

Characteristics of benign lymphadenosis of oral mucosa

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Abstract

AIM: To investigate the pathological characteristics and carcinogenesis mechanism of benign lymphadenosis of oral mucosa (BLOM).

METHODS: The expressions of Ki-67, CD34 and apoptosis were evaluated by immunohistochemical SP staining in 64 paraffin-embedded tissue samples. Of them, 9 were from BLOM with dysplasia, 15 from BLOM without dysplasia, 15 from oral squamous cell carcinoma (OSCC), 15 from oral precancerosis, and 10 from normal tissues. Cell proliferation, apoptosis and angiogenesis of tissue samples were also analyzed.

RESULTS: The expression of Ki-67 in BLOM with dysplasia, oral precancerosis and OSCC was significantly higher than in BLOM without dysplasia and normal mucosa. The microvascular density (MVD) in BLOM with and without dysplasia, oral precancerosis, and OSCC was significantly higher than in normal mucosa. Apoptosis in BLOM and oral precancerosis was significantly higher than in OSCC and normal mucosa.

CONCLUSION: Benign lymphadenosis of oral mucosa has potentialities of cancerization.

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Key words: Benign lymphadenosis of oral mucosa; Ki-67; Microvascular density; Apoptosis

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INTRODUCTION

Benign lymphadenosis of oral mucosa (BLOM), which could be commonly found clinically, usually occurs in the mucosae of lips, cheeks, tongue or gingiva. Sometimes BLOM may develop in all of the oral mucosa and accompan-

other oral diseases. As far as we know, few reports have ever mentioned BLOM, and there has been no international definition about it. Most researchers considered it as a kind of reactive proliferation. Since 1970s, our department has been studying BLOM from the aspects of morphology and immunopathology. Ultrastructural changes of this disease is similar to that of leukoplakia^[1,2]. Clinically, BLOM has malignant trend^[3,4]. At present, it is regarded as a kind of precancerous lesion^[5]. To investigate the pathologic characteristics and carcinogenesis mechanism of BLOM we studied cell proliferation, cell apoptosis and angiogenesis of different types of oral mucosa diseases, including BLOM with dysplasia and without dysplasia, OSCC, oral precancerous and normal tissues by immunohistochemistry method.

MATERIALS AND METHODS

Materials

A total of 64 paraffin-embedded tissue samples were from Pathological Research Laboratory, School of Stomatology, Peking University. Of them, 9 were from BLOM with dysplasia, 15 from BLOM without dysplasia, 15 from oral OSCC (8 in stage III, 7 in stage I or II), 15 from oral precancerosis, and 10 from normal tissues around the neoplasms. Streptavidin-biotin-immunoperoxidase system kit was purchased from Fuzhou Maixin Immunotech Corporation, China; mouse monoclonal antihuman Ki-67 antibody was provided by Beijing Immunotech Zhonghshan Corporation, China; and cell death detection kit was from Boehringer Mannheim, Germany.

Immunohistochemistry protocol for Ki-67 and CD34

Five micrometer thick sections were dewaxed with xylene, and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by immersion of slides in methanol with 0.03% hydrogen peroxide for 10 min. In order to retrieve antigenicity, these sections were then heated in a pressure cooker in 10 mmol/L citrate buffer (pH 6, 90 s after water boiled). After washing in distilled water, these sections were rinsed in phosphate-buffered saline (PBS) and incubated for 60 min at room temperature with CD34 or Ki-67 mAb. Reaction was visualized with a streptavidin-biotin-immunoperoxidase system using diaminobenzidine (DAB) as chromogen. All sections were then counterstained with hematoxylin.

Positive staining for CD34 or Ki-67 was regarded as positive control. For negative control, the primary antibodies were replaced with PBS. Staining was measured as the percentage of positively stained nuclei (Ki-67, labeling index, LI) or cytoplasm (CD34, microvascular density, MVD). Ki-67 reaction was scored at $\times 400$ magnification, a total of 1 000 epithelial cells (normal tissue and BLOM) or tumor cells

(OSCC) were evaluated. Reactive nuclei were considered positive, regardless of staining intensity. The percentage of Ki-67 positive was then calculated and positivity of lymphocytes was not evaluated. Criteria for counting included individual or cluster of cells with or without lumen positivity for CD34 immunoreactivity. Areas of inflammation, necrosis, and fibrosis were excluded. For determination of mean MVD, sections were first observed under low power ($\times 200$) for areas with dense vascularity. These were then counted in three different fields under high power ($\times 400$). The average counts were recorded as MVD for each case.

TUNEL protocol for apoptosis evaluation

Detection of apoptosis in tissue was determined by TUNEL assay, employing *in situ*. The procedure is based on the detection of chromatin DNA strand breaks, the most characteristic biochemical feature of apoptosis caused by activation of endogenous nuclease activity.

Sections were dewaxed, rehydrated, and washed in distilled water for 10 min. The nuclei in tissue sections were stripped from proteins with 4% pepsin diluted in sterile buffer (10 mm Tris-HCl, pH 7.4) for 60 min at 37 °C, followed by quenching of endogenous peroxidase. The sections were rinsed in 50 μ L of a label mixture containing the labeled nucleotide and enzyme, and incubated in a humidified chamber for 60 min at 37 °C. After washing with PBS, the sections were incubated for 30 min at 37 °C with alkaline phosphatase reagent. The substrate reaction was developed using diethyl chlorophosphate (DECP) and counterstained with nuclei fast red.

Positive staining for CD34 or Ki-67 was regarded as positive control. For negative control, the primary antibodies were replaced with PBS. A cell was considered apoptotic only when unequivocal nuclear labeling was observed in the areas with no inflammation. Cells exhibiting necrotic nuclear karyorrhexis as well as those in necrotic foci were excluded. TUNEL reactivity was always estimated in relation to characteristic histological criteria of apoptosis, including overall shrinkage, homogeneously dark basophilic nuclei, presence of nuclear fragments, sharply delineated cell borders and homogenous eosinophilic cytoplasm. To evaluate the different rates of TUNEL reactivity in each sample, 1 000 cells were counted randomly under high power ($\times 400$) and the percentage of positively stained nuclei (AI) was calculated.

Statistical analysis

The expression of Ki-67, MVD and extent of TUNEL reactive nuclei in different tissue samples was analyzed by *t*-test and χ^2 -test using SPSS10.0 for Windows. $P < 0.05$ was considered significant.

RESULTS

The histological characteristics of BLOM is that lymphoid follicles or dense lymphocytes foci could be seen in the basal cell layer. The expression of Ki-67, which was stained brown in nuclei, could be found in normal tissues, BLOM, BLOM with dysplasia, precancerosis and OSCC. Positive cells distributed dispersedly in epithelium of normal tissues and BLOM without dysplasia (Figure 1A). Meanwhile, many cells appeared positive in lymphoid follicles. The expression of Ki-67 obviously increased in BLOM with dysplasia and in some samples Ki-67 positively stained nuclei were seen in all layers of epithelium (Figure 1B). The expression of Ki-67 also increased in precancerous tissues and OSCC, in the center of which positive cells distributed diffusely (Figure 1C). The positive rates of Ki-67 expression in different groups are shown in Table 1.

Table 1 The positive rates of Ki-67 expression in different groups (mean \pm SD)

Group	Ki-67 positive rate (%)
Normal	14.19 \pm 6.18
BLOM	22.29 \pm 11.39
BLOM with dysplasia	45.42 \pm 9.89 *
Precancerosis	47.15 \pm 6.84 **
OSCC	45.80 \pm 9.00 **

* $P < 0.05$ vs normal, ** $P < 0.05$ vs BLOM, *** $P < 0.05$ vs BLOM with dysplasia.

The expression of CD34 was positive in all samples, and distributed punctately, located in the cytoplasm of vascular endotheliocytes (Figure 2A). Only a few microvessels in the connective tissues of normal mucosa could be seen. But in BLOM, BLOM with dysplasia and precancerosis, their number increased obviously (Figure 2B). A great amount of microvessels also could be found in OSCC. MVD values in different groups are shown in Table 2.

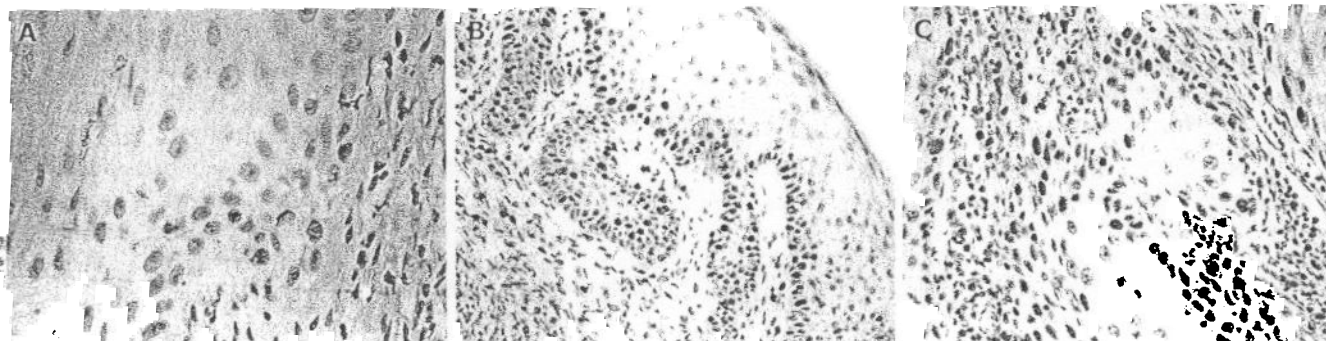


Figure 1 Ki-67 expression in A: BLOM without dysplasia (IHC $\times 400$), B: BLOM with dysplasia (IHC $\times 200$), and C: oral cancer (IHC $\times 200$).

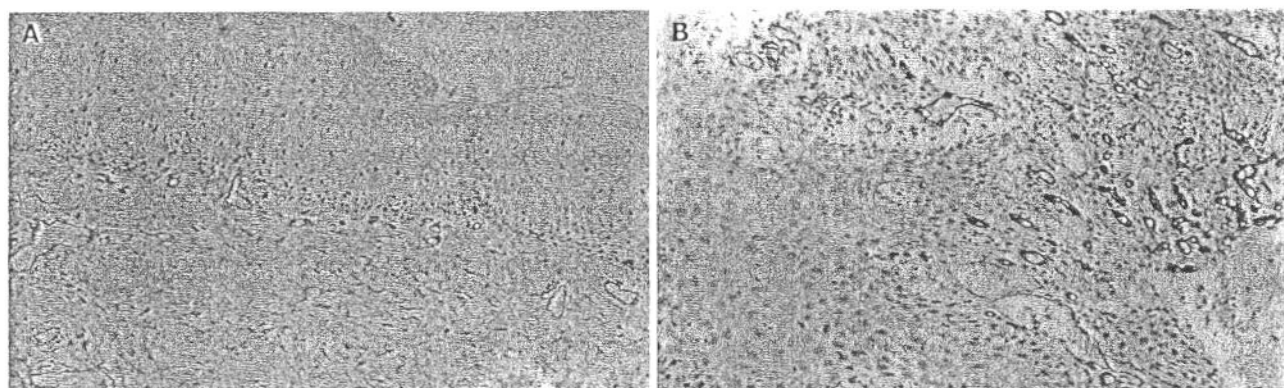


Figure 2 CD34 expression in A: BLOM without dysplasia (IHC $\times 100$) and

B: BLOM with dysplasia (IHC $\times 100$).

Table 2 MVD in different groups (mean \pm SD)

Group	MVD value
Normal	14.31 \pm 2.71
BLOM	44.81 \pm 10.20
BLOM with dysplasia	43.89 \pm 8.78*
Precancerosis	45.28 \pm 11.09* ^c
OSCC	62.73 \pm 8.13* ^{c,c}

* $P < 0.05$ vs normal, * $P < 0.05$ vs BLOM, * $P < 0.05$ vs BLOM with dysplasia.

Cell apoptosis was found in 2/10 normal tissues, 10/15 BLOM, 8/9 BLOM with dysplasia, 13/15 precancerosis and 8/15 OSCC. Apoptosis was seldom seen in the stromal layer of normal mucosa, but it increased obviously in BLOM, BLOM with dysplasia and precancerosis (Figure 3). At the same time, apoptosis could be found in connective tissues of BLOM, BLOM with dysplasia and precancerosis. The positive rate of apoptosis in OSCC was significantly lower than that in other groups except the normal mucosa.

DISCUSSION

Ki-67 is an acknowledged marker that indicates the proliferation capacity of cells, and many studies demonstrated that it was overexpressed in oral premalignancy and oral cancer^[6-9]. Tabor *et al.*^[10], studied 43 samples from patients who had undergone resection of their squamous cell carcinoma in oral cavity/oropharynx. With the consensus score, 12 samples were classified as normal, and 31 as dysplastic (21 mild, 6 moderate, and 4 severe). They found that there was a high coincidence between loss of heterozygosity (LOH) and the expression of Ki-67. The Ki-67 index in LOH-positive cases was significantly higher than that in LOH-negative cases. It was suggested that the expression of Ki-67 was correlated with other factors (beta-catenin, p53R2, *etc*)^[11-14]. Other scholars found there was a significant correlation between Ki-67 LI, stroma/tumor proportion and the degree of keratinization^[15,16]. In the present study, overexpression was found in the epithelium of BLOM with dysplasia, precancerosis and OSCC. But the expression in epithelium of BLOM without dysplasia was similar to that in the normal mucosa and there was an abundant expression in lymphoid follicles. This suggested that the epithelial cells of BLOM without dysplasia had not undergone genetic

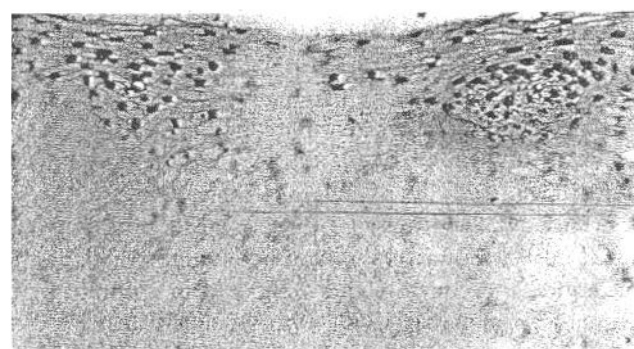


Figure 3 Apoptosis in BLOM with dysplasia (TUNEL $\times 200$).

transformation, and the cells of germinal center had a strong proliferation capacity. In the connective tissues of BLOM and BLOM with dysplasia, a great amount of positive cells could be found in the center of lymphoid follicles. However, no obvious difference was revealed. This indicated that the center had a strong capacity of proliferation, but not a malignant trend, because the proliferation of lymphocytes was a kind of benign and reactive proliferation. Why we considered BLOM benign was also due to this reason. The number of Ki-67 positive cells and the staining in BLOM without dysplasia had no obvious difference from those in normal mucosa, indicating that epithelial cells did not appear abnormal. In the epithelium of BLOM with dysplasia and precancerosis, the number of positive cells obviously increased and the distribution of positive cells extended from the lower basal layer to the upper basal layer and prickle cell layer. In some samples, positive cells were distributed in all the layers of epithelium. This indicated that the proliferation capacity of basal cells was strengthened. Angiogenesis is a process involved in the development of new blood vessels, by the division and migration of existing vasculature^[17-20]. It is an important mechanism to sustain tumorigenic potential in neoplasm and in dysplastic lesions, indicating abundant capillaries leading to intense vascularization in cancer. Therefore, evaluation of angiogenesis may be of prognostic significance in the assessment of tumor progression. Iwasa *et al.*^[21], suggested that negative DNA instability lesions had a higher MVD in proliferating cell nuclear antigen (PCNA)-positive cells than positive DNA instability lesions.

Other scholars proved that in non-small cell lung cancer and oral cancer, the mean AIs of the lower-MVD and the higher-MVD patients were significantly different^[22-24]. Our study has confirmed that insufficient angiogenesis could accelerate apoptotic cell death. The angiogenesis in BLOM with and without dysplasia, and in precancerosis was significantly higher than in normal tissue, and new blood vessels were more abundant. Sauter *et al.*, suggested that the angiogenesis in precancerosis was between oral normal tissue and OSCC^[17].

Apoptosis is a programmed cell death under genetic control. A fine balance between the cell proliferation and cell death is very important to normal development and maintenance of tissue size and shape. In another word, the imbalance between the positive and negative regulation of cell growth could cause a series of diseases including neoplasm. In neoplasm, the disorder of homeostasis may result in the development of tumor^[25-27]. Some studies showed that epidermal growth factor receptor blockade, cyclin dependent kinase inhibitor and 5-FU could induce cell apoptosis^[28-30]. Other scholars also found that the overexpression of p27(Kip1) and retinoic acid receptor could induce growth arrest and apoptosis in an oral cancer cell line^[31,32]. Results of the present study indicated that the apoptosis was significantly higher in BLOM, BLOM without dysplasia, and precancerosis than in normal tissue. However, it was lower in tumor tissue (OSCC). This suggested that the body tried to control the balance of cell number by increasing cell apoptosis with the increase in cell proliferation. However if the body fails, tumor develops. Similar result could be found in the study of Guan *et al.*^[33,34].

Previous studies also showed that BLOM should be regarded as a kind of precancerous lesion. In the present study, the cell proliferation, angiogenesis and apoptosis of BLOM were similar to that of precancerosis, and in BLOM without dysplasia the angiogenesis was significantly higher than that in normal oral tissue. But the expression of Ki-67 was similar to that in normal tissue. Therefore, the changes of cell proliferation, cell apoptosis, and angiogenesis in connective tissue were earlier than in epithelium; BLOM had the potential of carcinogenesis. Many factors may induce the proliferation of cancer cells, enhance their survival by escape of apoptosis, and provide abundant nutrients during early-stage carcinogenesis of oral precancerous lesions. Comparison of gene expression profiles between head and neck squamous cell carcinoma and normal tissues showed that altered expression levels of genes were involved in the control of cell growth and differentiation, angiogenesis, apoptosis, cell cycle, and signaling, most of which had not been previously described in head and neck squamous cell carcinoma^[35]. Which factor contributes to cell proliferation, and inhibits cell apoptosis during the carcinogenesis of BLOM is still a subject to be investigated in future.

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