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자극성 섬유종에서 TGF-β 1, MMP-1 및 TIMP-1 발현

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〈ABSTRACT〉

Expression of TGF-β 1, MMP-1 and TIMP-1 in Irritation Fibroma

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Irritation fibroma(IF) is the most common tumor-like lesion. IF is characterized by over-production of collagen and, thus, resembles scar tissue. TGF-β1, MMP and TIMP play an essential role in remodeling extracellular matrix during scar formation. This study investigates the pathogenesis of IF with respect to the coordinated expression of factors involved in wound healing. Proliferative activity and expression of TGF-β1, MMP-1 and TIMP-1 were observed using immunohistochemistry in 88 cases of IF and 9 cases of normal oral mucosa(NOM). Proliferative activity and expression of TGF-β1 and TIMP-1 were increased in IF compared to NOM. MMP-1 expression was not significantly increased in IF. We propose that IF is caused by increased expression of TGF-β1 and an imbalance in expression of MMP-1 and TIMP-1.

Key words : Irritation fibroma, Wound healing process, TGF-β1, MMP-1, TIMP-1, Proliferative activity

I. INTRODUCTION

Oral irritation fibroma (IF) is one of the most frequent tumorous lesions found in the oral cavity. The causes of IF include habitual cheek and lip biting and chronic irritation such as ill-fitting denture and calculus, which lead to over-growth of connective tissue under the epithelium. IF is most frequently encountered in adults and primarily occurs on the gingiva, lip, buccal mucosa, the lateral border of the tongue and the soft tissue in the plane of the occlusal line. Clinical features are a smooth mucosal surface of normal coloration and dome-like growth. Lesion size varies from 2 mm to several cm in diameter, and averages 1cm. Such lesions can be easily excised and recur rarely. IF is regarded as a simple reactive lesion rather than a true neoplasm[1)].

When oral mucosa is exposed to chronic irritation or trauma, signals are transferred to the submucosal connective tissue via the basement membrane. The process of wound healing is induced by the coordinated interactions of cells with extracellular matrix and secreted regulatory factors. When soft tissue receives

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trauma, an initial vascular reaction is followed by the emigration of inflammatory cells such as neutrophils, monocytes, lymphocytes and macrophages, and subsequent re-epithelialization of the wound site and formation of granulation tissue. Fibroblasts synthesize a large quantity of connective tissue components including fibronectin, glucosaminoglycan and collagen. Following a wound, collagen production constantly increases for 4 to 5 weeks, with type Ⅰ, Ⅲ, Ⅳ collagen produced in different ratios throughout. As the wound healing process progresses, the extracelluar matrix is degraded and remodeled to form normal mucosal surface. This process is mediated by coordinated, sequential intercellular interactions, growth factors and their receptors, matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP)[1-7].

Abnormalities in the wound healing process are associated with diseases such as chronic wound healing, proliferative scar, keloid, submucosal fibrosis, gingival overgrowth and palmar fibromatosis[8-12]. These diseases may result from an imbalance of growth factors and inhibitors involved in the wound healing process.

IF is thought to be the reactive lesion caused by collagen production that resembles scar tissue. Although IF is the most common lesion in the oral cavity, there has been no systemic study of the pathogenesis of IF. The present study addresses the role of factors involved in wound healing in the development of IF by studying the expression of TGF-β, MMP-1 and TIMP-1.

II. METHOD AND MATERIALS

Nine samples of NOM were obtained from adult patients with no pathologic lesions or smoking history in Yonsei University Dental Hospital.

Eighty-eight cases of IF with good preservation of paraffin tissue and H/E slides were obtained from files of the Department of Oral Pathology, Yonsei University College of Dentistry, from January 2001 to December 2004. We classified these lesions as IF by the following criteria: (1) whether there was a history of chronic biting on the IF site, (2) whether on clinical examination, the lesion was determined to be nodular or sessile, smooth-surfaced and similar in color to the surrounding mucosa, and (3) whether by histopathological analysis, the lesion was determined to have a nodular mass of fibrous connective tissue containing collagen bundles covered by stratified squamous epithelium with neither odontogenic epithelium nor calcification foci. Follow-up was performed for the patients with IF (Table 1).

Table 1. Characteristics of IF patients

Number of patients		88
Age(years)		36.29±14.92
Sex	Male	30
	Female	58
Location	Maxilla	3
	Mandible	8
	Buccal mucosa	18
	Tongue	35
	Lower lip	17
	Hard palate	6

1. H/E stain and immunohistochemistry

NOM and IF tissue were fixed in 10% neutral formalin solution for 24 hours, washed for 20 minutes, dehydrated in ethyl alcohol, washed in xylene and embedded in paraffin blocks. Three ㎛ sections were obtained and submitted for routine hematoxyline-eosin staining. For immunohistochemical stain, 3 ㎛ tissue sections were prepared and subjected to the avidin-biotin complex method. Immunohistochemical staining was performed using primary antibodies (Table 2). Antigen retrieval for TGF-β1 was achieved by using 0.4% trypsin solution (0.04g trypsin in 10mL of distilled water; 20 minutes at 37℃). The sections were visualized with freshly prepared substrate DAB (3,3'-diamonobenzidine tetrahydrochloride), counterstained with Mayer's hematoxylin, mounted and examined under light microscopy (Olympus, BH-2, Tokyo, Japan). Positive controls were: oral squamous cell carcinoma for PCNA, the placental tissue of trimester

Table 2. Primary antibodies used in immunohistochemical studies

Antibody	Dilution ratio	Clone	Source
TGF-β1	1:50	sc-146	Santa Cruz[a]
MMP-1	1:100	41-1ES	Oncogene[b]
TIMP-1	1:50	102D1	Neomarkers[c]
PCNA	1:100	PC10	Oncogene[b]

* [a]Santa Cruz Biotechnology, Santa Cruz, CA, USA
* [b]Oncogene Comp., SanDiego, CA, USA
* [c]Neomarkers Comp., Fremont, CA, USA

period for TGF-β1 and MMP-1, and breast cancer tissue for TIMP-1.

For each slide 10 non-overlapping fields were randomly selected (5 fields for epithelium and 5 fields for connective tissue) and photographed using light microscopy with a digital camera (Olympus, BX51T, Tokyo, Japan, ×200).

The expression of TGF-β1, MMP-1 and TIMP-1 was evaluated in a semi-quantitative manner[13]. Levels of immunoreactivity were graded into four easily reproducible subgroups: (a) No detectable expression (Grade 0), (b) positive expression in less than 〈30% cells (Grade 1), (c) positive expression in 30-70% of cells, indicating immunopositive sub-populations (Grade 2), and (d) positive expression in greater than 70% of cells (Grade 3).

To evaluate the proliferative index, the total number of epithelial cells and the number of nuclei with positive proliferating cellular nuclear antigen (PCNA) expression were counted using Image-Pro (ver. 3.0). The proliferative index was calculated by the following formula:

$$\text{proliferative epithelial index} = \frac{\text{total number of positive cells}}{\text{total number of epthelial cells}} \times 100$$

$$\text{proliferative index of fibroblast} = \frac{\text{total number of positive cells}}{\text{total number of fibroblasts}} \times 100$$

2. Statistical analysis

Because the data were not normally distributed and the scale of variables was an ordinal scale, for non-parametric analysis the Mann-Whitney U test was used to analyze differences between IF and NOM. To evaluate the correlation of each variable, the Spearman pair-wise correlation coefficient was calculated. Statistical analysis was performed using the Window SAS (statistical analysis system) 8.2 statistical package (SAS Institute, Inc. U.S.A).

Table 3. Comparison of TGF-β 1 expression between NOM and IF

Positive range of fibroblast	NOM	IF*
〈 30%	66.7%	16.3%
30 ~ 70%	22.2%	24.4%
〉 70%	11.1%	59.3%

* : $p < 0.05$ by Kruskal-Wallis test

III. RESULTS

1. Histological features of IF

The overlying epithelium and fibrous portions of IF were histologically examined. The overlying epithelium of IF showed atrophic change in 42 cases (47.7%) without rete ridge elongation. Epithelial proliferation was observed in 46 cases (52.3%). The connective tissue of IF showed variable cellularity with thick acidophilic collagen fibers. The fibroblasts were well-differentiated spindle cells with simple nuclei and neither atypia nor abnormal mitosis (Fig. 1a, b).

2. Immunohistochemical staining

1) TGF-β 1 expression

The fibrotic portion of IF showed significantly higher TGF-β1 expression compared to the submucosal portion of the NOM (Fig. 2a, b), with positivite staining in more than 70% of cells in 59.3% of IF cases compared to 11.1% cases in NOM (Table 3).

2) MMP-1 expression

In both NOM and IF, MMP-1 was expressed in most cells including fibroblasts, inflammatory cells and blood vessels (Fig 3a, b).

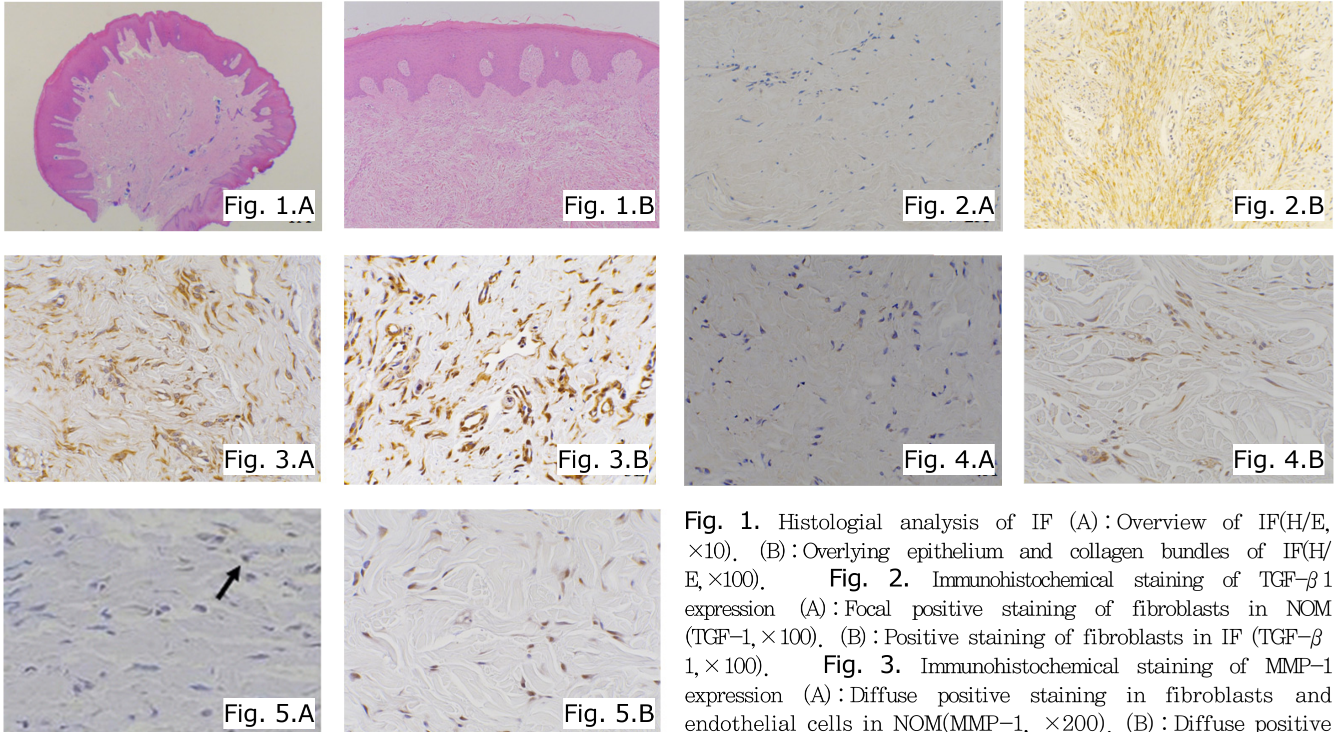

Fig. 1. Histologial analysis of IF (A) : Overview of IF(H/E, ×10). (B) : Overlying epithelium and collagen bundles of IF(H/E, ×100). **Fig. 2.** Immunohistochemical staining of TGF-β1 expression (A) : Focal positive staining of fibroblasts in NOM (TGF-1, × 100). (B) : Positive staining of fibroblasts in IF (TGF-β1, × 100). **Fig. 3.** Immunohistochemical staining of MMP-1 expression (A) : Diffuse positive staining in fibroblasts and endothelial cells in NOM(MMP-1, ×200). (B) : Diffuse positive staining in fibroblasts and endothelial cells in IF(MMP-1, ×200). **Fig. 4.** Immunohistochemical staining of TIMP-1 expression (A) : Focal positive staining in fibroblasts in NOM(TIMP-1, × 200). (B) : Positive staining of most fibroblasts in IF (TIMP-1, × 200). **Fig. 5.** Immunohistochemical staining of PCNA expression (A) : A few scattered positive staining fibroblasts (arrow) in NOM(PCNA, ×200). (B) : Most fibroblasts show PCNA positive reactions in IF (PCNA, ×200).

There was no detectable difference between NOM and IF in the incidence of MMP-1 positive fibroblasts, although the intensity of staining was much stronger in IF.

3) TIMP-1 expression

In NOM, a positive reaction was seen in a few fibroblasts, blood vessels and inflammatory cells in the connective tissue (Fig. 4a, b). Five (55.6%) cases of NOM had focal expression of TIMP-1. In 61 cases (70.9%) of IF, diffuse TIMP-1 positive expression (more than 70% positive cells) was demonstrated, while only one case of NOM showed diffuse positive staining. This difference in expression was statistically significant ($p<0.05$) (Table 4).

4) Proliferative activity

In NOM, positive cells were distributed mainly in the basal layer and the lower spinous layer of the epithelium, and a few cells in the submucosal portion showed a positive reaction (Fig. 5a).

In IF epithelium, a positive reaction was seen in the basal layer and in the lower spinous layer, as in NOM, while in the fibrous portion, positive cells were frequently found among the collagen fiber bundles (Fig. 5b).

The proliferative index of NOM epithelium was 4.04 and that of IF was 3.94, which was not statistically significant. The proliferative index of the fibrous portion of IF was three times higher (13.09) than that of NOM (4.00), which was statistically significant ($p<0.05$) (Table 5).

5) Spearman coefficient

The spearman coefficient of TGF-β1, MMP-1, TIMP-1 and proliferative activity showed no statistical significance.

Table 4. Comparison of TIMP-1 expression rate between NOM and IF

Positive range of fibroblast	NOM	IF*
〈 30%	55.6%	12.8%
30 ~ 70%	33.3%	16.3%
〉 70%	11.1%	70.9%

* : $p < 0.05$ by Kruskal-Wallis test

Table 5. Comparison of proliferative index between NOM and IF

	Normal	IF	P-value
Epithelium	4.04 (0.03)	3.94 (0.33)	-
Fibroblast	4.00 (0.07)	13.09 (0.13)	0.03

IV. DISCUSSION

IF has been traditionally thought to be a reactive lesion caused by chronic injury. True neoplasm is defined as autonomous proliferation of tumor cells, while reactive lesions demonstrate limited growth[1]. IF is considered a reactive lesion by this criteria. This study addresses the pathogenesis of IF with respect to the wound healing process.

TGF-β1 plays a key role in the wound healing process by promoting fibroblastic proliferation and collagen synthesis. Administration of TGF-β to wounded tissue promotes the process of bone and tissue healing[14]. TGF-β also promotes synthesis of fibronectin, elastic fibers and TIMP, and inhibits production of collagenase[15-17]. It has been reported that TGF-β expression in dermal fibroblasts of keloid or hypertropic scar is higher than that of normal dermis. In fibroblasts, increased levels of TGF-β1 causes an increase in type Ⅰ procollagen mRNA. Levels of TGF-β are also increased in hereditary gingival fibromatosis and drug-induced gingival hyperplasia[18-23].

The present study found that TGF-β levels in IF fibroblasts was markedly increased compared to that of NOM. TGF-β1 over-expression may be considered a major cause of IF through the stimulation of fibro

blasts to produce collagen, thus setting up an autocrine effect that leads to tumourous swelling. The proliferative index of fibroblasts in IF was much higher than in NOM, reflecting active mitotic activity of fibroblasts with collagen synthesis capacity in IF.

In cutaneous disorders such as palmar and plantar fibromatosis and malignant solitary fibrous tumor causing excessive collagen synthesis, an increase of the PCNA/Ki-67 index was reported[18, 29-31]. Andrade has reported that inhibition of TGF-β1 in hereditary gingival fibromatosis caused a decrease in the PCNA index in fibroblasts[18]. The accordance of increased proliferative activity of fibroblasts of IF and increased TGF-β1 expression support the proposal that increased levels of TGF-β1 accelerate fibroblast proliferation and collagen synthesis. However, we could not find statistical significance for the positive correlation between these two factors.

In the early stages of normal wound healing, MMP-1 levels are increased by inflammatory cytokines. This is followed by increased TGF-β1 expression, which is associated with normal synthesis of type I collagen, decreased production of MMPs, and enhanced expression of TIMPs. Next, during the remodeling phase, turn-over of collagen fibers occurs. The majority of fibroblasts in IF showed strong positive reaction for MMP-1. However, comparing the MMP-1 positive cells in fibroblasts between NOM and IF, the number of cells showing a positive reaction was not significantly different (Fig. 3a, b).

In the case of keloid formation, disruption of the remodeling mechanism occurs. TGF-β1 and TGF-β2 are both synthesized in excess by keloid fibroblasts.

In the liver, decreased MMP-1 synthesis and increased TIMP-1 and -2 function promote fibrosis[34]. Similarly, in oral submucosal fibrosis, MMP-2 and

MMP-9 synthesis and activity decrease, and increased TIMP-1 synthesis has been reported[19]. In hereditary gingival fibromatosis, Martelli-Junoir reported that MMP-1 and MMP-2 secretion decreased as TGF-β1 secretion and type Ⅰ collagen synthesis increased[35]. When fibroblasts are stimulated by secretion of TGF-β1 during the wound healing process, synthesis of extracellular matrix is initially stimulated, followed by synthesis of MMP and TIMP, which act together to remodel the extracellular matrix. In this study, TIMP-1 expression increases more in IF than in NOM, while MMP levels are not significantly different, suggesting that an imbalance between the degradation by MMP and its inhibition by TIMP may cause IF.

TGF-β controls expression of MMP, and TIMP has been known to decrease secretion of MMP-1. Following the sequence of normal wound healing, we calculated the Spearman coefficient between these cytokines and proliferative activity. In this study, there was no statistically significant relationship between these factors.

The present study has several limitations for understanding the pathogenesis of IF: First, the number of NOM cases is too small to analyze the results statistically, Second, it was not possible to evaluate the sequential expression pattern of MMP and TIMP.

Taken together, these results indicate that the increased expression of TGF-β1, followed by fibroblast proliferation and collagen synthesis, are contributing factors to the formation of IF. TIMP-1 over-expression may be associated with decreased degradation of collagen produced during the remodeling process which would result in tumourous swelling. To test this hypothesis directly, it will be necessary to follow temporally the biochemical and molecular changes that occur during the formation of IF.

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