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Increased mast cell density and protease content in actinic cheilitis

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BACKGROUND: Actinic cheilitis (AC) is a pre-malignant lesion caused by ultraviolet (UV) radiation and characterized by epithelial and connective tissue alterations. Mast cells (MCs), key contributors to solar elastosis in murine UV-irradiated skin, were characterized in order to assess their potential contribution to connective tissue degeneration in AC.

METHODS: Actinic cheilitis ($n = 15$) and normal lip ($n = 8$) biopsies were stained immunohistochemically for tryptase and enzymehistochemically for chymase to determine MC density and protease content. MC subpopulations (i.e. MC_T containing only tryptase, and MC_{TC} containing chymase and tryptase) and their distribution were also determined.

RESULTS: Mast cells and their proteases were increased in AC as compared with normal lip ($P < 0.0001$), and appeared degranulated especially around elastotic areas. MC_T predominated over MC_{TC} in AC and normal lip ($P < 0.05$). However, in AC MC_T were increased in the epithelium/connective junction and connective area ($P < 0.05$), while in normal lip MC_T predominated in connective and submucosal areas ($P < 0.05$).

CONCLUSION: The results suggest that increased MC density and protease content may contribute to elastosis formation in AC. In addition, changes in MC_T distribution may favor AC malignization.

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Keywords: actinic cheilitis; chymase; mast cells; proteases; solar elastosis; tryptase

Introduction

Actinic cheilitis (AC) is a pre-malignant lesion that can transform into squamous cell carcinoma (SCC) of the lip (1, 2). It affects mainly the lower lip of older, fair-skinned people, excessively exposed to sunlight (3, 4). Mild to severe epithelial and connective tissue alterations are usually found (1, 3). Epithelial changes include thickening of the epithelium and keratin layer, ulceration, acanthosis, and in more severe cases mild to severe dysplasia (3). In the connective tissue, solar elastosis and in most cases inflammation are found (3, 5). Solar ultraviolet (UV) radiation, known for its sunburning and immunomodulatory properties, has been recognized as the main etiological agent of AC (5–8).

Several studies have shown that mast cells (MCs) are significantly increased in UV-irradiated skin (9, 10). In addition, MCs have been implicated in the development of solar elastosis and connective tissue inflammation in UV-irradiated skin (9, 11, 12). It has been shown that UV light induces MC release and synthesis of several mediators capable of modulating, directly or indirectly, extracellular matrix (ECM) production and degradation (13–15). Among them are the serine proteases chymase and tryptase that directly degrade the ECM and activate collagenases (16–19). Human MCs are classified according to protease content in MC_T phenotype, if they only contain tryptase, and in MC_{TC} if they contain both tryptase and chymase (15, 20). Variations in MC phenotypes have been found in several malignant and pre-malignant lesions (21–23). Dermal MCs are also effector cells of UVB-induced immunosuppression, which can increase the susceptibility for skin cancer (8, 24, 25). Products of MC degranulation such as histamine and, tumor necrosis factor (TNF)-α, are key mediators of this UVB-induced immunosuppression (24, 26).

Despite the evidence implicating MCs in the development of photodamaged skin, MCs have not been yet studied in AC. Therefore, it was hypothesized that MCs were significantly increased in AC and could be key contributors to the connective tissue alterations found in AC. In order to assess this hypothesis, 15 lip biopsies of AC were analyzed for the content and distribution of MCs and their proteases chymase and tryptase, and compared with normal lip tissue.

Material and methods

Lip biopsies

Biopsies of lip vermilion from 15 non-smoker patients (three women and 12 men; ages ranged from 25 to 74,

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with a mean of 54 ± 14 years) with AC were obtained from the archives of the Oral Pathology Laboratory, Facultad de Odontología, Universidad de Concepción. Normal lip vermilion biopsies were also evaluated (two women and six men; ages 25–63, with a mean of 38 ± 14). Informed consent was obtained from all subjects. This study was approved by the Ethics Committee of the Universidad de Concepción. All specimens were fixed in 10% buffered formalin and paraffin-embedded within 24 h. Serial sections, 4 μm thick, were taken from the tissue blocks and processed for routine histopathologic and subsequent immunohistochemical studies.

Histopathologic studies

Tissue sections were deparaffinized through xylene and descending grades of ethanol to phosphate-buffered saline (PBS) and subsequently stained with hematoxylin and eosin. Sections were assessed for histopathologic signs of AC, including epithelial changes (hyperkeratosis, hyperplasia, atrophyl, acanthosis, ulceration and dysplasia) and connective tissue alterations (solar elastosis and inflammation).

Metachromatic staining of mast cells

Mast cells were detected by the classical staining of sulfated proteoglycans in secretory granules. Briefly, tissue sections were dewaxed, rehydrated, and immersed in 0.1% toluidine blue (Sigma, St Louis, MO, USA) in 1% NaCl for 5–10 min. Slides were then rinsed in distilled water, dehydrated, and mounted.

Immunohistochemical staining of tryptase-positive mast cells

Tissue sections were stained for tryptase-positive MCs as previously described (21) with some modifications. Briefly, sections were deparaffinized in xylene and descending grades of ethanol to PBS. Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in absolute methanol for 10 min, followed by a 30-min incubation in 10% goat serum in 1% bovine serum albumin–Tris buffered saline (BSA–TBS). Slides were then incubated with the primary antibody, monoclonal mouse-anti human MC tryptase (1:2000; Chemicon International, CA, USA), in 1% BSA–TBS for 1 h at room temperature, followed by incubation for 30 min with the secondary antibody, goat anti-mouse IgG-poly-HRP (Chemicon International, CA, USA). The reaction was developed for 5 min with 3-3′-diaminobenzidine (DAB; Chemicon International) and 3 ml/ml H_2O_2 in 50 mM Tris (pH = 7.6). Slides were then counterstained with Meyer's hematoxylin, dehydrated and mounted. Between steps the slides were washed three times in 1%BSA–TBS. Lip cancer sections were used as positive control, and omission of the primary antibody was used as negative control.

Enzymehistochemical staining of chymase-positive mast cells

Chymase-positive MCs were stained by using an incubation mixture containing the substrate N-acetyl-l-methionine alpha-naphtyl ester (u-N-O-Met) as previously described (27). Briefly, sections 4 μm thick were deparaffinized, rehydrated, and washed in 0.15 M phosphate buffer, pH 7.1. Sections were then incubated at room temperature for 10 min with a mixture of 5 mg of u-N-O-Met dissolved in 0.2 ml dimethyl formamide (Sigma), made up to a final volume of 25 ml with 0.15 M phosphate buffer, containing 3.2 mM Fast Blue salt (Sigma) as a capture reagent. The reaction was stopped by incubating with 1% cupric sulfate for 5 min. The slides were then washed with phosphate buffer, counterstained with saphranine-O, and mounted in aqueous medium (Paramount, DAKO, Carpinteria, CA, USA). As a negative control, the substrate was omitted from the incubation mixture.

Counting of tryptase- and chymase-positive mast cells

Tryptase- and chymase-positive cells were counted separately in serial sections of normal lip and AC using a Nikon Diaphot 300 microscope equipped with an OC-M calibrated eyepiece micrometer and connected to an ImagePro analysis program 4.0.1(Media Cibernetics, Atlanta, GA, USA). MCs were counted in 30 counting fields per section at 40× magnification (counting field area = 0.4 mm^2). In order to assess distribution, the counting fields were distributed in three areas located at the epithelial/connective tissue (E/C) junction, connective tissue and submucosa (10 adjacent counting fields per area) by two calibrated observers blinded to the objectives of this study. Results were expressed as MCs/mm^2 (mean ± SEM).

Statistical analysis

All data were tabulated and statistical tests were performed with JMP-IN 3.2.1 (SAS Institute Inc., Cary, NC, USA). Significant statistical differences between groups were examined using unpaired *t*-test and one-way ANOVA. The nonparametric, Wilcoxon and Kruskal–Wallis tests were used when variables did not

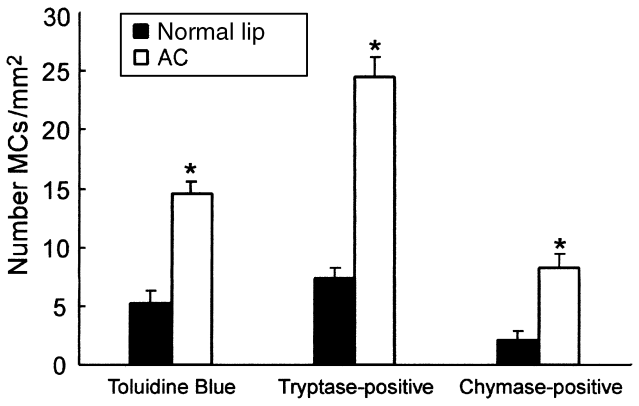

Figure 1 Mast cell (MC) density and protease content in actinic cheilitis (AC). Slides were processed for toluidine blue staining of MC proteoglycans, immunohistochemical detection of tryptase-positive MCs (also used for total MCs), and enzymehistochemical detection of chymase-positive MCs. Results are expressed as mean MCs/mm^2 ± SEM ($n = 15$ for AC samples, and $n = 8$ for normal lip samples). *$P < 0.0001$ (*t*-test), as compared with normal lip.

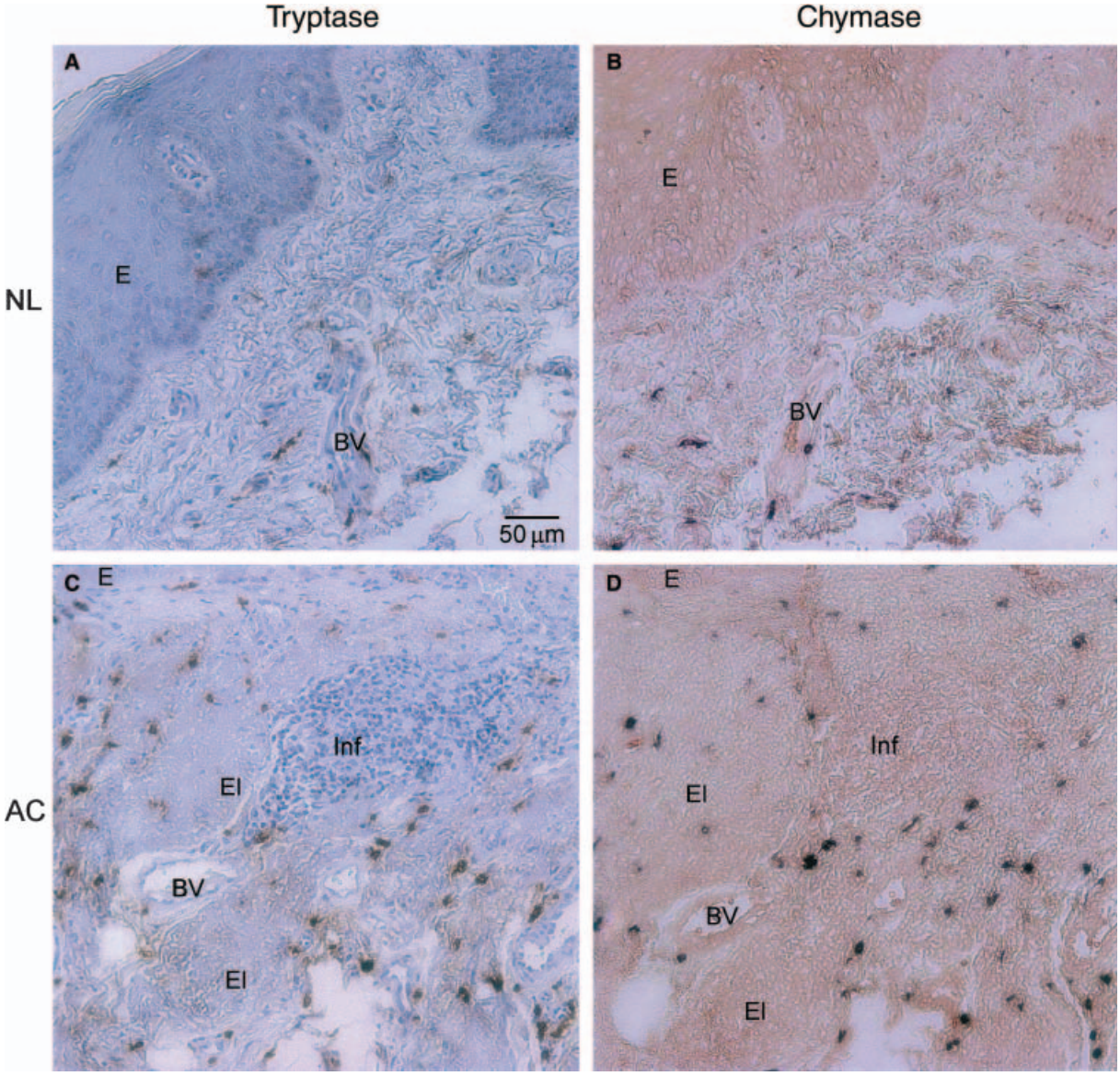

Figure 2 Staining of tryptase- and chymase-positive mast cells (MCs) in serial sections of actinic cheilitis (AC) and normal lip. Immunohistochemical staining of tryptase-positive MCs is shown for normal lip (A) and AC (C). Enzymehistochemical staining of chymase-positive MCs is shown for normal lip (B) and AC (D). AC, actinic cheilitis; NL, normal lip; E, epithelium; El, elastosis; Inf, inflammation, BV, blood vessel.

have a normal distribution. Differences were considered statistically significant when $P < 0.05$.

Results

Histopathologic analysis of AC biopsies

The histopathological findings in the 15 AC specimens were as follows: epithelial changes such as hyperkeratosis (in six of 15 AC samples), acanthosis (seven of 15), atrophyl (five of 15), ulceration (one of 15) and hyperplasia (two of 15) were found combined or alone in the AC samples. One case of epithelial dysplasia combined with hyperkeratosis and atrophyl was also found. All the samples presented basophilic connective changes or solar elastosis, and chronic inflammation. The normal lip vermillion biopsies had absence of both inflammation and detectable actinic damage.

Mast cell density and protease content in AC

As the first approach to determine MC density in normal lip and AC samples, the classic metachromatic method of MC proteoglycans staining with toluidine blue was used. MC protease content was determined by enzymehistochemical staining of chymase and immunohistochemical staining of tryptase. As all MCs contain tryptase (MCs containing only chymase are extremely scarce) (20, 21), this method was also used to determine total MC density.

Regardless of the method, toluidine blue or tryptase staining, a significant increase in total MC density was

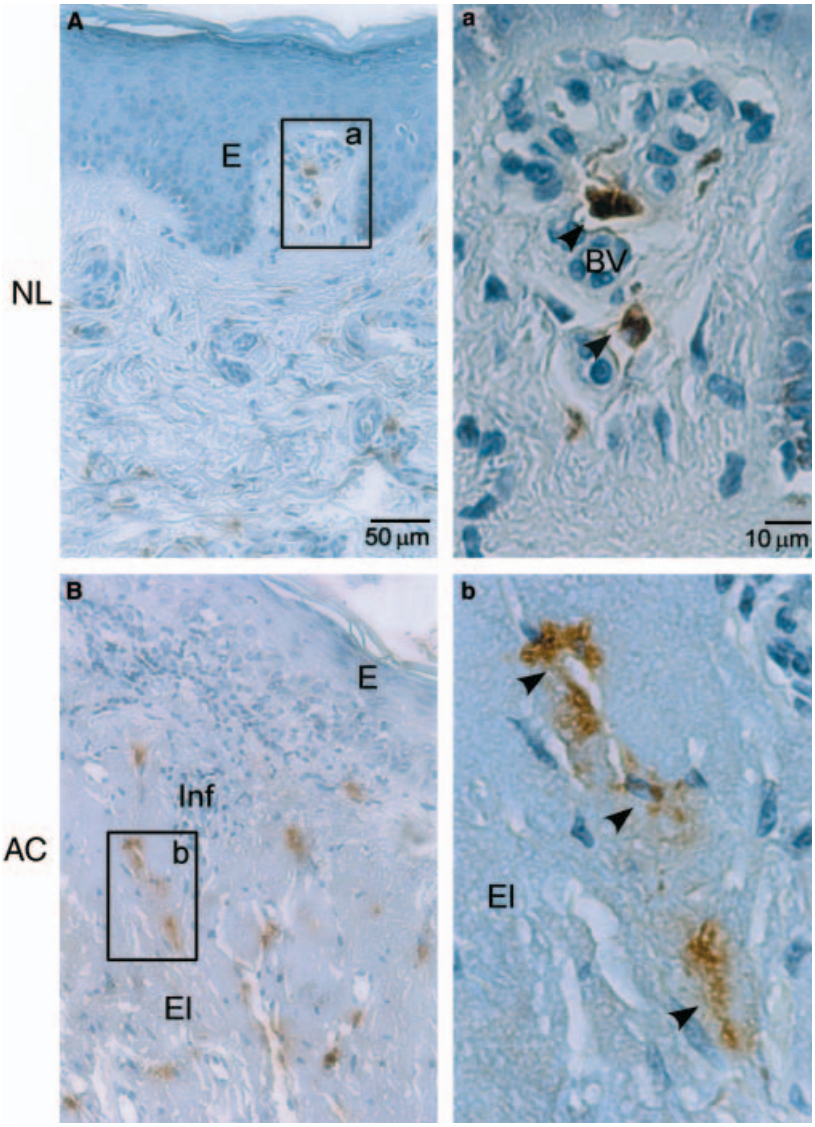

Figure 3 Mast cell morphology and location in actinic cheilitis (AC) and normal lip. (A, a) Inactivated tryptase-positive MCs located around blood vessels in normal lip. (B, b) Degranulated tryptase-positive MCs infiltrating areas of elastosis in AC. MCs are indicated by arrowheads. AC, actinic cheilitis; NL, normal lip; E, epithelium, Inf, inflammation, El, elastosis, BV, blood vessel.

found in AC as compared with control lip ($P < 0.0001$, *t*-test Fig. 1). However, using immunohistochemistry against tryptase, higher MC numbers were detected in the AC samples ($P < 0.0001$) as compared with AC samples stained with toluidine blue. No significant differences in MC numbers were found in the normal lip samples stained with either toulidine blue or anti-tryptase antibody. MC densities for metachromatic staining were 5.2 ± 1 for normal lip vs. 14.6 ± 0.9 for AC, and for tryptase-positive MCs were 7.3 ± 0.9 for normal lip vs. 24 ± 1.7 for AC.

In relation to protease content, both, tryptase- and chymase-positive cells were significantly increased in AC as compared with normal lip ($P < 0.001$, Figs 1 and 2). Regarding location and morphology, in AC MCs were found in close proximity to the areas of elastosis, and they appeared enlarged and in a state of degranulation (Fig. 3B). On the contrary, MCs in normal lip looked smaller, without signs of activation, and were found located around blood vessels and nerves (Fig. 3A).

Determination of MC subpopulations and their distribution in AC

To determine MC subpopulations (MC_T, MC_{TC}) in AC and normal lip, the formula (Total MCs = MC_T + MC_{TC}) was used as previously described (21), where Total MCs = tryptase-positive MCs and MC_{TC} = chymase-positive MCs. Results were confirmed by co-localization of serial sections stained either for tryptase- or chymase-positive MCs. It was found that the MC_T subpopulation predominated both in normal lip and in AC ($P < 0.05$, Wilcoxon test; Fig. 4).

To study distribution of total MCs and their subpopulations in AC vs. normal lip, MC density was determined in 30 counting fields located in three areas within each section, the area of epithelium/connective (E/C)

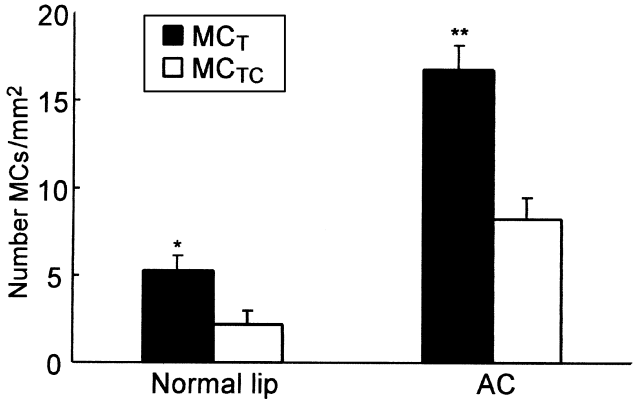

Figure 4 Determination of mast cell (MC) subpopulations in normal lip and actinic cheilitis (AC). MC subpopulations were determined using the formula (Total MCs = MC_T + MC_{TC}). Results are expressed as mean MCs/mm^2 ± SEM (n = 15 for AC samples, and n = 8 for normal lip samples). *P < 0.02 (Wilcoxon and Kruskal–Wallis) as compared with MC_{TC} in normal lip. **P < 0.0005 (Wilcoxon and Kruskal–Wallis) as compared with MC_{TC} in AC.

junction, connective tissue (where elastosis was found in all samples), and the underlying submucosa. In both, normal lip and AC, total MCs and MC_{TC}, were significantly higher in the connective tissue area as compared with both the E/C junction and submucosa (P < 0.03, Wilcoxon and Kruskal–Wallis tests; Fig. 5a,c). However, MC_T subpopulations had a different distribution pattern in both normal lip and AC. In normal lip, MC_T were increased in both connective tissue and submucosa as compared with the E/C junction (P < 0.02, ANOVA and Tukey–Kramer tests; Fig. 5b), whereas in AC, MC_T were increased in the E/C junction and the connective tissue as compared to the submucosa (P < 0.01, ANOVA and Tukey–Kramer tests; Fig. 5b).

Discussion

Mast cells have been recognized as important effector cells of the deleterious effects of UV light on the skin (8, 9). The results of this study showed that MCs and their proteases, tryptase and chymase, were significantly increased in UV-induced lesions of the lip, such as AC. Furthermore, enlarged, degranulating MCs were found infiltrating and/or surrounding areas of elastosis, which may suggest a role for MCs in the ECM changes found in AC.

In AC, as well as in chronically UV-irradiated skin, the connective tissue presents degenerative changes known as basophilic degeneration or elastosis (3, 5). This process is characterized by collagen degeneration and replacement of the ECM by a network of altered elastic material (28, 29). Studies have shown that several proteases of macrophage and fibroblast origin, including cathepsin G and gelatinases A and B, are increased in elastotic tissues (30–32). MC products have also been associated with elastosis formation (9, 10). Gonzalez et al. (9) found that MC-deficient mice did not develop solar elastosis in UV irradiated skin as compared with controls. Several preformed and newly formed MC mediators, released

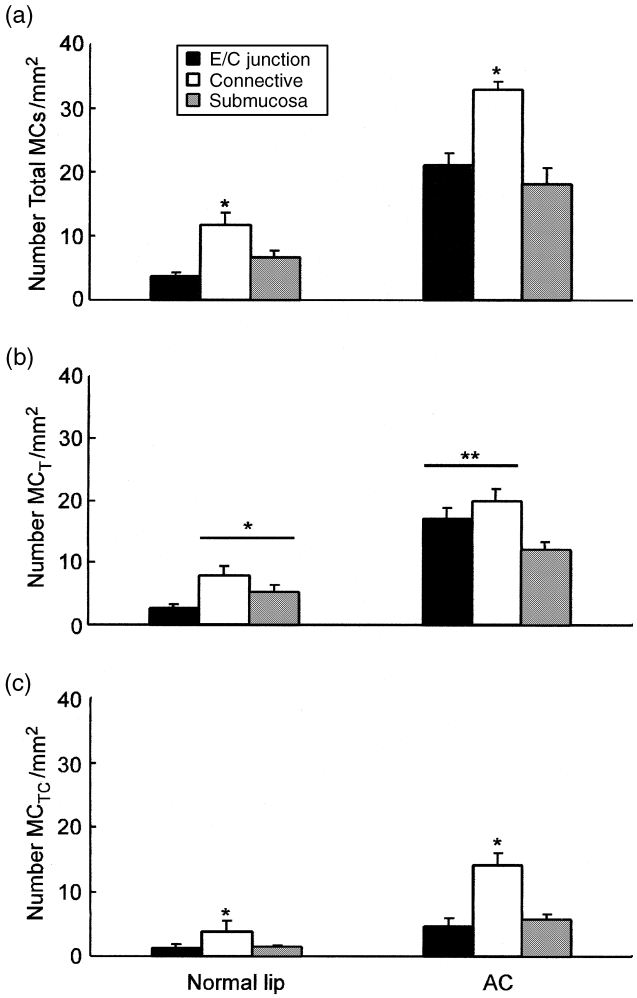

Figure 5 Distribution of mast cell (MC) subpopulations in normal lip and actinic cheilitis (AC). Number of total MCs (a), MC_T (b), and MC_{TC} (c) were determined at the epithelial/connective (E/C) junction, connective tissue, and submucous tissue. Results are expressed as mean MCs/mm^2 ± SEM (n = 15 for AC samples, and n = 8 for normal lip samples). (a and c) *P < 0.05 (Wilcoxon and Kruskal–Wallis) as compared with E/C junction and submucosa. (b) *P < 0.05 (ANOVA and Tukey–Kramer) as compared with E/C junction. **P < 0.05 (ANOVA and Tukey–Kramer) as compared with submucosa.

upon activation, are important modulators of ECM degradation and apposition (15, 19, 33–36). For example, MC_T and MC_{TC}, contain tryptase, a potent pro-angiogenic enzyme, that has been recently recognized as a potent ECM-degrading gelatinase-type enzyme (35, 37). The MC_{TC} subset contains chymase, which participates in ECM remodeling by modulating collagenase activity and fibroblast function (38, 39). In addition, enzymes linked to elastosis formation, such as cathepsin G, carboxypeptidase, and gelatinases A and B are also found in MC_{TC} (15, 40). As both MC subsets, MC_T and MC_{TC}, were significantly increased in AC, and in close relation to elastotic tissue, it may be possible that MCs play an important role in mediating the connective tissue alterations found in this lesion.

Actinic cheilitis is a pre-malignant lesion that could transform into SCC of the lip (5, 41, 42). However the events leading to AC transformation remain to be

elucidated. Several studies have shown that MC subpopulations and their distribution could vary depending on the malignancy of the lesion (21–23). In SCC of the lip, Yang and colleagues (22) found that MC_{TC} predominated. The present study showed that MC_T was the predominant phenotype in AC, which was similar to normal lip. However, a different distribution pattern of MC_T was found in AC as compared with normal lip. While in normal lip MC_T predominated in the connective and submucosal areas, in AC MC_T were significantly increased in the area of epithelium and connective tissue junction as well as in the connective tissue zone. This may suggest that alterations in the epithelial cells as a result of actinic damage, may lead to increased numbers of MC_T in the adjacent area, or vice-versa. The mediators involved in the changes in MC subpopulation distribution and density in AC remain to be elucidated.

In several cancers, such as breast cancer, the MC_T phenotype has been associated with inflammation and angiogenesis, which in turn favor cancer malignization and spreading (21, 23, 43, 44). Therefore MCs, in addition to contribute to connective tissue degeneration, may also be stimulating angiogenesis and inflammation in AC, along with their well-known immunosuppressive role in UV-irradiated tissues (24, 26). These effects may alter the microenvironment around the UV-damaged epithelium, which may contribute to malignization and spreading of the AC lesion.

In summary, this study showed that MCs were significantly increased in AC, and that active tryptase- and chymase-positive MCs were found specially around areas of elastosis. In addition, MC_T was the predominant subpopulation in AC, which was similar to normal lip, but with a different distribution pattern. Future studies should further characterize the contribution of MCs to elastosis in AC, and their possible role in AC malignization.

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