.001
20	Strong	213.75 ± 69.61	

*Number of positive cells/mm^2.
†Student *t* test.

the literature.[34,35] On the other hand, Oliveira-Neto et al.[36] found a decrease of tryptase$^+$ MCs in oral squamous cell carcinoma (OSCC) and leukoplakia. Studies have pointed to an increase in the number of MCs, including tryptase$^+$ ones, in solar radiation–exposed skin.[9,37-40] This may explain the differences in MC densities found in SCCs and ACs compared with OSCCs. Chronic exposure to radiation, particularly UV, has been described as one of the main risk factors related to AC and SCC development.[35,40]

Our results also showed a significant increase of tryptase$^+$ MCs in SCCs compared with ACs, unlike the findings of Costa et al.,[34] where similar MC densities were found in both lesions. ACs present a variety of histologic changes that primarily include varying degrees of keratosis, solar elastosis, epithelium atrophy or hyperplasia, and the absence or presence of dysplasia.[1] All cases studied by Costa et al. were classified as mild epithelial dysplasia, whereas in our study only 5 cases were so classified. That may explain the different MC densities observed in ACs between these studies.

In the present study, a higher MC density was observed in the stroma compared with the tumor parenchyma. MCs may accumulate in the stroma around the tumor and take part in the inflammatory reaction that happens at the tumor edge and in the local tumor immunity.[5] Nevertheless, the increase of stromal tryptase$^+$ MCs has also been reported as an important factor for tumor invasion on cancers in various anatomic sites.[41,42] Therefore, these cells may contribute to the defense against tumors as well as to their progression.

Our results also pointed to a significant increase in c-Kit$^+$ MC density in SCCs and ACs compared with control samples. These findings differ from the results of Costa et al.[34] who found a similar cell density for the 3 groups. c-Kit (CD 117) is a transmembrane receptor tyrosine kinase type III that acts in cellular signal transduction in various cell types. It is usually activated by binding to its ligand, stem cell factor (SCF). In this scenario, it promotes phosphorylation and activation of the intracytoplasmic signaling cascade which is essential for embryogenesis, hematopoiesis, development, proliferation, and migration. c-Kit is expressed in normal human tissue, melanocytes, breast epithelium, interstitial cells of Cajal, and MCs. SCF stimulates directional motility of both mucosal and connective tissue–type MCs. It

also plays a role in MC survival and activation via up-regulation of TNF-α, chemokine production, and the induction of histamine release.[43]

MCs are long-lived cells that can restart the cell cycle, proliferate, and be recruited after an appropriate stimulation. This stimulus may contribute to population expansion of these cells. Kim et al.[9] suggest that the stimuli derived from sunlight (either UV or infrared) or heat may act as extrinsic factors in regulating MC quantity and distribution in human skin. These authors postulate that these cells are recruited to sites of irradiated skin by several mechanisms. Ultraviolet or infrared radiation may both directly and indirectly stimulate MC proliferation in human skin. Indirect stimulation happens through keratinocytes or fibroblasts that can produce and release chemotactic factors for MCs. These factors recruit and activate inflammatory cells to sites of irradiated skin. Thus, MCs can migrate due to chemotactic factors, such as SCF, TGF-β, vascular endothelial growth factor, and IL-8.

On the other hand, other authors also point out that tryptase/chymase$^+$ MCs can promote ECM degradation and tumor progression at the invasive front in SCCs. Earlier studies suggest that tryptase has the ability to contribute to the process of tissue remodeling through pro–MMP-1, -2, -3, and -9 activation.[22,44-46] Tryptase may indirectly act in ECM degradation, activating latent forms of MMPs, as well as directly, degrading substrates such as collagens type I and IV and fibronectin.[22,38,46]

Prolonged exposure to UV radiation in the normal process of aging may also enable pro-MMPs, leading to ECM degradation.[47] A study reported a possible link between exposure to UV radiation and the degradation of the basement membrane by gelatinases.[48]

MMPs mediate tumor angiogenesis, malignant transformation, proliferation, and apoptosis through the degradation of the basement membrane, of cell adhesion molecules, and several matrix components and through the activation of chemokines and growth factors.[49] MMP-9 overexpression has been associated with tumors in various anatomic sites[25,50,51] as well as with tumor progression and skin metastasis.[10,11] A highly significant difference was observed in the present study between tryptase$^+$ and c-Kit$^+$ MC densities and MMP-9 expression. MMP-9 was strongly expressed both in tumor cells and in stromal inflammatory and endothelial cells. These findings agree with earlier studies.[52,53] Inflammatory stimulation may induce MMP-9 expression in various cells, such as endothelial cells, macrophages, fibroblasts, and MCs.[11]

The high tryptase$^+$ MC density observed in our study in SCCs may be related to the stimulus produced by sun exposure. This exposure may contribute to MC proliferation at irradiated sites. In the present study, MC migration did not differ in SCC compared with control samples, as shown by the c-Kit$^+$/tryptase$^+$ ratio. The strong MMP-9 expression observed in SCCs suggests that tryptase$^+$ MCs present in these lesions may take part in ECM degradation and tumor progression by means of activating this gelatinase. Furthermore, tryptase$^+$ MCs, predominantly located in the stroma, are also important for angiogenic stimulation, which is essential for growth and survival of tumor cells. Therefore, we may conclude that the increase in MC density in SCCs suggests that these cells, associated with MMP-9 expression, may contribute to the progression of these tumors.

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