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Immunohistochemical Characterization of the Human Sublingual Mucosa

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The sublingual locus has recently received great attention as a delivery site for various immunotherapies, including those that induce allergen-specific tolerance, and for vaccines that generate protective immunity. To further understand the immune functions of the human sublingual mucosa, we characterized the distribution of various immunocytes therein by immunohistochemistry. We identified professional antigen presenting cells (APCs), including Langerhans cells (LCs) and macrophages. CD1a$^+$ and langerin$^+$ LCs were further found to be distributed in the basal and supra-basal layers of the epithelium, and macrophages were identified in the lamina propria. HLA-DR$^+$ cells were observed in both the epithelium and the lamina propria, which mirrors the tissue distribution of LCs and macrophages within these tissues. CD3$^+$, CD4$^+$, and CD8$^+$ T cells were found to be distributed along the basal layer of the epithelium and also in the lamina propria. Although B cells, plasma cells, and Foxp3$^+$ regulatory T cells (Tregs) were only occasionally observed in the human sublingual mucosa in the absence of inflammation, they did show enrichment at inflammatory sites. Hence, we have further elucidated the immune cell component distribution in human sublingual mucosa.

Key words: sublingual mucosa, immunohistochemistry, dendritic cells, macrophages, T cells, B cells, Treg cells

Introduction

The sublingual mucosa has recently received great attention for its potential role as an attractive site for the delivery of drugs, immunotherapeutics, and vaccines (Holmgren et al., 2005). Due to its accessibility, high permeability, and high degree of vascularization, the sublingual mucosa presents a non-invasive alternative to subcutaneous antigen delivery (Moingeon et al., 2006). The oral mucosal immune system, like mucosal immunity at other body sites, must continuously maintain tolerance to innocuous antigens, such as food and commensal microbes, yet mount appropriate protective immune responses against pathogens. Many published studies support the idea that sublingual delivery of allergens is a suitable treatment for allergic rhinitis in adult individuals and is safer than subcutaneous desensitization (Holmgren et al., 2005; Moingeon et al., 2006; Novak et al., 2008). Furthermore, sublingual vaccination of mice with the influenza virus induced systemic cytotoxic T lymphocyte (CTL) responses and antibody production, both systemically and in mucosal tissue-specific sites, including the oro-gastrointestinal and respiratory tracts, thus conferring protection against infection (Song et al., 2008). However, the regulatory mechanisms that control sublingual mucosal immunity remain poorly understood. In this study, we use immunohistochemical techniques to identify the subpopulations of immunocytes in specimens from human sublingual mucosa.

Materials and methods

Specimen

The use of human materials was approved by Institutional Review Board at Seoul National University Dental Hospital. Eight sublingual mucosal tissues were obtained from patients

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with oral tumors during surgery under an informed consent. Small pieces (3 mm × 12 mm × 3 mm) of sublingual mucosa were excised avoiding tumor mass and fixed with formalin-free zinc fixative (BD Bioscience, San Diego, CA).

Antibodies

Primary antibodies and dilution used for immnohistochemistry are listed in table 1.

Immunohistochemistry

Serial paraffin-embedded sections (4 μm) were deparaffinized in xylene, hydrated, and then incubated in Tris/EDTA pH 9.0 buffer at 125°C for 3 min to expose antigens. After washing with running water, sections were immersed in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity, and then subjected to immunohistochemistry using a Autoimmunostainer (LAB VISION 2D). Briefly, the sections were incubated with primary antibodies at room temperature for 1 hour, washed with TBS (Tris-buffered saline and tween 20, pH 7.6) for five minutes three times, incubated with either anti-mouse EnVision+ system-HRP (DAKO, Denmark) or biotinylated anti-rat IgG (1:500, DAKO) followed by Streptavidin-HRP (DAKO) for 30 minutes, washed with TBS again, and then visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAKO). The sections were counterstained with Mayer's hematoxylin and mounted. Primary antibodies and dilution used for immunohistochemistry are listed in table 1. Immunolabeled cells were counted in three high power fields (× 400) per section that were chosen from the areas without inflammatory infiltrates, and the mean of three quantifications was obtained for each patient. The number of each immunolabeled cell subpopulation in table 2 was expressed as the mean and range of eight patients.

Results

Distribution of antigen-presenting cells (APCs)

Transverse sections of human sublingual mucosal tissue reveal a non-keratinized epithelium with occasional regions of para-keratinization (Fig. 1A and B). The distribution of APCs in the sublingual mucosa was evaluated by immunohistochemistry. $CD1a^+$ cells were distributed normally in the epithelium, but were absent from the lamina propria. Although most $CD1a^+$ cells were found in the basal and supra-basal layers of the epithelium, a few $CD1a^+$ cells were observed in more superficial epithelial layers (Fig. 1C). A very similar pattern of distribution was found for $langerin^+$ cells (Fig. 1E), which suggests that sublingual mucosa DCs are in fact Langerhans cells, an observation that has been reported elsewhere (Allam *et al*., 2008). Furthermore, the percentages of $CD1a^+$ and $langerin^+$ cells out of total keratinocytes were quite similar: 3.96% and 3.34%, respectively. Migrating $CD1a^+$ and $langerin^+$ cells were observed in both the lamina propria and the epithelium at sites of inflammation (Fig. 1D and F). Macrophages, which are restricted to the lamina propria in the absence of inflammation, were also found in inflammatory foci within the epithelium (Fig. 1G and H). $HLA\text{-}DR^+$ cells were observed in both the epithelium and in the lamina propria, a pattern that directly correlates with the distribution of LCs and macrophages in these two

Table 1. Primary antibodies used for immunohistochemistry

	Clone	Isotype	Dilution	Company
CD1a	O10	Mouse IgG1	1:25	Serotec
Langerin	306G9.01/HD24	Mouse IgG1	1:200	Dendritics
Macrophage/histiocyte	3A5	Mouse IgG2b	1:50	Serotec
HLA-DR	YE2/36-HLK	Rat IgG2a	1:100	Serotec
CD20	7D1	Mouse IgG1	1:50	Serotec
CD138	B-A38	Mouse IgG1	1:100	Serotec
CD3	CD3-12	Rat IgG1	1:100	Serotec
CD4	4B12	Mouse IgG1	1:50	Monosan
CD8	4B11	Mouse IgG2b	1:50	Serotec
FOXP3	221D/D3	Mouse IgG1	1:500	Serotec

Table 2. The number of immune cell subpopulation in human sublingual mucosa[a]

	DC		Macro-phage	HLA-DR	B cell		T cell			
	CD1a	Langerin			CD20	CD138	CD3	CD4	CD8	Treg
Ep	11.1 (4.7-20)	9.3 (5-16.3)		5.9 (2.7-11)			12.8 (5-20.3)	7 (1-17)	10.3 (3.3-16.7)	0.7 (0-2.7)
LP			13.7 (4-26)	7.5 (1.7-14.3)	0.7 (0-2.3)	3.6 (0-13.3)	20.6 (6.3-40.7)	12 (2.3-29.3)	12.3 (2-27.3)	0.2 (0-0.7)

[a]number per high power field (× 400) expressed as the mean and range of eight samples.

loci (Fig. 1I). Based on the total counts of langerin$^+$ cells, macrophages, and HLA-DR$^+$ cells, we calculated that approximately 52% of macrophages and 63% of LCs appear to express HLA-DR (Table 2). These results indicate that LCs and macrophages are the most abundant APC subsets in the human sublingual mucosa.

Distribution of lymphocyte subsets

We also examined the distribution of lymphocytes in the sublingual mucosa. In the absence of inflammation, CD3$^+$ T cells were the most abundant lymphocyte population present in the sublingual mucosa (Table 2), and were found to be distributed equally both along the basal layer of the

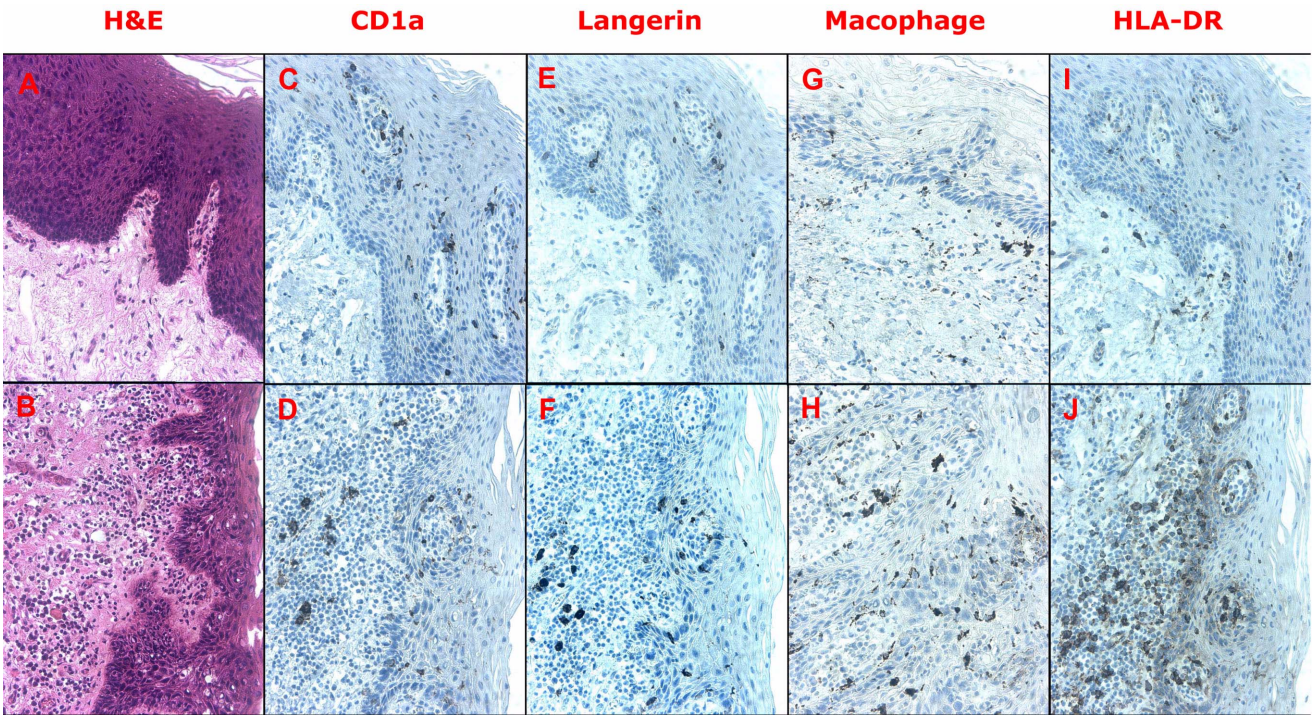

Fig. 1. Paraffin embedded sections of sublingual biopsies were subjected to H&E staining (A, B) and immunohistochemistry using antibodies for CD1a (C, D), langerin (E, F), macrophage marker (G, H), and HLA-DR (I, J). Areas without inflammation (A, C, E, G, I) and those with inflammatory infiltrates (B, D, F, H, J) were photographed.

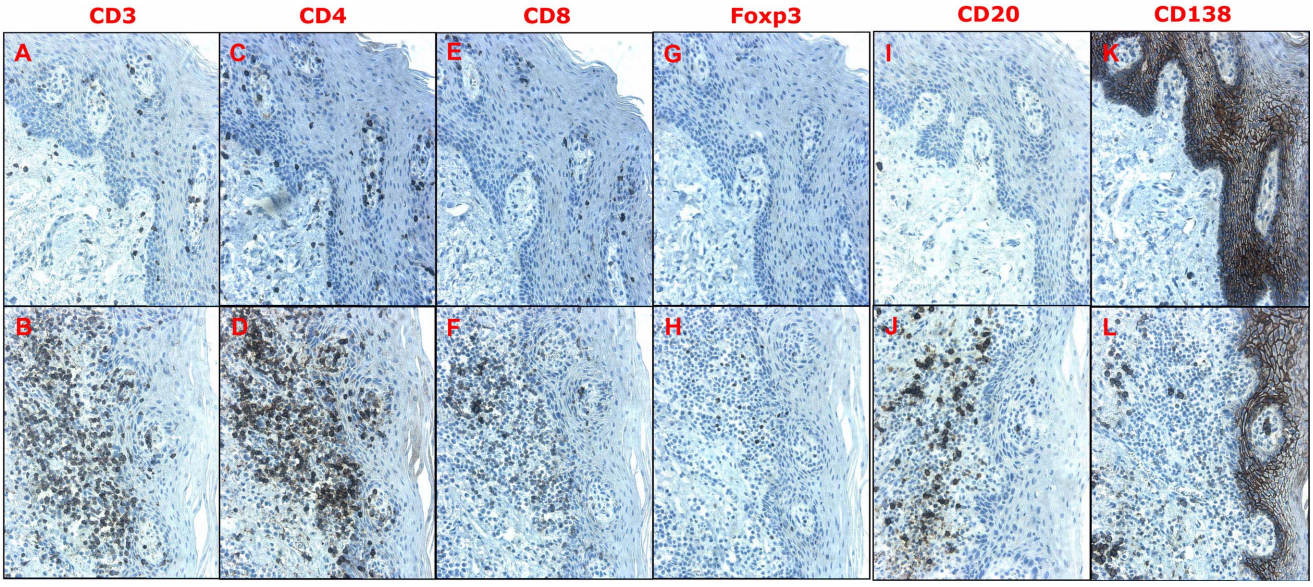

Fig. 2. Paraffin embedded sections of sublingual biopsies were subjected to immunohistochemistry using antibodies for CD3 (A, B), CD4 (C, D), CD8 (E, F), FOXP3 (G, H), CD20 (I, J), and CD138 (K, L). Areas without inflammation (A, C, E, G, I, K) and those with inflammatory infiltrates (B, D, F, H, J, L) were photographed.

epithelium and within the lamina propria (Fig. 2A). Furthermore, $CD3^+$ T cells comprised a large proportion of the infiltrating immunocytes at inflammatory foci (Fig. 2B). The pattern of distribution for $CD4^+$ and $CD8^+$ T cells was similar to that of $CD3^+$ cells (Fig. 2C-F), and the ratio of $CD4^+$ T cells to $CD8^+$ T cells was close to 1 (Table 2). Tregs were detected using an antibody against the transcription factor FOXP3 (Nik *et al*., 2008). Whereas antibodies targeted against CD markers stained the plasma membrane of cells, the anti-FOXP3 antibody stained their nuclei specifically (Fig. 2G and H). $FOXP3^+$ cells were occasionally observed in the epithelium or lamina propria of healthy sublingual mucosal tissue (Fig. 2G), but were more frequently found within the population of infiltrating inflammatory lymphocytes (Fig. 2H).

In contrast to the pattern of distribution of T cells, $CD20^+$ B cells were rarely observed in either the epithelium or the lamina propria of non-inflamed tissue. However, many B cells were found within the fraction of inflammatory infiltrates (Fig. 2I and J). The plasma cell marker CD138 (syndecan-1) was also strongly expressed on human oral epithelial cells, as reported by others (Sanderson *et al*., 1989; Soukka *et al*., 2000). Thus, this abundance of CD138 staining precluded our ability to determine an accurate assessment of the frequency of plasma cells in the epithelium. Like B cells, plasma cells were rarely observed in the sublingual mucosal lamina propria of non-inflamed tissue (Fig. 2K), but were readily detected around salivary glands (data not shown) and at inflammatory loci (Fig. 2L).

These results indicate that T cells are the predominant lymphocyte population in the immune compartment of non-inflamed sublingual mucosa. Regulatory T cells, B cells, and plasma cells, however, are detected in these tissues only in the context of inflammation.

Discussion

We evaluated the distribution of various immune cell subsets within the human sublingual mucosa and found that LCs, macrophages, and T cells, but not Tregs, B cells, or plasma cells, populate this tissue under normal conditions.

Our data demonstrate that LCs and macrophages are the principle APCs present in the human sublingual mucosa. However, it is possible that intraepithelial LCs, not lamina propria macrophages, are the cell type responsible for the uptake of antigens that are administered to the sublingual mucosa. Although most LCs are found in the basal and supra-basal layers of the epithelium, it has been reported that the dendrites of LCs residing in the gingival epithelium are able to extend to the tissue surface (Ito et al., 1998). In addition, a thin and permeable epithelium in the sublingual mucosa might enable LCs to sample antigens more efficiently. The distribution of $HLA\text{-}DR^+$ cells indicates that professional APCs with antigen presenting ability reside in the human sublingual mucosa. Allam et al. reported that LCs isolated from the sublingual oral mucosa express high levels of both MHC class II and the DC maturation marker CD83, and are able to stimulate allogeneic T cells to proliferate (2008). The presence of both LCs and T cells along the basal layer of the sublingual mucosal epithelium opens the possibility that these cells are in direct contact with each other for antigen presentation within this tissue *in vivo.* However, the lack of B cells within the sublingual mucosa suggests that the antigens are more likely to be delivered to the draining lymph node for presentation to both B and T cells. Whether antigen presentation occurs within the sublingual mucosa or in draining lymph nodes is an interesting question that still needs to be answered.

Regulatory T cells have been implicated in pro-tolerogenic mechanisms of the oral mucosa (Moingeon et al., 2006; Novak et al., 2008). Since Tregs are rarely observed in the sublingual mucosa in the absence of inflammation, they likely migrate into this tissue in response to inflammatory cytokines and chemokines. Tregs might be recruited to sites of inflammation to prevent excessive tissue destruction during an inflammatory response. However, Tregs do not appear to be involved in the maintenance of tolerance to food antigens and commensal microbes within the sublingual mucosa, at least at the peripheral site.

Conflict of Interest

Authors declare there are no conflicts of interest.

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