Increased subepithelial vascularization and VEGF expression reveal potentially malignant changes in human oral mucosa lesions

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Objective. The objective of this study was to provide evidence that the magnitude of angiogenesis induced by oral mucosa epithelium with potentially malignant lesions is related to the degree of epithelial aggressiveness.

Methods and results. We evaluated 96 biopsies that included: (1) leukoplakia with and without dysplasia, (2) nontumoral borders adjacent to squamous cell carcinomas with and without dysplasia, and (3) normal oral mucosa. Number, size, and localization of vessels labeled immunohistochemically for the antigen CD34 were assessed by image analysis using a software developed “ad hoc.” All vascular sections and those localized immediately below the epithelium (sub-basal vessels) were separately evaluated in areas 30-μm deep. Vascular endothelial growth factor (VEGF) expression was labeled immunohistochemically and evaluated semiquantitatively against a standard. Leukoplakia and nontumoral borders adjacent to carcinomas exhibited an increase in VEGF expression and in subepithelial vascularization. This increase was significantly greater in leukoplakia with dysplastic changes than in leukoplakia without dysplasia. Conversely, no differences were observed between epithelia with and without dysplasia adjacent to carcinomas.


The sequential or simultaneous observation of various potentially malignant or malignant lesions in the oral cavity of a single patient gave rise to the concept of field cancerization.1 This concept was later unequivocally supported by the demonstration of molecular changes in the clinically healthy oral mucosa of tumor-bearing patients.2,3 However, it is not yet possible to determine whether field cancerization will lead to the development of a carcinoma or whether a dysplastic lesion will progress to malignancy.4 Thus, the evaluation of the risk of malignant transformation of potentially malignant conditions or of lesions of the oral mucosa using objective, nonepidemiologic criteria is a standing challenge in oncology. Within this context, the search for indicators that can contribute to prognosis and the optimization of therapy becomes pivotal.5

Similarly to other solid tumors, head and neck squamous cell carcinoma must induce neovascularization directly or indirectly to grow and metastasize.6 The vascular endothelial growth factor (VEGF) is a potent mitogen of the vascular endothelial cells that plays a central role in vasculogenesis, angiogenesis, tumor angiogenesis, and blood metastasis. This factor is expressed by several normal and tumor cells.7-11

Angiogenic switch from a prevascular to vascular phase can be an early event during carcinogenesis.12 The existing data on the evaluation of tumor and pretumor angiogenesis in oral mucosa are still controversial. Some studies have reported a correlation between vascular density in tumor stroma and the degree of malignancy,13-17 whereas other authors have reported no correlation.18

Various studies have demonstrated a correlation between VEGF expression and tumor aggressiveness and lymph node metastases. VEGF expression has also been identified as a prognostic indicator of survival.19-21 Whereas some authors described a reduction in immunohistochemical expression of VEGF as head and neck carcinogenesis progresses,22 others reported that angiogenesis would be associated with progression to malignancy in the oral cavity.23-25 Other authors consider that VEGF plays a physiological role in oral

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mucosa that would be unrelated to angiogenesis, field cancerization, or the transition to dysplasia.\textsuperscript{26}

Controversial data are available on the correlation between VEGF expression and the increase in vascular density. Most of the studies have not found a correlation in the case of dysplasia and squamous cell carcinoma; however, some have reported that an increase in vascularization is correlated with VEGF epithelial expression.\textsuperscript{11,16,20,27,28}

The apparent discrepancy of the data could be attributable to differences in the methods of analysis. Furthermore, it is difficult to discriminate between vascularization induced by epithelia undergoing malignant transformation and that elicited by mediators of inflammation, usually present in potentially malignant lesions and in tumor stroma.

The aim of this study was to demonstrate that the magnitude of angiogenesis induced by oral mucosa epithelium with potentially malignant lesions is related to the degree of epithelial aggressiveness. Within this context, the observation of subepithelial vascularization could be included in the histopathological analysis as an aid in the determination of the severity of oral mucosa alterations.

To this end, we developed a software program to analyze vascularization in inflammation-free areas underlying potentially malignant epithelia to obtain objective, numerical indicators that can be analyzed in terms of their potential correlation with VEGF epithelial expression. We analyzed cases of leukoplakia as examples of the entity that is most representative of potentially malignant lesions of the oral mucosa\textsuperscript{4} and epithelia adjacent to carcinomas as a model of field cancerization.\textsuperscript{3,29,30} Areas of carcinomas in the same specimen were evaluated for comparison purposes.

**MATERIAL AND METHODS**

**Potentially malignant lesions**

Twenty-nine cases of oral leukoplakia available in the archives of the Laboratory of Oral Pathology, School of Dentistry, University of Buenos Aires, were analyzed. All the cases corresponded to patients who smoked tobacco and/or drank alcohol, albeit with a wide range of consumption patterns. Only the cases with scarce inflammatory infiltrate were included, and only if the infiltrate was far from the basal membrane, leaving an inflammation-free subepithelial band of at least 40 \(\mu\)m. A single pathologist (A.K.) classified the cases into 2 groups according to the presence or absence of epithelial dysplasia (\(n = 18\) and \(n = 11\), respectively), regardless of the degree of dysplasia. A case was considered dysplastic if it exhibited at least one of the following features: polymorphism, dyskeratosis, mitosis in the suprabasal layers, or atypical mitosis. Borderline or equivocal cases were excluded.

**Tumor lesions**

Forty-five biopsies of oral squamous cell carcinomas with nontumoral epithelial borders within the same specimen were selected. The areas of epithelium adjacent to tumor were divided into “borders with dysplasia” (\(n = 20\)) and “borders without dysplasia” (\(n = 25\)), using the same criteria as for leukoplakia.

**Controls**

Twenty-two specimens of clinically and histologically normal oral mucosa of patients who did not drink alcohol or smoke tobacco were selected for evaluation. The samples were obtained during surgery for pathologic processes occurring in-depth and lined by healthy oral mucosa. The samples with epithelial alterations or some degree of inflammatory infiltrate were excluded.

**Methods**

Six serial sections of each case were obtained. The first two (5-\(\mu\)m thick) were routinely stained with hematoxylin-eosin (HE), the following two (10-\(\mu\)m thick) were used to demonstrate vascular walls and the remaining sections (7 \(\mu\)m thick) were used for VEGF labeling.

**Immunohistochemical technique for vascular wall labeling**

Antigen retrieval was performed in citrate buffer pH 6.0, in a microwave oven for 2 minutes. The sections were incubated with an anti-human CD 34 raised in mouse (Biogenex, \# QBEnd/10, San Francisco, CA), for 24 hours at 4°C in a damp chamber, followed by labeling with a streptavidin-peroxidase detection kit (Biogenex). The sections were lightly counterstained with hematoxylin to enable the observation of structures and provide enough contrast for image analysis.

**Immunohistochemical demonstration of VEGF**

All the staining series included a section of human kidney (from the same paraffin block) as a positive control. Antigen retrieval was performed by incubating the sections in citrate buffer (pH 4) in a microwave oven at maximum power for 5 minutes. The sections were then incubated with the primary antibody anti-human VEGF raised in goat (VEGF-147, 200 \(\mu\)g/mL, Santa Cruz Cinasa; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:20 dilution for 12 hours, followed by incubation with the streptavidin-peroxidase detection kit (Biogenex).

**Quantitative evaluation of vascular walls**

Image analysis was performed using a light microscope Zeiss MPM 80 microscope (Carl Zeiss, Jena, Germany) online with an image analyzer IBAS-Kontron Electronic image analyzer with a Hitachi DK...
Image analysis software based on standard morphometric software was developed ad hoc to quantify the vascular sections contained in subepithelial areas (100 × 100 μm).

The software allows for the separate evaluation of the vessels immediately underlying the epithelium (sub-basal vessels) in fields 30-μm deep. The working hypothesis that led to the evaluation of these particular blood vessels was that they would indicate neovascularization potentially induced by epithelium in inflammation-free areas. The system also allows discrimination of vessels smaller than 8 μm in diameter. This value was fixed arbitrarily to, conceivably, distinguish more recently formed blood vessels. Eight to 10 areas were evaluated in each section and only 1 section was used in each case. In carcinoma specimens, areas with no invasive epithelium localized at least 2 mm from the tumor were selected. This arbitrary distance was set to minimize the influence of tumor factors on surrounding tissue. Within the tumor, 10 areas containing stroma immediately underlying the tumor cords were selected at random. The end points evaluated were:

- **Tn** (total number): number of vessel sections per 10,000 μm² of connective tissue.
- **%Sv** (percentage of small vessels): percentage of vessels less than 8 μm in diameter, in the total number of vascular sections.
- **Nbv** (number of basal vessels) (number of sub-basal vessels): number of sections of vessels adjacent to the basal membrane or contained in a band of connective tissue 30-μm deep underlying the basal membrane. The value was expressed as the number of blood vessel sections per 100 linear μm of basal membrane.
- **% Sbv** (percentage of small sub-basal vessels): percentage of sub-basal vessels less than 8 μm in diameter, of the total number of sub-basal vascular sections.

The first 2 parameters are indicators of total vascularization, whereas the last 2 parameters refer to the subepithelial vascularization.

### Evaluation of the VEGF reaction

The VEGF reaction is diffuse and cytoplasmatic, making image analysis unreliable. Densitometric evaluation in our hands led to methodological errors that were difficult to control. Within this context we undertook semiquantitative evaluation, comparing with the corresponding kidney section in each staining batch. The VEGF reaction was scored as 0: negative, 1: faint or moderate when the intensity was less than the corresponding kidney control section, and 2: intense when the intensity was equal to or greater than the corresponding control.

### Statistical analysis

Data from vascular measurements were compared by analysis of variance (ANOVA) followed by Bonferroni contrasts between groups and between field measurements in each case.

For VEGF expression, percentages of cases with different reaction intensity in each group were compared by the χ² test. Differences were considered significant at P less than .05.

The relation between subepithelial vascularization and expression of VEGF was evaluated independently in leukoplakia and borders adjacent to tumor by Spearman Rank correlations.

### RESULTS

#### Normal mucosa

Vascularization parameters and VEGF grading values of normal mucosa were compared separately with those corresponding to borders adjacent to tumors and leukoplakia.

The Tn value, which yields information on total vascularization, was 0.90 ± 0.3. This value implies that in histologic sections, 1 vascular section is observed on average in each square field of side 100 μm. Approximately one third of the vessels have a diameter smaller than 8 μm (%Sv = 38 ± 2). The measurement of sub-basal vessels yielded an Nbv value of 0.35 ± 0.03. This value implies that approximately 1 vascular section was observed for each 300 μm of epithelial basal membrane. About 25% of these vessels were smaller than 8 μm in diameter (%Sbv = 25 ± 10).

In 15 of the 22 samples of oral mucosa (68.18%), the VEGF reaction was completely negative. In the remaining
cases (31.81%), the reaction was fainter than in the control kidney sections and was classified as intensity grade 1.

**Vascularization in carcinomas and adjacent borders**

The reaction for CD34 revealed marked vascularization in the tumor stroma and numerous labeled blood vessels underlying the epithelial basal membrane both in tumor and in adjacent nontumoral epithelia (Fig. 1). The values of the parameters related to total connective tissue vascularization in nontumoral borders were intermediate between tumor and normal mucosa values. The difference between nontumoral borders adjacent to carcinomas and normal mucosa were not statistically significant. However, the differences among the 3 groups of samples reached statistical significance when only the percentage of vascular sections smaller than 8 μm in diameter was considered.

Vascular parameters did not exhibit statistically significant differences between the borders of carcinoma with and without dysplasia. Sub-basal vascularization showed statistically significant differences between tumors and nontumoral borders and between nontumoral borders and normal mucosa (Fig. 2).

**Vascularization in leukoplakia**

Conversely, to the findings in borders adjacent to carcinoma, marked differences in vascularization were observed between leukoplakia without dysplastic changes and leukoplakia with epithelial dysplasia.

Both the values of total vascularization and sub-basal vascularization were higher in leukoplakia with epithelial dysplasia than in normal mucosa. The values corresponding to leukoplakia without epithelial dysplasia were intermediate for all the end points evaluated (Fig. 3).

No statistically significant differences were found between the measured fields in each case of leukoplakia or border of carcinoma. However, differences were observed in some cases of tumor stroma (data not shown).

**Expression of VEGF in carcinoma and borders adjacent to carcinoma**

VEGF expression was diffuse and cytoplasmatic in the full epithelial thickness of all the samples. Reaction intensity varied with the lesions (Fig. 4).

Contrary to what was observed in normal mucosa, all the carcinomas and their borders exhibited a positive VEGF reaction. The proportion of cases with an intense reaction (grade 2) in the different groups increased with the severity of the lesions.

The statistical analysis of the data showed statistically significant differences between normal mucosa and the rest of the groups and between the tumor borders and the tumors. However, the difference between dysplastic and nondysplastic borders did not reach statistical significance (Fig. 5).

**VEGF expression in leukoplakia**

All the leukoplakia biopsies exhibited a positive reaction for VEGF. All the cases of simple leukoplakia,
without epithelial dysplasia, corresponded to intensity grade 1, whereas 83.33% of leukoplakia with dysplasia showed the highest grade of reaction intensity. Conversely, to borders adjacent to carcinoma, leukoplakia values exhibited statistically significant differences between the groups with and without dysplasia (Fig. 5).

**Relation between vascularization and the expression of the VEGF**

The number of sub-basal vessels plotted against VEGF grades in a pool of cases of normal mucosa and dysplastic and nondysplastic leukoplakia clearly show a relationship between both variables. Spearman’s rank correlation coefficient was $r_s = 0.7846$ (Fig. 6). A similar
relationship was found in a pool of cases of normal mucosa and tumoral borders: $r_s = 0.8372$ (Fig. 7).

**DISCUSSION**

There is enough evidence in the literature of the increase in vascularity in carcinomas and potentially malignant disorders of human oral mucosa.\textsuperscript{5-7,11,14,17,18,20,23,25} However, in premalignant lesions, the degree to which angiogenesis is induced by epithelial cells undergoing malignant transformation remains to be established. This issue is of direct practical relevance to the evaluation of lesion prognosis. However, it has not been elucidated to date partly because the studies fail to specify if vascular density was evaluated independently of the inflammatory component that releases angiogenic factors.
and is usually present in biopsies of oral lesions. Furthermore, studies on the production of angiogenic factors in potentially malignant epithelia have yielded conflicting results.\textsuperscript{21}

The data on expression of VEGF, the most specific angiogenic factor, are varied and often conflicting (as described at the beginning of this article). A study by Carlile et al.\textsuperscript{26} showed that the reactions for VEGF described at the beginning of this article). A study by angiogenic factor, are varied and often conflicting (as yielded conflicting results.\textsuperscript{31}

The evaluation of VEGF expression was optimized by including a standard in all the reaction batches. In our hands, the expression of VEGF, detected with an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 1 to 140 of VEGF of human origin, showed an increase with disease progression associated with increased vascularization. In leukoplakia, angiogenic activity increased with the aggressiveness of the lesions. The values corresponding to most of the vascular parameters evaluated and intensity of the VEGF reaction were higher in dysplastic lesions than in nondysplastic lesions. The subepithelial vascular density was higher in noninvasive epithelia adjacent to carcinomas than in normal mucosa, and was even higher in actual carcinomas. However, no differences were observed between adjacent epithelia with or without dysplasia. This finding is interesting within the context of the biological behavior of these epithelia. The epithelia of leukoplakia without dysplasia can remain as such for a long time, less frequently revert to a normal epithelium or, much less frequently, evolve toward more aggressive lesions. Dysplastic lesions are considered to have a higher risk of malignant transformation. In this way, leukoplakia with and without dysplasia would be distinct nosologic entities. The borders adjacent to carcinomas, regardless of whether they exhibit dysplasia at a histologic level, are constituted by cancerized epithelia in which molecular changes that are characteristic of malignant transformation have been demonstrated.\textsuperscript{2,3} These findings support the use of noninvasive borders of carcinoma as a model of field-cancerization to test early markers of malignant transformation, in spite of existing variations in the characteristics and magnitude of these changes.

Although vascularization was measured in areas distant from the tumor within the specimens available for study, vascularization might be affected by factors produced by the tumor. The finding that the subepithelial vascularization and VEGF expression increase in leukoplakia and even more in cases with dysplasia, is particularly relevant in that it reveals that both these histochemical reactions can be used as a diagnostic aid in the evaluation of the severity of potentially malignant lesions. In addition, the fact that these markers can be demonstrated in the biopsy material used for routine histopathological diagnosis is an added practical advantage. Moreover, if the increase in sub-basal vascularization is sufficient in magnitude, it can be assessed subjectively by the pathologist in routine sections stained with HE or periodic acid-Schiff reaction. Thus, the mere observation of this detail, even in the case of epithelia with no histologic alterations, can contribute to the evaluation of the severity of the lesions. In the case of leukoplakia, the oral mucosa lesion with the highest risk of transformation,\textsuperscript{4} the inflammatory infiltrate that impairs observation in other lesions, such as lichens, occurs deeper in the connective tissue. This fact allows for the distinction between vascularization induced by inflammatory factors and by epithelial factors.

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