

Original

Morphometric analysis of the mucosal vasculature in oral submucous fibrosis and its comparison with oral squamous cell carcinoma

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Abstract: Oral submucous fibrosis (OSMF) is a chronic fibrotic disorder in which the degree of vascularity has always been a matter of dispute; however, morphological studies of the blood vessels in OSMF have been sparse. This study was performed to assess the mucosal vasculature in normal oral mucosa, early and advanced OSMF, and well differentiated squamous cell carcinoma (WDSCC) using morphometry. The study included histologically diagnosed cases of early ($n = 30$) and advanced ($n = 30$) OSMF, and WDSCC ($n = 30$), with normal oral mucosa ($n = 10$) as a control. Morphometric image analysis of blood vessels was performed on H&E-stained sections for evaluation of vascular density, vascular luminal diameter, area and percentage area. A significant increase in all of the parameters was noted in the test groups relative to the controls. The mean vascular density and mean vascular percentage area were significantly increased in early OSMF and WDSCC relative to controls, and also in advanced OSMF and WDSCC in comparison with early OSMF. The vascularity increased progressively from normal to premalignancy and malignancy, emphasizing the importance of angiogenesis in tumor development and progres-

sion. The vascularity was increased in early OSMF and reduced in advanced OSMF, suggesting that inflammation may play a role in the early stages while progressive fibrosis may predispose to atrophy of the epithelium and subsequent malignant changes. (J Oral Sci 56, 173-178, 2014)

Keywords: oral submucous fibrosis; well differentiated squamous cell carcinoma; morphometry; vascularity; ischemia; malignant potential.

Introduction

Oral submucous fibrosis (OSMF) is a chronic disorder characterized by fibrosis of the lining mucosa of the upper digestive tract involving the oral cavity, oropharynx and hypopharynx and the upper third of the esophagus (1,2). A more serious aspect of this disease is the risk of development of oral carcinoma (3). The important histopathological characteristics of this disease include epithelial atrophy with loss of rete ridges, reduced vascularity, chronic inflammatory infiltration and hyalinization of the submucosal tissue (4).

The epithelial atrophy observed in OSMF is presumed to be secondary to reduced vascularity seen in the adjoining connective tissue, especially in the advanced stages (5-7). However, a prominent vascular response is commonly seen in the early stages of OSMF due to inflammation (8). A combination of normal, dilated and constricted blood vessels have often been observed in the same section (6). The degree of vascularity of the

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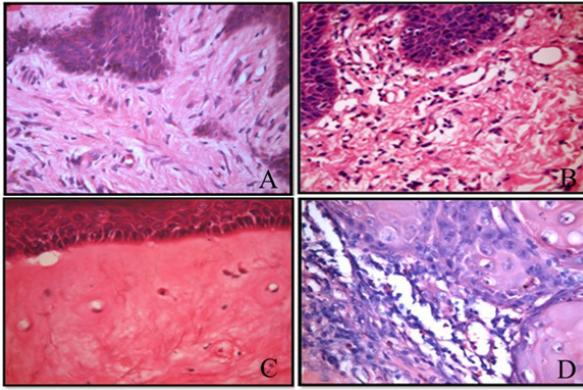


Fig. 1 Photomicrograph demonstrates normal mucosa with epithelium and underlying connective tissue without any inflammation (A; $\times 400$). Early OSMF shows thickened epithelium with rete ridges and varying degrees of inflammation and vascularity (B; $\times 400$). The epithelium is atrophic with sub-epithelial hyalinization and dense fibrosis evidenced in the connective tissue of advanced OSMF (C; $\times 400$). WDSCC demonstrates dysplastic epithelial islands dispersed in inflamed connective tissue (D; $\times 400$).

diseased mucosa in OSMF has always been a matter of considerable dispute (9).

Morphometry has been shown to have a great degree of precision and to be efficient for quantifying the morphologic characteristics of cells and tissues in two-dimensional planes. It also permits identification of variations from normal (10). Morphometry has been used in some previous studies for studying the vasculature in OSMF (4,11-13), but the morphological details of blood vessels have remained inconclusive, and therefore details of the pathogenesis of epithelial atrophy based on persistent ischemia and defective perfusion require further clarification.

The aim of the present study was to characterize and quantify the mucosal vasculature in OSMF tissues in comparison with oral squamous cell carcinoma, which has been considered the final stage of all precancerous lesions and conditions.

Materials and Methods

Following approval of the protocol from the Institutional Ethical Review Board (Approval No. 968, 2012), a total of 100 paraffin-embedded tissue blocks comprising histologically diagnosed cases of oral submucous fibrosis ($n = 60$), well differentiated squamous cell carcinoma ($n = 30$) and normal oral mucosa ($n = 30$) were retrieved from the archives of the Department of Oral Pathology and Microbiology. Histologically diagnosed oral submucous fibrosis was classified on the basis of Pindborg and Sirsat (14). Very early and early cases were categorized

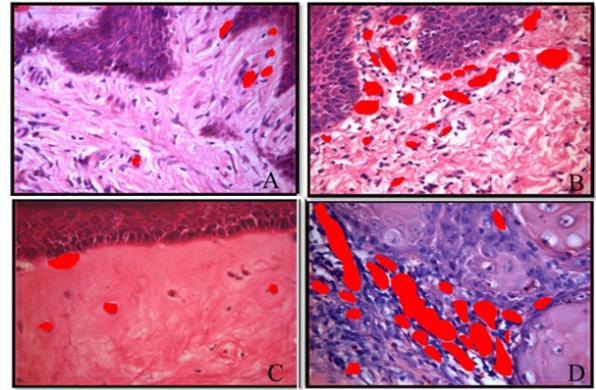


Fig. 2 Photomicrographs depict blood vessels marked for morphometric analysis in the subepithelial region of normal mucosa (A; $\times 400$), early oral submucosal fibrosis (B; $\times 400$), advanced oral submucosal fibrosis (C; $\times 400$) and well differentiated squamous cell carcinoma (D; $\times 400$).

as early oral submucous fibrosis (EOSMF) (30 cases), and the moderately advanced and advanced OSMF cases were categorized as advanced oral submucous fibrosis (AOSMF) (30 cases). Thirty cases of well differentiated oral squamous cell carcinoma diagnosed histologically on the basis of Broder's grading criteria were also included. The control group, comprising 10 sections of normal oral mucosal tissue, was employed to establish baseline data for normal vasculature. These specimens had been obtained from gingival and vestibular mucosa after extraction of impacted teeth.

These biopsied tissues were sectioned and stained with hematoxylin and eosin. Stained 4- μ m-thick sections were subjected to histomorphometric analysis using Q-Win standard (Leica) image analysis software (version 3.5.0, Leica Microsystems, Wetzlar, Germany) with a research microscope (Leica DM2500, Leica Microsystems, Wetzlar, Germany).

Using a CCD color video camera (Leica DFC 320) attached to the research microscope, the images of five representative fields in the sub-epithelial region from each section were captured in a stepwise manner by moving the microscope stage from left to right. The images were visualized and stored in a computer for further analysis. A cursor was used to outline the blood vessels at a magnification of $\times 400$. The blood vessels were measured for number, luminal diameter, area, and percentage area. The image analysis software has an inbuilt measurement tool for all of the parameters chosen, i.e. number, luminal diameter, and mean vascular area, while the percentage

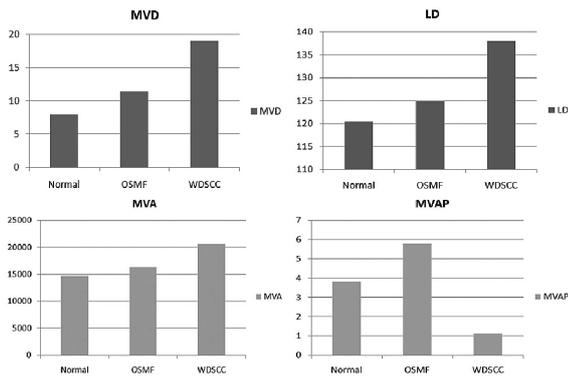


Fig. 3 Graph demonstrating the vascular parameters in normal mucosa, OSMF and WDSCC.

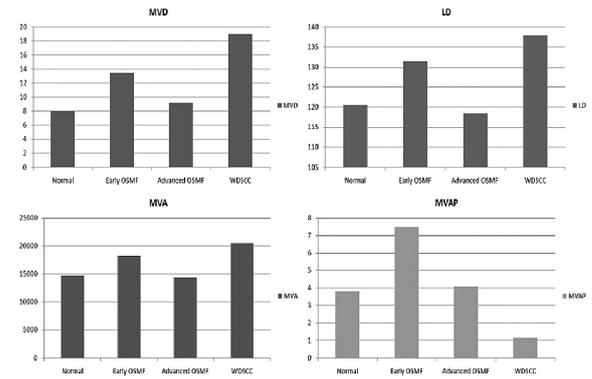


Fig. 4 Graph demonstrating the morphometrically measured vascular parameters including MVD (mean vascular density), LD (luminal diameter), MVA (mean vascular area) and MPVA (mean percentage vascular area) in normal controls, early OSMF, advanced OSMF and OSCC.

Table 1 Comparison of the various parameters of blood vessels in the three study groups i.e. normal, OSMF, and oral squamous cell carcinoma using Bonferroni multiple comparison test

	MVD	Luminal diameter	MVA	MVAP
Normal & OSMF	0.128	1.000	1.000	0.300
Normal & WDSCC	0.000 ⁺	0.144	0.117	0.000 ⁺
OSMF & WDSCC	0.000 ⁺	0.051 ⁺	0.044 ⁺	0.000 ⁺

$P < 0.05$ = Statistically significant (S), $P > 0.05$ = Statistically non-significant (NS), where P = probability; OSMF oral submucous fibrosis; WDSCC well differentiated squamous cell carcinoma

area signifies the area occupied by blood vessels in the entire field evaluated.

Statistical analysis

The mean and standard deviation were calculated for all of the parameters measured, and statistical analysis was performed using analysis of variance test (ANOVA) and Bonferroni multiple comparison test.

Results

In normal mucosa, the epithelium with underlying connective tissue showed no inflammation. EOSMF showed a thickened epithelium with rete ridges and varying degrees of inflammation and vascularity. In AOSMF, the epithelium was atrophic with sub-epithelial hyalinization and dense fibrosis in the connective tissue. WDSCC demonstrated dysplastic epithelial islands dispersed in an inflamed connective tissue (Fig. 1). Morphometric analysis of the vasculature for parameters including mean vascular density, mean vascular luminal diameter, mean vascular area (MVA) and mean vascular percentage area (MVPA) was undertaken in all of the cases in the various study groups (Fig. 2).

A progressive increase in the number of blood vessels

(MVD) from normal ($8 \pm 2.26 \mu\text{m}$) to OSMF ($11.35 \pm 4.34 \mu\text{m}$) and WDSCC ($18.99 \pm 6.04 \mu\text{m}$) was noted. The luminal diameter was $99.7\text{--}153.1 \mu\text{m}$ (mean $120.46 \mu\text{m}$) in the control group, $84.31\text{--}189.49 \mu\text{m}$ (mean $124.94 \mu\text{m}$) in OSMF, and $88.12\text{--}205.76 \mu\text{m}$ (mean $137.90 \mu\text{m}$) in WDSCC. An increase in MVD was noted in the test groups relative to the controls. The area of the blood vessels was increased in OSMF ($16,297 \pm 6,955.1 \mu\text{m}^2$) and WDSCC ($20,537 \pm 9,251.7 \mu\text{m}^2$) relative to normal mucosa ($14,718 \pm 5,728.8 \mu\text{m}^2$). In general, all of the parameters showed a progressive increase in OSMF cases relative to the control group. The highest mean values were noted in WDSCC. ANOVA test revealed statistical significant differences for MVD, LD, MVA, and MVAP among the study groups i.e. between normal, OSMF, and WDSCC (Fig. 3).

Pairwise comparisons of the study groups using the Bonferroni multiple comparison test are depicted in Table 1. None of the parameters demonstrated significant differences in comparison with the normal and OSMF groups. The MVD and MVAP were significantly larger in WDSCC as compared to normal controls while MVD, MVD, MVA, and MVAP were all significantly larger in WDSCC as compared to the OSMF group.

Table 2 Comparison of the various parameters of blood vessels in the normal, EOSMF, AOSMF, and WDSCC groups using Bonferroni multiple comparison test

	Count	Luminal diameter	MVA	MVAP
Normal & EOSMF	0.007 (S)	1.000 (NS)	1.000 (NS)	0.014 (S)
Normal & AOSMF	1.000 (NS)	1.000 (NS)	1.000 (NS)	1.000 (NS)
Normal & WDSCC	0.000 (S)	0.265 (NS)	0.217 (NS)	0.000 (S)
EOSMF & AOSMF	0.003 (S)	0.215 (NS)	0.267 (NS)	0.001 (S)
EOSMF & WDSCC	0.000 (S)	1.000 (NS)	1.000 (NS)	0.000 (S)
AOSMF & WDSCC	0.000 (S)	0.011 (S)	0.011 (S)	0.000 (S)

$P < 0.05$ = statistically significant (S), $P > 0.05$ = statistically non-significant (NS), where P = probability; OSMF oral submucous fibrosis; WDSCC well differentiated squamous cell carcinoma

Figure 4 demonstrates that the MVD increased from normal mucosa to EOSMF, decreased in AOSMF, and increased again in WDSCC. A similar trend was also noted for MVLD, MVA and MVAP. These differences observed in the various parameters among the four study groups were statistically highly significant (ANOVA test, $P < 0.001$).

Pairwise comparisons of the four study groups using Bonferroni multiple comparison test are depicted in Table 2. Normal mucosa and AOSMF showed no significant differences in any of the parameters. Significant differences in MVD and MVAP were evident between normal mucosa and EOSMF, normal mucosa and WDSCC, EOSMF and AOSMF, and EOSMF and WDSCC; the AOSMF and WDSCC groups demonstrated significant differences in all of the parameters.

Discussion

Oral carcinoma is a global health problem demonstrating a rising prevalence and mortality rate, with 3,000,000 new cases being diagnosed annually worldwide (15). Many oral squamous cell carcinomas are preceded by clinically evident potentially malignant oral disorders such as hyperkeratosis or epithelial hyperplasia, epithelial dysplasia, erythroplakia, and OSMF (16).

OSMF is characterized by inflammation and progressive mucosal fibrosis. Epithelial changes include hyperplasia in the early stage and atrophy in the later stage. The changes in connective tissue vary from fibrosis to hyalinization (17).

Angiogenesis plays an essential role in tumor progression and there is increasing evidence that the process of angiogenesis commences in the premalignant stages of most cancers, serving as an alternative marker of tumor development, as the majority of cases do not progress to malignancy (18).

The present study, morphometric analysis of the vasculature in normal oral mucosa, early and advanced

oral submucous fibrosis and well differentiated squamous cell carcinoma was conducted to determine for number, luminal diameter, area and percentage area of microvessels.

In normal oral epithelium and premalignant lesions, microvessels are mainly located just underneath the epithelium (19). Therefore, we counted five microscopic fields at $\times 400$ magnification with a one-field depth from the basement membrane of the epithelium, from the tumor-invasive margins, or from the tumor nests, and calculated the mean values of these five fields in each specimen. Necrotic areas within the tumor sections were avoided.

In the present study, MVD increased progressively from normal mucosa to OSMF, and to WDSCC, thus confirming the findings of Sabarinath et al. (13) and Rajiv et al. (4). In contrast, the study conducted by Rajendran et al. (6) showed that MVD was more or less the same in both the test and control groups. In the present study, an increase of MVD in the early stage of OSMF relative to the advanced stage was noted. This was in accord with previous studies by Singh et al. (12) and Fang et al. (11), who also found that the MVD increased in the early stages and decreased in the advanced stage of OSMF. In contrast, the study by Sabarinath et al. (13) showed that the mean MVD had a tendency to increase as the disease progressed, although the increase among the various stages of OSMF was not statistically significant. Singh et al. (12) and Fang et al. (11) found a significant increase of MVD in the early stage of OSMF in comparison with the other stages. Our present results suggest that the rise in MVD noted in the early stage of OSMF is probably due to neoangiogenesis in the mucosa to compensate for the hypoxia created by fibrosis (6). As the disease advances and the stroma becomes more and more hyalinized, the mediators of angiogenesis seem to diminish, which could explain the decrease in MVD observed in the late stage of OSMF. A significant rise in MVD was noted

in WDSCC relative to normal mucosa and OSMF. An angiogenic switch seems to be triggered in these early stages of epithelial malignant transformation due to the increased demand for blood to provide nutrition for the proliferating tumour cells, and this could account for the increased MVD.

The mean vascular luminal diameter (MVL) showed a decreasing trend in OSMF as the disease progressed, in accordance with the findings of Rajiv et al. (4) and Singh et al. (12). In contrast, Rajendran et al. (6) observed an increasing trend in MVL as the disease progressed and concluded that the vascular dilatation occurred as an adaptive response to tissue ischemia or hypoxia, although MVD was similar in the test and control samples. In the present study, ANOVA demonstrated significant differences in MVL among the four study groups.

Bonferroni test demonstrated no significant differences among the groups except between the AOSMF and WDSCC groups ($P < 0.005$). The present study suggests that a compensatory response to ischemia in the form of an increase in the number of blood vessels, i.e. MVD, rather than vascular dilatation, is more apparent in EOSMF. Thus the old concept that progressive fibrosis compresses blood vessels and causes a reduction of luminal diameter with disease progression, appears to be valid.

An exponential increase in mean vascular area (MVA) was noted with disease progression. Similar results have been obtained in previous studies where the tumor microvessel area was found to be higher in tumors than in normal mucosa (20). Comparison of the mean area using the Bonferroni multiple comparison test showed significant differences between the AOSMF and WDSCC groups, but not among any of the other groups. Though no previous studies have compared OSMF and WDSCC, a few studies have demonstrated a significant increase of MVD in WDSCC relative to normal oral mucosa (21,22).

An increase in mean percentage vascular area (MPVA) was noted in OSMF relative to controls. In contrast, the study by Rajendran et al. (6) showed that the MVAP tended to increase in OSMF as the disease progressed. They suggested that the usual tissue reaction in response to ischemia or hypoxia did not appear to operate in OSMF. In this situation, where neoangiogenesis/vasculogenesis is not feasible, vasodilatation remains the only alternative. However, our results contradicted this hypothesis, and demonstrated a progressive decrease with disease progression in OSMF, consistent with previous studies (4,12,13).

This is further corroborated by the findings related to proliferative activity, which demonstrated increased

proliferation in OSMF relative to normal mucosa, especially in EOSMF associated with inflammation, whereas a progressive decrease in proliferative activity in AOSMF may correlate with the decreased vascularity observed in our study. WDSCC has been reported to show increased proliferative activity with associated enhanced vascularity (23).

The present study corroborates previous findings that vascularity increases progressively from normal mucosa to premalignancy and malignancy, suggesting that angiogenesis is an important factor in tumor development and progression. With regard to OSMF, the vascularity was increased in EOSMF but showed a reduction in the advanced stage. The increased vascularity in the early stages could be due to inflammation and a compensatory attempt of the mucosa to cope with ischemia. However, with progressive fibrosis, the vascularity is reduced, which may predispose the tissue to atrophic changes in the overlying epithelium and subsequent malignant changes. The results of this study need to be evaluated using a larger sample with long-term follow-up for carcinomatous changes in OSMF patients.

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