

Immunohistochemical study on expression of mast cell and macrophage in irritation fibroma

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자극성 섬유종에서 비만 세포와 대식 세포의 면역조직화학적 발현

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초록

연구목적 : 자극성 섬유종은 만성자극에 의해 발생하는 구강내 증식성 병변이다. 상처 치유의 초기 과정에서는 비만 세포와 대식 세포가 섬유모세포의 이주, 증식, 아교질 합성 등에 연관되어 있는 성장인자와 사이토카인을 분비하여 상처 치유에 중요한 역할을 한다. 저자들은 자극성 섬유종을 조직학적 특성에 따라 세분하고, 각각의 조직학적 아형에서 비만 세포와 대식 세포의 발현을 조사하여 자극성 섬유종의 발생 기전을 이해하고자 하였다.

연구방법 : 본 연구에서는 82예의 자극성 섬유종을 조직 소견에 따라 4가지 유형으로 분류하였으며, 자극성 섬유종과 10예의 정상 구강점막에 톨루이딘 블루 염색과 CD 68 면역조직화염색을 시행하였다. 이를 통계화하여 자극성 섬유종의 조직학적 아형에 따른 비만 세포와 대식 세포의 분포 정도를 관찰하였다.

연구결과 : 통계 결과 비만 세포와 대식 세포의 분포는 자극성 섬유종에서 현저히 증가하였으며, Spearman 상관계수는 0.693이었다.

결론 : 조직의 섬유화에 관여하는 비만 세포는 자극성 섬유종의 cellular type에서 유의하게 증가하였으며, 대식 세포도 자극성 섬유종의 모든 아형에서 유의하게 증가하였다. 따라서 자극성 섬유종의 형성 과정에는 비만 세포와 대식 세포의 증가가 중요한 역할을 하는 것으로 생각되었다.

Key Words : fibrosis, irritation fibroma, macrophage, mast cell, wound healing

색인 : 대식 세포, 비만 세포, 상처 치유, 자극성 섬유종, 섬유화

Introduction

Oral irritation fibroma (IF) is one of the most common proliferative diseases of the oral cavity¹⁻³. Chronic cheek and lip biting, occlusive force, ill-fitting dentures, and calculus are the most common contributing factors, and they stimulate the proliferation of connective tissue^{1,2,4}. IF mostly afflicts in female, and primarily occurs on the gingiva, lips, buccal mucosa and the lateral border of tongue¹⁻³. The clinical characteristics of IF consists of a dome-shaped growth pattern covered with a normal-colored smooth mucosal surface¹⁻³. Biopsy specimens of IF demonstrate that the proliferation of connective tissue results in subepithelial mass formation while the overlying epithelium atrophies into a thin layer or shows a downward growth pattern into connective tissue layer^{1,2}. As a final point, IF is considered as reactive lesion formed by the hyperproliferation of fibroblasts and collagenous products caused by chronic irritation^{1,2}.

Mast cells, first described by Paul Erlich in 1878⁵, are multi-functional long-lived secretory cells that play a major role in hypersensitivity reactions⁶. In addition, they are involved in various physiologic conditions and diseases through their activities in the host immune reaction to tissue repair and regeneration, angiogenesis after injury, bacterial, parasitic, and viral infection^{7,8}. Moreover, they can promote many inflammatory diseases including tumor growth, rheumatic arthritis, psoriasis, and lung fibrosis^{8,9}.

Macrophage is derived from myeloid cell in the bone marrow, and then differentiated into monocyte cell⁸. These cells exist in various human organs, can be changed from one functional subpopulation to another by an appropriate stimulus, and possess diverse functions⁸. Besides the role as inflammatory cells in the immune mechanism, macrophages are involved in the control of wound healing, including wound closure as well as fibrosis associated with scar formation¹⁰. In an animal model with macrophage deficiency, wound healing was delayed due to defects in multiple steps, including re-epithelialization, granulation tissue formation, and angiogenesis¹¹.

The mechanism of chronic wound healing induced by chronic irritation or trauma contains very complex and elaborate reactions, in which epithelial keratinocytes, fibroblasts, polymorphonuclear leukocytes (PMNLs), macrophages, lymphocytes and mast cells cross-react^{12,13}. Abnormalities in the

wound healing process have been associated with conditions and diseases such as chronic wound healing, proliferative scarring, keloid, oral submucosal fibrosis, gingival overgrowth, and palmar and plantar fibromatosis^{13,15}. These may result from the imbalance of growth factors and inhibitors involved in wound healing process. Because both macrophages and mast cells are implicated in early wound healing process, the dysregulation of these cells is a candidate mechanism for oral IF. The authors address the role of wound healing-related factors in the development of irritation fibroma by studying mast cell distribution and the immunoexpression of macrophage.

Patients and Methods

1. Patients and tissue samples

Tissue samples from 10 cases of normal oral mucosa (NOM) and 82 cases of oral IF were investigated in this study. The samples of NOM were obtained during third molar extraction from adult patients with neither pathological lesions nor smoking history. The NOM and IF tissue samples were obtained from the Department of Oral Pathology, Yonsei University College of Dentistry from files dating between January 2001 to December 2004; cases were selected that had good preservation of both paraffin-embedded tissue and hematoxylin and eosin (H & E) slides. The diagnostic criteria of IF were based on the following conditions : clinical diagnosis, nodular mass of dense fibrous connective tissue containing mature collagen bundles, resembling scar tissue, and the overlying epithelium of the mass characterized by normal, atrophic, or having slight hyperkeratosis. In order to exclude any effects of inflammatory cells, cases of IF that had congregations of inflammatory cell infiltration were omitted.

Clinical data included age, sex, location of affected tissue, size, recurrence, clinical characteristics, patients symptoms, and clinical diagnosis at the time of biopsy and data were collected through a review of patients' clinical records.

2. H & E staining and pathological examination

NOM and IF tissues were fixed in 10% neutral buffered formalin solution for 24 hours, washed for 20 minutes, dehydrated in ethyl alcohol, washed in xylene, and embedded in paraffin

blocks at room temperature. Tissue sections of 3- μ m-thickness were obtained and submitted for routine H/E staining. Two pathologists examined histopathological reevaluation for each case.

3. Toluidine blue staining and immuno-histochemical staining

To observe the expression of mast cells, toluidine blue stain was performed. For this stain, more 3- μ m-thickness tissue sections were prepared, deparaffinized in 3 changes of xylene for 5 minutes each in which constantly dipped. Next, they were gradually hydrated in 100%, 90%, 70% ethyl alcohol solutions and in diluted water, and these sections were treated in 0.5% toluidine blue solution for 20 minutes and washed in running tap water. Finally, the sections were dehydrated in a change of 95% ethyl alcohol followed by a change of 100% ethyl alcohol, and were mounted with glycerin-jelly.

For the immunohistochemical stains, more 3- μ m-thick tissue sections were prepared and treated with an avidin-biotin complex (Vectastain Universal Elite ABC Kit, Vector, Burlingame, CA, USA). Immunohistochemical staining was performed using the primary antibody, CD 68 (1:200 dilution, Oncogene, San Diego, CA, USA). The sections were visualized with freshly prepared DAB (3,3'-diaminobenzidine tetrahydrochloride, Vector) as the substrate, counterstained with Mayer's hematoxylin (Sigma, St. Louis, MO, USA), dehydrated, and mounted with Canada Balsam.

For each slide, ten non-overlapping fields were randomly selected and photographed during examination by light microscopy with a digital camera (Olympus, BH-2, Tokyo, Japan), and then positive cells were counted in each field.

4. Histopathological subclassification of IF

The authors examined the H/E slides thoroughly, confirmed the histopathologic characteristics of the IF cases, and classified them based on characteristics of overlying epithelium, the interface between the mass and the surrounding normal tissue, the fibroblasts density, and the blood vessel density in the IF mass. IF was classified into nodular, spread, cellular, and vascular subtypes. Nodular-type IF was characterized by a prominent rete ridge of the overlying epithelium and distinct separation between the IF mass and the surrounding connective

tissue. Spread-type IF had no apparent rete ridge of the overlying epithelium, indistinguishable separation between the IF mass and the surrounding connective tissue, and low density of fibroblasts in the IF mass. Cellular-type IF was characterized by a high density and regular direction (and sometimes intersection) of pump fibroblasts. Vascular-type IF demonstrated numerous dilated and congested blood vessels in the IF mass.

5. Statistical analysis

Statistical analysis for the staining results was performed using PASW version 21.0 (SPSS Inc., NY, USA). One-way ANOVA was carried out to compare the expressions of mast cells and CD 68 and analyze the differences between IF and NOM tissues. In addition, correlations between mast cell distribution and CD 68 immunoexpression in IF were calculated by Spearman's Correlation Coefficients. A p-value ≤ 0.05 was considered statistically significant.

Results

1. Histopathologic characteristics of the IF masses

The histopathologic characteristics of the IF masses in the present study were shown in (Tables 1) and (Fig.1).

2. Mast cell expression and CD 68 immunoexpression

In the NOM tissue samples, there were very weak levels of mast cell expression and CD 68 immunoexpression (Fig. 2, 3). On the contrary, in IF tissue samples, the stains were expressed strongly throughout the mass (Fig. 2, 3). Both toluidine blue and CD 68 expressions in all four IF subtypes were significantly higher in IF masses than in NOM tissue (Fig. 4).

3. Correlations between mast cell distribution and CD 68 immunoexpression

Bivariate comparisons of mast cell distribution and CD 68 immunoexpression levels revealed a significant correlation in cellular-type of IF (Spearman correlation coefficient = 0.693, $p=0.038$).

Table 1. Histopathologic characteristics of IF masses

	Number of cases	Elongation of rete ridge	Cellularity	Fibroblast	Thickness of collagen bundle	Formation of blood vessel	Inflammatory cells
Nodular	38	—	+	resting	+++	+	—
Spread	10	+	+	active	++	+	—
Celluar	11	+/-	+++	ovoid/myofibroblast	+	+	—
Vascular	23	+/-	+	resting	++	+++	—

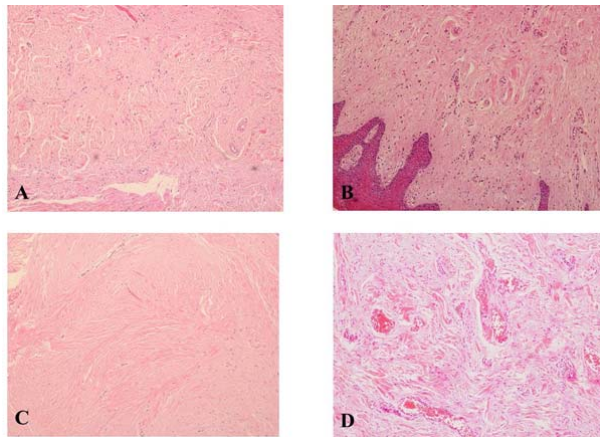


Fig. 1. Histopathological subtypes of IF

A - nodular, B - spread, C - cellular, D - vascular

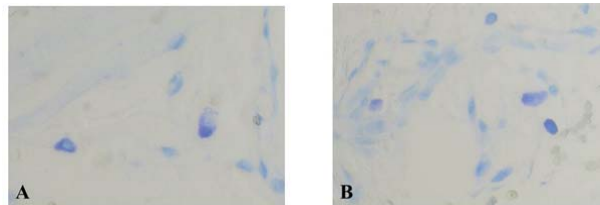


Fig. 2. Mast cell expression

A - NOM, B - IF (toluidine blue stain, X200)

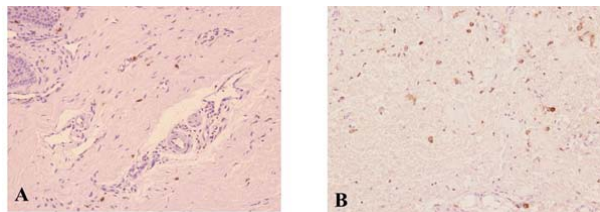


Fig. 3. Immunexpression of CD 68

A - NOM, B - IF (Immunohistochemistry, X200)

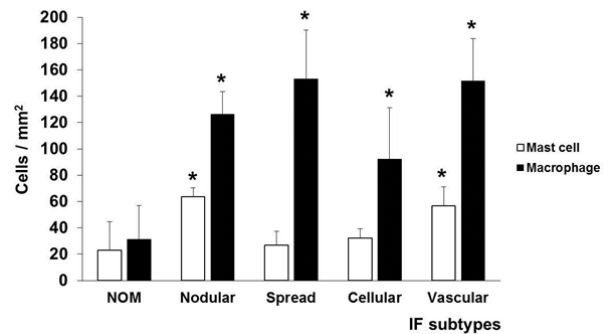


Fig. 4. Distribution of mast cells and macrophages by IF subtype

Discussion

Wound healing is a very complex biologic process in which various amounts of resident cells and inflammatory cells interact, including epithelial cells, fibroblasts, PMNs, macrophages, lymphocytes and mast cells¹²⁾. These cells usually play key roles in immune responses and in regulation of other cells by secretion of specific cytokines^{12,16)}. Several reports have revealed that mast cells and macrophages are essential elements in the wound healing process. Wound healing consists of multiple steps, and are divided into hemostasis, inflammation, neovascularization, fibroplasia, contraction, and remodeling^{16,17)}. Throughout these steps of wound healing, wound-breaking strength tends to increase proportional to the amount of mast cells in the dermis¹⁷⁾. In the skin healing process, mast cells are implicated in epithelial cell growth and restoration, repair of blood supply through neoangiogenesis, and collagen repair, which provides dermal strength¹⁷⁾.

Mast cells are associated with tissue homeostasis, remodeling, repair, and fibrosis⁶⁾. In 1878, Paul Ehrlich first reported

that the proximity of mast cell in early stages of fibrosis and tissue remodeling⁵⁾. In connective tissue, mast cells secrete growth factors and cytokines that stimulate fibrosis and motivate myofibroblasts, molecules that include histamine, heparin, tryptase, fibroblast growth factor-2 (FGF-2), tumor necrosis factor- α (TNF- α), and transformation growth factor- β (TGF- β)¹⁷⁻¹⁹. In hypertrophic scars and keloids, mast cells are considered very important in fibrosis as well, because human mast cells can modulate human fibroblasts directly and change the fibrotic phenotype in skin fibrotic lesions¹⁸⁾.

Mast cells secrete tryptase and platelet-derived growth factor (PDGF) in the wound lesion, so it is clear the cells affect the wound healing mechanism even in the early steps of wound healing²⁰⁾. In one animal wound healing model, a positive reaction of FGF-2 in the fibroproliferative stage was observed by increase of mast cell²¹⁾. Tryptase can stimulate the synthesis and secretion of collagen in cultured fibroblasts, and also prompt secretion of collagenase. However, we found no significant correlations in cellular-type IF in our results. Because the fibrotic process is a very complex mechanism, it is possible that another mechanism is at work in the fibrosis of IF.

Another special role of mast cells is the control of angiogenesis in the wound site. Angiogenesis is very complex and multistep process that consists of angiogenesis, migration and proliferation of endothelial cells, tube formation, and cell survival²²⁾. Greater-than-normal mast cell levels are associated with angiogenesis, and the mast cells accumulate in the neo-vascularized area²³⁾. Mast cells contain various biochemical mediators such as FGF-2 and vascular endothelial growth factor (VEGF), which affect the angiogenesis^{18,23)}. In fact, in many cases, mast cells are plentifully observed in pathological areas as containing numerous capillaries and lymphatic vessels. Moreover, increases in mast cells are known to be associated with angiogenesis in the cases of hemangioma, nasal polyps, wound healing, and ovulation²⁴⁾. In particular, tryptase from mast cells stimulates the growth of human endothelial cells to facilitate the formation of vascular tubes, and plays an important role in angiogenesis of human neoplasias, including B-cell non-Hodgkin lymphoma, multiple myeloma, myelodysplastic syndrome, chronic lymphoblastic leukemia, and malignant melanoma²⁵⁾. In vascular type of IF, mast cell and CD 68 immunoexpression were significantly increased compared with NOM and with other IF subtypes. These remarkable mast cell

and macrophage distribution levels support the notion that macrophages are strongly associated with angiogenesis.

Macrophages play an important role in the phagocytosis of apoptotic cells, and are also notable as the source of growth factors, which accelerate the proliferation and regeneration of injured tissue²⁵⁾. After 2 days postinjury, macrophages begin to be recruited in the wound site, and become involved in the inflammatory process, collagen production, angiogenesis and re-epithelialization^{10,11,26)}. Various cytokines secreted by macrophage play complex roles in inflammatory cell control, migration, and stimulation of keratinocytes and fibroblasts²⁶⁾. The overexpression of TGF- β 1 and TGF- β 2 by macrophage especially can stimulate hypertrophic scar formation¹²⁾.

According to Shaker, macrophages are the second common immune cell in keloid tissues, and CD 68 immunoexpression can be found in the cytoplasm adjacent normal fibroblasts²⁷⁾. Therefore, this phenomenon indicates a close relationship between macrophages and fibroblasts whose relationship may play a certain role in exaggerated fibrotic tissue formation²⁷⁾. Because these cells interact through a positive feedback mechanism, they are considered as important in abnormal scarring²⁷⁾. Additionally, macrophages secrete multiple cytokines, such as transforming growth factor (TGF)- β , interleukin-1, interleukin-6, interleukin-8, interferon, and tumor necrosis factor (TNF)- α ^{27,28)}. These cytokines are known to appear in abnormal scarring as well²⁸⁾. In the present study, there was a significant correlation between mast cell and macrophage expressions in the cellular subtype. While mast cell did not show a significant increase in IF tissue of the cellular subtype, macrophages did, and it seems that macrophages play a certain role in the fibrotic mass formation observed in IF. Moreover, because the macrophage count increased significantly (compared with NOM tissue) for all subtypes of IF, we suspect that macrophages affect the pathogenesis of IF in some way.

The result showed that CD 68 immunoexpression increased significantly in IF tissue. Furthermore, a correlation between mast cell distribution and CD 68 immunoexpression was demonstrated in cellular-type of IF, so these two cell types seem to be related with each other in at least some IF cases. However, since there was no significant correlation between mast cell distribution and CD 68 immunoexpression in total cases irrespective of subtype, further research is necessary to

elucidate the exact mechanism of these cells in IF pathogenesis.

Conclusions

The aim of the present study is to elucidate the mechanism of IF pathogenesis through the immunoeexpression of mast cells and macrophages, and compare their levels among IF subtypes. IF cases were classified by their histopathologic characteristics of IF mass. Toluidine blue and CD 68 immunohistochemical staining were performed for 10 cases of normal oral mucosa (NOM) and 82 cases of IF.

In conclusion, the authors suggest that, from among the cells involved in wound healing, mast cells and macrophages in particular increase in concentration, and this phenomenon could contribute to the pathogenesis of IF.

Mast cell increased significantly in the nodular and vascular subtypes of IF in comparison to NOM tissue; we consider these cells are likely involved in the fibrosis of oral tissue.

Macrophage count increased significantly in all subtypes of IF, compared with NOM tissue, while macrophage distribution showed no significant differences among IF subtypes. This increase of macrophages, especially in the cellular subtype, points to a major role for macrophages in tissue fibrosis.

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