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Comparison of Macrophage Frequency in Common Oral Cavity Reactive Lesions

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ABSTRACT

Background and Objective: Oral reactive lesions are the most common oral disease. Recent studies have insisted on the presence of macrophages as a crucial component of oral reactive lesions' microenvironment. The aim of the present study was to compare the macrophages frequency in three common oral reactive lesions by marker CD68 in order to determine the relationship between the frequency of macrophages and the type of the lesion.

Methods: In this cross-sectional analytic study, 20 samples of each of the three groups of pyogenic granuloma (PG), irritation fibroma (IF), and peripheral ossifying fibroma (POF), which were prepared by excisional biopsy, were retrieved from the Department of Oral Pathology of Isfahan Dental School. Clinical information including age, gender, and location of the lesions was extracted. In order to determine the frequency of macrophages, immunohistochemical staining for CD68 was performed and the expression level was determined by two oral pathologists with a light microscope blindly and simultaneously based on the SID (Staining intensity distribution) index.

Findings: The mean SID±SD indices for PG, IF and POF were 9.15±3.86, 2.2±3.69 and 6.4±4.55, which showed a statistically significant difference regarding the type of the lesions ($p<0.001$). Furthermore, there was a significant difference between CD68 expression of PG with IF, PG with POF and POF with ($p<0.001$, $p=0.027$, $p<0.001$).

Conclusion: According to the results of this study, macrophages are present as an important part of PG, POF and IF microenvironments in different levels. Therefore, they might be associated with the development of the disease, as well as being used in identifying the type of oral lesion.

Keywords: *Macrophage, Oral, CD68, Pathology*.

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Introduction

Oral reactive lesions are the most common oral disease, which could be observed in all parts of the oral cavity, with the highest frequency in the gingiva and alveolar mucosa. They are connective tissue hyperplasia formed in response to local and chronic irritations or low-grade trauma (1). Dental plaque, calculus, food accumulation, faulty restoration, hormonal fluctuations and iatrogenic damage such as broken teeth, overhanging repair and ill-fitting dental/oral appliances are reported to be associated with the formation of the lesions (2). However, the exact pathogenesis and mechanism of the lesion's development is yet to be fully understood (1, 3, 4). Oral reactive lesions have a high potential for recurrence if not completely removed or if the underlying irritating factors are not totally eliminated (4, 5). Recent studies suggest that removing the lesions by CO2 laser along with oral and dental hygiene and regular follow-ups can be useful in reducing the recurrence rate (5, 6).

Although oral reactive lesions are benign, their clinical appearance could mimic the neoplastic types (2). In addition, they show diverse clinical and histopathological features which can further complicate the diagnosis process (1). Therefore, understanding the mechanism of the lesion's formation could aid in formulating the diagnosis. In terms of histopathology, these lesions can be divided into vascular types (such as PG) and fibrous types (such as IF and POF) (7).

Recent studies on the pathogenesis of various lesions have focused on the microenvironmental factors (components of the stroma such as fibroblasts and immune cells) and inflammatory processes. Interactions between different cells such as monocytes, lymphocytes, fibroblasts, and epithelial cells with different kinetics are suggested to be the key factor in developing reactive lesions. Inflammatory cells synergize and cooperate with stromal and epithelial cells by local production of stimulating factors. Cellular communication occurs through a complex network of intercellular signaling pathways that causes the disease progression (8, 9). Macrophages are the primary antigen-presenting cells in the lesions' microenvironment that affect the pathogenesis of diseases through the production of inflammatory mediators and chemotactic factors. They could be immunohistochemically detected by their surface marker called CD68 (10, 11).

Tumor-associated macrophages (TAMs) are an important part of the tumor microenvironment and are considered as one of the main targets as immune regulators in the treatment of lesions. TAMs can cause tumorigenic and antitumor effects due to separate polarization in M1 and M2 chains. M1 macrophages release proinflammatory cytokines and can strengthen the responses of Th1 and destroy tumor tissue. On the other hand, activation of M2 macrophages causes Th2 responses and promotes regeneration, angiogenesis, suppression of the immune system, and tumor growth. CD68 is one of the identifying markers of TAM (12). Macrophages have the potential to exert various effects and produce various cytokines. In fact, apart from inflammation and stimulation of the immune system, macrophages have an important anti-inflammatory role and can reduce the immune response through the release of cytokines (12).

Considering that in order to have a suitable treatment, it is very important to know the possible origin and nature of the cells; therefore, new treatment methods without surgery and creating defects in the tissue, especially in patients with special systemic conditions such as pregnancy, are suggested by many researchers. Many studies have been conducted on the role of macrophages in many oral reactive lesions, especially in central giant cell granuloma (CGCG) and peripheral giant cell granuloma (PGCG), but few studies have been conducted about other common fibrotic lesions, including IF. Macrophages have the potential to exert various effects and produce various cytokines and have an important anti-inflammatory role and can reduce the immune response; therefore, it seems necessary to identify the effects and compare them in common lesions. Considering the prevalence of reactive oral lesions in the Iranian population and

the importance of these lesions due to their significant growth potential and the possibility of their recurrence, the need for the present study has been noted. Considering the prevalence of reactive oral lesions and the importance of these lesions due to their significant growth potential and the possibility of their recurrence, as well as their possible clinical similarity with neoplastic lesions, a correct diagnostic method is necessary in time to provide optimal treatment. Considering these cases, the necessity of the present study was considered and it was conducted with the aim of comparing the frequency of macrophages by analyzing the level of CD68 expression in three cases of the most common reactive oral lesions.

Methods

This cross-sectional study was approved by the Research and Ethics Committee of Isfahan University of Medical Sciences with ethics code IR.MUI.RESEARCH.REC.1400.186. The study had a total of 60 samples (this number of samples is determined based on the formula and opinion of the statistics consultant and can be generalized to the society), including 20 samples of IF, 20 samples of POF, and 20 samples of PG retrieved from the Department of Oral Pathology of Isfahan Dental School. Samples that were prepared under excisional biopsy and their paraffin blocks had sufficient and quality tissues for immunohistochemical staining (IHC) were selected and the definitive diagnosis of the type of lesion was confirmed by two oral pathologists. Samples with incisional biopsy, lacking the necessary clinical information, lacking appropriate block quality and suspicious for the diagnosis and insufficient tissue for specific staining were excluded from the study. Furthermore, in samples with recurrence, the original sample was studied. In addition, it was tried to homogenize the investigated groups regarding gender in order to eliminate the effect of this variable. In addition, in the case of lobular PG, which is sometimes considered as capillary hemangioma, samples were excluded from the study. All procedures performed in the study were in accordance with the ethical standards of the national research committee and with the 1964 Helsinki Declaration and its later amendments and comparable ethical standards.

For the detection of macrophages in tissue samples, immunohistochemical staining for CD68 was done. The following steps were taken according to the manufacturer guidelines:

1.Preparation of a 3-micron section of blocks
2.Placing the slides at 74°C (50 minutes) for paraffin removal
3.Placing the slides in two containers of xylene (5 minutes) and two containers of 96% alcohol (2 minutes) for antigen retrieval
4.Placing the slides in citrate (citral / Hce buffer, 10 mmol) for one hour in Ben Marie, pH: 6
5.Placing the slides in saline phosphate buffer and then 3% oxygenated water for 10 minutes
6.Rinsing with saline phosphate buffer
7.Pouring monoclonal mouse anti-human CD68 primary antibody (Dako Cytomation, Glostrup, Denmark) on the slides for 60 minutes
8.Placing the slides in saline phosphate buffer and then in Envision + Qual Link solution for one hour
9.Application of secondary antibody and the Streptavidin- Biotin- Peroxidase HRP complex (Envision/ HRP)
10.Placing slides diaminobenzidine hydrochloride (DAB) for 3-5 minutes (chromogenic)
11.Washing with distilled water
12.Inserting slides into Hematoxylin
13.Dehydration steps (taken in alcohol)
14.Placing the slide and gluing the slides with special IHC glue.

To analyze immunohistochemical staining, all slides were examined by two oral pathologists with a light microscope (Olympus BX41TF, Tokyo, Japan) blindly and simultaneously at the magnification rate of ×400 in 10 random fields to determine CD68 expression. Tissue samples with cytoplasmic staining, nuclear staining, or both in brown background cells were considered positive (13). Macrophages cells were evaluated using a semiquantitative scale: 0 (negative, without any immunostained cells), +1 (1% to 25% immunostained), +2 (26% to 50%), +3 (51% to 75%) and +4 (>75%). Furthermore, staining intensity was evaluated by the following scores: 0 (without immunostained cells), +1 (very low staining), +2 (moderate), +3 (moderate to high), and +4 (high). Staining intensity distribution (SID) score was calculated by multiplying the distribution by staining intensity (14).

The data obtained from clinicopathological and immunohistochemical studies were analyzed by SPSS Version 24 (SPSS, Inc., Chicago, IL). Categorical variables were reported as frequency and percentage, and quantitative variables as mean±standard deviation (SD). Kruskal-Wallis and Mann-Whitney tests were used to compare the CD68 expression rate between the three types of lesions. Distribution of location and age were compared between the groups using Fisher's exact and ANOVA tests, respectfully. $P<0.05$ was considered significant.

Results

In this study, the highest mean age was seen in patients with irritation fibroma and then POF and PG respectively (mean±SD= 46±15.55, 42±17.96, 39.15±14.79), but there was no significant difference in the mean age between the studied samples (Table 1). The most common location of occurrence for PG and POF was alveolar mucosa and for IF was buccal mucosa. There was a significant difference between PG with IF, PG with POF and IF with POF based on location ($p=0.021$, $p=0.006$, $p<0.001$) (Table 2). The mean SID±SD indices for PG, IF and POF were 9.15±3.86, 2.2±3.69 and 6.4±4.55, respectively. CD68 expression rate was significantly associated with the type of lesion ($p<0.001$) (Table 1). Furthermore, there was a significant difference between PG with IF, PG with POF and IF with POF based on CD68 expression ($p<0.001$, $p=0.027$, $p<0.001$) (Figure 1).

Table 1. Mean age and SID indices for CD68 of studied samples

Lesion Variable	PG Mean±SD	IF Mean±SD	POF Mean±SD	Total Mean±SD	p-value
Age	39.15±14.79	46±15.55	42±17.96	42.65±16.13	ANOVA 0.412
CD68	9.15±3.86	2.2±3.69	6.4±4.55	-	Kruskal-Wallis <0.001

Table 2. Distribution of studied groups based on gender and location

Lesion Variable	PG Number(%)	IF Number(%)	POF Number(%)	Total Number(%)	p-value
Gender					
Female	7(35)	10(50)	10(50)	27(45)	Not Applicable
Male	13(65)	10(50)	10(50)	33(55)	(homogenized)
Location					
Buccal Mucosa	3(15)	11(55)	0(0)	14(23.33)	Fisher exact <0.001
Alveolar Mucosa	11(55)	7(35)	20(100)	38(62.66)	
Tongue	6(30)	2(10)	0(0)	8(13)	

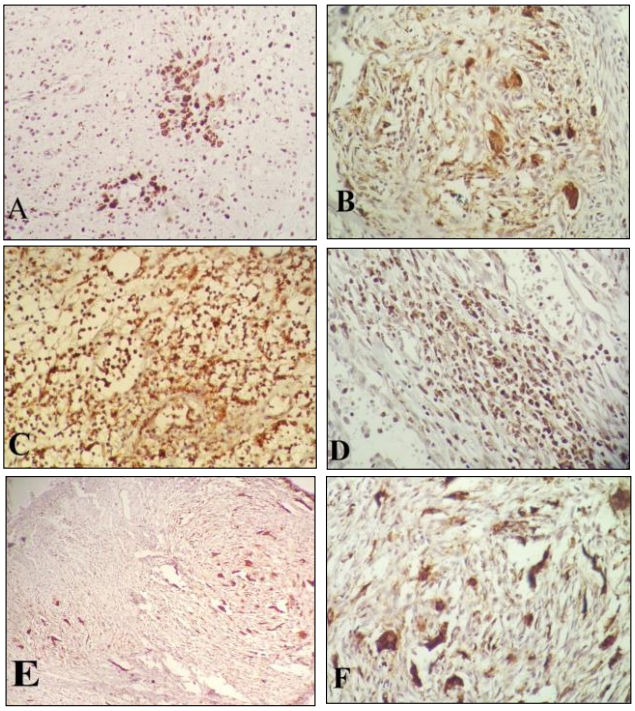

Figure 1. (A) Medium CD68 expression in POF (IHC staining, ×100), (B) Expression of CD68 in POF (IHC staining, ×400), (C) CD68 expression in PG (IHC staining, ×100), (D) High expression of CD68 in PG (IHC staining, ×400), (E) Low CD68 expression in IF (IHC staining, ×100), (F) Expression of CD68 in IF (IHC staining, ×400)

The level of CD68 expression based on the SID index was the highest in pyogenic granuloma and the lowest in excitatory fibroma, and the level of CD68 expression was significantly related to the type of lesion ($p<0.001$) (Table 1). Moreover, there was a significant difference between PG with IF, PG with POF and IF with POF based on CD68 expression ($p<0.001$, $p=0.027$, $p<0.001$) (Figure 1). As a result, the highest frequency of macrophages is observed in PG, then in POF and finally in IF.

Discussion

In this study, the highest frequency of macrophages based on CD68 expression was observed in PG, POF and IF, respectively, and the difference between all lesions was significant according to statistical analysis. However, in the study of Aghbali et al., CD68 expression was observed in PGCG, POF, PG and IF in descending order (13). In the study of Lázare et al., about half of the POF samples reported positive for CD68 (15). However, in the study of García de Marcos et al., all POF samples expressed CD68 (16). In the study of Han et al., CD68 expression was reported positive in all samples of IF and were significantly higher than normal mucosa tissue (17). Based on the study of Han et al., the frequency of macrophages was higher in the vascular and spread types of histopathological features of IF than in the cellular and nodular types. Based on the total results of the studies, it can be concluded that increased macrophage (CD68) index is associated with high vascularity. Regarding the different results observed in the current and previous studies, perhaps the selected samples of POF in the present study had higher vascularity or it was a pyogenic granuloma that was present in the mouth for a long time and calcification was also created in them.

Macrophage is the second most common immune cell in keloid tissue and the CD68 marker is expressed in the cytoplasm of adjacent normal fibroblasts. Therefore, a relationship between fibroblasts and macrophages in the development of fibrotic lesions and abnormal scaring was known (18). The secretion of different cytokines such as transforming growth factor β (TGF-β), interleukin-1, interleukin-6, interleukin-8, interferon and tumor necrosis factor (TNF-α) by macrophages is essential in fibrotic mass formation (17). CD68 is an intracellular glycoprotein that recognizes both M1 and M2 macrophages according to its occurrence in the granules of macrophage lysosomes, which is evaluated in this study (19).

The clinical appearance of oral reactive lesions reflects the different stages of their development; usually in the early stages, they are red with ulcerated surfaces and bleed spontaneously or with light touch. In the final stages, they appear as hard, mature fibrotic lesions with very few blood vessels. During the maturation stages of reactive lesions, fibrotic tissues with other tissue components such as multinucleated giant cells, calcified material, or hyperplasia of small vessels are also observed. Histopathological diagnosis is based on the observation of normal, atrophic, hyperplastic or wound patterns of mucosal lining epithelium, the type of inflammatory infiltrate, and the presence of extensive capillary or cavernous vascular proliferation, the presence of minerals, multinucleated giant cells and loose or stiff connective tissue. All types of oral reactive lesions with dense fibrous connective tissue can actually be the final stages of reactive lesions that were initially seen as vascular lesions (15). Therefore, the increase in the frequency of macrophages and their essential role at the beginning of the maturation process of oral reactive lesions in such a way that it reaches its maximum level in pyogenic granuloma with the minimum expression in IF can be justified by this, which is well shown in the present study. On the other hand, macrophages have an important role in angiogenesis, because they produce multiple growth stimulators and inhibitors, proteolytic enzymes and cytokines that modulate the angiogenesis process. Tumor-associated macrophages (TAMs) play an important role in angiogenesis in different in-vivo model systems. These findings suggest that macrophages have a fundamental impact on the proliferation of granulomas and play an important role in the pathogenesis of oral reactive lesions (20). This study suggests further studies regarding the ratio of M1 macrophages to M2 macrophages or in fact the ratio of CD163 to CD68 expressions in these lesions to further understand their natures.

Furthermore, this study showed that PG and POF mostly occurred in the alveolar mucosa, but IF mostly occurred in the buccal mucosa. These results are consistent with studies by Błochowiak et al. and de Almeida et al. (21, 22). The majority of affected patients in this study were in their fourth and fifth decades of life. However, in the study of Kadeh et al., the most common locations were more frequent in the 21-40 years age group (4). In the study of Błochowiak et al., the sixth decade and in the study of Soyele et al., the fourth decade of life had the highest frequency of oral reactive lesions (21, 23). These minor differences in results of these studies may be a result of small sample size or racial and geographical differences.

The presence of macrophages with high density was observed in all the studied oral reactive lesions. The significant difference between these 3 types of lesions may indicate that CD68 expression can be used as a method to differentiate between these lesions. Higher expression rate of CD68 in PG indicates that even more attention should be paid to this lesion's microenvironment to understand the exact pathogenesis of this lesion and whether it can be used to suggest a new treatment in future studies.

Conflict of interest: The authors have no relevant financial or nonfinancial interests to disclose.

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