Abstract: Lichen planus (LP) is a chronic T-cell-mediated mucocutaneous inflammatory disease that targets stratified epithelia, including those lining the oral cavity. The intraoral variant of LP (OLP) is associated with interferon (IFN)-γ production by infiltrating T lymphocytes; however, the role of epithelial cells in the etiopathogenesis OLP is not completely understood. There is however a growing body of evidence regarding the involvement of epithelial-derived cytokines, immune receptors, and costimulatory molecules in the pathobiological processes that promote and sustain OLP. In the present study, we used a reverse transcriptase-polymerase chain reaction assay to assess whether CD40—a receptor found mainly on antigen presenting cells—and the costimulatory molecule CD86 were expressed in oral keratinocytes (three strains of primary normal oral keratinocytes and the H357 cell line) in the presence or absence of IFN-γ. To further characterize the involvement of CD40 in OLP, expression and distribution of receptor and ligand (CD40/CD154) in tissues from OLP were evaluated by immunohistochemistry. The present results are the first to show that both CD40 and CD86 are constitutively expressed at low levels in oral keratinocytes and that their expression was enhanced by IFN-γ stimulation. The intensity of CD40 staining in OLP tissues was strong. Taken together, the results strongly suggest that CD40 and CD86 play a role in the pathophysiology of oral inflammatory diseases such as OLP.

Keywords: CD40; CD86; oral lichen planus; oral inflammation; regulatory T-cells; mucosa.

Introduction

Lichen planus (LP) is a chronic T-cell-mediated mucocutaneous inflammatory disease whose etiology and pathogenesis are not completely understood. To clarify the pathological regulatory mechanisms associated with LP, we investigated expressions of CD40 and CD86 in oral keratinocytes and their role in inflammatory disease of the oral cavity.

CD40 is a 45-50 kDa phosphorylated type I integral membrane glycoprotein that belongs to the tumor necrosis factor receptor superfamily, which is expressed on various hematopoietic and non-hematopoietic cells (1-11). CD40 is involved in several biological functions, such as cell-mediated immunity and cell growth regulation. The ligand for CD40 is CD154, also known as CD40 ligand (CD40L), a cell surface molecule mainly expressed by activated T-cells. Through interaction with its receptor, CD154 is pivotal in T-cell-dependent humoral response and cell-mediated immunity and
inflammation. Thus, it is a key element in the pathogenic process of chronic inflammatory diseases and autoimmune disorders and has additional implications for the many pathogenic steps of tumorigenesis (10,12,13).

Interferon (IFN)-γ increases CD40 expression in a number of epithelial cell lines, including buccal oral epithelium (1,7). CD40 expression increases after proinflammatory cytokine treatment; thus, it is not surprising that CD40 epithelium expression is enhanced in some inflammatory conditions (4).

In oral epithelium, CD40 is involved in many aspects of cell homeostasis, as it is constitutively expressed by oral keratinocytes and can stimulate T lymphocytes by reacting further with several costimulatory molecules, including B7.2 (or CD86), one of the most important B7 molecule family members (14). CD86 can bind to the ligand CD28 or cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4), located on resting and activated T-cells, thus determining respective upregulation of T-cell stimulation and clonal expansion via multiple cytokines stimulation, or downregulation of this process (15,16).

Increasing evidence indicates that CD40 expression on epithelium plays a role in immune response by stimulating production of cytokines and chemokines. Previous studies reported that these chemokines are upregulated in oral lichen planus (OLP) and that OLP is characterized by large numbers of activated T-cells localized near the epithelium or infiltrating basal epithelium (7,9).

This study investigated the effect of IFN-γ treatment on CD40 mRNA expression in oral epithelial cells. Antigen presentation by keratinocytes is believed to have a role in OLP pathogenesis, and data on CD86 expression are inconsistent (1,17-24). Thus, we investigated CD86 mRNA expression in oral epithelial cells because of its crucial role in this process. In addition, to characterize CD40/CD154 cell expression and distribution, CD40/CD154 were additionally evaluated by immunohistochemistry in OLP tissue. The results are the first to show that CD40 and the costimulatory molecule CD86 are constitutively expressed at low levels in oral keratinocytes and that their expression is enhanced by IFN-γ stimulation.

Materials and Methods

Patients
OLP tissue (n = 12) was collected from patients attending the Oral Medicine Clinic of Eastman Dental Institute or the Royal Dental Hospital of Melbourne. In all cases, the diagnosis of OLP was confirmed by a pathologist; patients with other systemic diseases were excluded. Normal oral mucosa was obtained from patients attending the Oral Surgery Clinic of Eastman Dental Institute for routine extraction of third molars. This study was approved by the internal Ethical Committee of the UCL Eastman Dental Institute and the University of Melbourne Human Research Ethics Committee in 2008 (0827052. 1).

Cell culture techniques

Culture of normal human oral keratinocytes

Normal oral mucosal tissue was obtained from healthy patients, and three normal human oral keratinocyte (NHOK) strains (NHOK1-3) were isolated by separating connective tissue from excised normal tissue. The samples were cut into approximately 1-mm² pieces and cultured at 37°C/5% CO₂ in keratinocyte basal medium-2 containing the recommended growth supplements (Biowhittaker, Wokingham, UK). The epithelial cells were then detached using 0.25% trypsin-1 mM EDTA. The viability of the keratinocytes was confirmed by trypan blue exclusion.

H357 cell culture

The oral squamous cell carcinoma cell line H357 was established by Prime et al. (25) from a primary explant of a tongue squamous cell carcinoma. This cell line was grown in the same medium used for NHOK.

CD40/CD86 reverse transcriptase-polymerase chain reaction

mRNA derived from H357 cells and primary oral epithelial cells treated with 1,000 U/mL IFN-γ for 48 h was investigated. Primers specific for human CD40 mRNA and CD86 mRNA were generated for this reaction, namely, CD40 forward 5’-CTGGGCTAGCGATACAGGAG-3’ and reverse 5’-GGAAATTTCTGTTGGCCAAATCCA-3’ and CD86 forward 5’-AGACGCGGCTTTTATCTTCA-3’ and reverse 5’-AACTCCAGCTCTGCTCCGTA-3’ (Genosys-Sigma, Poole, UK), and reverse transcriptase-polymerase chain reaction was performed. Magnesium concentration was optimized for each primer as follows: 1 μL of cDNA was added to 4 μL dNTP (2.5 mM), 5 μL 10× buffer, 0.225 μL AmpliTaq (5.0 U/μL) (Perkin Elmer, Wokingham, UK), 4 μL of each specific primer (5 μM), 1.5, 3.0, or 4.5 mM MgCl₂ in each reaction and dH₂O, to yield a final volume of 50 μL. The thermocycler (Techne Genius; Cambridge, UK) parameters varied in relation to the primers used, and the annealing temperature of the reaction depended on the guanine-cytosine content of the primers. The general parameters used were 94°C for 45 s, annealing temperature (57 to 60°C) for 45 s, and 72°C for 45 s, repeated for 35 cycles.

The products were separated on a 2% agarose
(GibcoBRL Life Technologies, Paisley, UK) gel and visualized by ethidium bromide (Sigma, Poole, UK) staining. Specific bands were visualized by ultraviolet transillumination in a Multimage Light Cabinet (Alpha Innotech Corp., Cannock, UK), and digital images were acquired and stored using Alphalmager Software (Alpha Innotech Corp.). The primers used for the study of housekeeping expression encoded a region of 18S ribosomal RNA (5'-TTTCGGAACTGAGGCCATGA-3', 5'-GCATGCCAGAGTCTCGTTCG-3').

IFN-γ cell treatment assay

In a modification of the method described by Altenburg et al. (26), the primary oral epithelial cells and H357 cell line (at the second or third passage) were seeded at 8 × 10^4 cells/well in a Falcon 6-well plate (Becton Dickinson, Oxford, UK) with 3 mL of KBM-2 medium containing no hydrocortisone. The cells were incubated for at least 3-5 days until the cell culture was 60 to 80% confluent. We used dose-response curves from preliminary experiments to determine the optimal experimental conditions. Medium containing 1,000 U/mL IFN-γ was added to three wells, and control cell culture medium only was added to the remaining three wells. The cells were incubated for 48 h or for 3, 6, 9, 24, 48, or 72 h for the H357 time course. The supernatant was extracted, centrifuged, and stored at −70°C. The adherent cells were washed with phosphate-buffered saline (Gibco Life Technologies, Paisley, UK) before the addition of 0.5 mL of TriReagent (Sigma). The suspension was then removed and stored at −70°C.

Immunohistochemical methods

Paraffin section preparation

Archival paraffin-embedded formalin-fixed (PEFF) OLP specimens were used. Normal non-diseased skin (leg) sections and oral mucosa (tonsil) sections were used as positive and negative control samples. A microtome was used to cut the paraffin blocks to a thickness of 4 μm, and the specimens were mounted on Superfrost Plus slides (Thermo Scientific, Paisley, UK).

Antigen retrieval methods

Sections were dewaxed with a xylene wash (BDH, Poole, England) followed by ethanol washes. The CD154 sections were boiled in citric acid on a hot plate for 20 min and cooled at room temperature for 20 min; the CD40 sections were boiled in citric acid on hot plate for 10 min and cooled at room temperature for 20 min. Then the sections were washed in PBS, and the endogenous peroxidase was quenched with 3% H_2O_2 in methanol for 5 min.

Antibodies for immunohistochemistry

The primary antibodies were purified anti-human CD154 (C-20) antibody (sc-978, rabbit polyclonal IgG, Santa Cruz Biotechnology Inc., Dallas, TX, USA; diluted 1:50 in PBS) and purified anti-human CD40TNFRSF5 antibody (PA5-32325, rabbit polyclonal IgG, Thermo Fisher, Paisley, UK) diluted 1:50 in PBS. The secondary antibody was Biotinylated Universal (PK-6200, anti-mouse IgG/rabbit IgG) antibody (Vectastain Universal, Elite, ABC kit, Vector Laboratories, Inc, Burlingame, CA, USA).

3', 3'-diaminobenzide staining and image acquisition

The study slides containing OLP sections and positive control were incubated with the primary antibody for 30 min at room temperature. The negative control was incubated with no primary antibody (PBS only) for the same time at room temperature. The slides were then washed in PBS three times and incubated with the secondary antibody for 30 min at room temperature. The slides were then incubated with Avidin DH/horse biotinylated horseradish peroxidase solution (Vectastain Universal, Elite, ABC kit, Vector Laboratories, Inc). The slides were washed three times with PBS, and 3', 3'-diaminobenzide (DAB; Dako Australia Pty. Ltd, North Sydney, Australia) was applied for 5 min. After this, the slides were washed with PBS, counterstained in Mayer’s Hematoxylin (Amber Scientific, Midvale, Australia), and mounted. Consecutive sections of the same samples were also stained with hematoxylin and eosin without immunohistochemistry. Images were obtained by using an Aperio CS2 Digital Pathology Slide Scanner (Leica Biosystems, Mount Waverley, Australia).

Statistical analysis

Statistical significance was determined with the unpaired t-test.

Results

Time course study of CD40/CD86 expression in H357 cells treated with IFN-γ

CD40/CD86 production in oral mucosal keratinocytes was first assessed over time in preliminary experiments using the keratinocyte cell line H357 (Fig. 1). After 3 h of IFN-γ treatment, very low levels of CD40 mRNA and CD86 mRNA were detectable in H357 cells. After 6 h, expressions of CD40 mRNA and CD86 mRNA were slightly higher in cells treated with IFN-γ than in the control cells. Interestingly, CD40 and CD86 mRNA
expression was virtually undetectable in control cells at 9 h. IFN-γ-treated cells had detectable CD86 mRNA levels up to 72 h, whereas CD86 mRNA transcripts were present for up to 48 h in control cells. Taken together, these findings indicate that CD40 and CD86 expressions in H357 cells are enhanced by IFN-γ stimulation.

Costimulatory molecule expression in oral epithelium in vitro

CD40 was expressed constitutively in three primary oral epithelial cell lines but was increased by IFN-γ treatment in vitro (Fig. 2a, b). Statistical analysis revealed that mRNA expression was significantly higher ($P < 0.001$) in the IFN-γ group (0.853 ± 0.025) than in the control group (0.419 ± 0.0134) (Fig. 2c). The pattern was similar for the costimulatory molecule CD86 on the same cell lines. Constitutive CD86 expression was very low but increased after IFN-γ treatment, although the difference was not statistically significant (Fig. 2c).

CD40/CD154 expression in OLP tissue

In sections of OLP lesional tissue, concentrated CD40 staining was associated with cells within OLP infiltrate, although staining of individual cells near the epithelium were visible, as was moderate epithelial staining (Fig. 3). Staining was particularly intense within band-like inflammatory infiltrate (Fig. 4). Cells in the basal area had relatively high expressions, although positive cells were also seen in the suprabasal area. Certain focal areas of the basal epithelium had particularly high expression.
levels. CD154 was expressed on infiltrating cells in OLP lesions but not within the epithelial layers (Fig. 5). Positive cells tended to be located near epithelial cells at the epithelial-connective tissue junction, especially in focal areas of cell damage.

**Discussion**

The intensity of CD40 staining in OLP was strong, particularly on cells within infiltrate, which may be CD40-positive Langerhans cells. In addition, CD40 expression was seen in oral epithelial cells. CD40 expression has been reported in other epithelial cell types in vivo and appears to increase during other inflammatory disorders. Furthermore, CD40 expression was increased on primary oral keratinocytes after IFN-γ incubation in the present study, which is consistent with the findings of other studies (1). This cytokine may thus be important in the stimulation and inflammation of oral epithelium.

CD86 mRNA expression was enhanced after IFN-γ treatment of oral keratinocytes, and the implications of this expression in oral inflammatory disease may be of great importance, as discussed below.

Past and present findings suggest that CD40/CD86 ligation is involved in inflammatory enhancement. In OLP, T-cells appear to be activated and thus are likely to express CD154 (27), the ligand for CD40. The proximity of infiltrating T-cells and epithelium in OLP, which sometimes facilitates interactions between T-cells and epithelium, suggests CD40 and CD154 ligation in OLP. This ligation induces or enhances multiple effects in epithelial cells, including increasing the production of proinflammatory cytokines (5,28) and chemokines (5,29-31). Increased production of these molecules in the epithelial area in OLP likely increases inflammation. Specifically, increased expression of CXC-ELR-chemokines in OLP is due in part to ligation of CD40 on oral epithelial cells, as was shown previously in cervical carcinoma cells (26). Furthermore, increased production of RANTES by keratinocytes in OLP (32) might be influenced by CD40 ligation (29,33).

IFN-γ may also be important in inflammation of oral epithelium, as it can increase CD40 expression on epithelial cells and induce expression of chemokines such as CXCL10 (Marshall et al., manuscript in preparation). These mechanisms presumably promote migration into the area of activated T-cells, which may interact with epithelial cells and bind CD40. These effects would synergize to produce more chemokines and exacerbate the inflammatory status of conditions such as OLP (34-36).

Induction of B7 molecules on keratinocytes increases immunogenicity to antigenic stimulants, which results in a large influx of stimulated T-cells. An increase in the delayed-type hypersensitivity response to reencountered antigens in B7 transgenic mice has many similarities to the pathogenic mechanisms of OLP, including the large T-cell influx visible in OLP and the chronic nature of the disease. CD86 is upregulated on oral epithelium by IFN-γ in vitro, and there are many features suggestive of IFN-γ stimulation on keratinocytes in OLP. These findings suggest that this molecule is expressed on cells involved in increased immunogenicity to antigens, including common oral commensals such as Candida. Furthermore, CD86 expression on oral epithelium may be relevant to cell migration, as CD28 ligation of CD4+ cells can alter chemokine receptor expression (37).

Although we have shown that CD86 is induced on oral epithelium in vitro, its expression in OLP is unknown. Simon et al. (38) investigated the expression of CD28 and B7 in cutaneous LP lesions and found that the B7-1 molecule was focally expressed on keratinocytes within the lesion. However, the antibody for B7-1 was also detected in the major histocompatibility complex (MHC) molecule (39), and we suspect that it was this molecule that was detected in LP.

CTLA-4 and CD28 binding affect Th1/Th2 differentiation but would appear to have a larger effect on naive cells than on memory cell interactions (40), which occur in OLP.

Because of the increase in costimulatory molecules in epithelium after inflammatory signals, including oral epithelial cells (as shown in this study), such molecules may act as nonprofessional antigen-presenting cells in OLP (41). Keratinocytes have the genes necessary for antigen presentation (42); however, if oral keratinocytes were capable of antigen presentation, it would likely have to be immunogenic antigen presentation in OLP, to cause
the observed reaction. However, it has proven difficult to identify the factors that provide even professional antigen-presenting cells with tolerogenic or activating signals to T-cells in the periphery (43).

Like bronchial and intestinal epithelium, oral epithelium is capable of antigen presentation, although by other mechanisms (3,44-46). In particular, an increase in the expression of costimulatory molecules, e.g., during inflammation, is required for oral epithelial antigen presentation. It is possible that the antigens present at high doses—and thus more likely to elicit an effective T-cell response (16)—are those with persistently high levels in oral mucosa, such as betel nut antigens in chronic users and amalgam antigens of patients with these fittings. If these antigens were combined with inflammatory signals, active antigen presentation of these antigens might yield an immunogenic rather than tolerogenic response.

Proposed activation/tolerance theories regarding dendritic cells—i.e., that stimulatory function is either enhanced by toll-like receptors on dendritic cells recognizing microbial products and upregulating costimulatory molecules (47) or that damage in other cells and production of molecules such as heat shock proteins act as “danger signals” (48) that activate dendritic cells—may also be true for epithelial cells (49-52).

Induction of costimulatory molecules on the epithelium does not result in T-cell activation, as these molecules may actually bind to CTLA-4, which is thought to be important in providing tolerance (53). In fact, dysregulation of CTLA-4 in OLP patients may be responsible for a breakdown in tolerance for oral keratinocyte antigens (54). Although CTLA-4 cells were clearly expressed in most samples of cutaneous LP tissue, there was no such expression in a variety of other skin inflammatory disorders (55). The implications of this finding are unclear; however, the fact that many infiltrating T-cells in LP express CD28 (38) suggests that these cells interact with CD86-expressing keratinocytes, thereby leading to specific clonal activation of these cells (56).

A subset of CD4+ T-cells that express CD25 represents regulatory T-cells that might be involved in the suppression of autoimmunity (57-59). Interestingly, CD25+ cells are upregulated in OLP (58). However, these cells are also thought to involve in infection persistence, perhaps in order to permit long-term immunity (57). Therefore, these cells might be important in disease chronicity in persons with low-level (or undetectable) infection. OLP chronicity and the presence of CD25+ cells may therefore be related.

If oral keratinocytes are capable of antigen presentation, it would be nonprofessional antigen presentation, i.e., the cells would be unable to migrate to lymph nodes to stimulate naïve T-cells. This suggests that it is predominately memory cells that are activated in this manner. In fact, memory T-cells are activated by a range of different antigen-presenting cells and require less costimulatory function than do naïve cells (60). Interestingly, most infiltrating cells in OLP lesions are memory T-cells. Furthermore, the possible increase in chemokine production by T-cell ligation of keratinocytes in OLP would likely promote further migration of memory cells into this area. This suggests that Langerhans cells are important in initiating naïve T-cell responses, whereas, in secondary response, keratinocytes may play a role in reactivating memory T-cell responses. However, resident tissue APCs are implicated in presenting self-antigen to Th1 cells in autoimmune conditions (61); thus, keratinocytes may also play a role in initiating the presentation process to Th1 cells.

The reaction against oral keratinocytes in OLP suggests a failure in tolerance to self-antigens of oral epithelial cells. A number of mechanisms could affect this potential breakdown of self-tolerance in oral mucosa, including molecular mimicry (62) and epitope spreading (63).

Because of the number of agents associated with OLP onset and chronicity, which may be caused by the different shift of epitopes detected in the disease, epitope spreading is a possible pathogenetic mechanism of this disease. Furthermore, CD80/86 blockade (64) or CD40-CD154 blockade (65) inhibits epitope spreading and eases ongoing autoimmunity in animal models. The fact that CTLA-4+ve cells enable epitope spreading (54) suggests that CD25+ cells observed in OLP may down-regulate immune reaction. However, further epitope changes might promote a wave of inflammatory T-cells. Epitope spreading is implicated in chronic diseases like multiple sclerosis, which exhibit relapse and remission. Therefore, this pathogenic pattern might be present in OLP.

Despite the large number of CD4+ cells present in lesions, CD8+ cells are localized near the epithelial area in OLP. This suggests the presence of MHC class I restricted presentation within OLP lesions. However, it may be that prior class II presentation by keratinocytes assists in initiating a cytotoxic T lymphocyte reaction. Interestingly, CD40 ligation of antigen-presenting cells is important in the generation of cytotoxic T lymphocytes (66, 67). Therefore, if keratinocytes are presenting antigen through the MHC class II pathway, ligation of CD40, which is classically associated with antibody-mediated reactions, may amplify inflammation in the area and facilitate induction of specific cytotoxic T lymphocytes.
A group of CD8+ effector memory cells is preferentially located in non-lymphoid tissue. These cells rapidly expand after activation (68) and thus may not require further T helper cells. Furthermore, CD8+ memory cells have a limited requirement for B7 costimulatory signals. However, because autoreactive CD4+ and CD8+ T-cells often have a weaker affinity to antigen, they may require B7 stimulation (40). Thus, expression on oral epithelium may still be relevant.

The process of cross-tolerance (69) is thought to be important in acquiring self-tolerance to apoptotic cells. In fact, there is a subset of intestinal dendritic cells that transports apoptotic epithelial cells to lymph nodes (70), a process thought to provide exposure and induction of tolerance to “self” antigen. Furthermore, Langerhans cells are capable of phagocytosis of vaginal apoptotic epithelial cells (71). These findings are of particular interest, as it is thought that apoptotic keratinocytes are present in OLP (9). Transport of antigen from these cells to lymph nodes may induce an immunogenic response to these antigens instead of a tolerizing effect (72).

This process might produce T-cells autoreactive to keratinocytes. Additionally, ligation of CD40 on epithelial cells causes growth inhibition and increases apoptosis of cells in ovarian carcinoma cell lines (5). This finding is of interest because of increased apoptosis of this cell type in OLP (in conjunction with associated CD40 expression). This may induce transport of apoptotic cells to lymph nodes for cross-presentation, a process that was found to be age-related (73).

Molecular mimicry and epitope spreading is thought to occur in class I restricted antigens and in class II antigens, so it may have an effect on direct MHC class I presentation, thereby exerting a cytotoxic effect in OLP.

Accumulating evidence regarding the presence of molecules on epithelial cells suggests that they may sometimes be capable of antigen presentation. Whether this antigen presentation produces a tolerogenic or immunogenic response is unknown, although the presence of MHC-II and CD40, potential CD86 expression on oral epithelial cells, and CD28 in T-cell infiltrate of OLP suggests that “activating” antigen presentation might occur. Antigen presentation may take the form of “sampling” antigens on oral mucosa, such as bacterial antigens. The implications for antigen presentation may be very important in conditions such as OLP, as there appears to be a breakdown in tolerance for “self” keratinocyte antigens. This breakdown could be caused by a number of mechanisms, including molecular mimicry or epitope spreading. However, CD8+ cells appear to be acting cytotoxically in OLP; therefore, the class I pathway may be more important in the disease process. The presence of inflammatory signals during cross-presentation of apoptotic keratinocytes might cause normal tolerogenic CD8+ cells to become autoreactive for these cells. Interestingly, ligation of CD40 on keratinocytes (which is expressed in OLP) is thought to cause apoptosis of this cell type.

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Conflict of interest
None declared.

References


antigen and CD40 triggering are sufficient to induce primary and memory cytotoxic T cells. J Immunol 164, 725-732.


Supplementary Files
Supplementary Fig. 1
Please find supplementary files; doi: 10.2334/josnusd.16-0334.