Original Article

Exploring the varied expressions of basic fibroblast growth factor in different histopathological grades of oral submucous fibrosis

Geetpriya Kaur[1], Vijay Wadhwan[2], Kiran Kumar[3], Aparna Pathak[4], Farnaz Y. Shah[1], Pallak Arora[5]

[1]Department of Oral Pathology and Microbiology, Institute of Dental Studies and Technologies, Modinagar, Uttar Pradesh, [2]Department of Oral and Maxillofacial Pathology, Subharti Dental College, Swami Vivekanand Subharti University, Meerut, Uttar Pradesh, [3]Department of Oral and Maxillofacial Pathology, SDM College of Dental Sciences and Hospital (Constituent Unit of Shri Dharamasthala Manjunatheshwara University, Dharwad), Dharwad, Karnataka, [4]Consultant Oral Pathologist, Paradise Diagnostics, Gujranwala Town, New Delhi, India, [5]Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, Boston, MA, USA

Abstract

Context: Oral submucous fibrosis (OSMF) is a chronic disorder with multi-factorial aetiology. The OSMF pathophysiology includes the homeostatic equilibrium disruption between synthesis and degradation of extracellular matrix. Thus, various growth factors produced by activated inflammatory cells may promote fibrosis by inducing fibroblast proliferation, collagen synthesis upregulation, and reduced collagenase production.

Aims: To correlate the role of basic fibroblast growth factor (bFGF) in the endothelial cells, fibroblasts, and connective tissue stroma in varying grades of OSMF. The bFGF expression was also correlated with the amount of inflammation.

Settings and Design: This retrospective study was designed to evaluate bFGF expression in 30 histopathologically diagnosed cases of OSMF from the Department of Oral and Maxillofacial Pathology, I.T.S CDSR, Muradnagar.

Materials and Methods: We included 30 cases, ten each of early, intermediate, and advanced stages of OSMF. Immunohistochemical staining using bFGF antibody was performed, and bFGF expression was noted in the blood vessels, fibroblasts, and connective tissue stroma in all the study cases.

Statistical Analysis Used: Different variables were analysed using the ANOVA test, *post hoc* test, and Bonferroni test.

Results: The bFGF-labelled blood vessels and fibroblasts were significantly higher in early OSMF cases than in the intermediate and advanced groups. bFGF expression was significantly observed in the connective tissue stroma in most of the cases.

Conclusions: The bFGF intensity was mild, moderate, and severe in early, intermediate, and advanced OSMF cases, respectively. Moreover, bFGF expression was noted in the blood vessels, fibroblasts, and connective tissue stroma in the majority of the cases.

Keywords: Chronic inflammation, extracellular matrix, fibrogenic cytokines, growth factor

Address for correspondence: Dr. Pallak Arora, Department of Oral Medicine, Infection and Immunity, Harvard School of Dental Medicine, 188 Longwood Ave, Boston, MA, USA.
E-mail: pallak_arora@hsdm.harvard.edu

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INTRODUCTION

Pindborg JJ and Sirsat SM in 1966 defined oral submucous fibrosis (OSMF) as a chronic insidious disease affecting any part of the oral cavity and sometimes the pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with juxta-epithelial inflammatory reaction followed by a fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat.[1] OSMF is a chronic oral mucosal condition that occurs predominantly among Indians and people of Indian origin living outside India, occasionally in other Asians, and sporadically in Europeans. The prevalence rate in India is 0.2 to 1.2%.[2] The majority of the studies have shown female preponderance with the maximum number of cases reported between 20 and 40 years.[3]

The aetiology is believed to be multi-factorial. A number of factors trigger the disease by causing a juxtaepithelial inflammatory reaction in the normal oral mucosa.[4] These include local irritants such as betel quid, capsaicin, tobacco, pungent and spicy food, and alcohol. Other possible aetiological agents are chronic iron and vitamin B deficiency, viruses, and candida.[2,4] The increased frequency of human leucocyte antigen (HLA) haplotypic pairs A10/DR3, B8/DR3, and A10/B8 suggested an MHC-mediated immunological derangement operating in the disease.[5] Initial events of OSMF start when oral mucosa is in direct contact with betel quid due to the habit, resulting in a chronic inflammatory process characterised by the presence of inflammatory cells like T-cells and macrophages.[4]

These cells stimulate the synthesis of various cytokines and growth factors like interleukin 6, tumour necrosis factor, and basic fibroblast growth factor (bFGF) that might promote fibrosis.[4] FGF (fibroblast growth factor) is a member of a family of polypeptides that are potent regulators of cell proliferation, differentiation, and function.[6] In OSMF, varied expressions of bFGF in fibroblasts, endothelial cells, and fibrous stroma are observed, which could be related to receptor stabilisation by cytokines.[7]

Hence, this study aimed to correlate bFGF expressions in fibroblasts, endothelial cells, and connective tissue stroma in varying grades of OSMF as well as hypothesise the role of FGF in the pathogenesis of OSMF. We also tried to correlate the immunohistochemical expression of bFGF in the connective tissue stroma with the grade of inflammation in varying grades of OSMF.

SUBJECTS AND METHODS

This is a retrospective analysis of 30 OSMF archived cases from the Department of Oral and Maxillofacial Pathology, I.T.S Centre for Dental Studies and Research, Muradnagar. After grading specimens according to the criteria enlisted by Utsunomiya *et al*.,[8] the cases were divided into three groups:

GROUP I (Early stage) – 10 cases showing a large number of lymphocytes in the sub-epithelial zone along with myxoedematous changes.

GROUP II (Intermediate stage) – 10 cases showing granulation changes close to the muscle layer and hyalinisation in the subepithelial zone where blood vessels were compressed by fibrous bundles along with reduced inflammatory cells in the subepithelial layer.

GROUP III (Advanced stage) – 10 cases with marked fibrosis areas and hyaline changes from sub-epithelial to superficial muscle layers. Atrophic, degenerative changes were observed in muscle fibres with a few inflammatory cells.

GROUP IV (Control group) – 10 cases of normal mucosa from healthy patients.

All cases were fixed in 10% buffered formalin, embedded in paraffin wax, serially sectioned, and stained with haematoxylin and eosin. Immunohistochemical staining was done by de-paraffinisation of tissue sections taken on polylysine-coated slides. Quenching of endogenous enzymes was done by 3% H_2O_2. After antigen retrieval, monoclonal anti-fibroblast growth factor-Basic Clone FB-8, mouse IgG (Biogenex Ind. Pvt. Ltd) antibody was used. For secondary antibody application, the slides were incubated with SS-labelled poly-HRP reagent. Slides were then incubated in freshly prepared 3,3-diaminobenzidine (DAB) solution and dipped once in Harris Haematoxylin for counterstaining. Moreover, bFGF expressions were evaluated in the blood vessels and fibroblasts in varying OSMF grades [Tables 1 and 2]. Furthermore, the inflammation grades were correlated with various grades of OSMF [Graph 1], while inflammatory cell count was assessed in different OSMF grades [Table 3].

Selection of field for counting cells

From all positively stained areas, ten representative fields at 40x were selected and counted that were not in a continuum with each other to minimise any possible errors. The images of each of the ten fields were obtained using a binocular light microscope (x40) and then transferred to a grid for

Table 1: Quantitative assessment of bFGF expression in blood vessels in varying grades of OSMF

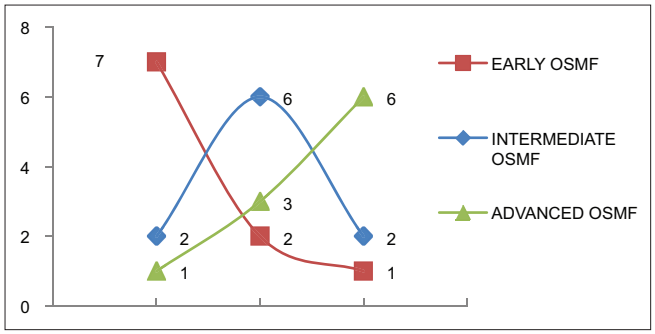
Blood vessels	Early OSMF (*n*=10)	Intermediate OSMF (*n*=10)	Advanced OSMF (*n*=10)	*P* (*P*<0.05) significant
Mean±Std deviation	5.940±2.4019	3.450±1.5522	2.850±1.1453	0.003

Table 2: Quantitative assessment of BFGF expressions in fibroblasts in varying grades of OSMF

Fibroblasts	Early OSMF	Intermediate OSMF	Advanced OSMF	*P* (*P*<0.05)
Mean±Std deviation	24.530±12.9730	13.420±7.0177	10.980±5.402	0.004 significant

Table 3: Inflammatory cell counts in varying grades of OSMF

Inflammatory cells	Early OSMF (Mean±SD)	Intermediate OSMF (Mean±SD)	Advanced OSMF (Mean±SD)	*P* (<0.05 is significant)
Mean±SD	42.46±17.67	30.20±10.05	24.62±10.97	0.027 Non-significant



Graph 1: Correlation of the grade of inflammation with bFGF expressions in connective tissue in different OSMF grades

analysis. The immunoreactive score was then obtained by the assessment of bFGF-labelled cells and their intensity along with different histological grades.

Statistical analysis

The resulting data were analysed using SPSS (Statistical Package for the Social Sciences). Differences between the mean of the two independent groups have been observed by the T-test in normally distributed data. Differences between the different variables were analysed using the ANOVA test and *post hoc* test followed by the Bonferroni test. Pearson's Chi-square test was carried out to determine the level of correlation between the study groups. *P* value < 0.05 was considered to be statistically significant.

RESULTS

With the increase in the severity of OSMF, there was a decrease in bFGF expression in the number of blood vessels. Blood vessel expression was the highest in early OSMF (5.940 ± 2.4019), followed by a gradual decrease in intermediate OSMF (3.450 ± 1.5522), and the least in advanced OSMF (2.850 ± 1.1453), as seen in photomicrographs (1a-c). Therefore, as the grade of OSMF increased, the expression of blood vessels reduced, which was statistically significant. This indicates that there is proliferation of blood vessels in early OSMF and a decrease is seen with the progression of the lesion [Table 1].

As the OSMF grades increased, there was a reduction of bFGF expressions in fibroblasts. Fibroblast expression was higher in early OSMF (24.530±0.12.9730), followed by a gradual decrease in intermediate OSMF (13.420 ± 7.0177) and the least in advanced OSMF (10.980 ± 5.4024) cases; however, this difference was statistically significant, as observed in photomicrographs (2a-c). Thus, this indicated that there is proliferation of fibroblasts in early OSMF and a decrease was observed as the lesion progressed [Table 2].

The scatter graph [Graph 1] shows the intensity of bFGF expression in connective tissue stroma in different grades of OSMF. Out of ten cases in early OSMF, the intensity of bFGF expression was mild (+) in seven cases, moderate (++) in two cases, and severe expression (+++) in one case. Out of ten cases in intermediate OSMF, the intensity of bFGF expression was mild (+) in two cases, moderate (++) in six cases, and severe expression (+++) in two cases. Out of ten cases in advanced OSMF, the intensity of bFGF expression was mild (+) in one case, moderate (++) in three cases, and severe expression (+++) in six cases, displayed in photomicrographs (3a-c). Therefore, as the grade of OSMF increases, the intensity of bFGF expression in stroma increases.

With the increase in the severity of OSMF, there was a decrease in the number of inflammatory cells. The inflammatory cell count was higher in early OSMF (42.46 ± 17.67), followed by a gradual decrease in intermediate OSMF (30.20 ± 10.05) and the least in advanced OSMF (24.62 ± 10.97) cases. This indicated that there are numerous inflammatory cells in early OSMF and a decrease in the number of inflammatory cells was observed with the progression of the lesion [Table 3].

DISCUSSION

In our study, we found that there was a proliferation of blood vessels in early OSMF, and a decrease was seen thereafter with the progression of the lesion [Table 1].

Our results were in accordance with the study by Pandiar and Shameena,[9] who demonstrated that the mean vascular density reduced significantly as the lesion progressed. Furthermore, significantly enhanced vascular supply was noticed in cases of OSMF which turned into malignant lesions.

Singh *et al*.[10] also analysed OSMF cases of Grades I, II, III, and IV morphometrically concerning the epithelium, vasculature, and fibrosis and concluded that the mean blood vessel area and the mean vessel diameter showed a marked increase in grade II and a marked decrease in grades III and IV, respectively. They also stated that microvessel hyperplasia occurs during the early stage of OSMF. On the contrary, Rajendran[11] found that mean vascular density was similar in all the grades. They observed an increase in mean vascular dilatation and considered it an adaptive response to compensate for tissue ischaemia.

In our study, we found that there was a proliferation of fibroblasts in early OSMF, and a decrease was observed as the lesion progressed [Table 2]. Our results were in accordance with Meghji *et al*.,[12] who compared human fibroblasts from normal and oral submucous fibrotic tissues. In normal tissue, the fibroblast activity is modulated by the interaction of cells with local factors, whereas in OSMF tissues, betel nut extracts and cytokines from inflammatory cells stimulate fibroblast proliferation and collagen synthesis. Thus, there is fibroblast proliferation in the early stages of OSMF.

Jeng *et al*.[13] studied the pathobiological effects of betel quid extracts on cultured human buccal mucosal fibroblasts and suggested that betel contained cytotoxic as well as genotoxic agents and could stimulate cellular proliferation. Thus, fibroblast proliferation can be observed in the early OSMF stage. Another study by Van *et al*.[14] suggested that arecoline at higher concentrations markedly suppresses the proliferation of fibroblasts and phagocytotic activities in fibroblasts in OSMF. Thus, there is a reduction of fibroblasts in advanced OSMF cases.

Graph 1 shows that as the grade of OSMF increased, the intensity of bFGF expression in connective stroma increased. This is similar to the study by James *et al*.,[15] which evaluated the role of bFGF in the progression of OSMF and connective stroma changes as the lesion progressed. Thus, an increase in bFGF expression in early OSMF is associated with the injury state caused by areca consumption. Another study by Bishen *et al*.[7] suggested that increased bFGF expression in the early stages could be a result of an initial injury phase because of areca nut; this leads to cellular activation by chemotactic cytokines and fibrosis.

With the increase in the severity of OSMF, there was a decrease in the number of inflammatory cells. The inflammatory cell count was higher in early OSMF (42.46 ± 17.67), followed by a gradual decrease in intermediate OSMF (30.20 ± 10.05) and the least in advanced OSMF (24.62 ± 10.97) cases. The difference between the means was non-significant. This indicates that there are numerous inflammatory cells in early OSMF and a decrease is seen as the lesion progresses [Table 3].

Our study results showed that with the increase in the severity of OSMF, a reduction was observed in the number of inflammatory cells. Ankle *et al*.[16] stated that in early OSMF, mast cell-derived tryptase causes increased production of type–I collagen and fibronectin, thereby leading to fibrosis. Bishen *et al*.[7] also stated that local and systemic upregulation of inflammatory and fibrogenic cytokines and downregulation of antifibrotic cytokines are described to be central to the pathogenesis of OSMF.

The pathophysiology of OSMF could be described as overlapping, yet sequential events of biochemical alterations occurring at the cellular level, where there is initially a cellular activation and injury phase. During this phase, the endothelium facilitates the migration of mononuclear cells into the interstitium and activated fibroblast begins to populate the interstitium. Hence, our findings of increased bFGF expression in both endothelial cells and fibroblasts could be explained by understanding these fundamental factors of cell kinetics.

While bFGF is auto-repressive and catabolic, TGF-β has shown to be auto-inductive and anabolic, thus representing a part of the feedback mechanism controlling stromal growth and extracellular matrix accumulation. Additional studies to test the effect of bFGF and TGF-β alone and in combination on cultured fibroblasts from OSMF tissues may prove beneficial as these studies may provide greater insights into its pathogenesis and offer novel options for relevant therapeutic interventions.

CONCLUSION

To conclude, bFGF expression was noted in the blood vessels, fibroblasts, and connective tissue stroma in the majority of the study cases. The count of bFGF-labelled blood vessels among the Early OSMF subgroup was significantly higher than in the intermediate and advanced OSMF groups. The count of bFGF-labelled fibroblasts among early OSMF subgroups was found to be significantly

higher than that of the intermediate and advanced OSMF groups. The intensity of bFGF expression in connective tissue stroma was mild in early OSMF, followed by moderate intensity in intermediate OSMF and severe intensity in advanced OSMF.

As seen in the present study, bFGF expressions in both endothelial cells and fibroblasts could be described as overlapping, sequential events occurring at the biochemical level which are responsible for cellular activation. Increased bFGF expression in the stroma was observed in conjunction with increased fibrosis as fibrosis is a complicated process where several cellular and molecular mediators interact with each other. Chemotactic cytokines act as a stimulus for cellular activation and extracellular matrix deposition. Further studies are required to generalise the findings of our study and validate our results for positive patient outcomes.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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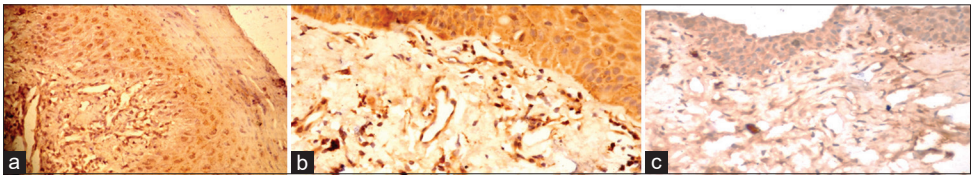
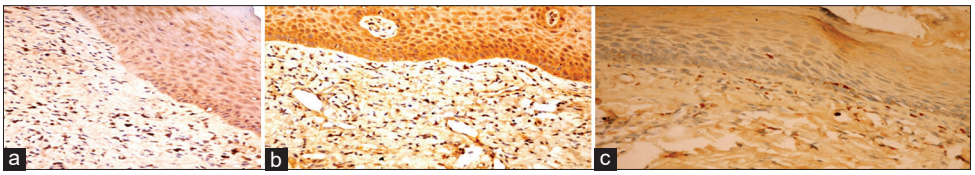
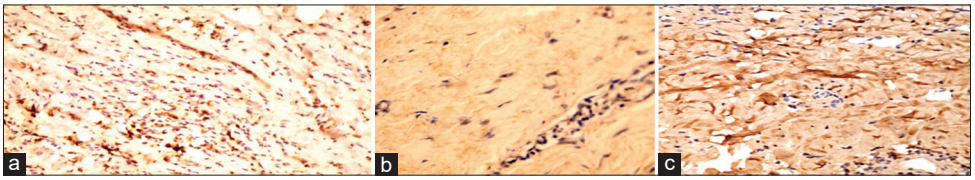
SUPPLEMENTARY FILES



Photomicrograph 1: (a) Section showing bFGF expression in blood vessels in Early OSMF (IHC, x40). (b) Section showing bFGF expression in blood vessels in Intermediate OSMF (IHC, x40). (c) Section showing bFGF expression in blood vessels in Advanced OSMF (IHC, x40)



Photomicrograph 2: (a) Section showing bFGF expression in fibroblasts in Early OSMF (IHC, x40). (b) Section showing bFGF expression in fibroblasts in Intermediate OSMF (IHC, x40). (c) Section showing bFGF expression in fibroblasts in Advanced OSMF (IHC, x40)



Photomicrograph 3: (a) Section showing bFGF expression in connective tissue stroma in Early OSMF (IHC, x40). (b) Section showing bFGF expression in connective tissue stroma in Intermediate OSMF (IHC, x40). (c) Section showing bFGF expression in connective tissue stroma in Advanced OSMF (IHC, x40)