IL-1 Alpha Regulate CXCL1, CXCL10 and ICAM1 in Network Form in Oral Keratinocytes

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Abstract

Keratinocytes are the major constituent of epithelial cells at skin and mucosal surfaces such as oral mucosa. In our previous study, it was indicated that oral keratinocytes play active roles in oral mucosal adaptive immune response and an IL-α sub-network was identified. In the past decades IL-1, a revolutionarily ancient cytokine, has attract our attentions for its important roles in innate and adaptive immune responses. In this study, we first testified that IL-1α regulated its sub-network genes including CXCL1, CXCL10 and ICAM1 mRNA levels in time dependent manners but not in concentration dependent manners. Our following immunohistochemistry study showed that IL-1α and CXCL1 were expressed in OLP tissues, which were only detected in tissue transudate and whole unstimulated saliva in previous study. A complete characterization of IL-1α sub-network will shed light on the exploration of IL-1 as the therapeutic target in OLP and help to illuminate the multiple regulatory functions of keratinocytes in oral mucosa or even in other mucosa sites.

Keywords: IL-1 alpha, CXCL1, CXCL10, ICAM1, Oral keratinocytes, Oral lichen planus

Introduction

Keratinocytes are the major constituent of epithelial cells at skin and mucosal surfaces, which cover organs, internal cavities and the body. Accumulated researches of skin, airway and gastrointestinal tract have demonstrated that keratinocytes function in the development of the immune system, promotion of pathological inflammation and even impose diverse decisions on immune cells [1-4]. During the immune responses, keratinocytes were challenged to trigger discrete pathways and secrete specific cytokines and chemokines which alter the molecules displayed at the epithelium. In order to define all the epithelial molecules that direct the actions of immune cells, a new subject ‘epimimunome’ has emerged, the novel and pleiotropic roles of keratinocytes in the initiation, regulation and resolution of immune responses at skin and several mucosa sites will be disclosed [2].

Lichen planus is a chronic, immunological mucocutaneous disorder of the stratified squamous epithlia with a wide range of clinical manifestation. The oral mucosa is commonly involved and may be the only site of involvement. Oral lichen planus (OLP) is the most common non-infectious oral mucosal diseases affecting one to two percent of the adult population [5]. The histology of OLP is characterized by a dense subepithelial lymphocytic infiltration and basal keratinocytes degeneration. However, the etiology and pathogenesis of OLP is controversial and largely unknown. What roles do oral keratinocytes play in OLP lesion formation and chronicity? What induce the T cells to migrate to oral mucosa? What trigger keratinocytes apoptosis? Answers to these questions may help produce a cure for complete understanding of OLP.

Compared with keratinocytes in other mucosa sites, relevant researches on oral keratinocytes are scattered and how they participate in immune response is largely unknown. With the development of high-throughput technologies it is possible to gain a systematic view of biological systems to understand the regulatory roles of oral keratinocytes in immune response. In previous study, we applied microarray technique on oral keratinocytes from our established oral DTH murine model [6]. And the following bioinformatic analysis of gene regulatory networks help us to reveal specific sub-networks that lead to the in-depth understanding of the regulatory roles of keratinocytes such as IL-1a sub-network. IL-1α was a general amplifier of T-cell responses in several epithelial tissue immune responses [7,8]. Relevant researches from our group and other groups demonstrated that there was a higher expression level of IL-1α and its target genes CXCL1, CXCL10 and ICAM1 in OLP lesion, tissue transudate and whole unstimulated saliva [9-15], but the regulatory effect of IL-1α on its target genes have not been reported. In this study, we first testified whether IL-1α regulated its sub-network genes including CXCL1, CXCL10 and ICAM1 mRNA level in time and concentration dependent manners. And then we investigated IL-1α and CXCL1 expression in OLP tissue, which were only detected in tissue transudate and whole unstimulated saliva in previous study. A complete characterization of IL-1α sub-network will shed light on the exploration of IL-1 as the therapeutic target in OLP. Moreover, it will help to illuminate the multiple regulatory functions of keratinocytes in oral mucosa or even in other mucosa sites.

Material and Methods

Cell cultures and treatments

Keratinocytes from C57BL/6 mice tongue mucosa were collected as described previously [6]. After that keratinocytes were cultured in Keratinocyte-SFM (Gibco, Invitrogen Co., Carlsbad, CA, USA) containing 0.2 ng/ml epidermal growth factor (Invitrogen) and 25 ng/ml bovine pituitary extract (Invitrogen) at an initial density of 2.0×10⁵ cells/well in a 6-well plate precoated with mouse collagen type IV (BD Biosciences, Bedford, MA, USA) collagen-coated. Cells were incubated at 37°C, 5% CO2 in humidified air. Medium was changed...
every other day. Cells were grown to approximately 70-80% confluence and treated with medium only or Recombinant Mouse Interleukin-1 alpha (Prospec-Tany Technogene, Rehovot, Israel) as indicated. All experiments were performed at least three times with similar outcomes.

**RNA isolation and quantitative real-time PCR**

Total RNA of keratinocyte was extracted with Trizol reagent (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions. The RNA quality was assessed by formaldehyde agarose gel electrophoresis and quantitated spectrophotometrically. PCR assays used primers specific for IL-1α, CXCL1, CXCL10, intercellular adhesion molecule-1 (ICAM1), IL-1 receptor 1 (IL-1r1) and IL-1 receptor antagonist (IL-1ra). The primer sets for these genes were designed to amplify approximately 100 bp segments and primers specific for GAPDH were used as a housekeeping gene control. All primers were custom-designed from GenBank mRNA sequences and prepared by DaAn Gene Company (Guangzhou, China). PCR primer sequences are as follows: for IL-1α, F: 5'-TACTCTGCGGGAGGAAGCA-3' and R: 5'-TTCTGGCGACCACTCA-3'; for CXCL1, F: 5'-TGTCGATGCTGGCATGACCA-3' and R: 5'-GGGCTATGCTTCCCCGTTGGGT-3'; for CXCL10, F: 5'-GTCTGGGTCTGATGGGGACTC-3' and R: 5'-GCTTCCTATGGCGCCCCTATT-3'; for ICAM1, F: 5'-CAATTCTCATGCGCCGACACG-3' and R: 5'-CTGGAAGATCGAAGATCCGG-3'; for IL-1r1, F: 5'-CTGTCGCTGGAGATTTGAGTA-3' and R: 5'-GGTGTCGCCGCCGTCAATTT-3'; for IL-1ra, F: 5'-AAAGTGCAAGGCTCTGAGGCT-3' and R: 5'-CTCCGTTGAAGATCTCCACCCG-3'; for GAPDH, F: 5'-CGTGTCCTGAGATTTGAGTA-3' and R: 5'-TGTCGATGCTGGCATGACCA-3' and R: 5'-TGTGATGCTGCTGCCGTTGGGT-3'. cDNA was synthesized from 4 μg RNA samples using oligo dT to prime the reverse transcriptase reaction. RT-PCR was carried out on the ABI 9700 instrument (ABI company, Foster City, CA, USA) with the QPCR SYBR green detection reagent (ABI company, Foster City, CA, USA) for 3 min at 93°C for the initial denaturing, followed by 40 cycles of 93°C for 30 seconds, 55°C for 45 seconds and 72°C for 45 seconds. Cell threshold values for each gene were determined and fold induction compared with medium only (Figure 1). IL-1r1 mRNA has the similar expression pattern of genes mRNA which indicated that IL-1α regulate its sub-network genes mRNA level in time dependent manners (data not shown).

**Human oral mucosa sample collection**

Human OLP samples were obtained from patients diagnosed with OLP (n=31) based on the modified WHO diagnostic criteria (2003) both clinically and pathologically [17]. Informed consent was obtained from patients who were involved in this experiment visiting the Department of Oral Medicine, School of Stomatology, Sun Yat-sen University. The characteristics of the subjects are summarized in table 1. None of the subjects had previously received topical or systemic medication within 6 months and they did not have other systemic autoimmune diseases or severe tumors. All the mucosa biopsys were localized in areas that did not have an erosion lesion. Ten control cases were evaluated by dissociation curves to confirm single amplification and R: 5΄-GGTGTCGCCGTGCATTTTAT-3΄; for CXCL10, F: 5΄-CTGCGCTGGAGATTTGAGTA-3΄ and R: 5΄-GGTGTCGCCGCCGTCAATTT-3΄; for ICAM1, F: 5΄-CAATTCTCATGCGCCGACACG-3΄ and R: 5΄-CTGGAAGATCGAAGATCCGG-3΄; for IL-1r1, F: 5΄-CTGTCGCTGGAGATTTGAGTA-3΄ and R: 5΄-GGTGTCGCCGCCGTCAATTT-3΄; for IL-1ra, F: 5΄-AAAGTGCAAGGCTCTGAGGCT-3΄ and R: 5΄-CTCCGTTGAAGATCTCCACCCG-3΄; for GAPDH, F: 5΄-CGTGTCCTGAGATTTGAGTA-3΄ and R: 5΄-TGTCGATGCTGGCATGACCA-3΄ and R: 5΄-TGTGATGCTGCTGCCGTTGGGT-3΄. cDNA was synthesized from 4 μg RNA samples using oligo dT to prime the reverse transcriptase reaction. RT-PCR was carried out on the ABI 9700 instrument (ABI company, Foster City, CA, USA) with the QPCR SYBR green detection reagent (ABI company, Foster City, CA, USA) for 3 min at 93°C for the initial denaturing, followed by 40 cycles of 93°C for 30 seconds, 55°C for 45 seconds and 72°C for 45 seconds. Cell threshold values for each gene were determined and fold induction compared with medium only (Figure 1). IL-1r1 mRNA has the similar expression pattern of genes mRNA which indicated that IL-1α regulate its sub-network genes mRNA level in time dependent manners (data not shown).

**Immunohistochemistry**

In brief, the IL-1α sub-network from our previous array data, time-course experiments involving IL-1α stimulation were performed using primary mouse oral keratinocyte. Keratinocytes were collected at several time points (3h, 6h, 12h, 18h and 24h) after IL-1α treatment. mRNA levels of IL-1α sub-network genes including CXCL1, CXCL10 and ICAM1, further more IL-1α, its receptor 1 (IL-1r1) and receptor antagonist (IL-1ra) were determined using real-time RT-PCR. mRNA levels of CXCL1, CXCL10, ICAM1 and IL-1r1 were found to be time dependent (Figure 1). Interestingly, the kinetics of the observed expression changes differed for the four mRNAs. CXCL1 mRNA levels gradually changed and peaked at 12h. At the 12-hour time-point CXCL1 mRNA levels were over 10-fold higher (P<0.05) in 10.0 ng/ml IL-1α treated primary keratinocytes than in primary cells treated with medium only (Figure 1). IL-1r1 mRNA has the similar expression kinetic which peak at 12h. In contrast to the gradual increase in CXCL1 mRNA levels, a decline trend which reach the lowest point at 12h-18h time points was observed in both CXCL10 and ICAM1 mRNA expression pattern (P<0.05).

In order to eliminate the influence of the fluctuation of IL-1α concentration in keratinocyte culture medium, we repeated the experiment and changed the medium with initial 10.0 ng/ml IL-1α medium at 12h time-point. The results showed the similar expression pattern of genes mRNA which indicated that IL-1α regulate its sub-network genes mRNA level in time dependent manners (data not shown).

**IL-1α did not regulate its sub-network genes mRNA levels in concentration dependent manners**

After determining IL-1α regulate its sub-network genes mRNA level in time dependent manners, different concentration of IL-1α (2.5 ng/ml, 5.0 ng/ml, 10.0 ng/ml, 20.0 ng/ml, 40.0 ng/ml) were added to 478. doi:10.4172/scientificreports.0704172

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Table 1: Clinical variable of the patients with OLP and health volunteers.
into the culture medium to investigate whether IL-α can stimulate its sub-network genes in concentration dependent manners. We were unable to detect significant difference in CXCL1, CXCL10, ICAM1 and IL-1r1 mRNA levels among different concentration groups (Figure 2). In contrast, IL-1ra mRNA levels significant decreased as the IL-α concentration increases from 2.5ng/mL to 20.0 ng/mL.

**IL-1α and CXCL1 expression in OLP**

The oral tissues collected in clinic exhibited characteristic histological features of OLP including epithelial atrophy, basement membrane zone degeneration and presence of a dense band of subepithelial inflammatory cell infiltration. Immunohistochemistry staining showed that IL-1α was distributed diffusely on lower spinous cell layers and basal keratinocytes in OLP epithelium while it was seldom detected in control samples (Figure 3a and 3c). In contrast to the IL-α staining, CXCL1 expressions were widely spread in whole oral epithelium both in control and OLP group (Figure 3b and 3d).

![Image](https://example.com/image.png)

**Figure 3**: IL-1α and CXCL1 expression in OLP biopsys.

In particular the increased staining of CXCL1 was detected in basal keratinocytes in OLP biopsys.

**Discussion**

Our previous study has indicated that oral keratinocytes play active roles in oral mucosal adaptive immune response [6]. In our results an IL-α sub-network which was composed of hub gene IL-1α and other five node genes including CXCL1, CXCL10, ICAM1, adrenomedullin (ADM) and prostaglandin-endoperoxide synthase 2 (PTGS2) was identified [6]. Relevant researches from our group and other groups demonstrated that there was a higher expression level of IL-1α and its target genes CXCL1, CXCL10 and ICAM1 in OLP lesion, tissue transudate and whole unstimulated saliva [9-15], but the regulatory effect of IL-1α on its target genes have not been reported. OLP is a chronic, immune-mediated condition characterized by persistent T lymphocytes accumulation and epithelial cell damage. Although OLP has been defined as an oral precancerous condition by WHO [18], its etiology and potential of malignant transformation are largely unknown and controversial. Therefore, in this study we focused on testing the regulatory function of IL-1α on CXCL1, CXCL10 and ICAM1 in OLP keratinocytes, hoping to illuminate the multiple regulatory functions of keratinocytes in oral mucosa and may be of practical relevance for future treatment of OLP.

Our results demonstrated that IL-1α regulated its sub-network genes including CXCL1, CXCL10 and ICAM1 mRNA levels in time dependent manners (Figure1), which is consistent with the biological association network constructed from our microarray data [6]. But IL-1α sub-network genes mRNA levels did not alter according to the variation of IL-1α concentration (Figure 2). In order to get more details about this biological response, the expression of IL-1 receptor 1 (IL-1r1) and IL-1 receptor antagonist (IL-1ra) were also detected in our experiments. The recent description of IL-1 antagonist confirmed that, for its significant homology with IL-1, IL-1 receptor antagonist was an
inducible antagonist in experimental inflammation and human disease binding to IL-1 receptor 1 to inhibit the action of IL-1 [19]. In our concentration dependent experiments, CXCL1, CXCL10 and ICAM1 mRNA did not but IL-1ra mRNA level did vary according to IL-1α stimulation (Figure 2). This finding suggested that the reason for IL-1α’s inability to induce its sub-network genes expression in concentration dependent manner may be the IL-1ra's significant role in inhibiting IL-1α’s action. On the other hand, in IL-1α time dependent experiment, CXCL1, CXCL10, ICAM1 and IL-1r1 mRNA expression varied while IL-1ra did not. The explanation for this observation may be the quantity of IL-1ra is insufficient to prevent the exaggerated IL-1 response in oral keratinocytes. The competition between IL-1α and IL-1ra may be one of the principal modulatory mechanisms to restore this homeostatic state in oral keratinocytes. Once IL-1α outweighs IL-1ra it will elicit biologic responses such as inducing cytokine secreting and exaggerating the inflammatory response.

Unlike CXCL10 and ICAM1 which have been reported to be expressed on OLP lesion, IL-1α and CXCL1 were only detected in tissue transudate and whole unstimulated saliva in previous study [9-15]. So in order to analyze the distribution patterns of IL-1α and CXCL1 on OLP lesion, immunohistochemistry staining was applied on OLP biopsys. The expression pattern of IL-1α in OLP is intriguing that it is presented only on the basal cell layer where the keratinocytes proliferation took place (Figure 3C). Similar to skin, oral mucosa is a typical keratinized multilayered epithelium. Within the epidermis, proliferation takes place in the basal layer of keratinocytes which are covered by layers of gradually differentiating cells. Keratinocytes that attached to the underlying basement membrane leave the basal layer and undergo terminal differentiation as they moving towards the surface. OLP is a chronic T cell mediated inflammatory oral mucosal disease characterized by degeneration of the basal layer keratinocytes of unknown etiology. Many controversies exist about the pathogenesis of OLP. One of these hypotheses is direction migration hypotheses. It is said that an altered cytokine environment in basal cell layer attribute to basal cell degeneration [5]. In particular cytokines secreted by the keratinocytes direct the T cells to migrate into the epithelium and trigger the following keratinocytes apoptosis and basement membrane disruption. The function of IL-1α and its mechanism of localization at the basal keratinocytes in oral mucosa are significant issues to be addressed concerning its unique role in the pathogenesis of OLP. Although CXCL1 were also expressed in control group, CXCL1 staining was much more obvious in basal keratinocytes in OLP biopsys. This increased staining of CXCL1 in basal keratinocytes may be the result of IL-1α stimulation, but the exact connection between them still remains to be elucidated.

In this study, we firstly verified that IL-1α stimulated CXCL1, CXCL10 and ICAM1 mRNA expression in time dependent manners. And then our following immunohistochemistry study demonstrated that IL-1α and CXCL1 protein were expressed in OLP. Another two target genes in this sub-network ICAM1 and CXCL10 which induce leucocytes to leave the circulation to patrol to inflammatory tissue have been detected in OLP in previous histochemistry studies [10,11]. In combination, IL-1α and its target genes CXCL1, CXCL10 and ICAM1 are important immune regulators in the pathogenesis of OLP. If this IL-1α sub-network also exists in OLP tissue in vivo, IL-1α may be an important gatekeeper of inflammation in OLP. It is promising to develop IL-1α as the therapeutic target in OLP treatment. Recently a pure receptor antagonist (Anakirna) binding tightly to the type I IL-1 receptor (IL-1r1) and preventing activation of IL-1 has been widely used in a broad spectrum of inflammatory diseases [19]. Further studies will focus on the potential involvement of this IL-1α sub-network in vivo and OLP lesion. It is essential to understand the fundamental role of IL-1α in the pathogenesis OLP and will shed light on the mechanisms of keratinocytes directing the initiation and progression of oral mucosal adaptive immune response.

Acknowledge
This work was supported by grants from the National Natural Science Foundation of China (91029712).

References