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ORIGINAL RESEARCH

Vascular Endothelial Growth Factor Expression in Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma

[1]Sujatha Varma, [2]PM Shameena, [3]Sudha Sivasankaran, [4] KP Manoj Kumar, [5]Aniruddha A Varekar

ABSTRACT

Background: Among the molecular events involved in carcinogenesis, neovascularization or angiogenesis is considered to be crucial for tumor growth and progression. VEGF is an angiogenic cytokine involved in endothelial cell proliferation, migration and differentiation.

Aim: The purpose of this study was to investigate the immunohistochemical expression of VEGF in normal oral mucosa (NOM), epithelial dysplasia and oral squamous cell carcinoma (OSCC) and to correlate this expression with histologic features of tumor progression.

Materials and methods: In this retrospective study, we examined the immunohistochemical expression of VEGF in 46 oral mucosal biopsy samples which consisted of 5 normal oral mucosal specimens, 17 specimens with varying histologic degrees of epithelial dysplasia and 24 invasive OSCC specimens with varying grades of tumor differentiation.

Results: Statistical analysis indicated an upregulation of VEGF during the transition from NOM through epithelial dysplasia to OSCC whereas a significant correlation could not be established between various grades of dysplasia. An increased and intense expression of VEGF was observed in different histologic grades of squamous cell carcinoma with well differentiated OSCC showing the lowest mean VEGF percentage and intensity scores and poorly differentiated OSCC, the highest.

Conclusion: Our results suggest that VEGF is present in elevated levels in dysplasias and OSCCs when compared with normal oral mucosal specimens. Increased levels of this angiogenic factor could enhance tumor growth by promoting neovascularization.

Keywords: Angiogenesis, Vascular endothelial growth factor, Epithelial dysplasia, Oral squamous cell carcinoma, Immunohistochemistry.

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[1]Assistant Professor, [2,4]Professor and Head
[3]Associate Professor, [5]Senior Lecturer

[1-3]Department of Oral Pathology, Government Dental College Calicut, Kerala, India

[4]Department of Oral and Maxillofacial Surgery, KMCT Dental College and Hospital, Calicut, Kerala, India

[5]Department of Oral Pathology, Dental College and Hospital Bharati Vidyapeeth Deemed University, Sangli, Maharashtra, India

Corresponding Author: Sujatha Varma, Assistant Professor Department of Oral Pathology, Government Dental College Calicut, Kerala, India, Phone: +91-9847705213, e-mail: varma_sujatha@yahoo.com

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INTRODUCTION

Cancer is a leading cause of death worldwide. The magnitude of the problem in the Indian subcontinent is somewhat alarming. Oral squamous cell carcinoma is among the commonest cancers seen in Indian men and women.[1] Despite the advances made in diagnosis and treatment, the mortality rates due to oral cancer have not changed significantly during the past few decades. The current treatment modalities for OSCC are based up on clinical staging and histopathologic grading of the disease.[2] There is increasing evidence that effectiveness of these methods is less reliable.

Researches in the field of oncology have identified several molecular changes occurring during carcinogenesis. The histologic progression of cancer is believed to reflect the accumulation of these changes. Hence, it becomes increasingly important to consider the nature of subcellular alterations in tumor cells for predicting the biological behavior of tumors.

Angiogenesis or neovascularization is the process of new blood vessel formation.[3] It is considered as an essential requirement for physiologic processes as well as tumor growth and progression. The process of angiogenesis is complex and is mediated by the delicate balance between proangiogenic and antiangiogenic molecules.[3-5]

VEGF is a potent angiogenic cytokine involved in the development of blood supply. It has been known to induce both physiologic and pathologic angiogenesis.[3] The mechanism by which VEGF induces angiogenesis includes increased vascular permeability, leakage of proteins like fibrinogen which forms a substrate for endothelial cell proliferation, promotion of endothelial cell growth, migration and differentiation.[3,6,7] *In vitro* studies have shown that VEGF can prevent endothelial cell apoptosis. VEGF has an essential role in embryonic vasculogenesis and angiogenesis. It has been found to be a mediator of angiogenesis and increased vascular permeability in wound healing, several inflammatory disorders like rheumatoid arthritis and psoriasis.[3]

Regulation of VEGF gene expression has been found to be influenced by factors like oxygen tension and the

presence of major growth factors like EGF, TGF-α, β, PDGF and FGF.[3,4] VEGF is found to be expressed under hypoxic conditions and may promote metastasis by exposing the tumors to a greater endothelial surface area thus increasing the likelihood of hematogenous spread.[8] The role of VEGF in pathological conditions has been studied extensively. The factors in the tumor environment and signal transduction pathways that regulate VEGF production are complex.[3,9] Though tumor cells usually present the major source of VEGF, tumor associated stroma is also an important site of VEGF production. The chemotactic signals from tumor cells recruit stromal cells, which also produce VEGF and other angiogenic factors.[3]

Several studies have examined VEGF protein immunohistochemical expression in oral squamous cell carcinomas (OSCC). The results of the studies are conflicting due to the variability in definitions, measurements and experimental procedures.[10] In the literature, VEGF over expression has been shown in OSCCs and tumors with this feature have shown some what aggressive behavior.[11,12] A positive correlation between lymph node metastasis and VEGF upregulation has been observed in some of the studies.[6,11,12]

In the oral cavity 10 to 20% of dysplastic lesions have been found to progress in to invasive carcinomas.[13,14] A significant increase in vascularity occurs during the transition from normal oral mucosa through different degrees of dysplasia to invasive squamous cell carcinoma.[13,14] This increase in vascularity has been associated with tumor progression from early to late carcinoma and lymph node metastasis. However, there is a paucity of literature regarding the role of VEGF in varying grades of dysplasia and squamous cell carcinoma.

The present study aims to investigate the immunohistochemical expression of VEGF in normal oral mucosa, epithelial dysplasia and OSCC and to correlate this expression with histologic features of tumor progression.

MATERIALS AND METHODS

The institutional ethics committee approved the study.

A total of 46 cases were included in this study. Normal oral mucosal tissue specimens were obtained from healthy subjects with informed consent (n = 5). Tissue samples were obtained from patients who underwent diagnostic biopsies for leukoplakia and squamous cell carcinomas of oral cavity in our institution during the period 2006 to 2009.

The histopathologic diagnosis of all cases was established through routine H&E staining and light microscopic examination. Patients with a prior history of diagnostic biopsy or with a previous history of treatment for OSCC were excluded from the study.

All specimens were fixed with 10% formalin for 24 hours, after which they were processed for routine paraffin embedding. Sections of 5 micron thickness were cut from paraffin embedded tissues for hematoxylin and eosin staining. Diagnosis was established by examining the sections under light microscope.

VEGF immunostaining was carried out using rabbit polyclonal anti VEGF antibody (Biogenex, San Ramon, CA, USA).

VEGF expression was mainly confirmed by the presence of brownish, granular staining of cytoplasm of the epithelial cells. The immunoreactivity was strongest in the spinous cell layer of epithelium. Positive staining was also detected in small numbers of fibroblasts, a few endothelial cells and inflammatory cells. When present, salivary glands, muscle and occasionally associated vessels showed positive staining. Immunolocalization of VEGF was examined in normal oral mucosal specimens, different grades of oral epithelial dysplasias and different histologic grades of OSCCs. A specimen was considered to be VEGF positive when at least 50% of epithelial cells showed positive immunostaining.

VEGF immunostaining was quantified as: (1) percentage of epithelial cells stained. (2) Intensity of staining. In each section, three high power light microscopic fields (40 × magnification) were randomly selected. Two observers independently recorded the percentage of VEGF positive cells in each field and mean values for VEGF positivity were calculated. The intensity of VEGF staining was calculated by comparing it with precalibrated control slides. Invasive duct carcinoma of breast was taken as a positive control. As described above, two observers independently recorded the intensity scores. When staining intensity of the VEGF positive specimens matched with that of the invasive duct carcinoma specimens, a score of 2 was given. When staining intensity of the specimen was less than the positive control, it was given a score of 1 and when the intensity of staining was stronger than the control specimen, the score was given as 3. When no staining was evident the scores were given as 0. A mean value of the intensity scores obtained by the two observers was taken as the final VEGF intensity score.

RESULTS

Statistical analysis of the data was carried out with the help of SPSS software using ANOVA analysis and Pearson's Chi-square test. The results were considered significant when $p \leq 0.05$.

In normal oral mucosal (NOM) specimens 40% showed VEGF immunoreactivity (i.e. ≥ 50% VEGF positive cells). Within the mild dysplasia (MED) group, 66.6% expressed VEGF positivity. In moderate dysplasia (MODD) specimens

75% showed VEGF positivity. All severe epithelial dysplasia (SED) specimens showed VEGF positivity. In well differentiated squamous cell carcinoma (WDSCC), 42.8% cases were VEGF positive. In moderately differentiated carcinomas (MDSCC), 88.8% cases were VEGF positive whereas in poorly differentiated squamous cell carcinomas (PDSCC), 100% were VEGF positive (Table 1).

MEAN VEGF PERCENTAGE (TABLE 2)

The mean value for VEGF percentage was 21.5 in the normal mucosal specimens whereas the mean values for VEGF percentage was 57.4 and 64.4 in epithelial dysplasia and OSCC respectively. A significant correlation was observed between mean VEGF percentage in NOM, epithelial dysplasia and OSCC (p = 0.009).

Within the dysplasia group the mean values for VEGF percentage were as follows: MED = 55.1, MODD = 53.9, SED = 64.6. Statistical analysis showed no significant correlation between VEGF percentage and grades of epithelial dysplasia (p = 0.6).

In the OSCC group, the mean values for VEGF percentage were as follows: WDSCC = 44.04, MDSCC = 72.03, PDSCC = 74.3. There was significant correlation between VEGF expression and the histologic grades of squamous cell carcinoma (p = 0.03).

Table 1: VEGF immunoreactivity

Factors	*No. of cases*	*VEGF positive (%)*	*VEGF negative (%)*	*p-value*
Histologic diagnosis				
NOM	5	2 (40)	3 (60)	0.05
Dysplasia	17	13 (76.4)	4 (23.6)	
OSCC	24	19 (79.9)	5 (21.1)	
Grade of dysplasia				
Mild (MED)	6	4 (66.6)	2 (33.3)	0.5 (NS*)
Moderate (MODD)	8	6 (75)	2 (25)	
Severe (SED)	3	3 (100)	0 (0)	
Tumor differentiation				
WDSCC	7	3 (42.7)	4 (57.3)	0.017
MDSCC	9	8 (88.8)	1 (11.2)	
PDSCC	8	8 (100)	0 (0)	

NS*: Not significant

Table 2: Mean VEGF percentage

Factor	*No. of cases*	*Mean VEGF percentage*	*p-value*
Histological diagnosis			
NOM	5	21.5	0.009
Dysplasia	17	57.4	
OSCC	24	64.4	
Dysplasia grade			
MED	6	55.1	0.6 (NS*)
MODD	8	53.9	
SED	3	71.3	
Histopathologic differentiation			
WDSCC	7	44.04	0.03
MDSCC	9	72.03	
PDSCC	8	74.3	

NS*: Not significant

INTENSITY OF VEGF STAINING (TABLE 3)

Mean value of VEGF intensity in NOM was 0.8 where as the mean values for VEGF intensities were 2.05 and 2.1 in epithelial dysplasia and OSCC respectively. Significant difference (p = 0.01) was observed between VEGF intensities in NOM, epithelial dysplasia and OSCC (Figs 1A to F). Within the dysplasia group the mean values for VEGF intensity was 2.08, 1.99 and 2.01 in MED, MODD, SED respectively. There was no statistically significant correlation between the grades of dysplasia and VEGF intensities (p = 0.9). In the OSCC group the mean values for VEGF intensities were 1.4, 2.3 and 2.4 in WDSCC, MDSCC and PDSCC respectively. The correlation between VEGF intensities and histologic grades of OSCC was statistically significant (p = 0.02).

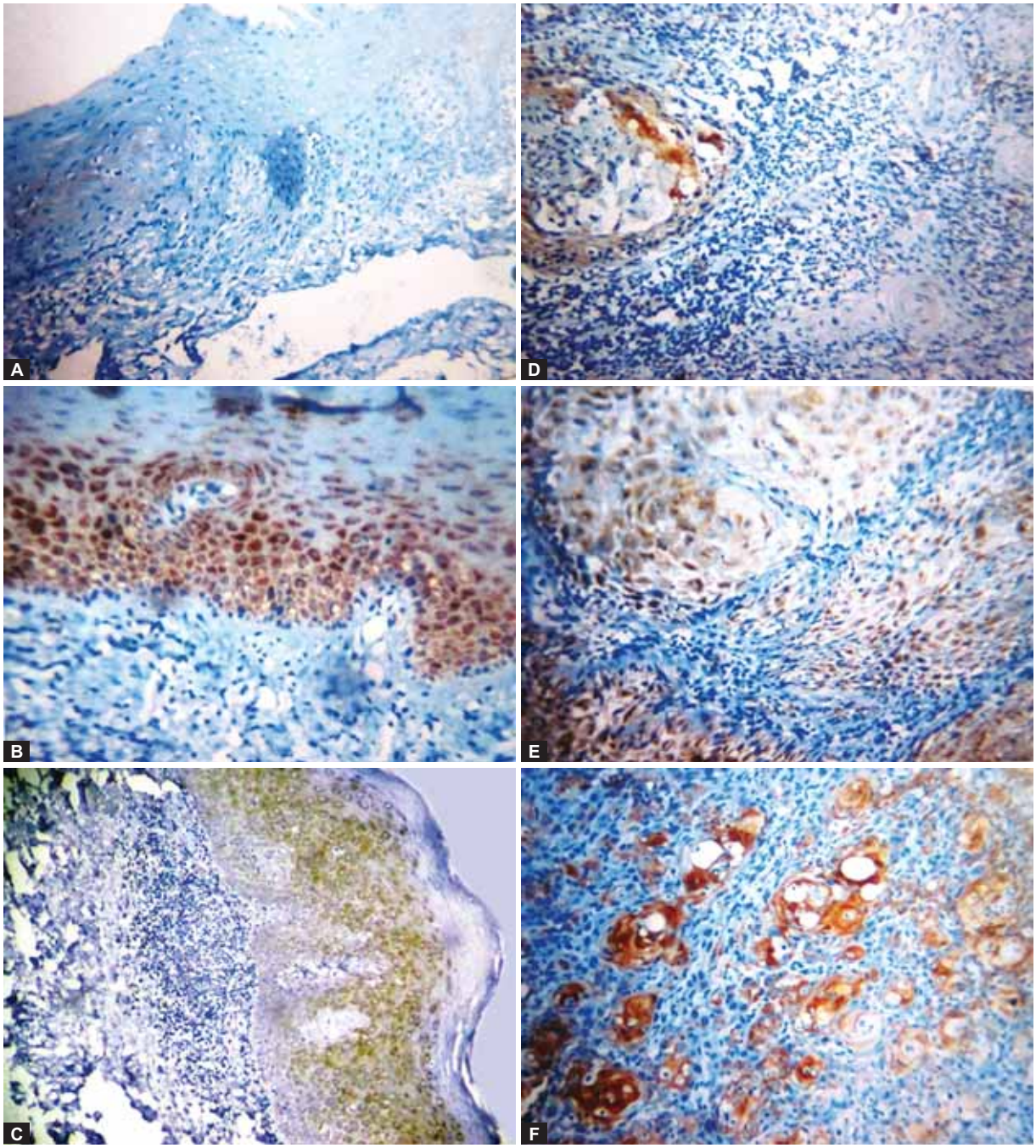
DISCUSSION

Carcinogenesis is a sequential and multi-step process. The neoplastic transformation of squamous epithelial cells is mediated by deregulation of crucial molecular pathways including angiogenesis. Angiogenesis is absolutely necessary for the continued growth and survival of solid

Table 3: Intensity of VEGF staining

Factor	*No. of cases*	*Mean VEGF stain intensity scores*	*p-value*
Histological diagnosis			
NOM	5	0.8	0.01
Dysplasia	17	2.05	
OSCC	24	2.1	
Dysplasia grade			
MED	6	2.08	0.9 (NS*)
MODD	8	1.99	
SED	3	2.01	
Histopathologic differentiation			
WDSCC	7	1.4	0.02
MDSCC	9	2.3	
PDSCC	8	2.4	

NS*: Not significant



Figs 1A to E: Immunohistochemical staining of VEGF in NOM, (A) mild epithelial dysplasia, (B) moderate epithelial dysplasia, (C) well differentiated squamous cell carcinoma, (D) moderately differentiated squamous cell carcinoma, (E) poorly differentiated squamous cell carcinoma (F) (100x)

neoplasms.[3,15] New vessels also increase the opportunity for tumor cells to enter the circulation. Thus, after a tumor has attained a size of 1 to 2 mm, further expansion requires recruitment of new capillaries.[15,16]

Angiogenesis is the outcome of an imbalance between positive and negative angiogenic factors produced by normal cells.[3,5] There is considerable experimental evidence to indicate that tumor growth is dependent on angiogenesis. Researchers have identified the angiogenic effects of various factors including VEGF, IL-8, TGF-α, TGF-β, FGF, TNF-α and angiogenin.[3,4] For the past two decades, the role of VEGF in angiogenesis has been extensively investigated. It has been recognized as a critical angiogenic cytokine involved in the development of blood supply in several different tumors.[9,15] The multiple roles of VEGF are based on its ability to induce various responses by endothelial cells

during vascular development including cell proliferation, migration, specialization and prevention of endothelial cell apoptosis.[3]

Most of the literature on VEGF evaluates the relationship of VEGF with factors like lymph node metastasis and prognosis.[8,10] A great number of them have stated that overexpression of the protein correlates with poorer outcome. The high levels of VEGF expression in tumors are expected to be associated with increased angiogenesis and poor prognosis.[8] Relatively little has been done to explore the role of VEGF expression in epithelial dysplasia and varying histologic grades of OSCC. Because of the obvious importance of angiogenesis for cancer growth and the conflicting data in the literature, we examined the correlation between VEGF expression in normal oral mucosa, epithelial dysplasia and different histologic grades of invasive oral squamous cell carcinoma.

Like most normal healthy tissues, there is usually no neovascularization with in the normal oral mucosa and its underlying stroma.[4] The observations in our study showed that normal oral mucosal specimens did not express or expressed very low levels of VEGF. The low levels of VEGF expression seen in some normal specimens in our study may be due to the presence of mild inflammation as they were obtained from the impacted third molar area.

Johnson[13] and Carlile[14] have found that a significant up regulation of VEGF from NOM to dysplasia to OSCC. In the present study, we observed that VEGF expression was intense and increased throughout the entire thickness of epithelium in severe epithelial dysplasia. Although a statistically significant correlation could not be established between VEGF expression and different degrees of epithelial dysplasia, we have observed that the shift to angiogenic phenotype occurs as early as mild dysplasia, which is sustained through the development of invasive squamous cell carcinoma. This may be due to the fact that as cells transform from normal to dysplastic, the balance between proangiogenic and antiangiogenic factors is altered and the epithelial tumor cells themselves acquire transient angiogenic properties.[4,5]

We found that only 42.7% of WDSCC specimens showed VEGF expression with low mean VEGF intensity scores. In our opinion, the low VEGF values obtained in our cases may be due to the fact that the tumor cell population in WDSCC exhibits a tendency for a terminally differentiated phenotype, which is somewhat similar to the normal keratinocytes. According to Lingen normal keratinocytes were found to secrete high levels of angiogenesis inhibitory factors.[4] High levels of angiogenic factors like TGF-β, produced by these tumors have antiangiogenic properties, resulting in inhibition of angiogenesis. Expression of thrombospondin, a potent negative regulator of angiogenesis has been associated with significantly favorable prognosis.[5] However, we have not investigated the role of the angioinhibitory factors in our study. To obtain more precise results and to assess the overall angiogenic phenotype, the expression of negative regulators of angiogenesis should also be considered along with the VEGF status.[5,10]

Overexpression of VEGF has been correlated to poor survival rate in esophageal, colorectal, pancreatic, lung, gastric and breast carcinomas. Penfold and Eisma has correlated the increased VEGF expression with metastasis and poorer outcome in HNSCC. VEGF secreted by the tumor cells does not stimulate tumor growth directly, but leads to increased growth and permeability of endothelial cells.[17,18] As vascular permeability increases, the microvessels in the tumor environment may become leaky, thereby making them more penetrable by tumor cells. The fragmented basement membranes and endothelial cells at the tips of growing capillaries which secrete collagenases and plasminogen activators in turn increases the likelihood of metastatic process.[18]

We observed that all of the poorly differentiated squamous cell carcinoma specimens and majority of moderately differentiated SCC specimens expressed VEGF significantly with moderate to strong staining intensity. We are of the opinion that the tumor cells in the poorly and moderately differentiated squamous cell exhibit an angiogenic phenotype which could be a reflection of their deregulated genotype.

The results of our study implicate that there is up regulation of VEGF expression from NOM to dysplasia to OSCC, indicating an increase in angiogenesis. The increase in VEGF expression supports the idea that VEGF is involved in increasing vascularity with the progression of disease. VEGF expression has been found to correlate with histopathologic differentiation of invasive OSCC as well. These observations are in agreement with the previous studies.[13,17,18]

CONCLUSION

Our results suggest that VEGF is present in elevated levels in dysplasias and OSCCs when compared with normal oral mucosal specimens. Increased levels of this angiogenic factor could enhance tumor growth by promoting neovascularization. Hence, VEGF expression may be a useful marker in the assessment of angiogenesis in developing precancerous lesions and invasive OSCCs. Thus, the examination of VEGF expression before treatment may be useful in assessing the biologic behavior of tumors and there by suggesting antiangiogenic therapies.

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