Vascular Endothelial Growth Factor Is a Marker of Tumor Invasion and Metastasis in Squamous Cell Carcinomas of the Head and Neck

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ABSTRACT

Angiogenesis has been linked to increased metastasis formation and decreased overall survival in patients with various tumors, including and neck squamous cell carcinomas (HNSCC). Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis. In the present study, we evaluated VEGF expression and microvessel density (MVD), a quantitative means of angiogenesis, in both experimental and clinical models of HNSCC. Analysis of VEGF RNA expression in cell lines of keratinocyte origin [HNSCC, facial skin squamous cell carcinoma (SCC), and transformed but nontumorigenic keratinocytes] and normal skin keratinocytes revealed two VEGF transcripts corresponding to proteins of 165 and 121 amino acids in length, with the transcript for the 165-amino acid species predominating. Six of eight SCC cell lines showed increased levels of one or both transcripts, and seven SCC cell lines and the transformed keratinocyte cell line showed increased protein expression. We then evaluated VEGF protein expression in human head and neck specimens containing normal epithelium (n = 10), dysplasia or carcinoma in situ (CIS; n = 15), early invasive SCCs (n = 9), advanced primary SCCs (n = 10), lymph node metastases (n = 3), and s.c. tumors or cysts (n = 7) formed in severe combined immunodeficient mice. Intense VEGF staining was found in the majority of advanced primary SCCs, lymph node metastases, and human SCCS in severe combined immunodeficient mice, whereas no dysplasia, CIS, or early SCCs showed intense immunostain. A highly significant increase (P = 0.0001) in VEGF expression was seen in the advanced SCC versus dysplasias and CIS lesions, as was the difference between SCC versus normal epithelium from nonsmokers (P = 0.01). VEGF expression in advanced primary cancers was greater (P = 0.002) and, in early cancers, marginally greater (P = 0.05) than adjacent normal mucosa. MVD increased with the progression of preinvasive disease (P = 0.04). VEGF expression and MVD (both, P = 0.003) were directly associated with tumor aggressiveness in experimental tumors. These findings suggest a role for VEGF in both clinical and experimental HNSCC.

INTRODUCTION

Angiogenesis is required for solid tumor growth (1) and facilitates tumor progression and metastasis (2–4). Angiogenesis is thought to be regulated by a number of growth factors, including bFGF (5), TGF-β (5), and VEGF. Whereas tumor-derived bFGF and TGF-β have both autocrine and paracrine functions, VEGF seems to be produced by human tumors solely for the stimulation of tumor vascularization. VEGF is a disulfide-linked dimeric glycoprotein that increases blood vessel permeability, endothelial cell growth, proliferation, migration, and differentiation (6). VEGF shares significant amino-acid sequence homology with platelet-derived growth factor (7). Four different transcripts that have been identified encode proteins of 206, 189, 165, and 121 amino acids through alternative splicing (8). Only the 165- and 121-amino acid proteins are secreted to a significant extent.

Although VEGF and MVD have documented importance in both the development and progression of tumors in multiple models (6), their roles in HNSCC carcinogenesis are not clear. In other tumor systems, it seems that the timing (early versus late in tumor development and progression) of increased expression of VEGF is tumor-specific. In the breast [in which increased VEGF expression is seen in ductal CIS (9)], in the ovary [in which increased expression has been identified in borderline tumors (10)], and in the vulva [in which stage III vulval intraepithelial neoplasias show increased expression (11)], VEGF seems to be up-regulated before tumor invasion. In other tumor types, including colon carcinomas [in which increased VEGF expression is associated with the metastatic phenotype (12)] and prostate cancer [in which enhanced expression is associated with invasive carcinoma (13)], VEGF up-regulation seems to occur later in progression.

VEGF can be constitutively expressed by human tumors, or it can be induced by other growth factors and cytokines includ-
VEGF Increases with SCC Progression

Changes in environmental conditions such as episodes of hypoxia seem to be significant for VEGF induction (15). Thus, expression of VEGF may depend on a variety of endogenous and exogenous factors that can vary greatly during tumor progression. Studies in non-SCC lesions have demonstrated a direct correlation between VEGF expression and MVD (16), which suggests that VEGF is the predominant growth factor for tumor vascularization. High MVD is associated with increasing tumor aggressiveness as demonstrated by Weidner et al. (2), who found a nearly linear relationship between microvessel counts and metastatic potential. However, this close relationship between MVD and metastatic potential has not been detected universally in human tumors (17), nor do all human tumors demonstrate increased expression of VEGF. These differences in observations may reflect: (a) high tumor vascularization in dysplasia or biologically early malignancy, leading to insignificant increases in VEGF with progression; (b) inconsistency of VEGF expression because hypoxia may occur only intermittently, with the tumors removed at times of low VEGF expression; or (c) changes in the microenvironment that lead to decreased expression of VEGF-inducing growth factors and cytokines.

In the present study, we evaluated VEGF expression and MVD in head and neck epithelial lesions containing cysts, dysplasia, CIS, early and advanced SCC (LNM) and in cell lines representing human precancerous, primary and metastatic cancer, and metastatic stages. VEGF expression generally increased with progression, and the most aggressive lesions showed the highest expression levels. These findings suggest a role for VEGF in HNSCC that drives the progression of HNSCC toward an invasive and aggressive phenotype.

**MATERIALS AND METHODS**

**Tissue and Cell Lines**

Tissue from 10 adult nonsmokers with histologically normal epithelium, as well as from 34 adult subjects with dysplasia or invasive SCC of the head and neck who underwent surgical excision at the Fox Chase Cancer Center (Philadelphia), was evaluated. Specimens included 9 moderate dysplasias, 6 CIS, 9 stage I or II (early) SCCs, and 10 stage III or IV (advanced) invasive carcinomas. LNM from 3 of the 10 advanced invasive carcinomas were also evaluated. None of the subjects with biopsies demonstrating only normal mucosa or dysplasia received preoperative chemotherapy/radiation to the oral cavity. Two of nine subjects with Stage I/II tumors received preoperative chemotherapeutic or radiation therapy, and 1 of 10 subjects with Stage III/IV tumors received preoperative chemotherapeutic or radiation therapy. In addition, nine cell lines derived from squamous epithelium (six HNSCC lines—FaDu, Det562, A253, SCC 4, SCC 9, SCC 25; two lines from SCCs of the facial skin—SCC 12, SCC 13; and one spontaneously transformed line from skin epithelium—HaCaT) were also studied. Cell lines FaDu, Det562, and A253 were obtained from the American Type Culture Collection (Rockville, MD); SCC 4, SCC 9, SCC 12, SCC 13, and SCC 25 were kindly provided by Dr. J. Rheinwald (Boston, MA); and HaCaT was a gift of N. Fuesenig (Heidelberg, Germany). Normal human epidermal keratinocytes were isolated from neonatal foreskins and grown in serum-free keratinocyte medium supplemented with 5 ng/ml epidermal growth factor and 50 μg/ml bovine pituitary extract (Keratinocyte-SFM, Life Technologies, Inc., Grand Island, NY). Keratinocytes were obtained when 70–80% confluent using trypsin/EDTA and RNA, and protein extracts were made from passages 2–4. All of the tumor cell lines were grown in serum-free MCDB 201/L15 (4:1) medium (Sigma, St. Louis, MO) supplemented with 5 μg/ml insulin.

**Experimental Tumors**

Monolayer cells at 70–80% confluence were trypsinized and counted, and 2 × 10⁶ cells in 50 μl of culture medium were injected s.c. in the dorsum of SCID mice. Each group of five mice was monitored twice weekly for the development of tumors. Mice were killed when tumors reached a minimum diameter of 5 mm, or if no tumor was observed at 100 days after tumor cell injection. Tumors were excised, fixed overnight in 10% neutral buffered formalin, and embedded in paraffin.

**RNA Isolation and Northern Blot Analysis**

RNA was isolated from epidermal keratinocytes and cell lines using TRIzol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Total RNA concentration was determined based on absorbance. Total RNA (15 μg) from each specimen was loaded onto 1.2% agarose-formaldehyde gels containing 10 ng/ml ethidium bromide, subjected to denaturing electrophoresis, transferred to Hybond-N nylon membranes (Amersham Life Science, Arlington Heights, IL), and prehybridized in 6× SSC, 2× Denhardt’s solution, and 0.5% SDS. Membranes were probed with an α-[32P]dATP-labeled fragment containing the entire open reading frame of the human VEGF gene. After washing, blots were exposed to Kodak X-OMAT AR film with an intensifying screen at −80°C.

**Protein Isolation and Western Blot Analysis**

Cell cultures grown to 70–80% confluence were lysed with an SDS-based single lysis buffer. The lysate was centrifuged at 13,000 rpm, and the supernatant was collected. Total protein concentration of the soluble cell extract was determined using the Pierce BCA Protein Assay Reagent Kit (Rockford, IL). Total protein (100 μg) of each sample was labeled in the presence of SDS before the addition of 2-mercaptoethanol and applied to a discontinuous 10% polyacrylamide gel containing 10 ng/ml ethidium bromide, subjected to denaturing electrophoresis, transferred to Hybond-N nylon membranes (Amersham Life Science, Arlington Heights, IL), and immunoblotted with an antibody to human VEGF (1:200 mouse monoclonal Ab-3, Calbiochem, Cambridge, MA) followed by a horseradish peroxidase-conjugated goat antimouse IgG (Jackson ImmunoResearch, West Grove, PA), and washed. The substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium were added for signal detection.

**Quantitation of Secreted VEGF**

The concentration of VEGF protein in supernatants that were collected 3 days after culture medium change of 1.0 × 10⁵ immortalized cells was determined. Normal keratinocyte me-
dium was not collected because it contains: (a) growth factors not present in the SCC medium that stimulates VEGF (18); and (b) a relatively high percentage of senescent keratinocytes whose cell membranes are degrading, which results in the measurement of cellular VEGF in addition to secreted VEGF. A VEGF ELISA kit (R&D Systems, Minneapolis, MN) was used to measure VEGF protein levels in the SCC supernatants according to the manufacturer’s instructions.

**Immunohistochemistry**

Paraffin-embedded, formalin-fixed normal epithelium, dysplasias, CIS, invasive SCCs, LNM from primary HNSCC, and tumors from SCC cell lines in SCID mice were cut in 5-μm-thick sections and placed on poly-l-lysine-coated glass slides. After boiling with distilled water for 10 min, slides were incubated with VEGF antibody Ab-3 (1:50). Reactions were detected using the Super Sensitive detection system with the substrate Fast Red (Biogenex, San Ramon, CA); levamisole was added to the substrate to block endogenous alkaline phosphatase activity. Skeletal muscle in the section served as a positive control, and a corresponding tissue section without primary antibody Ab-3 served as a negative control.

To determine MVD, slides were incubated sequentially with a mouse monoclonal antibody to Factor VIII-related antigen (Dako Corp., Carpinteria, CA), a horse antimouse IgG (1:200 dilution; Vector Labs, Burlingame, CA), and avidin-biotin-peroxidase complex (1:25 dilution; Vector Labs). The chromagen 3′-diaminobenzidine was used to visualize antibody binding, and microvessels were counted as described below.

Each evaluation of VEGF and Factor VIII was performed simultaneously and agreed upon by at least two of the investigators (E. R. S., J. C. W., and A. K-S.) using a double-headed light microscope. VEGF staining was scored as 0 = none; 1 = weak; 2 = moderate; or 3 = intense.

**Image Cytometric Quantitation of Factor VIII Immunostain**

In normal epithelium and preinvasive lesions, the dermis below the epithelial lesion was evaluated. For invasive lesions, peritumoral vessels were counted. After the area of interest was identified, individual microvessels were counted in 3–5 (as available for any given lesion) noncontiguous, randomly selected fields (×200) using a computerized image analyzer (Roche Pathology Work Station; 0.104 mm² per field). Any brown-staining endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumor cells, and other connective-tissue elements was considered a single, countable microvessel. Vessel lumens, although usually present, were not necessary in defining a structure as a microvessel, and red cells were not used to define a vessel lumen. All of the counts were performed simultaneously and in agreement by two investigators (J. C. W. and A. K-S.) without knowledge of the patient’s diagnosis. MVD was expressed as number of microvessels per mm². Because MVD is often heterogeneous among tumors, average MVD was determined after the lowest and highest counts were omitted; this gave values more representative of the histology as a whole and improved reproducibility.

**Statistical Analysis**

**VEGF Expression in Clinical Specimens.** Fisher’s exact test (two-sided) was used to test the hypothesis that VEGF immunoreactivity increased with increasing lesion severity. Specimens with Stage I/II cancer did not have different staining intensity than specimens with normal histology, whereas Stage III/IV specimens stained more intensely for VEGF (P = 0.01). There was not a significant difference identified between lesions with moderate dysplasia versus CIS, between moderate dysplasia versus Stage I/II invasive cancer, or between moderate dysplasia and CIS versus Stage I/II invasive cancer. The difference between moderate dysplasia and CIS versus invasive cancer (Stage I-IV) was marginally significant (P = 0.05), whereas specimens with Stage III/IV cancer had significantly higher staining intensity (P = 0.0001) than specimens containing moderate dysplasia and CIS. VEGF levels in histologically normal mucosa adjacent to lesions did not differ from lesion levels for samples taken for either moderate dysplasia or CIS. However, VEGF level in Stage I/II lesions marginally exceeded that in adjacent mucosa (P = 0.05) and significantly exceeded adjacent mucosa in Stage III/IV lesions (P = 0.002).

**VEGF Expression in Experimental Tumors.** Cells with VEGF intensity of 2 are less aggressive than those with VEGF intensity of 3. In mice with 2 million cell innocula, neither mouse with a VEGF intensity of 2 developed a tumor within the 100-day observation period. Each of the 4 mice with a 2-million-cell inocula with VEGF intensities of 3 developed a tumor within the observation period. We ranked the number of days until tumor could be observed. The VEGF 2 mice thus received ranks 5 and 6, whereas the VEGF 3 mice received ranks 1, 2, 3, and 4. Thus, the rank sum of VEGF 2 mice in this group is 5 + 6 = 11. On the hypothesis that the VEGF 2 mouse ranks are chosen randomly from the numbers 1, 2, 3, 4, 5, and 6, the chance of this observation or one more extreme (there are none more extreme) is 1/15. This result is not statistically significant in its own right but was combined with our results from 5 mice inoculated with 10 million cells.

As Table 2 shows, one VEGF 2 mouse in the 10-million-cell category did not develop a tumor within the 100-day period. The other mouse did develop a tumor at 89 days. All of the 3 VEGF 3 mice in this group developed a tumor within 14 days of inoculation. Again, ranks assigned to the VEGF 2 mice would be 4 and 5. VEGF 3 mice are assigned ranks 1, 2, and 3. Thus, the rank sum of VEGF 2 mice in this group is 4 + 5 = 9, again the most extreme that is possible for this group. The chance of this rank sum, assuming random assignment, is 1/150. This result is not statistically significant.

An overall assessment of statistical significance is obtained by summing the two rank sums from the two VEGF 2 groups. This gives an overall rank sum of 11 + 9 = 20, the highest it could possibly be. There is only one way to arrive at so large a rank-sum total, and its probability under the null hypothesis of random assignment is 1/150, with a significant association (P = 0.003; Fisher’s exact test) between VEGF level and tumor aggressiveness.

**MVD.** The Wilcoxon rank-sum test was used to determine the significance of increased MVD comparing histologically abnormal versus adjacent normal epithelium and then comparing lesion subtypes (normal epithelium, dysplasias, early...
VEGF Increases with SCC Progression

SCCs, advanced SCCs, LNM, and tumors formed in SCID mice). Lesions with CIS had a higher MVD \((P = 0.04)\) than lesions with less severe dysplasia or than normal epithelium. Fisher’s exact test was used to evaluate the association between the rate of formation of experimental tumors and MVD. The association was significant \((P = 0.003)\).

**RESULTS**

**VEGF RNA and Protein Expression**

**Cell Lines.** Northern blot analysis (Fig. 1) demonstrated a 2-fold or greater increase in VEGF RNA (compared with GAPDH) in six of the eight cells using normal keratinocytes as a control. Western blotting (Fig. 2) demonstrated a higher level of VEGF protein in all of the SCC cell lines than in normal human keratinocytes. All of the cell lines had been cultured in serum-free medium with insulin as the only mitogen. The lowest protein level was found in HaCaT cells, which are spontaneously immortalized but nontumorigenic keratinocytes (19). An ELISA was performed on day-3 supernatants of each immortalized cell line, with VEGF concentrations ranging from 121 to 1817 pg/ml. SCC 9 and HaCaT cells had the lowest VEGF protein level was found in HaCaT cells, which are spontaneously immortalized but nontumorigenic keratinocytes (19). An ELISA was performed on day-3 supernatants of each immortalized cell line, with VEGF concentrations ranging from 121 to 1817 pg/ml. SCC 9 and HaCaT cells had the lowest VEGF protein level was found in HaCaT cells, which are spontaneously immortalized but nontumorigenic keratinocytes (19). An ELISA was performed on day-3 supernatants of each immortalized cell line, with VEGF concentrations ranging from 121 to 1817 pg/ml. SCC 9 and HaCaT cells had the lowest VEGF protein level was found in HaCaT cells, which are spontaneously immortalized but nontumorigenic keratinocytes (19). An ELISA was performed on day-3 supernatants of each immortalized cell line, with VEGF concentrations ranging from 121 to 1817 pg/ml. SCC 9 and HaCaT cells had the lowest VEGF

**Normal Epithelium and Lesions from Clinical Specimens.** Lesions in the progressive steps of SCC, i.e., dysplasia, CIS, early SCC (stages I and II), advanced primary SCC (stages of III and IV), and metastatic lesions in lymph nodes, as well as normal adjacent epithelium, were tested for VEGF expression by immunostaining (Table 1). In normal epithelium from non-smokers as well as in normal epithelium adjacent to dysplasias and SCC, expression was weak or absent in all of the specimens. Median staining intensity in lesions was moderate in dysplasias, CIS, and early primary SCC, but intense in advanced primary and metastatic SCC (Table 1; Fig. 3). The differences in VEGF staining intensity for advanced SCC versus dysplasias and CIS lesions were highly significant \((P = 0.0001)\), as was the difference between SCC versus normal epithelium from nonsmokers \((P = 0.01)\). When VEGF expression was compared in lesions adjacent to histologically normal mucosa, expression was significantly greater in the Stage III/IV tumors \((P = 0.002)\), marginally greater in the Stage II/III tumors \((P = 0.05)\), and not different in the preinvasive lesions. No significant differences were found between LNM and advanced SCC or tumors grown in SCID mice.

**Tumors and Cysts in SCID Mice.** Seven tumors or cysts generated in SCID mice showed moderate-to-intense cytoplasmic VEGF staining, with five tumors intensely staining (Table 2). The two specimens with moderate intensity either did not form a tumor (HaCaT cells) or required a larger inoculum to form a tumor (\(10^3\) cells instead of \(2 \times 10^6\) cells for SCC 12). By contrast, the cell lines with the highest VEGF expression (SCC 13, Det 562, A253, and FaDu) showed the most rapid tumor growth in SCID mice. Tumor formation was accelerated when \(10^3\) cells were inoculated. The association between VEGF expression and rapid tumor growth was significant \((P = 0.003)\). Intensity of VEGF staining also correlated for vessel formation \((P = 0.003)\). The highest mean MVD was seen for FaDu and A253, the two cell lines that had (a) the highest VEGF expression in Western analysis (Fig. 2); (b) intense expression in the developing s.c. tumor; and (c) the shortest latency period until tumors were visible after injecting \(2 \times 10^6\) cells (Table 2).

**MVD and Invasive Potential**

Comparison of MVD in dysplasia versus CIS revealed a significantly increased MVD \((P = 0.04)\) in CIS (median MVD, 173 vessels/mm²) compared with moderate-severe dysplasia (median MVD, 112 vessels/mm²) and normal epithelium (median MVD, 88 vessels/mm²; Fig. 4). On the other hand, there was no association between peritumoral MVD and disease pro-

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**Table 1**  VEGF immunostaining intensity in head and neck lesions

<table>
<thead>
<tr>
<th>Histology of lesion</th>
<th>No. of specimens</th>
<th>Normal epithelium</th>
<th>Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal epithelium</td>
<td>10</td>
<td>1.0 (1.4 ± 0.5)</td>
<td>1.0 (1.0 ± 0.5)</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>9</td>
<td>1.0 (1.33 ± 0.50)</td>
<td>2.0 (1.56 ± 0.53)</td>
</tr>
<tr>
<td>CIS</td>
<td>6</td>
<td>1.0 (0.83 ± 0.75)</td>
<td>1.5 (1.50 ± 0.55)</td>
</tr>
<tr>
<td>Preinvasive</td>
<td>15</td>
<td>1.0 (1.13 ± 0.64)</td>
<td>2.0 (1.53 ± 0.52)</td>
</tr>
<tr>
<td>Stages I and II</td>
<td>9</td>
<td>1.0 (1.33 ± 0.58)</td>
<td>2.0 (1.86 ± 0.54)</td>
</tr>
<tr>
<td>Stages III and IV</td>
<td>10</td>
<td>1.0 (0.60 ± 0.53)</td>
<td>3.0 (2.40 ± 0.53)</td>
</tr>
<tr>
<td>LNM</td>
<td>3</td>
<td>N/A a</td>
<td>3.0 (2.67 ± 0.58)</td>
</tr>
</tbody>
</table>

a  Stain intensity scored as: 0 = no stain; 1 = weak; 2 = moderate; 3 = intense.

b  N/A, not applicable.
gression, with no significant difference in MVD of stage I and II lesions (median MVD 140, vessels/mm²) versus stage III and IV lesions (median MVD, 123 vessels/mm²; Fig. 4). Preinvasive lesions were not compared with invasive lesions because the latter were evaluated for peritumoral vessels, regardless of their relationship to the epithelium. An example of MVD staining in four different specimens is indicated in Fig. 5.

**DISCUSSION**

To evaluate a complete model of HNSCC carcinogenesis, we analyzed VEGF expression and MVD in clinical specimens ranging from normal epithelium of nonsmokers through preinvasive lesions, primary invasive and metastatic cancer. Advanced lesions (stage III and IV SCC and SCC formed in SCID mice) showed a significant increase in VEGF expression as compared with early lesions (dysplasias to early invasive SCC) and normal mucosa, and compared with adjacent histologically normal mucosa. Northern and Western analyses of VEGF expression in experimental HNSCC carcinogenesis revealed higher VEGF expression in all of the HNSCC cell lines than in normal keratinocytes, and HNSCC cells implanted in SCID mice.

**Table 2**  Association of VEGF with MVD and tumor aggressiveness in experimental tumors and cysts from human squamous cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>VEGF intensity</th>
<th>Days to visible tumor or cyst</th>
<th>Mean MVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaCaT</td>
<td>2</td>
<td>81 (cyst) 55 (cyst)</td>
<td>0.3</td>
</tr>
<tr>
<td>SCC 12</td>
<td>2</td>
<td>no tumor 89</td>
<td>3.3</td>
</tr>
<tr>
<td>SCC 13</td>
<td>3</td>
<td>89</td>
<td>N/A</td>
</tr>
<tr>
<td>Det 562</td>
<td>3</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>FaDu</td>
<td>3</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>A253</td>
<td>3</td>
<td>74</td>
<td>14</td>
</tr>
</tbody>
</table>

*a* All of the cell lines formed tumors except HaCaT, which formed a cyst.

*b* Cellular staining compared with negative control: 0 = negative; 1 = weak; 2 = moderate; 3 = intense.

*c* Number of cells injected at baseline.

*d* Mean number of vessels counted in three separate 0.83-mm² areas of tumor.

*e* N/A, not available.
mice formed tumors readily only in the lines with intense VEGF expression. Increased VEGF transcript levels were detected in four of nine cell lines evaluated (SCC 13, A253, Det 562, FaDu). Tumors that formed from these four cell lines when injected into SCID mice all showed intense VEGF staining, and tumors formed after a low tumor cell inoculum in each case. Taken together, these data suggest that VEGF expression is up-regulated in both clinical and experimental HNSCC, and that the increased expression occurs primarily in association with the ability of clinical tumors to metastasize and of experimental tumors to readily form tumors in immunodeficient mice.

Angiogenesis can be measured in a variety of ways, including the measurement of MVD and the expression of growth factors known to be important in stimulating angiogenesis, including bFGF, platelet-derived growth factor, and VEGF. Among the various growth factors thought to be important in the angiogenic process, VEGF is generally regarded as the most likely candidate for the induction of tumor growth because it is a soluble secreted endothelial cell mitogen and its receptor (KDR) is selectively expressed by activated endothelium (20). In experimental tumors, administration of anti-VEGF monoclonal antibody decreased the MVD and suppressed tumor growth (21).

Recent reports document the expression of VEGF both in HNSCC cell lines (22, 23) and in clinical specimens (24–27). What is not clear from these reports is the role of VEGF in either experimental or clinical HNSCC carcinogenesis. In one of the reports (27), subjects with normal mucosa, dysplasia, and invasive cancer were evaluated, but neither the degree of dysplasia (moderate, severe, or CIS) nor the stage of the invasive SCC was clear. Tumor metastases were apparently not evaluated. The authors concluded that VEGF expression is increased in high-grade dysplasia and SCC compared with normal epithelium, although no statistical support was described. A separate report using a polyclonal antibody to VEGF (25) documents an association of VEGF expression with LNM compared with non-metastatic SCC.

We considered the use of image analysis to quantitate VEGF immunostain but elected not to perform the procedure for a variety of reasons. First, image analysis has a number of problems that are difficult to circumvent. The problems include: (a) only a portion of a cell may be on a slide because of the thickness of the tissue section. This will affect the intensity scoring; (b) even if sections from tissue sections are cut at the same setting on the same microtome, the thickness of the sections can vary, potentially altering intensity scoring. This results in loss of precision in image analysis measurements that could neutralize its perceived advantages over the semi-quantitative immunostain method; (c) many tumor cells are nonspherical and vary in size and shape, which limits the ability to quantitate immunostain intensity (28); (d) staining heterogeneity—either true or induced—during tissue fixation and processing requires the subjective selection of fields, leads to inter-observer variability, and limits the reproducibility of image analysis results.

Fig. 5 MVD staining pattern in A, normal epithelium; B, CIS; C, stage I (early stage) SCC; and D, stage III (advanced) SCC. Arrows, examples of vessels.
Although a minority of subjects received preoperative chemotherapy, this treatment did not significantly influence VEGF expression. VEGF staining intensity was scored as 1 and 1, respectively, for the two Stage I/II specimens that had received prior treatment. The median score for all of the Stage I/II tumors was 1. VEGF intensity was 2 for the Stage III/IV specimen that had received prior treatment, compared with a median of 3 for the group.

MVD in invasive primary carcinomas has shown promise as a reliable prognostic marker in a number of tumor systems, including both node-positive and node-negative breast cancer (30). A variety of markers have been used to measure MVD. One of the more common, Factor VIII-related antigen, is expressed mainly on large blood vessels and also on the endothelial cells of lymph vessels (31). This staining of lymph vessels may be an advantage when evaluating HNSCC, given the propensity of these tumors to metastasize through lymphatics. Another marker, platelet/endothelial cell adhesion molecule, has the reported advantage of staining a higher number of small blood vessels as compared with Factor VIII (32). However, neither of these two markers is specific for tumor-stimulated blood vessels. An endothelial cell proliferation antigen, endoglin, may prove more specific for tumor-stimulated blood vessel growth (33).

In our normal epithelium and preinvasive lesions, stromal vessels deep in the basement membrane below the preinvasive lesion were measured. On the other hand, peritumoral vessels were counted in our tissue sections containing HNSCC. Thus, comparison of preinvasive with invasive lesions did not seem possible, and only normal versus dysplasia versus CIS and only early versus advanced SCC were compared. MVD was higher (P = 0.04) in CIS compared with dysplasia and normal mucosa (Fig. 4). Although there was no association between peritumoral MVD and disease stage, the effects of earlier treatment may have influenced MVD, especially in the advanced lesions. Radiatherapy, for example, is known to reduce the network of small blood vessels in tissue, leading to ischemia, atrophy, and occasionally necrosis (34). Unfortunately, complete clinical information is not available for most of the advanced lesions, but we do know that the preinvasive and early invasive lesions were not treated with radiation or chemotherapy before resection. This is consistent with the findings of Inoue et al. (25) that MVD is not a prognostic marker in oral SCC. On the other hand, we found a direct association between MVD and experimental SCC tumor formation (Table 2). Two prior studies evaluating experimental tumors of different origin (20, 35) reported similar findings.

We observed an increase in MVD in preinvasive lesions without a concomitant increase in VEGF, which was observed only much later in squamous cell carcinogenesis. It is well known that the induction of angiogenesis frequently precedes the formation of malignant tumors (36), which suggests that angiogenesis may be rate-limiting not only for tumor progression but also for the onset of malignancy. Skobe et al. (37), who analyzed the role of VEGF in both initiating and sustaining angiogenesis in malignant skin keratinocytes, demonstrated that, during the first 2 weeks after grafting benign and malignant variants of HaCaT cells on a collagen matrix, both types of cells express similar levels of VEGF mRNA. On the other hand, increased VEGF mRNA was detected 2–6 weeks after grafting in the more advanced lesions. The authors concluded that VEGF is crucial in sustaining, but not initiating, angiogenesis by malignant squamous cells, and that other angiogenic factor(s) mediates early-stage tumor angiogenesis. Both our clinical and our experimental data support this hypothesis.

In summary, VEGF expression seems to be associated with progression to a more aggressive phenotype both in experimental and in clinical systems involving HNSCC, with clinical evidence of lymphatic spread and with more rapid development of tumors in immunodeficient mice. Up-regulation of MVD seems to occur early in HNSCC carcinogenesis, although this observation awaits confirmation in a larger study.

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