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Original Article

Expression of keratinocyte growth factor and its receptor in oral lichen planus

Ling-Li Wang[1], Ren-Cin Wang[1], Ling-Zhen Wang[2], Li-Rong Sun[2], Xiang-Min Qi[3]

[1]Department of Stomatology, Weihai Municipal Hospital, Weihai, Shandong Province, China; [2]Department of Pediatrics, The Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, China; [3]College of Stomatology, Shandong University, Jinan, Shandong Province, China

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Abstract: This study aimed to investigate the expression of keratinocyte growth factor (KGF) and its receptor KGFR in oral lichen planus (OLP). Oral mucosa specimens from 30 OLP patients and ten healthy controls were collected. The expression of KGF and KGFR proteins was detected by immunohistochemistry and the expression of KGF mRNA was detected by in situ hybridization. We observed KGF protein expression but not KGF mRNA expression in the epithelium of both OLP and normal oral mucosa. The expression intensity of KGF protein was much lower in the epithelium of OLP than in that of normal oral mucosa. KGF protein was also expressed in the cytoplasm of some fibroblasts and vascular endothelial cells in the connective tissues underlying the epithelium for both OLP and normal oral mucosa, but the expression intensity of KGF was lower in the connective tissues for OLP. KGF mRNA was expressed in the cytoplasm of some fibroblasts and vascular endothelial cells in the connective tissues underlying the epithelium for both OLP and normal oral tissues. Although KGFR was expressed in vascular endothelial cells of connective tissue and in all epithelium of normal oral mucosa, it was only expressed in the basal layer and prickle layer of the epithelium and in vascular endothelial cells of the connective tissue of OLP. Compared to normal oral mucosa, OLP had lower expression of KGFR in the epithelium but higher expression of KGFR in the connective tissue underlying the epithelium. In conclusion, this study revealed significant differences in the expression intensity and distribution of both KGF and KGFR between OLP and normal oral mucosa tissues. KGF and its receptor KGRF may play an important role in the development and progression of OLP.

Keywords: Keratinocyte growth factor, keratinocyte growth factor receptor, oral lichen planus, immunohistochemistry, in situ hybridization

Introduction

Oral lichen planus (OLP) is a T-cell mediated chronic inflammation occurring in the oral mucosa [1-3]. The incidence OLP is 0.1-4% and the malignant transformation rate of OLP is 0.4-5.6% [4, 5]. The World Health Organization has listed OLP as a possible precancerous condition. The etiology for OLP is still unknown. Currently, there is no effective treatment for OLP in clinical practice [6, 7]. Therefore, there is an urgent need to better understand the pathogenesis of OLP in order to develop therapeutic approaches for OLP.

OLP has characteristic histological features such as subepithelial band-like inflammatory cell infiltration, epithelial basal cell destruction via liquefaction, and incomplete keratosis of epithelial cells. It has been reported that basal cell liquefaction observed in OLP was attributed to the apoptosis of keratinocytes and the breakdown of the epithelial basal membrane [8-10]. A variety of proteins and growth factors have been identified to be implicated in OLP [8, 11].

In the oral, keratinocyte growth factor (KGF) is secreted by both fibroblasts and vascular endothelial cells, and regulates the growth of fibroblasts in blood vessels and connective tissues in an autocrine manner. KGF also stimulates the proliferation and migration of oral mucosa epithelial cells in a paracrine manner [12]. KGF could accelerate the growth of epithelial cells during the morphological reconstruction of oral mucosa, but did not enhance the differentiation

of epithelial cells. In clinics, KGF has been successfully used to treat mucositis induced by radiotherapy and chemotherapy, and ulceration caused by epithelial damages through accelerating wound healing [13]. However, the role of KGF and its receptor (KGFR) in OLP remains elusive.

In the present study, we investigated the protein expression of KGF and KGFR in biopsies collected from OLP patients by immunohistochemistry. In addition, we detected the expression of KGF mRNA in these tissues by *in situ* hybridization. Our results demonstrated that there were significant changes in the expression of both KGF and KGFR in OLP biopsies.

Materials and methods

Clinical tissue samples

OLP tissue samples were obtained from the biopsies of 30 pathologically confirmed OLP patients (13 males and 17 females) who visited our hospital. These patients were 25-60 years old and had OLP for 30 days to four years. Thirteen OLP tissues were from cheek; six were from gums; six were from tongues and five were from lower lips. All 30 OLP patients had no systemic disease and did not receive any treatment in the past six months (OLP group). Normal oral mucosa specimens (Normal group, n=10) were collected from healthy donors (18-32 years old) when they had their teeth extracted or voluntary donors (24-63 years old) for comparison. OLP and normal oral mucosa specimens were pathologically confirmed by the Department of Pathology of Shandong University Dental Hospital. Approval of this study was obtained from institutional review board (IRB), and all subjects gave informed consent.

Immunohistochemistry

The fresh tissues specimens were fixed in 4% paraformaldehyde, 0.1 M phosphate buffered saline (PBS, pH 7.2-7.6), and 0.1% diethylprocarbonate (DEPC) for 1 h. After being washed with running water for 2 h, the fixed specimens were dehydrated in a series of ethanol (30, 50, 70, 90, and 100%). The dehydrated specimens were then air dried and soaked in xylene for 30 min to extract any residual ethanol. The cleared specimens were wax-embedded in paraffin and cut into 5 μm sections. The sections were then de-waxed and incubated in 3% H_2O_2 solution on glass slides for 5-10 min at room temperature to eliminate/inactive endogenous peroxidase. The sections were washed three times with distilled water and then incubated with goat serum at room temperature for 30 min. Next the sections were incubated with diluted primary antibody for KGF or KGFR (1:50-1:200, BosterBio, Wuhan, China) at 4°C overnight, followed by incubation with biotinylated goat anti-mouse IgG at 37°C for 20 min. The sections were incubated with SABC (horseradish peroxidase labeled streptavidin solution) at 37°C for 10-15 min, and diaminobenzidion (DAB) was used to visualize the sections. The sections were counterstained with hematoxylin, dehydrated, and sealed for observation under microscope. In the negative control, tissue sections were incubated with PBS instead of primary antibody.

Evaluation of immunohistochemical staining

Photo images were taken for immunohistochemically stained sections under a microscope. The images were analyzed using image analysis system to semi-quantify the staining of KGF and KGFR. Briefly, one typical field was randomly selected from each section of the quadruple in the image. The optical density (OD) for each selected field was measured, and the OD of stained epithelium or connective tissue in each tissue section was calculated to be the average of the OD measured for all four selected fields in the image.

In situ hybridization

KGF expression in tissue sections was detected using KGF *in situ* hybridization/detection kit (BosterBio, Wuhan, China) according to the manufacturer's instructions. The sequences for the anti-sense probes were 5'-CACAC AAGAA GTTAT GATTA CATGG AAGGA GGGGA-3' and 5'-GCCTT AAATC AAAAG GGGAT TCCTG TAAGA GGAAA-3'. The tissue sections were incubated with pepsin freshly diluted in 3% citric acid at 37°C for 30 min to expose mRNA fragments, and then washed with PBS and distilled water. The sections were fixed in 1% paraformaldehyde, 0.1 M PBS (pH 7.2-7.6) and 0.1% DEPC at room temperature for 10 min, washed with distilled water, and pre-incubated with pre-hybridization solution at 38-42°C for 2 h. The sec-

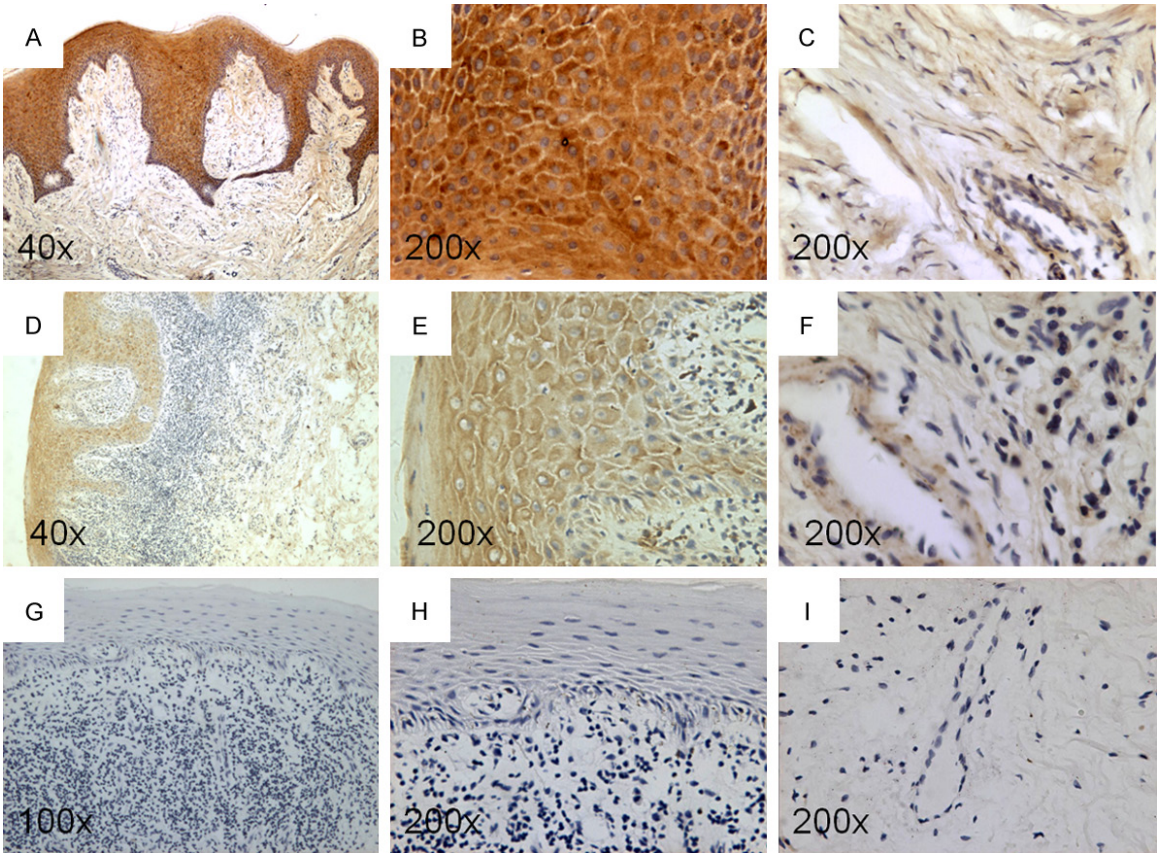

Figure 1. Typical images of immunohistochemical staining of KGF protein. A: Normal oral mucosa (magnification: 40×); B: Epithelium of normal oral mucosa (magnification: 200×); C: Connective tissue of normal oral mucosa (magnification: 200×); D: OLP (magnification: 40×); E: Epithelium of OLP (magnification: 200×); F: Connective tissue of OLP (magnification: 200×); G: Negative control of OLP (magnification: 100×); H: Negative control of epithelium in OLP (magnification: 200×); I: Negative control of connective tissue in OLP (magnification: 200×).

tions were then incubated with hybridization solution at 38-42°C overnight, and subsequently washed at 37°C with SSC (2×) twice (5 min each), SSC (0.5×) once (15 min), and SSC (0.2×) once (15 min). The sections were incubated with biotinylated mouse anti-digoxin at 37°C for 1 h, and washed with PBS for 4 times (5 min each). The sections were incubated with SABC at 37°C for 20 min, and diaminobenzidion (DAB) was used to visualize the sections. For the negative control, the sections were incubated with pre-hybridization solution without the probe) instead of hybridization solution containing the probe.

Evaluation of in situ hybridization

Under microscope, five fields were randomly selected from each stained tissue to count positively stained cells, and the average number of positively stained cells was calculated. The positively stained cells were further classified into fibroblast (spindle-shaped with oval nucleus) and vascular endothelial cells (flat and located at the vessel wall). Photo images were taken for sections stained under a microscope, and analyzed using image analysis system to semi-quantify the expression of KGF mRNA. One typical field was randomly selected from each section of the quadruple in the image. The optical density (OD) for each selected field was measured and the OD of the stained connective tissue in each tissue section was calculated to be the average of the OD measured for all four selected fields in the image.

Statistical analysis

Data were expressed as mean±standard deviation. The *t*-test in SPSS software was used to analyze the differences between groups. $P< 0.05$ was considered as significant difference.

Table 1. Immunoreactivity of KGF in OLP and normal mucosa tissues

Group	Average OD for positively stained epithelium	Average OD for positively stained connective tissues
Normal oral mucosa	0.37±0.04	0.17±0.05
OLP	0.21±0.05	0.16±0.05

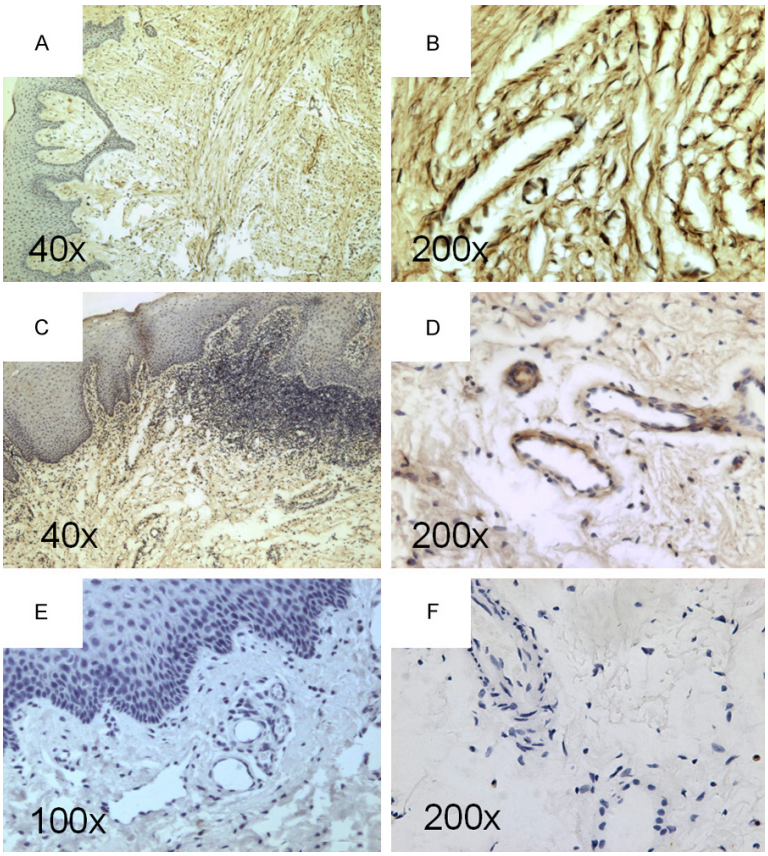

Figure 2. Typical images of in situ staining of KGF mRNA. A: Normal oral mucosa (magnification: 40×); B: Connective tissue of normal oral mucosa (magnification: 200×); C: OLP (magnification: 40×); D: Connective tissue of OLP (magnification: 200×); E: Negative control of normal oral mucosa (magnification: 100×); F: Negative control of connective tissue in normal oral mucosa (magnification: 200×).

Results

Expression of KGF in OLP specimens

In normal oral mucosa, KGF protein was expressed in the epithelium and connective tissues. This was indicated by strong staining in the cytoplasm and the nucleus of the epithelial cells and positive staining in the cytoplasm and/or the nucleus of connective tissue cells (**Figure 1A-C**). In the epithelium, the expression of KGF was relatively weak in the cytoplasm of the basal layer but was increased in the cytoplasm of prickle layer. Moreover, the keratinized layer showed brown staining. In the connective tissue underlying the epithelium, strong staining of KGF was observed in the cytoplasm of some fibroblast and vascular endothelial cells (**Figure 1C**).

In OLP specimens, the expression pattern of KGF protein in the epithelium was similar to that in normal oral mucosa (**Figure 1D**, **1E**). However, the intensity of staining decreased compared to normal oral mucosa. Positive expression of KGF was mainly observed in the cytoplasm of prickle layer, while the cytoplasm of basal layer had weak staining of KGF. In the connective tissue underlying the epithelium, positive staining (brown) was observed in the cytoplasm of some fibroblasts and vascular endothelial cells. OLP tissues had more positively stained vascular endothelia cells in the connective tissues compared to normal oral mucosa. However, the staining intensity was much weaker than that observed in normal oral mucosa. No brown staining was observed in the negative control group of the OLP samples (**Figure 1G-I**). Semi-quantitative analysis showed that the average ODs of KGF staining for the epithelium and connective tissues of OLP were both significantly lower than those of normal oral mucosa ($P<0.01$ for epithelium and $P<0.05$ for connective tissues) (**Table 1**).

Expression of KGF mRNA in OLP specimens

We observed no positive expression of KGF mRNA in the epithelium of both normal oral mucosa and OLP. However, positive staining (brown) was observed in the cytoplasm of some fibroblasts and endothelial cells in the connec-

Table 2. Expression of KGF mRNA in OLP and normal mucosa tissues

Group	Average OD for positively stained connective tissues	Cells positive for KGF mRNA per field
Normal oral mucosa	0.21±0.05	10.83±3.18
OLP	0.19±0.03	8.67±2.65

tive tissues of normal oral mucosa (**Figure 2A**, **2B**), and of OLP tissues (**Figure 2C**, **2D**). The negative control of normal oral mucosa showed no positive expression of KGF mRNA in both the epithelium and connective tissues (**Figure 2E**, **2F**). Quantitative analysis showed that fewer cells were positive for KGF mRNA in OLP than in normal oral mucosa (P<0.05). Furthermore, the average OD of the stained connective tissues was significantly lower in OLP than in normal oral mucosa (P<0.05) (**Table 2**).

Expression of KGFR in OLP specimens

In normal oral mucosa, KGFR were positively expressed in cell membranes of all epithelial cells and the cytoplasm of some epithelial cells. This was indicated by very strong staining (dark brown) in the basal layer and strong staining (brown) in the other layers (**Figure 3A**, **3B**). Brown staining was also observed in the cell membranes and cytoplasm of some vascular endothelia cells in the connective tissues (**Figure 3C**), but no brown staining was observed in the fibroblasts in the connective tissues, indicating that KGFR protein is not expressed in these fibroblasts.

In the epithelium of OLP, KGFR was only positively stained in the cell membranes and the cytoplasm of cells located in the basal layer and prickle layer (**Figure 3D**, **3E**). In the connective tissues of OLP, a large portion of vascular endothelia cells were positively stained in brown and the staining intensity was much stronger than in the connective tissues of normal oral mucosa. However, no brown staining was observed in the fibroblasts in the connective tissues of OLP, indicating that KGFR is not expressed in these fibroblasts. No brown staining was observed in the negative control group of OLP samples (**Figure 3G-I**). Semi-quantitative analysis showed that the average OD of KGFR staining was significantly lower in the epithelium of OLP than in that of normal oral mucosa (P<0.01), but the average OD of KGFR staining was significantly higher in connective tissue of OLP than in that of normal oral mucosa (P<0.05) (**Table 3**).

Discussion

KGF is secreted by mesenchymal-derived cells and KGFR is usually expressed on the cell membrane of epithelial cells. KGF plays an important role in the re-epithelialisation after injury through its binding to KGFR [14-16]. In this study, we investigated the expression of KGF and KGFR in OLP in order to determine the role of KGF and KGFR in the development and progression of OLP. Immunohistochemistry was used to characterize protein expression of KGF and KGFR in OLP biopsy tissues, and *in situ* hybridization was used to detect and locate the expression of KGF mRNA in OLP. Normal oral mucosa was used as the control.

Immunohistochemistry showed that the expression patterns of KGF in the epithelium were similar in OLP biopsy tissues and normal oral mucosa. However, the protein expression of KGF in the epithelium of OLP was significantly lower than that in normal oral mucosa. In the connective tissue underlying the epithelium, OLP contained more vascular endothelia cells expressing KGF protein compared to normal oral mucosa, although KGF expression intensity was much weaker than in normal oral mucosa.

It is well-known that the KGF protein is secreted by mesenchymal-derived cells such as fibroblasts. Our observation of KGF protein expression in the epithelium of both normal oral mucosa and OLP seemed to be controversy to earlier report that KGF protein was not expressed in the epithelium of other tissues [17]. Therefore, we further performed *in situ* hybridization to locate KGF mRNA in both normal oral mucosa and OLP. Interesting, KGF mRNA was only detected in the connective tissues but not in the epithelium for both normal oral mucosa and OLP. Apparently, vascular endothelial cells and fibroblasts in normal oral mucosa and OLP secreted KGF protein, which was diffused into the epithelium and subsequently internalized into the cytoplasm of epithelial cells [18].

Previous studies have shown increased expression of KGF in inflammatory tissues [14, 19].

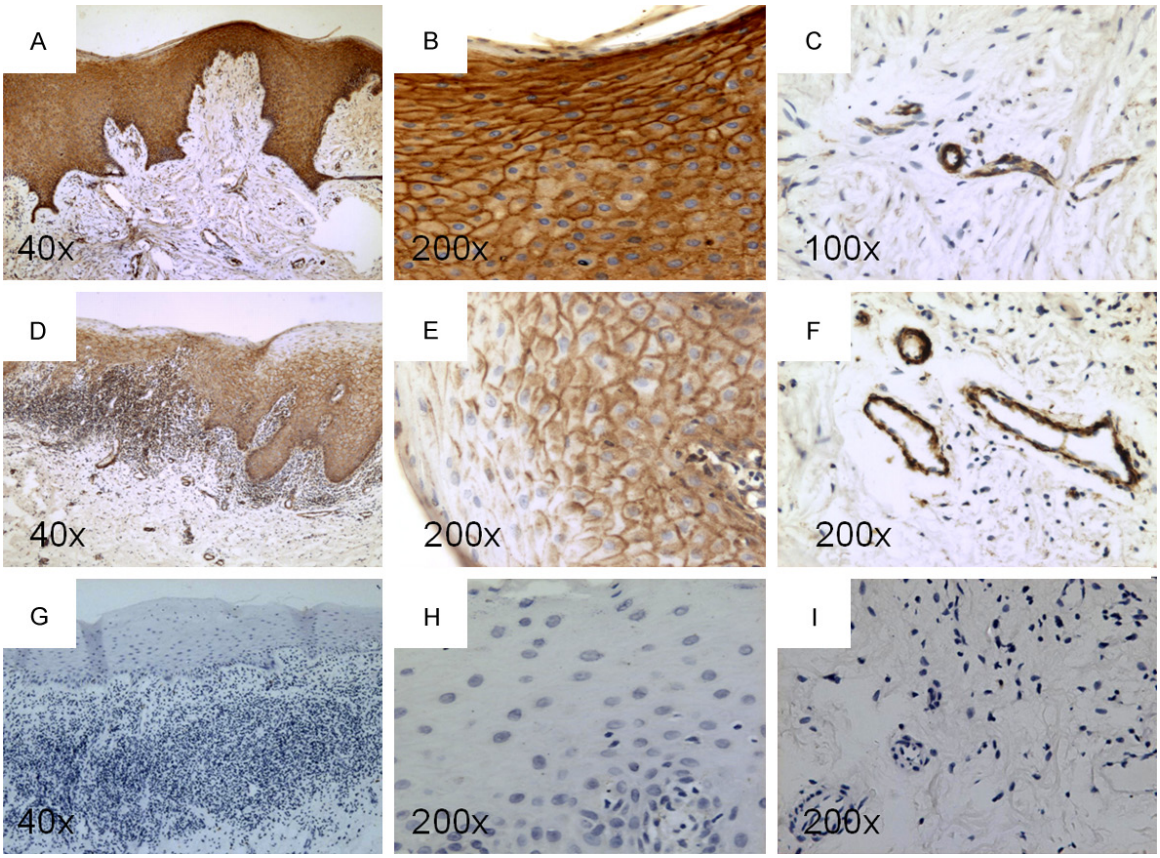

Figure 3. Typical images of immunohistochemical staining of KGFR protein. A: Normal oral mucosa (magnification: 40×); B: Epithelium of normal oral mucosa (magnification: 200×); C: Connective tissue of normal oral mucosa (magnification: 200×); D: OLP (magnification: 40×); E: Eepithelium of OLP (magnification: 200×); F: Connective tissue of OLP (magnification: 200×); G: Negative control of OLP (magnification: 40×); H: Negative control of epithelium in OLP (magnification: 200×); I: Negative control of connective tissue in OLP (magnification: 200×).

Table 3. Immunoreactivity of KGFR in OLP and normal mucosa tissues

Group	Average OD for positively stained epithelium	Average OD for positively stained connective tissues
Normal oral mucosa	0.36±0.04	0.15±0.03
OLP	0.26±0.04	0.19±0.04

However, our study showed that the expression of KGF was decreased in the biopsy tissues of OLP compared to normal oral mucosa. This may be attributed to the unique features of OLP. In OLP, the basal layer has liquefaction and the lamina propria has infiltration of T lymphocytes. It has been reported that the infiltrating T lymphocytes in the OLP lesion are the activated CD4+ and CD8+ T cells [20]. These two important subsets of T cells and the cytokines secreted by these activated T cells have a major impact on the development and progression of OLP lesions [20]. We speculate that some unknown cytokines may downregulate the expression of KGF in OLP, but further investigations are needed to test our hypothesis.

Next, we employed immunohistochemistry to detect KGFR in normal oral mucosa and OLP biopsy tissues. KGFR was expressed throughout the epithelium of normal oral mucosa. However, in the epithelium of OLP, KGFR was only expressed in the basal layer and prickle layer. KGFR was not detected at the most top of epithelium in OLP. The observation of KGFR staining in the whole epithelium of normal oral mucosa was consistent with earlier report on other types of tissues. The fact that KGFR was not expressed in the most top of epithelium in OLP might contribute to the persistence and further progression of OLP. Interestingly, although KGF mRNA was expressed in a large portion of vascular endo-

thelia cells in connective tissues of OLP, the expression intensity determined by *in situ* hybridization was much lower than the corresponding intensity in normal oral mucosa. Moreover, KGFR was not expressed in the fibroblasts in connective tissues of both OLP and normal oral mucosa.

All these observations suggest that KGF is not produced and secreted by epithelial cells in normal oral mucosa and OLP, consistent with the results of other reports [13, 14, 18]. Instead, KGF is secreted by fibroblasts and vascular endothelial cells, which may regulate the growth of connective tissue vascular endothelial cells and fibroblasts in an autocrine manner and stimulate epithelial cell proliferation in a paracrine manner. Taken together, this study demonstrates significant differences in the expression intensity and distribution of both KGF and KGFR between OLP and normal oral mucosa tissues and suggests that KGF and its receptor KGFR may play an important role in the development and progression of OLP.

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Disclosure of conflict of interest

None.

Address correspondence to: Xiang-Min Qi, College of Stomatology, Shandong University, 44 Wenhua West Road, Jinan 250012, Shandong Province, China. E-mail: qidoc105@sdu.edu.cn

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