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Expression profile of chemokines and chemokine receptors in epithelial cell layers of oral lichen planus

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BACKGROUND: To understand the immunopathological features of oral lichen planus (OLP), we analyzed the expression of chemokines in the epithelial cell layers.
METHODS: Epithelia from OLP or healthy gingiva were collected by laser microdissection. The chemokine and chemokine receptor expressions in the epithelia were analyzed by DNA microarray.
RESULTS: High levels of MIP-3α/LARC/CCL20 and its receptor CCR6 were expressed in the lesional epithelia. Furthermore, DC-CK1/CCL18, ELC/CCL19, SDF-1/CXCL12 and CXCR4 expressions were also increased. Immunohistologial analysis showed that high numbers of Langerhans cells (LCs) were present in the epithelia of OLP. Lesional epithelia also expressed high levels of the ligands specific for CXCR3 (e.g. MIG/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11) and CCR5 (e.g. RANTES/CCL5).
CONCLUSIONS: Infiltration of LCs is orchestrated by CCR6. Further, LCs residing in the lesional epithelia may be a mature phenotype. Moreover, infiltration of T cells in OLP could be mediated by signaling pathways through CXCR3 and CCR5.
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Keywords: chemokine; chemokine receptor; DNA microarray; epithelial cell layer; oral lichen planus

Introduction

Oral lichen planus (OLP) is a common chronic inflammatory disease of the oral mucosa characterized by a reticular white lesion with mucosal atrophy and erosions usually distributed bilaterally on the buccal mucosa, tongue and gingiva. Histopathological features include subepithelial band-like accumulation of mononuclear cells focused to the basal keratinocytes (1, 2). There is a consensus that chronic, cell-mediated, immune damage to basal keratinocytes occurs in OLP (1–6). Analysis of the lesional cell populations has revealed that T cells are the main component of the inflammatory infiltrate, with significant numbers of cytotoxic T cells in close proximity to the epithelial basement membrane (3, 4, 7).

It is now well established that the differential expression of chemokines, chemokine receptors, and adhesion molecules plays an important role in determining tissue-specific trafficking and the positioning of leukocyte subsets within both normal and inflamed tissues (8, 9). The human chemokine system currently includes more than 50 chemokines and 18 chemokine receptors. According to NH_2-terminal cysteine-motifs, the chemokines are divided into C, CC, CXC and CX3C subfamilies (9–11). Chemokines released by affected keratinocytes, and the associated inflammatory infiltrate, may play a crucial role in the selective recruitment of the T cell-dominated infiltrate through their chemokine receptors (12). T cell chemokine receptors are differentially expressed by type-1 (Th1, Tc1) and type-2 (Th2, Tc2) T cells. Type-1 and type-2 cells are distinguished according to the cytokines they secrete. Type-1 T cells are associated with CXCR3 and CCR5 expression, whereas type-2 T cells express CCR3, CCR4, and CCR8 (12–18). In this regard, previous studies have shown that lesional keratinocytes of OLP and other inflammatory dermatoses express MIG/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11, which are the ligands of CXCR3 (19, 20). In support of this, majority of T cells infiltrating in skin lichen planus express CXCR3 (19). Other studies have shown that lesional keratinocytes in OLP express RANTES/CCL5 and CCR1 (21, 22).

Langerhans cells (LCs) are dendritic cells residing in mucosal and skin epithelium. LCs can leave skin/oral mucosa and migrate to regional lymph nodes, where they initiate T cell responses (23). Previous studies have shown that increased numbers of LCs, which possess a mature phenotype and express CCR7 are found in the epithelium and stroma of OLP (24), suggesting that they

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may play a crucial role in the pathogenesis of OLP. These studies suggest that better understanding of the chemokine network in OLP may shed light on the pathogenesis of the disease. In the present study, we examined the expression profile of chemokines and chemokine receptors in the epithelium by comparing OLP with healthy tissue.

Materials and methods

Specimens

The patients without systemic diseases as well as periodontal diseases were selected. Biopsy specimens were obtained from lesional lingual gingiva of patients with OLP ($n = 3$). Diagnosis was made by clinical features and histopathological findings. In all cases, the lesions showed the characteristic clinical features of the erosive form of OLP. Biopsies from normal gingival tissue were obtained when the third molar was extracted by orthodontic treatment plan, and was used as control tissue ($n = 3$). This study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan. Under RNase-free conditions, specimens were embedded in OCT compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan) and frozen in isopentane cooled in liquid nitrogen and stored at −80°C until processing.

Immunohistochemistry

Cryostat sections (6 μm) were incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Sections were then incubated with anti-Langerin antibody (1 : 25, YLEM, San Francisco, CA, USA) for 1 h at room temperature in a humid chamber and with Simple Stain MAX-PO (Nichirei Corporation, Tokyo, Japan) for 30 min according to manufacturer's instructions. The sections were then incubated with anti-CD1a antibody (1 : 20, DAKO Cytomation Ltd., Copenhangen, Denmark) for 1 h at room temparature and with Simple Stain AP (Nichirei) for 30 min according to manufacturer's instructions. The diaminobenzidine (DAB) system (DAB Buffer tablet, MERCK, Dermstadt, Germany) was used to detect Langerin and a new fuchsine system (Histofine, Nichirei) was used to detect CD1a. The section was counterstained with hematoxylin before mounting.

Laser microdissection

Cryostat sections (8 μm) were quickly fixed in 100% methanol for 3 min and then stained with 1% toluidine blue (Fig. 1a). Laser microdissection system-PRO 300 (Cell Robotics, Inc., Albuquerque, NM, USA) was used for procurement of target epithelial cell layers. The epithelial cell layer bound on an ultrathin transparent supporter membrane was dissected with an ultraviolet laser (Fig. 1b). The target cell population was then collected by laser pressure (Fig. 1c,d).

Total RNA extraction

Total RNA was extracted using a modified acid-guanidinium thiocyanate-phenol-chloroform method (25). Isolated epithelial cell layers were homogenized by vortexing for 10 min in a denaturation guanidinium isothiocyanate-based buffer containing 25 mM sodium citrate, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol. RNA was isolated by sequential addition of 2 M sodium acetate, pH 4.0, chloroform-isoamyl alcohol mixture, and water-saturated phenol. Samples were vortexed and placed on ice for 15 min before centrifugation. The aqueous phase was transferred to a new microtube and an equal volume of isopropanol and 1 mg/ml glycogen added. The precipitated RNA was obtained by incubation at −80°C for 30 min followed by centrifugation. After precipitation, the RNA pellet was dissolved in 10 μl of DNase, RNase-free water (Invitrogen Life Technologies, Carisbad, CA, USA) and kept at −80°C until processing.

DNA microarray analysis

Total RNA was mixed with T7-oligo (dT) promoter primer, reverse-transribed to cDNA, and synthesized to the double-stranded cDNA with GeneChip® Expression 3′-Amplification Reagents Two-cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA, USA). The double-stranded cDNA was purified and served as a template in the subsequent *in vitro* transcription (IVT) using GeneChip® IVT Labeling kit (Affymetrix). The biotinylated cRNA target was then fragmented, and hybridized to the array (GeneChip® Human Focus Array; Affymetrix). Immediately following hybridization, the array was washed and stained by streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA). Each probe array was scanned and an average of the two images calculated, and the probe cells defined and computed intensity for each cell. Finally, the results were analyzed using the Microarray Suit Expression Analysis and Genespring software (Silicon Genetics, Redwood City, CA, USA).

Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described previously (26) with minor modifications. Briefly, total RNA was reverse transcribed with oligo $(dT)_{20}$ primer, dNTPs, and Thermoscript reverse transcriptase (Invitrogen Life Technologies). Two microliter of the reaction mixture was then mixed with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) for RT-PCR analysis by use of the gene-specific primers. The following primer sequences were used for amplification: +5-CCCCGTGCCCACATCAAGGAGTATTT-3 and −5-CGTCCAGCCTGG-GGAAGGTTTTTGTA-3 for CCL5; +5-AGTTTCCAAGCCCCAGCTCA-3 and −5-TGGGGGCTGGTTTCAGAATAGTCA-3 for CCL18; +5-AGACTGCTG- CCTGTCTGTGA-3 and −5-GCT TCATCTTGGCTGAGGTC-3 for CCL19; +5-GAAG GCTGTGACATCAATGCT-3 and −5-CAAGTCCAG TGAGGC- ACAAA-3 for CCL20; +5-CATGCTGGT GAGCCAAGCAGTTTGAA-3 and −5-CACTTCTGT GGGGTGTTGGGGACAAG-3 for CXCL9; +5-TGC AAG- CCAATTTTGTCCACGTGTTG-3 and −5-GCA GCTGATTTGGTGACC- ATCATTGG-3 for CXCL10;

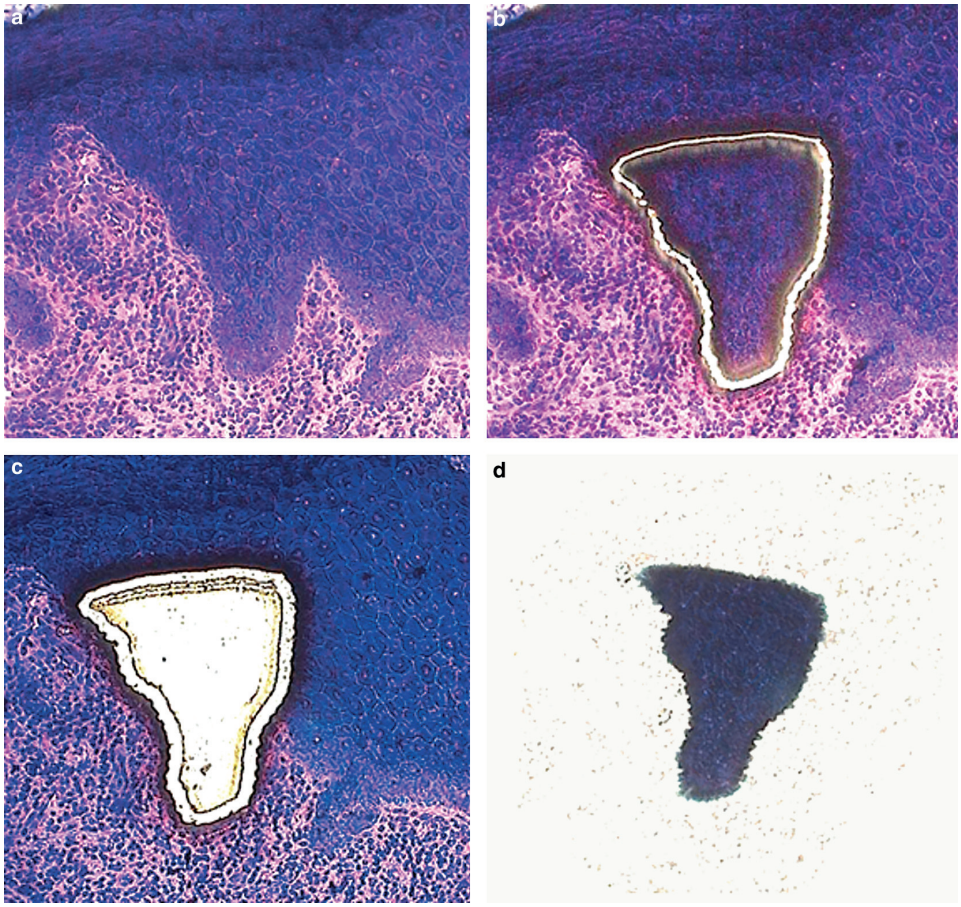

Figure 1 Use of laser capture microdissection to selectively harvest an epithelial cell layer of OLP. Eight micrometer sections from snap-frozen specimen are stained by toluidine blue to illustrate detailed histomorphology of OLP (a). Laser capture microdissection was performed on the epithelial cell layer (b). The tissue void created by laser capture microdissection-dissected epithelial cell layer (c). The laser capture microdissection-harvested epithelial cell layer (d). Original magnification was ×100.

+5-CGATGCCTAAATCCCAAATCGAAGCA-3 and −5-AATTGCTGGACTCCTTTGGGCAGTGG-3 for CXCL11; +5-TTCCAT- GGTGTGATCGTCTG-3 and −5-ACTGAGAGTCCAGCGAGGTT-3 for CXCL12; +5-CGACGTGAAGAAGCTGGAA-3 and −5-GGCG TTGTA- CCACTTGATGA-3 for CXCL14; +5-GAG CAATGTGTGGGCTGAAGA-3 and −5-AGCCCATG ACAGTACCTTCC-3 for CCR6; +5-CCCACAGACT CAAAT- GCTCA-3 and −5-CCAAGAGCTGAGTGC- ATGTC-3 for CCR7; +5-CCGCTTATTCCTTGGTAT GG-3 and −5-GGAAGTAAATAGCCTTCC- AGCA-3 for CCR9; +5-TGTGGCCAAGTTCTTAGTTGC-3 and −5-GGTGCTGAAATCAACCCACT-3 for CXCR4; +5-GACACCCCCAGC- TCATCTTA-3 and −5-GGG AATAGTCTTTGCCTTGC-3 for CCR4; +5-CTGGC CATCTCTGACCTGTTTTTC-3 and −5-CAGCCCTG TGCC- TCTTCTTCTCAT-3 for CCR5; +5-CAACG CCACCCACTGCCAATACAA-3 and −5-CAGGCGC AAGAGCAGCATCCACAT-3 for CXCR3; +5-ATG GAC- TGTGGTCATGAG-3 and −5-ATCACCATCT TCCAGGAG-3 for GAPDH. Amplification was carried out under following conditions: 5 min at 95°C; 40 cycles of 1 min at 95°C, 1 min at 60°C, 2 min at 72°C; followed by 15 min at 72°C as the final extension.

Results

Expression profile of chemokines and chemokine receptors in the epithelial cell layers of OLP

To identify the expression differences in chemokines and corresponding receptors between the lesional epithelial cell layers of OLP and those of normal gingival tissue, laser microdissection and high-density oligonucleotide array analysis were performed. Thus, target epithelial layers in OLP or healthy tissue from snap frozen biopsies were harvested by laser microdissection (Fig. 1a–d). Differential gene expression using GeneChip® analysis revealed that among the chemokines and chemokine receptors, the 25 probe set changed in the majority of cases (two-third or greater). Among the 25, 20 increased and five decreased in OLP, compared with normal samples. There were 12 probe sets that changed in all three cases. Interestingly, all 12 were found to be increased in OLP, compared with normal tissue (Table 1).

Analysis of chemokines and chemokine receptors relating to LC infiltration

As it has been suggested that LCs may play a crucial role in the pathogenesis of OLP, we have focused on the

Table 1 Chemokines and chemokine receptors whose expression changed in three of three cases*

	Name	*Average % fold changes*
Increase in OLP	RANTES/CCL5	294
	DC-CK1/CCL18	742
	ELC/CCL19	329
	MIP-3α/LARC/CCL20	427
	MIG/CXCL9	5848
	IP-10/CXCL10	5186
	I-TAC/CXCL11	1681
	SDF-1/CXCL12	258
	BRAK/CXCL14	177
	CCR6	213
	CCR9	199
	CXCR4	629

*No probe set was found to be decreased in the epithelial cell layers of OLP when compared with those of normal tissues.

expression profile of chemokines and those receptors which relate to the LC infiltration of OLP. The transcript of MIP-3α/LARC/CCL20 was significantly increased in the lesional epithelia of OLP when compared with normal tissue. Furthermore, the receptor of CCL20, CCR6 was also increased. In the case of the ligands of CCR7, ELC/CCL19, but not SLC/CCL21, was found to be increased in the epithelial layers of OLP (Table 1). However, alteration in CCR7 expression was not detected (data not shown). RT-PCR analysis confirmed the oligonucleotide array results and showed increases in the transcript of CCL18, CCL19, CCL20, CXCL12 and CCR6. In one patient, low signals were also noted in the transcripts of CCR7 (Fig. 2). In support of the results of the chemokine and chemokine receptor expression profiles, $CD1a^+$ $Langerin^+$ LCs were scattered throughout the epithelial cell layer of OLP (Fig. 3a). Furthermore, significantly higher numbers of LCs were found in the epithelia of OLP compared with the normal tissue (Fig. 3a,b). These results indicate that infiltration of LCs is mediated by signaling via CCR6, but not CCR7.

Interestingly, the expression of CXCR4 and its ligand SDF-1/CXCL12 was found to be increased in the epithelial cell layers of OLP compared with healthy tissue. Furthermore, expression of DC-CK1/CCL18 was also increased. Moreover, a slight, but statistically significant, increase was seen in the transcript of BRAK/CXCL14 in the epithelia of OLP compared with normal tissue (Table 1). RT-PCR analysis also showed that mRNA specific for CXCL12 and CCL18 was significantly higher than normal tissue (Fig. 2). On the contrary, the transcripts of CXCR4 and CXCL14 were detected in both normal and OLP epithelia, although the signals in OLP were stronger than those is normal samples (Fig. 2).

Chemokine expression relating to T cell infiltration

In the next series of studies, we analyzed the expression profiles of the chemokines and chemokine receptors that relate to T cell infiltration. The profiles of type-1 and type-2 T cell related chemokines showed that the

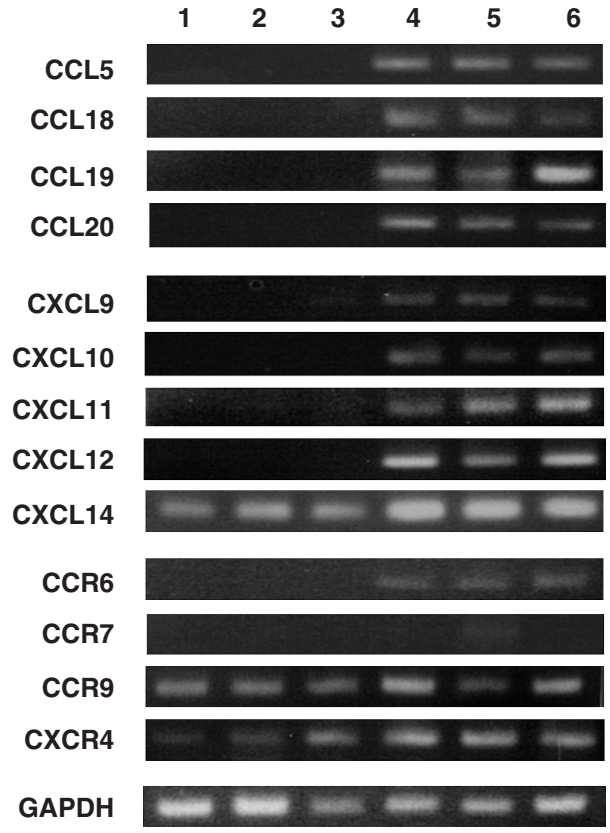

Figure 2 RT-PCR analysis for expression of chemokines and chemokine receptors in the epithelial cell layers of OLP. RT-PCR was performed with total RNA samples prepared from the epithelial cell layers of three normal gingival tissues (lane 1–3) and OLP (lane 4–6). The results are representative from two separate experiments.

significantly higher expression levels of MIG/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11, which are the ligands of CXCR3 were detected in the epithelial cell layers of OLP compared with normal tissue. The ligand of CCR5, RANTES/CCL5 was also found to be increased (Table 1). In contrast, the gene expression of the ligands of CCR3 (MCP-3/CCL7, MCP-4/CCL13, Eotaxin/CCL11) and CCR4 (TARC/CCL17 and MDC/CCL22) was not changed between OLP and healthy samples (data not shown). The results of the oligonucleotide array were confirmed by RT-PCR analysis demonstrating that CXCL9-, CXCL10-, CXCL11- and CCL5-specific messages were noted in the epithelia of OLP, but not in healthy, samples (Fig. 2). Further, mRNA specific for CXCR3 and CCR5, but not CCR4, was detected in the lamina propria of OLP (Fig. 4). These results suggest that CXCL9, CXCL10, CXCL11 and CCL5 induce the infiltration of T cells that express CXCR3 and CCR5.

Discussion

In the present study, to elucidate the mechanisms of the infiltration of mononuclear cells into the lesional epithelium and underling lamina propria of OLP, we

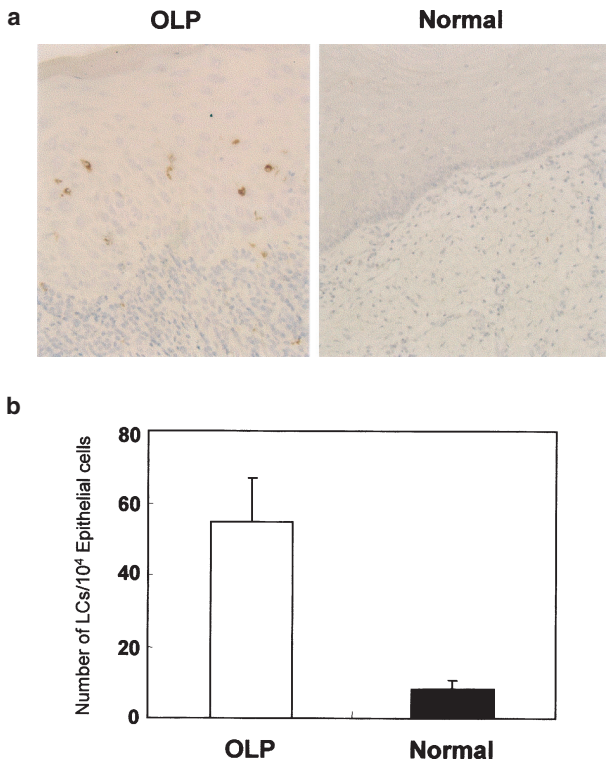

Figure 3 Immunohistological analysis of Langerhans cells in the epithelial cell layers of OLP. Langerhans cells were stained by anti-CD1a and anti-Langerin monoclonal antibodies (a). The number of $CD1a^+Langerin^+$ cells is expressed as mean ± SE from five tissue samples (b).

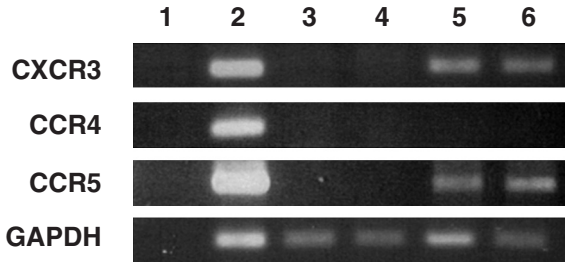

Figure 4 RT-PCR analysis for expression of chemokines and chemokine receptors in lamina propria of OLP. RT-PCR was performed with the reaction mixture without sample RNA (lane 1) as well as total RNA samples prepared from PHA-treated peripheral blood mononuclear cells as a positive control (lane 2), from lamina propria of two normal gingival tissue samples (lanes 3, 4), and lamina propria of OLP (lanes 5, 6). The results are representative from two separate experiments.

analyzed the expression profiles of chemokines and chemokine receptors in the epithelial cell layers of OLP using DNA microarray analysis. Oral mucosal tissues are complex heterogeneous structures, composed of numerous interacting cell populations. Further, the epithelium and underlying cells in the lamina propria interact with each other in a dynamic way, and each requires the other for development of OLP. For these reasons, approaches that can measure responses in specific cell populations without disrupting their network of interactions with other cells are required for elucidation of molecular events in the epithelial cell layers of OLP. In this regard, laser microdissection has been shown to isolate selected regions or cell populations from complex tissues such as the oral mucosal tissues with their native tissue context (27). Laser microdissection methods have become widely available over the past few years, and now offer the ease, precision and efficiency that is required for routine research (28). Thus, in the present study, we demonstrated the application of laser microdissection together with DNA microarray to harvest target regions from their native tissue environment, to provide a powerful means with which to decode the pathological events in the genesis and progression of OLP at the molecular level.

Our results showed that the expression levels of CCL20 and its receptor CCR6 were increased in the lesional epithelial cell layers of OLP when compared with normal tissue. It has been shown that the regulation of LC recruitment by mucosal epithelial cells is controlled by CCL20 (29, 30). This chemokine is the most potent chemoattractive molecule for immature LCs and acts via its cognate CCR6 (31). Indeed, significantly higher numbers of $CD1a^+$ $Langerin^+$ LCs were found in the epithelia of OLP when compared with that of normal tissue. These results suggest that the infiltration of LCs into the epithelial layers of OLP is mediated by the signaling pathways through CCR6.

Other studies have reached somewhat conflicting conclusions as to the infiltration of LCs into the epithelial cell layers of OLP. It has been shown that numbers of $CD1a\ (T6)^+$ LCs in the epithelia of OLP were identical to those of healthy samples (32, 33). The basis for this discrepancy is not known. In this regard, non-erosive legions were taken for the analysis in those studies, whereas this study used erosive form of OLP. Thus, type and stage of OLP may influence the infiltration of LCs into the epithelial cell layers.

DNA microarray analysis indicated that the expression levels of CXCL14 were slightly increased, but statistically significant, in the epithelia of OLP. RT-PCR analysis showed that epithelia from normal tissue also expressed this chemokine. CXCL14 is a new CXC chemokine with unknown function and receptor selectivity. In this regard, it has been shown that CXCL14 is expressed constitutively by a variety of epithelia including the basal keratinocytes of skin (34). Further, recent studies have demonstrated that the loss of CXCL14 in tumor cells and at tumor tissue is correlated with low or no attraction of dendritic cells (DCs) (35). Moreover, restoration of CXCL14 expression in tumor cells results in increased tumor infiltration by DCs, suggesting that CXCL14 is a potent chemoattractant of DCs (35). Thus, CXCL14 may be involved in the LC infiltration into the lesional epithelium of OLP in addition to the interactions between CCL20 and CCR6.

Interestingly, lesional epithelia of OLP showed higher expression of CXCL12 and its receptor, CXCR4 than normal tissue. Both molecules are known to be expressed on DCs (36–39). In this regard, previous studies have reported that expression levels of CXCR4

are increased in mature DCs when compared with those of immature phenotype (38), implying that LCs residing in the epithelia of OLP show a mature phenotype. In support of this, the expression of CCL18 and CCL19 was also found to be increased. CCL18 was shown to be expressed in DCs in germinal centers and to attract naïve T cells, $CD38^-$ mantle zone B cells (40, 41). Furthermore, a recent study has reported that CCL18 is expressed by antigen-presenting cells in the dermis and by LCs in the epidermis of atopic dermatitis patients, but not normal skin, and induces migration of atopic dermatitis-derived T cells (42). Moreover, CCL19 is preferentially expressed in DCs within the T cell zone of secondary lymphoid tissues, which are considered to be interdigitating DCs (37, 39). These studies together with our findings suggest that LCs residing in the epithelial cell layers of OLP may be of mature phenotype.

There is consensus that, in OLP, keratinocytes become the target of damage through foreign or altered self-antigen on their surface. Although the source of antigen has not been determined yet, it is likely that it will first be taken up by LCs in the epithelium. In this regard, it is well known that DCs migrate at the precursor stage into the site of inflammation following pathogen invasion. These immature DCs preferentially express CCR6. After antigen capture, DCs undergo a maturation process decreasing CCR6 and increasing CCR7 expression (37). The CCR7 and its cognate ligands are considered to mediate arrest and facilitate interaction between mature DCs in the secondary lymphoid tissues (43–47). In this regard, LCs present in the epithlia of OLP have been shown to be in a higher state of activation and the rate of trafficking between oral mucosal and lymphoid tissue appears to be increased (2, 48–50). However, our results indicated that although LCs residing in the epithelial cell layers of OLP possessed mature phenotype, the cells expressed CCR6, but not CCR7. These findings suggest that traffic of LCs from the lesional epithelia of OLP to the draining lymphoid organs may be controlled by other chemokine and chemokine receptor interactions but not CCR7. This interesting possibility is currently under investigation in our laboratory.

Our results showed that the epithelial cell layers of OLP exhibited significantly higher expression of CXCL9, CXCL10, CXCL11 and CCL5 compared with normal tissue. These chemokines especially attract activated T cells and CXCL9, CXCL10 and CXCL11 are known to be the ligands of CXCR3, whereas CCL5 is the ligand of CCR5. Both CXCR3 and CCR5 are expressed by type-1 T cells, e.g. Th1 and Tc1 (37). Indeed, RT-PCR analysis of the lamina propria of OLP demonstrated that strong expression of CXCR3 and CCR5, but not CCR4, was observed in OLP. In this regard, previous studies have demonstrated that CXCL10 and CXCL11 are mainly expressed by basal keratinocytes whereas CXCL9 is located predominantly dermal infiltrates in different types of inflammatory skin diseases including lichen planus (19). Furthermore, lesional keratinocytes of OLP express CXCL9, CXCL10, CXCL11 and CCL5 (20, 21). Our results further confirm previous studies and suggest that infiltration of T cells in OLP is orchestrated by signaling via CXCR3 and CCR5.

In summary, our results showed that the expression levels of CCL20 and its receptor CCR6 increased in the lesional epithelial cell layers of OLP. Immunohistological analysis showing high numbers of $CD1a^+$ $Langerin^+$ LCs in the epithelium supported the role of CCL20 and CCR6 interactions in the recruitment of LCs in the epithelial cell layers of OLP. Furthermore, LCs residing in the epithelia of OLP may be of mature phenotype, expressing CCL18, CCL19, CXCL12 and CXCR4. Epithelium also expresses high levels of the ligands specific for CXCR3 (CXCL9, CXCL10 and CXCL11) and CCR5 (RANTES/CCL5), which are known to be selectively expressed on type-1 T cells. The lamina propria of OLP expressed the mRNA specific for CXCR3 and CCR5, and thus infiltration of T cells in OLP appears mediated by signaling through CXCR3 and CCR5.

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