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Original Research

Determination of Mean Vessel Density Baseline for Normal Buccal Mucosa: A Histomorphometric Study

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Abstract:

Background: Angiogenesis or neovascularization, the process that leads to formation of the new blood vessels plays a major role not only in the embryogenesis, growth and development of normal tissues but also in various kinds of pathological disorders such as diabetic retinopathy, rheumatoid arthritis, lichen planus, and squamous cell carcinoma. Mean vessel density can be a measure of angiogenesis.

Materials and Methods: The study sample consisted of twenty cases of the normal buccal mucosa (NBM). The twenty samples from the archive were stained using hematoxylin and eosin (H and E) and Masson Trichrome (MT). The stained sections were analyzed using the Image analysis software. Statistical analysis used: SPSS 13.0 statistical software was used.

Results: The mean vessel density (MVD) of all the cases in H and E was combined at 0.1002 and for MT 0.2050. The difference of MVD between H and E and MT was statistically significant.

Conclusions: MVD can be used as an indicator of turn-on and turn-off phenomenon of angiogenesis. MVD can also be used as a supplement to other diagnostic techniques. The buccal mucosa baseline level MVD for was 11.5%. More statistics is required to standardize baseline levels for different sites of oral mucosa, age, and gender groups. Hence, the increase or decrease of angiogenesis can be easily compared to the baseline values.

Key Words: Angiogenesis, Masson's Trichrome, MVD

Introduction

Angiogenesis or neovascularization is a complex, multistep process involving cells of endothelium, their growth, structure of capillaries, and dissemination.[1] To grow and disseminate tumors require a rich supply of blood. A lot of studies have been done in recent years mainly upon the process through which tumors develop their supply of blood. We all know that tumors recruit attract new endothelial cells from the already present circulation by secreting various types of growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor, etc., from the cells of tumor. This has been proved in many human malignancies, for example in the lung, prostate, and breast cancers. However still, in some cancers the relationship between tumor angiogenesis and clinico-pathologic parameters of malignancies are still controversial.[2]

In clinical practice, a lesion can be said potentially malignant only when the malignant changes have been developed since there are no means of predicting with certainty the risk of cancerous transformation. Microscopy has been the best available guide. The oral mucous membrane is highly supplied by blood, and the degree of vascularity differs in different parts of the oral mucous membrane. Hence, this study used mean vascular density (MVD) as a diagnostic tool for diagnosing lesions undergoing angiogenesis.

Aims and objectives

The study attempts to:

1. Evaluate vascularity in normal buccal mucosa (NBM).
2. Evaluate the correlationship between morphometric analysis of blood supply in normal cheek mucosa by hematoxylin and eosin stain (H and E).
3. Evaluate the correlationship between morphometric analysis of blood supply in normal cheek mucosa by Masson's Trichrome (MT) stain.
4. Evaluate the interobserver variability of blood supply in H and E and MT.

Materials and Methods

The study used the 10% formaldehyde fixed tissues, which were embedded in routinely used paraffin embedding material. The study sample consisted of 20 cases of NBM. Suitable information regarding age, gender, area of biopsy site was obtained. Mean vessel density was assessed of H, and E and MT stained 30 normal cheek mucous membrane. Through light microscopy, those areas in the tissue sections, where the density of blood vessels were highest, were identified in ×40 (Figures 1 and 2), and counting was performed in ×400 field (Figure 3). The assessment was made by two trained observers. The images of areas of interest in the stained tissue sections were captured using Lawrence and Mayo (×75) microscope and Nikon digitized camera. After saving the images to the

computer, they were examined using high-resolution RGB display monitor, and the histomorphometric analysis was performed manually by using "Image-J" analysis software.

Following assessments were done:
The vascular tissue was identified by the presence of red blood cells and endothelial cells.

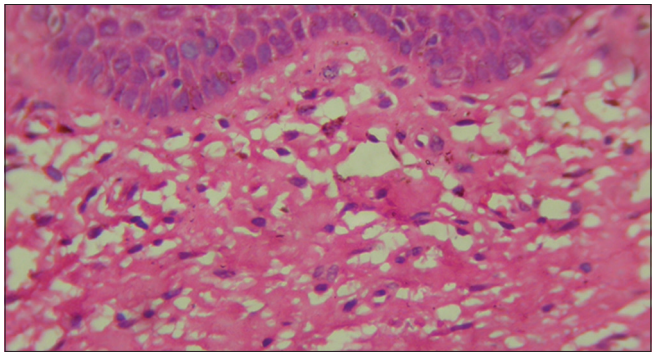

Figure 1: Photomicrograph of normal buccal mucosa showing hematoxylin and eosin stained section (×4.0).

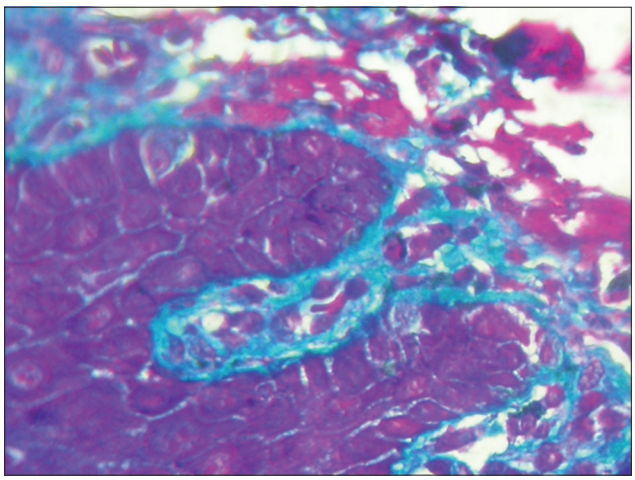

Figure 2: Photomicrograph of normal buccal mucosa showing Masson's trichrome stained section (×4.0).

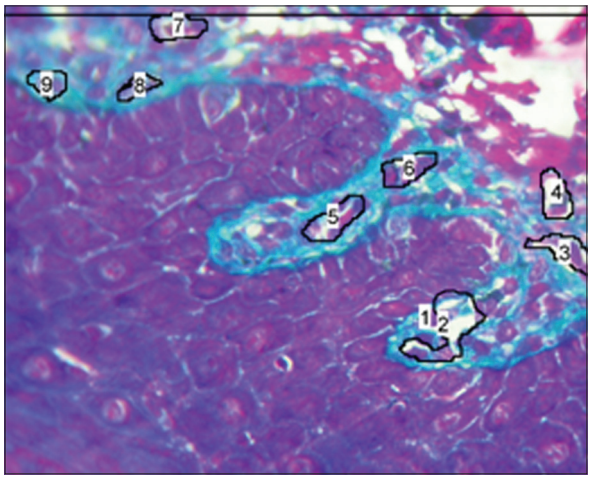

Figure 3: Photomicrograph of normal buccal mucosa showing morphometric analysis in Masson's trichrome stained section

a. For MT method, blood capillaries were surrounded by the red line; whereas lymphatic vessels showed the absence of this red line. The blood capillaries also showed red blood cells within them whereas lymphatic vessels showed the absence of red blood cells within them.
b. Areas rich in blood were found by light microscope in ×40.
c. Eight such fields were selected under ×40 for each slide.
d. The area is representing vascular tissue in the said digital image.
e. The area is representing total tumor tissue in the said digital image.
f. MVD – the ratio of vascular tissue to a total area in the said digital image.

Statistical analysis

For each group, as well as for normal cheek mucous membrane, following statistical analysis was calculated, i.e. mean, standard deviation; minimum and maximum values. To observe differences present in the slide, clockwise and mean vessel density between different groups, student's *t*-test was used.

ANOVA and Student's *t*-test were used for the variance between groups and variance within the group using SPSS 13.0 software, respectively.

For the 20 cases taken in the study, which were all from male subject's, the mean MVD of all the cases in MT staining was 0.2050 and in H and E staining it was 0.1002 with a statistically significant *P*-value of 0.0019 (Table 1). All the study samples were male.

The comparison of NBM in H and E and MT by one-way ANOVA test resulted in a *P*-value of 0.0005 and 0.0001, which was statistically significant (Tables 2 and 3).

Pairwise comparison of two groups by Student's *t*-test was studied for H and E and MT with a *P*-value of 0.0356 and 0.0019. The *P*-values for NBM in H and E was statistically not significant, whereas it was significant for MT group (Tables 4 and 5).

Discussion

The oral mucous membrane undergoes various types of reversible and irreversible changes. These changes have been observed both in the epithelial and connective tissues.[3] The changes in the connective tissues are seen by the presence of an inflammatory component, as well as an increase in blood supply.[4] For more than 100 years, tumors had been seen to contain a higher amount of blood vessels as compared to the

Table 1: Comparison of H and E and MT groups in the two groups.

Group	Mean±SD	*t*-value	*P*-value
H and E (*n*=20)	0.1002±0.1826	−3.1854	0.0019*
MT (*n*=20)	0.2050±0.1024		

H and E: Hematoxylin and eosin, MT: Masson Trichrome, SD: Standard deviation,
*: Significant value < 0.005 is stastically significant.

Table 2: Comparison of two groups (interobserver variability) of in H and E by one-way ANOVA.

Source of variation	Degree of freedom	Sum of squares	Mean sum of squares	*F*-value	*P*-value
Between groups	2	0.49	0.2536	8.2133	0.0005*

H and E: Hematoxylin and eosin, MT: Masson Trichrome, SD: Standard deviation, *: Significant value < 0.005 is stastically significant.

Table 3: Comparison of two groups (interobserver variability) in MT by one-way ANOVA.

Source of variation	Degree of freedom	Sum of squares	Mean sum of squares	*F*-value	*P*-value
Between groups	2	0.85	0.4259	13.7177	0.0000*

H and E: Hematoxylin and eosin, MT: Masson Trichrome, SD: Standard deviation, *: Significant value < 0.005 is stastically significant.

Table 4: Pairwise comparison of three groups using Student's *t*-test for H and E.

Group	Mean±SD	*t* value	*P* value
Normal (*n*=20)	0.0522±0.0716	−2.0009	0.0356*

H and E: Hematoxylin and eosin, SD: Standard deviation

Table 5: Pairwise comparison of three groups using Student's t-test for MT.

Group	Mean±SD	*t* value	*P* value
Normal (*n*=20)	0.1414±0.1378	−2.6660	0.0041*

MT: Masson Trichrome, SD: Standard deviation

physiological counterpart of the same tissues. This increased vascularity in the tumor was observed during surgery, and it was explained due to simple dilation of existing blood capillaries. The cause of dilation of blood capillaries was generally thought to be due to secretion of metabolites or due to necrotic products of tumor escaping from it. The characteristic of pathologic angiogenesis is regular growth of new blood capillaries (i.e., sustained neovascularization).[5] The value of vascularity increases markedly from normal oral mucous membrane to dysplasia and from dysplasia to carcinoma.[6] Various observations confirm that as disease progresses from normal oral mucous membrane to dysplasia and to carcinoma it is associated with increased angiogenesis in the connective tissue. Interestingly, high vascularity is found in that normal mucous membrane, which are adjacent to tumors than in normal mucous membrane without concurrent lesion.[7] In normal oral epithelium and premalignant lesions, micro-vessels are mainly located just beneath the epithelium.[8] Various methods have been developed to assess various characteristics of neovascularization in a tumor. These include alkaline phosphatase method in endothelial cells, India ink perfusion method, etc. Numerous workers have suggested that the number of vessels is a good indication of dissemination and clinical course.[6] Prolong generation of new blood vessels supports the progression of many neoplastic and non-neoplastic diseases. It is interesting to note that angiogenesis whether physiologic or pathologic, are focal. Newly formed blood vessels usually appear as a minute fraction or as a small "hot spot" of proliferating and migrating endothelial cells that arise from a single layer of endothelial cells. Mineo *et al.* have evaluated the impact of tumor angiogenesis on prognosis in those patients who had undergone radical treatment procedure for non-small cell lung cancer by assessing the VEGF, micro-vessel density and involvement of capillaries of tumor.[9] Rostalska *et al.* found out the relationship between micro-vessel density (MVD) and clinicopathological parameters. and stated that in patients less than 50 years of age the density of microvessel was lower.[5] An important parameter for metastasis and tumor growth is neovascularization, thus studying the factors related to it such as MVD can be helpful.[1] MVD is an excellent predictor of the tumor progression and survival rate;[10] however, some workers do not agree with it.[1,11] This discrepancy is due to differences in the techniques of research used in different studies. Other possible reasons could be the absence of a direct method of assessing MVD and the different MVD observed in different areas of the tumor.[12] Growing amount of studies in recent years have proved a marked association of increased MVD with poor prognosis in various types of neoplasms.[13]

The present study included 20 cases of NBM. The aim of the study was to find the morphometric correlation of vascularity, and its usefulness for the buccal mucosa.

Numerous research works on neovascularization considering various parameters which include mean vascular density (MVD), mean vascular volume, mast cell density, and immunohistochemistr.[14] However, none of the antibodies could differentiate between blood capillaries and newly formed capillaries of blood. In some studies α versus β3 integrin and transforming growth factor-β receptor complex were thought to differentiate pre-existing blood capillaries from newly formed blood capillaries, but, they were not found useful in the further studies.

Though the exact causes for inter-observer variability are unclear, the possible reasons could be attributed to the variations in the anatomy of vascularity in different locations of mouth, different methods of determining mean density of blood vessels, different staining procedures (Antigens used) methods of observation, and degree of angiogenesis.[15,16] Hence, digital method of determining the mean density of blood vessels in MT stained sections is thought to be suitable in developing countries due to economic factors and early detection of the tumor.[6]

Hence, in the present study, the results obtained were that the mean density of blood vessels of all the groups by MT staining was 0.2050 and the mean density of blood vessels of all the

groups by H and E staining was 0.1002. On comparing the values of both the staining methods the difference was found to be statistically significant ($P = 0.0019$). The interobserver variability was also statistically significant ($P = 0001$). This is consistent with the findings of Pujari *et al.*, where they showed that the mean MVD staining by MT was 0.2150 and by H and E it was 0.1112. On comparing both the values their results were also statistically significant ($P = 0.0017$).[6] Hence, MVD using MT can be used as a cost-effective tool to measure the rate of angiogenesis.

Numerous studies showed that the density of micro-vessel could be a reliable independent prognostic factor for tumor progression. It has also been reported that there is a direct relationship between increased micro-vessel density (MVD) and increased rate of tumor progression in epidermoid carcinoma.[17] A high vascular supply is essential to keep cancer tissues growing and to develop metastasis. Hence, determination of level of angiogenesis and lymphangiogenesis in terms of vessel density, i.e., micro-vessel density-MVD and lymphovascular density -LVD is one of the prognostic indicators for a malignant growth (Gleich *et al.*, 1996).[18,19] Researchers have reported that mean density of blood vessels may be considered as an independent indicator of prognosis to understand the size of tumor, its rate of growth, and the mode of metastasis.[17]

Conclusions

MVD can be used as an indicator of turn-on and turn-off phenomenon of neovascularization. Thus, MVD can also be used as an additional tool along with other diagnostic modalities. The baseline level of MVD for buccal mucosa was 11.5%. Further research works are required to set the standard baseline levels of MVD for various areas of mucous membrane of the oral cavity, age, and gender groups. Hence, the increase or decrease of angiogenesis can be easily compared to the baseline values.

References

1. Schimming R, Reusch P, Kuschnierz J, Schmelzeisen R. Angiogenic factors in squamous cell carcinoma of the oral cavity: Do they have prognostic relevance? J Craniomaxillofac Surg 2004;32(3):176-81.
2. Polverini PJ. Transfer of advances in science into dental education. J Dent Edu 2002;66(8):962-74.
3. Veda H, Vinay M. Mast cells, angiogenesis in oral epithelial dysplastic lesions and oral squamous cell carcinoma. Int J Med Res Health Sci 2015;4(1):46-52.
4. Raica M, Cimpean AM, Ribatti D. Angiogenesis in pre-malignant conditions. Eur J Cancer 2009;45(11):1924-34.
5. Kumar DV, Shaktidar PR, Hemavathy S, Sahana NS. Review on angiogenesis and its importance in tumor progression. IOSR J Dent Med Sci 2014;13(6):103-8.
6. Pujari RK, Vanaki SS, Puranik RS, Desai RS, Motupalli N, Halawar S. Histomorphometric analysis of vascularity in normal buccal mucosa, leukoplakia, and squamous cell carcinoma of buccal mucosa. J Oral Maxillofac Pathol 2013;17(3):334-9.
7. Carlile J, Harada K, Baillie R, Macluskey M, Chisholm DM, Ogden GR, *et al.* Vascular endothelial growth factor (VEGF) expression in oral tissues: Possible relevance to angiogenesis, tumour progression and field cancerisation. J Oral Pathol Med 2001;30(8):449-57.
8. Murgod VV, Kale AD, Angadi PV, Hallikerimath S. Morphometric analysis of the mucosal vasculature in oral submucous fibrosis and its comparison with oral squamous cell carcinoma. J Oral Sci 2014;56(2):173-8.
9. Mineo TC, Ambrogi V, Baldi A, Rabitti C, Bollero P, Vincenzi B, *et al.* Prognostic impact of VEGF, CD31, CD34, and CD105 expression and tumour vessel invasion after radical surgery for IB-IIA non-small cell lung cancer. J Clin Pathol 2004;57(6):591-7.
10. Marioni G, D'Alessandro E, Giacomelli L, Staffieri A. CD105 is a marker of tumour vasculature and a potential target for the treatment of head and neck squamous cell carcinoma. J Oral Pathol Med 2010;39(5):361-7.
11. Pazouki S, Chisholm DM, Adi MM, Carmichael G, Farquharson M, Ogden GR, *et al.* The association between tumour progression and vascularity in the oral mucosa. J Pathol 1997;183(1):39-43.
12. Tadbir AA, Ashraf MJ, Moradi M. Clinicopathological significance of CD105 expression in squamous cell carcinoma of the oral cavity. Middle East J Cancer 2014;5(1):7-12.
13. Margaritescu C, Simionescu C, Mogoanta L, Badea P, Pirici D, Stepan A, *et al.* Endoglin (CD105) and microvessel density in oral squamous cell carcinoma. Rom J Morphol Embryol 2008;49(3):321-6.
14. Li C, Shintani S, Terakado N, Nakashiro K, Hamakawa H. Infiltration of tumor-associated macrophages in human oral squamous cell carcinoma. Oncol Rep 2002;9(6):1219-23.
15. Sedivy R, Beck-Mannagetta J, Haverkampf C, Battistutti W, Hönigschnabl S. Expression of vascular endothelial growth factor-C correlates with the lymphatic microvessel density and the nodal status in oral squamous cell cancer. J Oral Pathol Med 2003;32(8):455-60.
16. Xuan M, Fang YR, Wato M, Hata S, Tanaka A. Immunohistochemical co-localization of lymphatics and blood vessels in oral squamous cell carcinomas. J Oral Pathol Med 2005;34(6):334-9.
17. Chowdhury CR, Kirita T, Jose M, Abdullah R. Angiogenesis and lymphangiogenesis in oral squamous cell carcinoma: Comparison of Japanese and Indian cases. Asian Pac J Cancer Prev 2012;13(7):3219-22.
18. Gleich LL, Biddinger PW, Pavelic ZP, Gluckman JL. Tumor angiogenesis in T1 oral cavity squamous cell carcinoma: Role in predicting tumor aggressiveness. Head Neck 1996;18:343-6.
19. Rao BK. Kaposi's sarcoma: Insights into its understanding. Int J Contemp Dent Med Rev 2014;2014:Article ID: 031114, 2014. doi: 10.15713/ins.ijcdmr.7.