J Oral Pathol Med (2005) 34: 134–9

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Expression of endothelial nitric oxide synthase and vascular endothelial growth factor in oral squamous cell carcinoma: its correlation with angiogenesis and disease progression

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BACKGROUND: Angiogenesis is a crucial step in the successful growth, invasion, and metastasis of a tumor. It has been popularly accepted that vascular endothelial growth factor (VEGF) is the most potent angiogenic factor in tumor angiogenesis. As another possible star molecule responsible for tumor angiogenesis, the role of nitric oxide (NO) in tumor biology has gained much attention in recent years. The aim of this study was to investigate whether the expression of endothelial nitric oxide synthase (eNOS) and VEGF in oral squamous cell carcinoma (OSCC) is associated with angiogenesis. The present study also made a preliminary exploration of the possible cross-talking existing between eNOS and VEGF during tumor angiogenesis.

METHODS: In this study, expression of eNOS and VEGF were studied immunohistochemically in tissue sections from 40 patients with OSCC and 20 normal controls. To exclude eNOS antibody cross-reactivity with inducible or neuronal nitric oxide (iNOS or nNOS), eNOS expression was confirmed by using an eNOS mRNA *in situ* hybridization kit. The intratumoral microvessels were highlighted by immunostaining with anti-factor VIII-related antigen monoclonal antibody and counted as well-established methods. Then, chi-square test or Student's t-test was performed to study the correlations between the expression of eNOS and VEGF, microvessel density (MVD), and various clinicopathologic factors.

RESULTS: Both eNOS and VEGF expression significantly increased in OSCC tissues, with a positive rate of 47.5% and 50%, respectively. The average MVD in OSCC tissues was 23.45 per high-power field (HPF), showing an obvious association with lymph node metastasis and clinical stages of patients with OSCC. Either eNOS or VEGF positivity was correlated with vessel involvement and OSCC progression. The mean MVD was significantly higher in eNOS- or VEGF-positive tumors than in eNOS- or VEGF-negative tumors. An obvious, correlation was also seen between eNOS and VEGF expression in OSCC tissues in this study.

CONCLUSIONS: Overexpression of eNOS and VEGF might make an important contribution to the tumor angiogenesis in OSCC. NO generation by eNOS might be implicated in the VEGF-associated angiogenic process. Further investigation of the possible cross-talking between eNOS and VEGF with respect to tumor angiogenesis is necessary.

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Keywords: endothelial nitric oxide synthase; oral squamous cell carcinoma; tumor angiogenesis; vascular endothelial growth factor

Introduction

Angiogenesis is a crucial step in the successful growth, invasion, and metastasis of a tumor. It is now well-established that tumors will not grow beyond 1–2 mm^3 volume unless an intratumoral capillary network is constructed (1, 2). According to the hypothesis of the angiogenic switch balance, both elevation of angiogenic factors and decrease of angiogenic inhibitors may make the switch open and initiate the angiogenic process (3, 4).

Recently, nitric oxide (NO) is thought to play an important role in assisting tumor growth, facilitating dissemination and actively promoting angiogenesis (5, 6). NO is synthesized by three isoforms of nitric oxide synthase (NOS), including neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (7). To our knowledge, most work published on oral cancer and dysplasia has focused on iNOS and concluded that overexpression of iNOS correlates with lymph node metastasis and increase of microvessel count in oral cancer. To date, however, there has only been one clinical paper published on eNOS expression in head and neck cancer and dysplasia (8). Therefore, it is difficult to comment the potential role of NO

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Accepted for publication May 18, 2004

produced by eNOS in angiogenesis of oral squamous cell carcinoma (OSCC) although eNOS was first found in endothelial cells.

As we know, vascular endothelial growth factor (VEGF) has been considered as the most potent candidate for the induction of angiogenesis in tumor growth (1, 9). Many studies have shown that VEGF expression is increased in several kinds of malignant tumors, acting as an endothelial cell mitogen and motogen and as a mediator of increased vascular permeability by binding to its receptors (10). One of our previous studies also demonstrated that elevated circulating levels of VEGF was closely related to progression of OSCC (1). Recently, several studies have shown that VEGF might up-regulate eNOS message, protein, and NO production in human endothelial cells and proposed that NO is an upstream signal for VEGF (11). However, *in vivo* role of eNOS expression in VEGF-induced angiogenesis is poorly understood.

The purpose of the present study was to evaluate the expression of eNOS and VEGF in OSCC and its association with tumor angiogenesis. The current study also made a preliminary investigation of the correlation between eNOS and VEGF expression in tumor angiogenesis.

Materials and methods

Study population

The study population consisted of 40 patients with OSCC and 20 normal individuals without any evidence of disease (e.g. liver dysfunction, diabetes, etc.). The patients ranged in age from 37 to 74 years old (average age: 56.4 years); 30 were men, and 10 were women. No patient had received chemotherapy or radiation therapy before surgery. Local Ethical Committee approval was granted for the use of surgical trimming and informed consent was also obtained from the patients before surgery. All patients were treated at our department and the diagnosis was pathologically conformed. Resected specimens were fixed in 10% formaldehyde solution and embedded in paraffin. Five micrometer thick sections were cut and mounted on glass slides.

Immunohistochemical determination of eNOS and VEGF expression

A-20 is an affinity-purified rabbit polyclonal antibody raised against a 20 amino acide synthetic peptide corresponding to residues 1–20 mapping at the amino-terminus of human VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). C-20 is an affinity-purified rabbit polyclonal antibody raised against a peptide mapping at the carboxy-terminus of eNOS of human origin (Santa Cruz Biotechnology, Inc.). A-20 and C-20 have demonstrated specific activity against human VEGF (12, 13) and eNOS (14, 15), respectively.

Sections were sequentially dewaxed through a series of xylene, graded ethanol, and water immersion steps. Sections were then incubated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Slides were then washed in phosphate-buffered saline (PBS) and incubated in 10% normal goat serum for 20 min to reduce non-specific staining. Specimens were then incubated with a 1:100 dilution of affinity-purified primary antibodies overnight at 4°C, followed by three washes. Slides were then treated with streptoavidin–biotin complex (SABC kit, Boster Biotechnology, Wuhan, China) for 30 min at a dilution of 1:100. After rinsing with PBS (three times for 5 min each), the sections were incubated with the secondary biotinylated goat antirabbit antibody for 30 min at room temperature and avidin-biotin-peroxidase complex for further 30 min. Immunostaining was visualized by developing the slides in diaminobenzidine (DAB) and counterstaining with methyl green. Finally, the sections were subjected to alcohol and xylene baths, then mounted for examination. To control the immunohistochemical specificity, controls were performed using the same immunoglobulin concentrations of rabbit polyclonal immunoglobulin and PBS. Additionally, eNOS expression was also confirmed by using an eNOS mRNA *in situ* hybridization kit according to the manufacturer's instructions.

Slides were interpreted for antigen expression by two investigators without any knowledge of the corresponding clinicopathologic data. The degree of polyclonal antibody reactivity with individual tissue sections was considered positive if unequivocal staining of the membrane or the cytoplasm was seen in more than 25% of tumor cells. At least three fields were assessed from both the invading tumor margin and the tumor center.

Microvessel staining and counting

The methods of microvessel staining and counting were performed according to Maeda et al. (16). Briefly, intratumoral microvessels were highlighted by immunostaining with anti-factor VII- related antigen monoclonal antibody in a 1:200 dilution and incubating at room temperature for 2 h. Any single brown-stained cell or cluster of endothelial cells that was clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements was considered a vessel. Branching structures were counted as a single vessel unless there was a discontinuity in the structure. The stained sections were screened at 5× magnification to identify the areas of the highest vascular density within the tumor. These high neovascular areas could occur anywhere within the tumor, but were most frequent at the margins of the carcinoma. Sclerotic areas, in which microvessels were sparse, and areas immediately adjacent to benign tissue were not considered in vessel counts. Vessels were counted in the five areas of highest vascular density at 200× magnification (20× objective and 10× ocular, 0.785 mm^2 per field). Microvessel count was expressed as the mean number of vessels in these areas.

Statistical analysis

Results were statistically analyzed by using SPSS 10.0 for windows software (SPSS, Inc., Chicago, IL, USA). The relationship between eNOS expression, VEGF expression, the various clinicopathologic factors, and

MVD was examined by chi-square test or Student's *t*-test. For all tests, a *P*-value <0.05 was considered significant.

Results

Our immunohistochemical data revealed that the expression of eNOS was detected in 19 (47.5%) tumors. Positive eNOS staining was mainly localized to the epithelial pearls, cancer nests (mainly in its membrane although sometimes you can see some cytoplasmic staining for eNOS), and microvessels in the stroma (Fig. 1). Tumor cells that stained strongly for eNOS were observed more often in the invasive front than in the tumor center. Positive eNOS staining was also seen on some endothelial cells adjacent to the nests. Endothelial NOS-negative control tumors had large necrotic areas that were not seen in those tumors expressing eNOS. *In situ* hybridization confirmed positive eNOS mRNA expression in tumor tissues (Fig. 2), without cross-reactivity with iNOS or nNOS. Similarly, VEGF expression can be seen in 20 (50%) tumors. Overexpression of VEGF was also seen in both cancerous nests and endothelial cells in the tumor stroma (Fig. 3). The most intense VEGF staining was found at the invading tumor margin. However, normal oral mucosa was not immunoreactive with an anti-VEGF antibody. None of these control stainings showed any immunoreactivity.

Table 1 showed the correlation between eNOS or VEGF expression and various clinicopathologic factors. There was no significant association between eNOS or VEGF expression and degree of differentiation. However, significant difference was noted with respect to nodal metastasis. Both eNOS and VEGF expressions were significant higher in patients with regional lymph nodal involvement than in those without lymphatic invasion. Similar results were obtained with regard to the correlation between eNOS or VEGF expression and clinical stage.

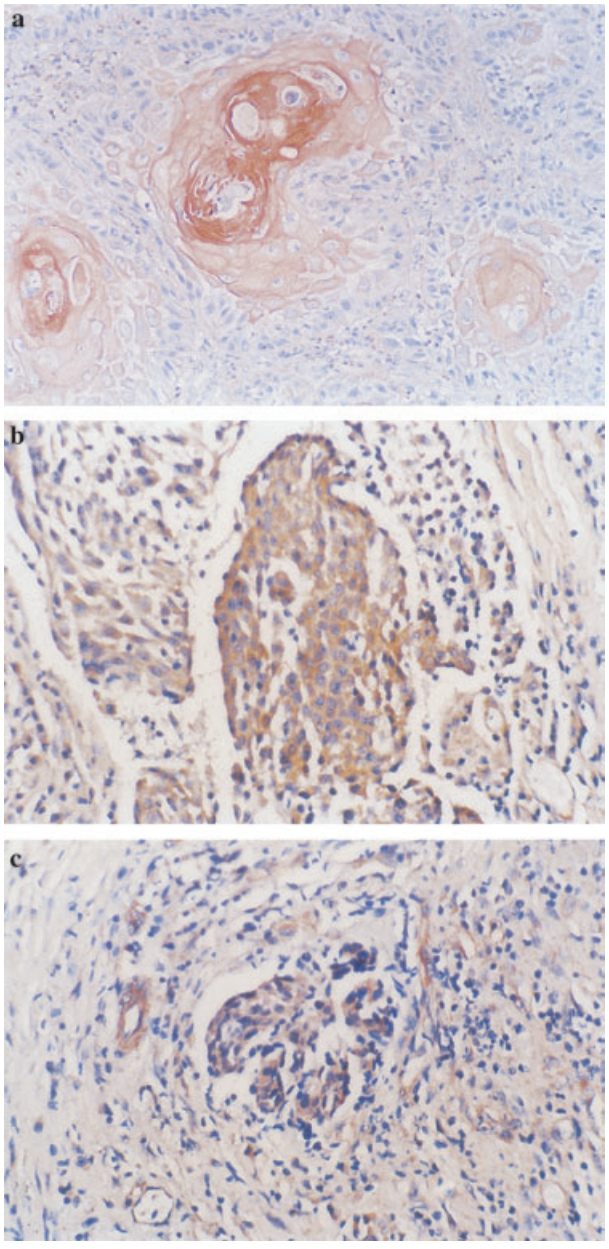

Figure 1 Positive eNOS expression in pearls (a; original magnification: ×400), nests and microvessels (b and c; original magnification: ×200).

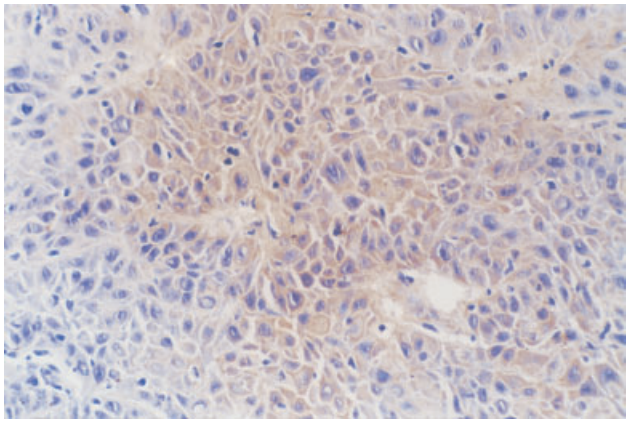

Figure 2 Positive eNOS mRNA expression in oral cancer tissues. (original magnification: ×400)

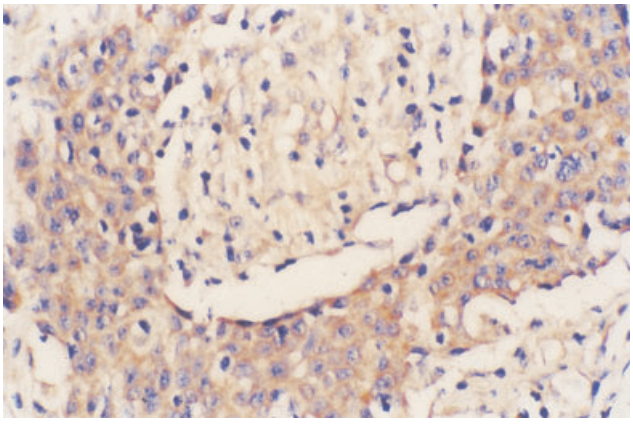

Figure 3 Positive VEGF staining in OSCC in oral cancer tissues. (original magnification: ×400)

Table 1 Expression of endothelial nitric oxide synthase (eNOS)/vascular endothelial growth factor (VEGF) and microvessel density (MVD) count in oral squamous cell carcinoma ($n = 40$)

Clinicopathologic factors	*n*	*eNOS* +	*eNOS* −	χ^2-*test*	*P-value*	*VEGF* +	*VEGF* −	χ^2-*test*	*P-value*	*MVD* ($\bar{x} \pm SD$)	*P-value (t-test)*
Nodal metastasis											
Positive	12	9	3	5.20	< 0.025	9	3	4.29	< 0.05	35.42 ± 9.35	< 0.001
Negative	28	10	18			11	17			18.32 ± 8.26	
Differentiation											
Higher	10	3	7	1.96	> 0.05	4	6	2.24	> 0.05	22.70 ± 5.34	> 0.05
Moderate	22	11	11			11	11			23.55 ± 5.16	
Lower	8	5	3			5	3			24.01 ± 6.80	
Clinical stage											
I–II	25	8	17	6.42	< 0.025	8	17	8.64	< 0.005	20.44 ± 6.28	< 0.002
III–IV	15	11	4			12	3			28.47 ± 7.96	

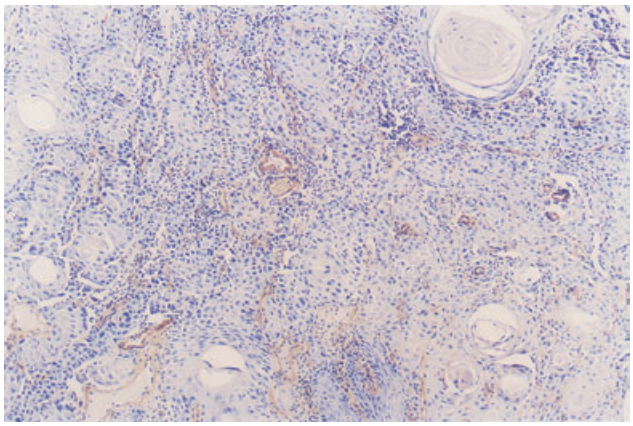

Figure 4 Microvessels staining in oral squamous cell carcinoma. (original magnification: ×200)

Table 2 Relationship between endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) expression and microvessel count

Items	*n*	*Microvessel density (MVD)* ($\bar{x} \pm SD$)	*Student's t-test*	*P-value*
eNOS				
Positive	19	27.11 ± 7.04	3.260	< 0.005
Negative	21	20.14 ± 6.42		
VEGF				
Positive	20	27.15 ± 6.65	3.594	< 0.001
Negative	20	19.75 ± 6.37		

The anti-factor VIII-related antigen expression in OSCC tissues was showed in Fig. 4. The average microvessel density (MVD) in OSCC tissues was 23.45 per high-power field (HPF). The mean MVD was 35.42 ± 9.35 per HPF in node-positive group and 18.32 ± 8.26 per HPF in node-negative group, with significant difference between the two groups. The mean MVD was also obviously correlated with different clinical stages of OSCC ($P < 0.002$), but not with the degree of differentiation of OSCC ($P > 0.05$).

As shown in Table 2, elevation of MVD in human OSCC is closely correlated with overexpression of both eNOS and VEGF. The mean MVD was 27.11 ± 7.04 per HPF in eNOS-positive group and 20.14 ± 6.42 per HPF in eNOS-negative group, with a significant elevation of the mean MVD in eNOS-positive OSCC tissues ($P < 0.005$). The mean MVD was 27.15 ± 6.65 per HPF in VEGF-positive group and 19.75 ± 6.37 per HPF in VEGF-negative group, indicating significantly statistical difference between the two groups ($P < 0.001$).The increased eNOS expression was statistically associated with an elevated VEGF expression ($\chi^2 = 8.120$, d.f. = 38, $P < 0.005$), suggesting that NO might take part in the VEGF-induced angiogenesis (Table 3).

Table 3 Correlation between endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) expression in oral squamous cell carcinoma (OSCC) ($n = 40$)

eNOS	*VEGF* + (20)	*VEGF* − (20)	χ^2-*test*	*P-value*
+ (19)	14	5	8.120	< 0.005
− (21)	6	15		

Discussion

Tumor angiogenesis, an important step in tumor growth and metastasis, could be induced and regulated by many factors. It is now becoming increasingly evident that NO appears to play a crucial, pivotal role in angiogenesis, since L-NAME, a competitive inhibitor of NOS effectively blocks the angiogenic process (6). There are three isoforms of NOS, each isoenzyme being the product of a distinct gene (7, 17–19). While the role of increased iNOS expression was discussed in many tumors, few studies have addressed whether or not expression of eNOS correlates with enhanced angiogenesis and tumor invasiveness. Bentz et al. (8) were the first to study the eNOS expression in head and neck cancer and dysplasia. They reported the immunohistochemical expression of eNOS in cases of hyperplasia, dysplasia and invasive cancer and found significantly increased eNOS staining intensity in cancer cases, compared with normal oral mucosa. The present study found that elevated expression of eNOS is significantly associated with lymph node metastasis and enhanced microvessel count. Furthermore, eNOS-negative control tumors had large necrotic

areas that were not seen in those tumors expressing eNOS. It was postulated that these areas of necrosis were due to deficient angiogenesis. NO increases vessel density in tumors grown by transfecting DLD-1 human colon adenocarcinoma cells with murine iNOS (20). It was also found that these tumors had a more invasive phenotype when compared with untreated controls.

The VEGF is known as one of the most pivotal angiogenic factors responsible for inducing tumor angiogenesis (1, 13, 21). The present study demonstrated that VEGF was overexpressed in patients with OSCC, and that the mean MVD in VEGF-positive group was obviously higher than that in VEGF-negative group. These results were consistent with our previous study, which revealed a significant association between elevated circulating levels of VEGF and lymph node status and clinical stages of OSCC (1). VEGF is a selective mitogen for vascular endothelial cells and may directly stimulate the development of new blood vessels. Elevation of VEGF can also result in extravasation of plasma proteins (e.g. fibrinogen) into the extravascular space through increasing microvascular permeability (16).

Although the true role of VEGF in tumor angiogenesis was well-established, it was poorly understood and controversial with regard to the transduction of angiogenic signal activated by VEGF in the endothelial cells (22, 23). As we know, eNOS is mainly localized within the endothelium. NO produced by eNOS is a signaling molecule in blood vessels, where a continuous formation from endothelial cells acts on the underlying smooth muscle to maintain vasodilation and nutritious blood flow (24). To our experience, however, there is no published data regarding the role of eNOS overexpression in VEGF-induced tumor angiogenesis in OSCC. This preliminary study revealed that increased eNOS expression was statistically associated with an elevated VEGF expression, both showing a close correlation with enhanced angiogenesis. The finding that neovascularization is most pronounced in eNOS- and VEGF-positive tumors suggested that an enhanced vascularity reflects an increased risk of metastasis. These results suggested that there might be a cross-talking existing between VEGF and NO synthesized from eNOS with respect to tumor angiogenesis. VEGF-eNOS pathway may induce greater number of tumor vessels, which in return increase the opportunity for tumor cells to enter the circulation (25). Moreover, newly formed capillaries have fragmented basement membranes and are leaky, making them more penetrable by tumor cells than mature vessels (26). In fact, some authors observed that exogenous VEGF could actively stimulate eNOS expression and NO production of endothelial cells *in vitro* (27–29). Our deduction may also gain part support from the concept that NO is an upstream signal for VEGF, which together with its receptors are mainly responsible for the mechanism of tumor angiogenesis (5, 10, 30, 31). Recently, some studies inferred that binding of VEGF to its receptors might activate the calcium-dependent eNOS, which then increased NO production in endothelial cells and finally initiate the proliferation and migration of endothelial cells (11, 29). Several authors also deduced that Akt phosphorylation plays an important role in VEGF-eNOS pathway in tumor angiogenesis (32). We are currently conducting some *in vitro* studies to investigate the VEGF-eNOS cross-talking during tumor angiogenesis and results should be available soon.

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Acknowledgments

This work was supported by National Natural Science Foundation of China grants (No. 30300390), and Wuhan University Scientific Research Project grants (No. 273106) awarded to Dr Shang.