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Role of angiogenic and non-angiogenic mechanisms in oral squamous cell carcinoma: correlation with histologic differentiation and tumor progression

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BACKGROUND: Angiogenesis has been demonstrated to associate with various measures of tumor aggressiveness in many human malignancies. However, studies of tumor angiogenesis in oral squamous cell carcinoma (SCC) are still unclear. Recent studies indicate non-angiogenesis mechanism (tumor-lined vessel) may exist in certain tumors. Therefore, we investigate microvessel density (MVD) and tumor-lined vessel in oral SCC.

METHODS: Peritumoral and intratumoral MVD were measured by immunohistochemical staining. Tumor-lined vessels were identified by double staining. Statistical analysis of peritumoral and intratumoral MVD and presence of tumor-lined vessels with clinicopathologic parameters was performed.

RESULTS: The results showed peritumoral MVD increased with disease progression and further increases of intratumoral MVD was detected by CD31 and CD34. Non-angiogenesis, tumor-lined vessel, presented in oral SCC and correlated significantly with tumor size, stage, and histologic differentiation.

CONCLUSION: Our results suggest at the initiation of oral SCC, increasing vascularity is observed at the periphery of the tumor. As the tumor continues to grow, further increases of intratumoral vascularity and the presence of tumor-lined vessels are associated with cancer progression.

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Introduction

Tumors require a blood supply for growth and dissemination (1). Considerable attention has been focused on the mechanisms by which tumors acquire their blood supply. It is a well-accepted paradigm that tumors recruit new blood vessels from the existing circulation (angiogenesis) by secreting growth factors from the tumor cells. This has been shown in many human malignances, especially lung, prostate, and breast cancers (2–4). Nonetheless, in some cancers the relationship between tumor angiogenesis and cancer clinicopathologic parameters are still controversial (5, 6).

Recent studies indicate that in addition to the angiogenesis-dependent mechanism, non-angiogenesis-dependent mechanisms such as vascular mimicry (7) and mosaic blood vessels (8) exist in certain tumors. With vascular mimicry, some tumor cells acquired trans-endothelial functions, and then participated in forming tumor-lined vessels to connect with endothelial cell-lined blood vessels. By this mechanism, tumor cells were less angiogenesis-dependent and they used these alternative vessels to grow and metastasize. In the study of mosaic blood vessels, cancer cells were found to locate in the walls of blood vessels, which could not be stained by endothelial markers. These studies implied that some vessels exist in cancer tissue were lined by tumor cells, which could contribute to the controversial relationship between tumor angiogenesis and clinicopathologic parameters.

In oral SCC, angiogenesis correlated with tumor metastasis, disease progression, and prognosis has been demonstrated (9, 10). However, some studies showed that tumor angiogenesis was not associated with tumor size, degree of differentiation, invasion, metastasis, recurrence, prognosis or survival (11–13). This discrepancy may be due to methodologic differences including (i) sensitivity of different endothelial markers for detecting tumor angiogenesis, (ii) selection of the vascular hotspot area, and (iii) presence of non-angiogenic

mechanisms. Thus, we first examined endothelial-lined vessels using immunohistochemical staining with the endothelial cell-specific markers of von Willebrand factor (vWF), CD31, and CD34. Non-angiogenic mechanisms were investigated using double-labeled immunohistochemical staining to identify tumor-lined vessels. Their correlations with clinicopathologic parameters were also evaluated to clarify the roles of angiogenic and non-angiogenic mechanisms in oral SCC.

Materials and methods

Specimens

The specimens obtained from the archives of Tri-Service General Hospital included 12 samples of normal mucosa, 28 samples of dysplastic epithelium, and 112 samples of oral SCC. Primary cancers sites consisted of the tongue in 55 patients, cheek in 30 patients, gingiva in 10 patients, floor of the mouth in four patients, hard and soft palates in seven patients, and lips in six patients. For all patients, the histologic sections exhibiting the most characteristic SCC features and avoiding areas of necrosis or hemorrhage in each specimen were selected. Serial sections were then cut for hematoxylin and eosin (H & E) staining and experimental studies. Histologic changes that contributed to a diagnosis of oral epithelial dysplasia were as defined previously (14). Clinical staging of oral SCC was made according to the American Joint Committee on Cancer (15).

Immunohistochemical staining

Specimens from the paraffin-embedded blocks were cut into 5-μm sections. Standard immunohistochemical staining was performed. The antibodies and concentrations used in this study were as follows: monoclonal mouse antihuman vWF (250 μg/ml, used at 1/200 dilution; Dako, Carpinteria, CA, USA), monoclonal mouse antihuman CD31 (250 μg/ml, used at 1/100 dilution; Dako), and monoclonal mouse antihuman CD34 (250 μg/ml, used at 1/200 dilution; Dako) were used for detection of endothelial cells (16) and monoclonal mouse antihuman pan-cytokeratin (CK, AE1/AE3) (250 μg/ml, used at 1/600 dilution; Dako) was used for detection of squamous cells. Immunostaining was performed with the avidin-biotin-peroxidase complex detection kit (Dako). Double-labeled immunostaining was performed with Dako EnVision Doublestain system.

Sections were dewaxed, retrieved by pressure cooking in 10 mM citrate buffer, pH 6.0 for 30 min (17, 18). Endogenous peroxidase activity and non-specific-binding were blocked by incubation with 3% hydrogen peroxide and non-immune serum, respectively. Slides were then incubated sequentially with primary antibody for 30 min, biotinylated secondary antibody for 10 min, and peroxidase-conjugated streptavidin for 10 min. Then, the chromogen aminoethylcarbazole (AEC) test was performed to localize positive staining by microscopy. For double-labeled immunostaining, primary antibody was visualized with diaminobenzidine (DAB). Doublestain Block (Dako) was applied for 3 min before second antibody incubation, followed by incubation of the labeled polymer alkaline phosphatase (AP) for 30 min. The second antigen stain was completed with 10 min of incubation with fast red substrate-chromogen. Sections were counterstained with hematoxylin and coverslipped.

Microvessel density and tumor-lined vessel determination

For determination of microvessel density (MVD), the stained sections were screened microscopically on low power (×100) to identify the areas of highest vascularization (hotspots) in the tumor tissue. Microvessel counts were performed at ×400 (0.09 mm^2) magnification with the use of an ocular grid (19). In addition, microvessels were counted throughout each of the tissue sections in 10 systematically selected fields of vision, to obtain systematic MVD values. The peritumoral and intratumoral MVD were counting separately, and the adjacent MVD in normal tissue also counted for comparison with the tumor tissue.

To identify tumor-lined channels, the sections were doubly stained to highlight tumor cells and endothelial cells, and a condensing lens was used to highlight RBCs. Tumor-lined channels were defined as vessels with CK-positive tumor cells in obvious contact with the lumen, as indicated by the absence of detectable overlying CD34 immunoactivity. Furthermore, to distinguish microhemorrhage from tumor-lined vessels, sections or area with severe hemorrhage were excluded in this study.

Statistical analysis

The MVD counts were presented as mean ± SD. MVD was examined in relationship to primary tumor size, clinical stage, and degree of differentiation. One-way ANOVA to analyze the correlation of MVD with clinicopathologic parameters was performed. Results of presence or absence of tumor-lined vessels were analyzed using the chi-squared test for a table comparison with tumor size, histologic type, and clinical stage. Statistical significance was set at $P < 0.05$.

Results

Correlation between MVD and clinicopathologic parameters

Vascular endothelial cells specifically stained red with vWF, CD31, and CD34. More hematopoietic cells and background staining was seen in CD34-stained specimens, and some inflammatory cells were co-stained by CD31 antibody. In general, CD31 and CD34 detected greater peritumoral MVD than did vWF. The peritumoral MVDs in all 112 tumors detected by vWF, CD31, and CD34 ranged from 8.0 to 29.0, 11.0 to 33.0, and 11.0 to 35.0, respectively, with mean values of 17.6, 20.4, and 22.2, respectively. The peritumoral mean MVDs obtained with vWF, CD31, and CD34, according to clinicopathologic parameters were shown in Table 1. Peritumoral MVD as determined with vWF, CD31, and CD34 showed no significant correlation with tumor size, stage, or histologic differentiation.

Table 1 Correlation between peritumoral MVD and clinicopathologic parameters

		Peritumoral MVD		
	$N = 112$	*vWF*	*CD31*	*CD34*
Tumor size				
T1	16	16.0 ± 3.8	18.4 ± 3.7	21.4 ± 5.1
T2	50	17.6 ± 3.5	20.1 ± 4.3	21.6 ± 5.2
T3	33	18.1 ± 2.7	21.4 ± 4.2	22.9 ± 5.1
T4	13	17.9 ± 1.9	21.5 ± 3.3	23.2 ± 5.3
ANOVA		$P = 0.179$	$P = 0.073$	$P = 0.542$
Differentiation				
Well	41	17.2 ± 3.3	19.6 ± 3.9	22.3 ± 4.6
Moderate	43	18.0 ± 3.2	21.1 ± 4.2	21.7 ± 5.4
Poor	28	17.3 ± 3.0	20.6 ± 4.4	22.7 ± 5.6
ANOVA		$P = 0.473$	$P = 0.227$	$P = 0.709$
Stage				
I	14	16.5 ± 3.7	18.9 ± 3.6	22.6 ± 4.3
II	36	17.6 ± 4.0	19.6 ± 4.2	20.5 ± 4.0
III	23	18.0 ± 2.8	21.6 ± 4.0	22.0 ± 5.4
IV	39	17.6 ± 2.3	21.0 ± 4.3	23.6 ± 5.8
ANOVA		$P = 0.561$	$P = 0.124$	$P = 0.066$

MVD, microvessel density; vWF, von Willebrand factor.

Similar to peritumoral MVDs, CD34, and CD31 revealed greater intratumoral MVD than did vWF. Most of the intratumoral vessels that were not stained with vWF, but stained with CD31 and CD34 were small vessels. The intratumoral MVDs determined with vWF, CD31, and CD34 ranged from 8.0 to 26.0, 10.0 to 29, and 11.0 to 35.0, respectively, with mean values of 14.8, 19.0, and 21.2, respectively.

The intratumoral mean MVDs revealed by vWF, CD31, and CD34, according to clinicopathologic parameters were shown in Table 2. MDV determined with vWF showed no significant correlation with tumor stage, tumor size, or histologic differentiation. When intratumoral MVDs were determined with CD31 and CD34, the value of MVD is significantly correlated with tumor size ($P = 0.002$ and $P < 0.0001$, respectively), as well as tumor stage ($P < 0.0001$ and $P < 0.0001$, respectively).

Table 2 Correlation between intratumoral MVD and clinicopathologic parameters

		Intratumoral MVD		
	$N = 112$	*vWF*	*CD31*	*CD34*
Tumor size				
T1	16	14.4 ± 3.5	16.9 ± 3.2	15.9 ± 3.2
T2	50	15.1 ± 3.4	18.1 ± 3.6	20.3 ± 4.5
T3	33	15.0 ± 2.2	20.7 ± 4.5	23.7 ± 4.9
T4	13	14.2 ± 3.9	20.3 ± 3.1	24.8 ± 3.0
ANOVA		$P = 0.181$	$P = 0.002$	$P < 0.0001$
Differentiation				
Well	41	14.1 ± 2.5	17.8 ± 3.6	19.8 ± 5.2
Moderate	43	15.6 ± 3.9	19.6 ± 4.4	22.3 ± 5.0
Poor	28	14.8 ± 2.4	19.7 ± 3.7	21.7 ± 4.8
ANOVA		$P = 0.079$	$P = 0.054$	$P = 0.065$
Stage				
I	14	14.5 ± 3.7	17.1 ± 3.4	16.4 ± 3.0
II	36	14.6 ± 2.4	17.4 ± 2.9	18.8 ± 3.1
III	23	15.6 ± 2.1	21.2 ± 3.7	20.8 ± 4.6
IV	39	14.7 ± 3.9	19.7 ± 4.5	25.4 ± 4.6
ANOVA		$P = 0.632$	$P < 0.0001$	$P < 0.0001$

MVD, microvessel density; vWF, von Willebrand factor.

Vascularity alternation with disease progression

The MVD values by vWF, CD31, and CD34, were 7.5 ± 1.6, 7.7 ± 1.7, and 7.6 ± 1.9, respectively, in the normal mucosa, and 16.4 ± 2.8, 17.4 ± 3.3, and 19.1 ± 5.0, respectively, in dysplastic epithelium. When peritumoral MVD was determined using vWF, it showed significantly increased vascularity in normal epithelium compared with dysplastic epithelium ($P < 0.0001$ by ANOVA). Peritumoral MVD as determined using CD31 and CD34 revealed significant increases in vascularity with increasing severity of disease from normal to dysplasia to early localized tumor ($P < 0.0001$ by ANOVA) (Fig. 1).

Identification of tumor-lined vessels

In preparation for the study of vascularity of oral SCC, CD34 was shown to be better than vWF and CD31 for detecting tumor vascularity. CD34 endothelial marker highlighted most of the vessels. Nonetheless, some RBC-containing vessels were not stained by CD34 (Fig. 2a). To further validate the observation that some vessels were lined with tumor cells, tissue sections were double-labeled for a marker associated with squamous carcinoma cells that was not expressed by endothelium. CK intermediate filament is typically expressed in epithelial cells and not by vascular endothelium. CD34 as endothelial marker and pan-CK as a squamous cell marker were chosen for double stain immunohistochemistry to identify the RBC-containing, CK-positive, tumor cell-lined vessels. As shown in Fig. 2b, tumor-lined vessels were readily identified by this method.

Correlation between tumor-lined vessels and clinicopathologic parameters

Correlation of tumor-lined vessels and clinicopathologic parameters by cross-table analysis was performed. The chi-squared test was used to examine the significance.

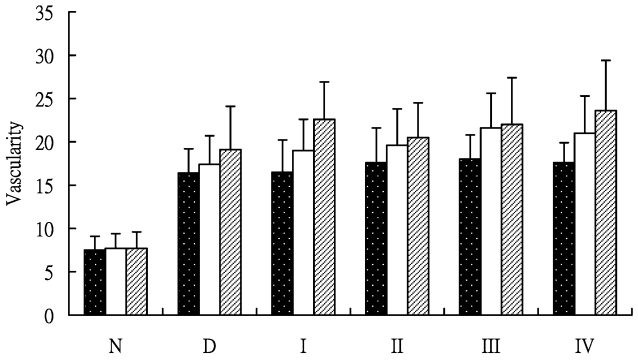

Figure 1 Vascularity alternation with disease progression. Microvessel density (MVD) measured in the subepithelial stroma of normal oral mucosa (N), dysplasia (D), and peritumoral tissue of stage I (I), stage II (II), stage III (III), and stage IV (IV) oral squamous cell carcinoma (SCC). von Willebrand factor (vWF) MVD (▩); CD31 MVD (□); CD34 MVD (▨).

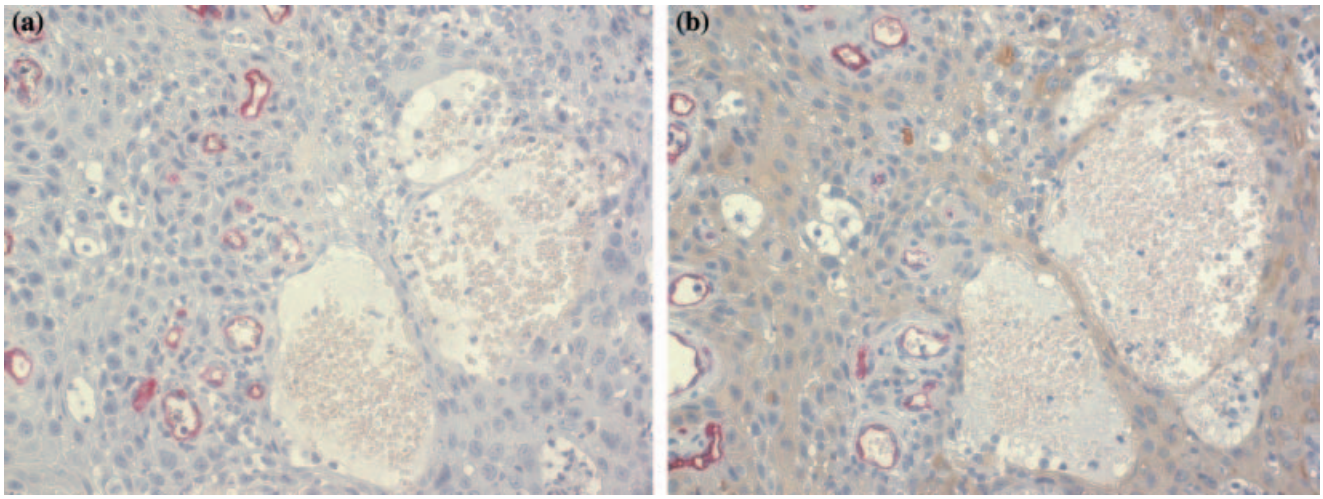

Figure 2 Identification of tumor-lined channel by double staining immunohistochemistry. (a) Endothelial cell-lined blood vessels were positive staining with CD34 when compared with those RBC-containing vessel which was CD34-negative. (b) In serial section from (a), these blood vessels lined by tumor cell were confirmed by CD34 and cytokeratin (CK) double staining (original magnification ×200 in a, b).

The presence of tumor-lined vessels was significantly correlated with tumor size, histologic differentiation, and stage ($P = 0.017$, $P = 0.001$, $P = 0.029$, respectively) (Table 3).

Discussion

It has been suggested that angiogenesis is necessary for continued tumor growth (2–4). In oral SCC, however, no consistent results between tumor angiogenesis and disease progression, prognosis, and metastasis were established (9–13). We evaluated the peritumoral and intratumoral MVD to compare both peritumoral and intratumoral angiogenesis to tumor size and stage and histologic differentiation. Our results demonstrated a significant increase in peritumoral vascularity during the transition from normal tissue through the dysplastic state to early cancer. This observation confirmed results of previous studies reporting that angiogenesis began in the early stage of oral carcinogenesis (the pre-malignant lesion stage) (9). Nonetheless, peritumoral vascularity did not increase with tumor growth (no significant difference in peritumoral vascularity in T1, T2, T3, and T4 tumors) or further disease progression (peritumoral vascularity was not significantly different between stages I, II, III, and IV). In contrast to peritumoral vascularity, intratumoral vascularity increased with tumor progression from early tumor to advanced tumor. Moreover, our study also confirmed that vascular hotspots of oral SCC were encountered predominantly at the peripheral tumor margin (10). For determining MVD, hotspots were defined as the most vascular areas in the tumor tissue. We demonstrated that peritumoral MVD was not consistent with intratumoral MVD during tumor progression. This might explain, in part, why angiogenesis in oral SCC remains controversial and suggested that peritumoral and intratumoral angiogenesis of oral SCC were important for tumor initiation and progression, respectively.

For detection of tumor vascularity, we utilized vWF, CD31, and CD34 to evaluate peritumoral and intratumoral MVD. Our data showed that CD34 and CD31 are more sensitive for evaluating the tumor blood vessels of oral SCC, and MVD determined using CD34 and CD31 correlated with tumor growth and disease progression. In a recent report on breast cancer using antibodies to CD31, CD34, and factor VIII-RA, the results also revealed that CD31 and CD34 were better than factor VIII-RA in associating with survival (20). Generally, CD31 or CD34 were utilized as the pan-endothelial markers of choice for paraffin-embedded sections (16, 21). Several reasons to explain MVD detection with vWF, CD31, and CD34 had discrepant correlation with clinical parameters presented in this and previous study (20). The tumor vessels often have irregular diameters, abnormal branching patterns, absence of the pericyte

Table 3 Correlation of tumor-lined vessel and clinicopathologic parameters

	Size				*Differentiation*			*Stage*			
Tumor-lined vessel	*T1*	*T2*	*T3*	*T4*	*W*	*M*	*P*	*I*	*II*	*III*	*IV*
Present ($n = 41$)	1	17	16	7	6	22	13	1	12	8	20
Absent ($n = 71$)	15	33	17	6	35	21	15	13	24	15	19
P-value	0.017				0.001			0.029			

W, well; M, moderate; P, poor.

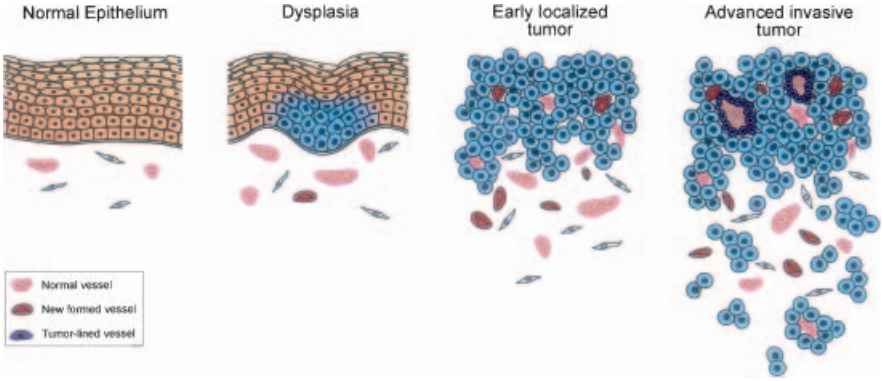

Figure 3 Model of the angiogenesis and non-angiogenesis in oral squamous cell carcinoma (SCC). Angiogenesis occur as epithelium transition from normal through dysplastic state to cancer. While cancer progression, intratumoral angiogenesis are predominant. Meanwhile, presence of non-angiogenesis (tumor-lined vessel) is also accompanied with tumor progression.

coat, and defective wall structure. In addition to the structural differences, the endothelial cells within the tumor are active, proliferating, and immature in contrast to their normal counterparts (22). Certain endothelial markers may not detect these aberrant vessels. Furthermore, CD34+ cell have been found in area of tumor angiogenesis and regulated angiogenesis (23, 24). The specificity of the antibodies used in present study could also explain that the increase SD of MVD from normal stroma to tumor periphery as disease progression. These may contribute to more MVD detected by CD34 than other endothelial markers in this and other studies.

We also identified that as tumors continued to grow, tumor-lined vessels appeared in oral SCC, and this non-angiogenic phenomenon correlated with tumor progression from early, localized to late, advanced cancer. It has been proposed that tumor cells acquire nutrition by these non-angiogenesis or angiogenesis-independent pathways (25, 26). In a study of non-small cell lung cancer, Pezzella et al. suggested that tumor cells might be able to grow without neovascularization if a suitable vascular bed was available (25). In breast cancer, evidence of a novel non-angiogenic mechanism for cancer cell metastasis has also been reported (26). In oral SCC, this could be the reason for the observation that oral cancer is less angiogenesis-dependent (11).

Meanwhile, tumor-lined vessels correlated with histologic differentiation of oral SCC and that they were readily found in advanced tumors and in poorly differentiated tumors. It has been reported that epithelial cancer cells could have switched differentiation lineage to mesenchymal cells (27). Molecular analyses of human cancer cells have also shown the expression of multiple molecular phenotypes, including epithelial, fibroblastic, endothelial, and stem cell types, reminiscent of a deregulated genotype and an embryonic-like phenotype. Endothelial cell markers or genes normally expressed in endothelial cells such as VEGFR, VE-cadherin, and $\alpha v\beta 3$ integrin were overexpressed in some tumor cells (28–30). These observations suggest that differentiation regulation of tumor cell may play an important role in the formation of tumor-lined vessels.

Our data, applied to the above model fit well; as epithelial cells transition from normal to dysplastic, angiogenesis occurred in the peripheral stromal areas. Intratumoral angiogenesis increased when disease progressed from early localized to advanced tumors. Furthermore, presence of tumor-lined vessels was observed when the tumor continued to grow and progress (Fig. 3).

In conclusion, we demonstrated that both angiogenic and non-angiogenic mechanisms are important for oral SCC progression. These two distinct mechanisms could facilitate tumor growth and metastasis. Further studies to disclose the molecular and genetic mechanisms of non-angiogenic tumor growth phenomena need to be investigated.

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