Angiogenesis and the Expression of Vascular Endothelial Growth Factors A and C in Squamous Cell Carcinoma of the Piriform Fossa

Jarrod J. Homer, MD, FRCS; Michael G. Prentice, MRCS; Lynn Cawkwell, PhD; Martin Birchall, MD, FRCS; John Greenman, PhD; Nicholas D. Stafford, FRCS

Background: Angiogenesis is essential for tumor growth and invasion. Vascular endothelial growth factor A (VEGF-A) is a prime mediator of tumor angiogenesis; VEGF-C, another member of the closely related VEGF family of proteins, has major effects on lymphatic endothelial cells and may be important in the process of lymphatic metastasis.

Objectives: To evaluate the expression of these cytokines in hypopharyngeal squamous cell carcinoma and to ascertain the effects of these proteins on lymphatic metastasis and vascular angiogenesis.

Design: Retrospective analysis of microvessel density and the expression of VEGF-A and VEGF-C.

Setting: An academic referral center.

Subjects: Thirty-four patients with stage T2 to T4 squamous cell carcinoma of the piriform fossa.

Interventions: Expression of VEGF-A and VEGF-C was determined by immunohistochemistry on formalin-fixed, paraffin-embedded biopsy specimens. Angiogenesis was measured as microvessel density by staining endothelial cells for platelet-endothelial cell adhesion molecule 1/CD31.

Results: Of the 34 tumors, 21 had clinicoradiologic evidence of lymphatic metastasis. Expression of VEGF-C was associated with lymphatic metastasis ($P < .001$), but not with microvessel density. The VEGF-A expression correlated with microvessel density ($P < .001$), but neither VEGF-A expression nor microvessel density was associated with lymphatic metastasis.

Conclusions: The expression of VEGF-C is associated with lymphatic metastasis in squamous cell carcinoma of the piriform fossa. This is not secondary to effects on vascular angiogenesis and is hypothesized to be due to effects on lymphatic endothelial cells.

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# Angiogenesis

**Angiogenesis** is the development of new blood vessels. Interest in tumor angiogenesis was initiated largely by the work of Folkman and colleagues, and it is now widely accepted that angiogenesis is essential for tumorogenesis and for many characteristics of a malignant tumor phenotype. It has been shown that inhibiting angiogenesis in vivo halts tumor growth and invasion. In addition, there are established correlations between angiogenesis, usually measured as microvessel density (MVD), and disease aggressiveness, as measured by variables such as tumor stage, grade, metastasis, survival, and disease recurrence in many solid tumors.

Folkman et al proposed that tumors secrete cytokines that act on endothelial cells and hence are responsible for angiogenesis. There is a great deal of evidence to suggest that vascular endothelial growth factor A (VEGF-A) is a critical proangiogenic cytokine. It enhances multiple steps of the angiogenic process via 2 receptors, VEGFR1 and VEGFR2, that are specifically expressed on endothelial cells and have no other reported role except angiogenesis. Like many proangiogenic and antiangiogenic factors, VEGF-A and VEGF-C work in a paracrine fashion. The crucial role of VEGF-A in angiogenesis is supported by studies on embryonic vasculogenesis and endometrial angiogenesis.

In neoplasia, most tumors express both VEGF-A and its receptors, and capillaries are observed clustered around the VEGF-producing tumor cells. Expression of VEGF-A is associated with poor prognosis in many solid tumors in humans and inhibition of tumor growth can be achieved by targeting either VEGF-A or VEGFR2.

In both normal and malignant cells, the principal stimulus for VEGF-A expression is hypoxia, but tumor cells additionally constitutively overexpress VEGF-A.
Vascular endothelial growth factor C is a closely related protein to VEGF-A, also belonging to the VEGF family, that binds to VEGFR2 and VEGFR3. It is expressed at a low level in many tissues, but is overexpressed in many tumors.12 There is some evidence supporting an effect of VEGF-C via VEGFR3 on lymphatic endothelial cells in tumors.13–16 The effects may be a result of lymphangiogenesis, lymphatic dilation, or an increase in permeability. Alternatively, VEGF-C may also have vascular angiogenic effects, most probably via VEGFR2.17

Vascular endothelial growth factor A is overexpressed in head and neck squamous cell carcinoma (HNSCC).18 The association between VEGF-A expression and angiogenesis, assessed by MVD and/or clinicopathologic measures, has been investigated previously by several groups. Most work suggests that VEGF-A is the prime mediator of angiogenesis by the finding of positive associations between VEGF-A expression and MVD. However, no consistent pattern associating VEGF-A expression with clinicopathologic variables or with outcome has been shown.19 Previously, our group demonstrated overexpression of VEGF-C by immunohistochemistry in early laryngeal cancer,19 and a recent study found high VEGF-C messenger RNA expression to be independently associated with lymph node metastasis in a heterogeneous group of 54 patients with HNSCC.20

The presence of lymph node metastasis is of critical importance in terms of treatment and prognosis in HNSCC. The aim of this article is to ascertain the relationship of angiogenesis and VEGF-C expression in the process of lymphatic metastasis in HNSCC. While it would be anticipated that the expression of VEGF-A is related to MVD, whether VEGF-C has effects on mainly lymphatic or vascular endothelial cells is not known. Our study used a homogeneous group of tumors limited to a specific subsite of the hypopharynx. To include a group in which a large proportion of tumors will have metastasized, the piriform sinus was chosen.

METHODS

A group of 34 previously untreated piriform sinus tumors (25 men and 9 women) was identified from 2 regional head and neck centers in England: the South-West Cancer Intelligence Unit in Bristol and the Royal Infirmary in Hull. The median age of the patients was 69.5 years (range, 49-92 years). Tumors were selected on the basis of being clinically staged T2 to T4, arising from the piriform fossa subsite, having complete and accurate database information, and having a formalin-fixed, paraffin-embedded biopsy specimen available. There were 5 T2 stage tumors, 6 T3 stage, and 23 T4 stage. Twenty-one tumors had clinical evidence of lymph node metastases (cN+), on the basis of clinical and radiologic assessment. Seven patients had well-differentiated tumors, 14 were moderately differentiated, and 13 were poorly differentiated. Ethical approval was granted for the study by the Hull and East Riding Ethics Committee. Clinicopathologic data were obtained from hospital records.

The biopsy specimens were immunostained for the following antigens: platelet-endothelial cell adhesion molecule (PECAM)–1/CD31, an established endothelial cell marker,21 VEGF-A, and VEGF-C. A commercial staining system (ImmunoCruz; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) was used except where mentioned. Briefly, 4-µm paraffin-embedded sections were dewaxed in xylene and rehydrated in 100% ethanol. Endogenous peroxidase was blocked in hydrogen peroxide (0.6% vol/vol) in methanol for 20 minutes. Epitope retrieval was carried out by boiling in a pressure cooker in 1% vol/vol antigen retrieval solution (Antigen Masking Solution; Vector Laboratories Ltd, Peterborough, England) in distilled water for 3 minutes. The slides were transferred to Tris-buffered saline and washed with 2 rinses of Tris-buffered saline for 5 minutes. Blocking of nonspecific sites was achieved by the addition of 1 × casein for 10 minutes. The slides were then incubated with the primary antibody (polyclonal goat anti–PECAM-1, Santa Cruz Biotechnology, Inc; polyclonal rabbit anti–VEGF-A, BioGenex Laboratories, Inc, San Ramon, Calif; or polyclonal goat anti–VEGF-C, Santa Cruz Biotechnology, Inc). The primary antibodies were diluted (PECAM-1, 1:100; VEGF-A, 1:20; and VEGF-C, 1:100) in 0.2% vol (vol/vol) casein in Tris-buffered saline and incubated overnight at 4°C. After a wash, the biotinylated secondary antibody was added for 30 minutes. After a further wash, the horseradish peroxidase–streptavidin complex was added for another 30 minutes. The slides were then immersed in a pot of diaminobenzidine substrate for 15 minutes. The staining was enhanced by means of copper sulfate (0.5% wt/vol). Sections were counterstained with methyl green and dehydrated in ethanol, cleared in xylene, and mounted. Negative controls used a primary antibody of the same species against a different species (ie, goat antirabbit or rabbit antigoat), while colorectal cancer tissue known to be positive for VEGF-A and VEGF-C proteins was used as a positive control.

Quantitative analysis of VEGF-A and VEGF-C staining was done with computer-based image cytometry (CAS 200; Recont Dickinson Co, Oxford, England). The use of this and other similar systems is well established for semiquantitative immunohistochemistry and correlates well with manual subjective assessment.22 The percentage of positive-staining cells in 15 representative high-power (×400, 1.885 mm2) fields was measured and averaged. The method used to calculate MVD is based on that first described by Weidner et al,23 which has become the gold standard in the measurement of angiogenesis.24 With this method, the slide is scanned at low power (×100) to identify “hot spots” of angiogenesis as detected by PECAM-1 staining. The 3 hot spots of greatest angiogenesis are then measured at ×400 magnification for PECAM-1 staining in the same way as described above, with the computer program summing the results of the 3 areas.

Analysis was primarily carried out to determine an influence on the presence of clinically and radiologically staged lymph node metastasis, the most important factor with respect to treatment and prognosis. Because some patients were treated by surgery to the neck, the pathologic nodal status was not known in these cases. Nevertheless, all 3 of the patients in the cN0 group who had neck dissection were pathologically negative and all of the 15 patients in the cN+ group who had neck dissection were positive. We used t tests if the data were parametric, as tested by the Kolmogorov-Smirnov test. Analysis was also carried out to ascertain whether there were any correlations between the expression of the different cytokines and MVD. All tests were 2-tailed, and differences were regarded significant if P<.05. Analysis was carried out with the SPSS 9.0 software package (SPSS Inc, Chicago, Ill).

RESULTS

The VEGF-A and VEGF-C were expressed by all tumors. Both antigens were localized to the cytoplasm of cells in positive sections. In tumor specimens, there was some staining of endothelial cells and other stromal cells (eg, macrophages or fibroblasts) for both antigens, but this was less than the staining on tumor cells (Figure 1 and Figure 2). There was some degree of heterogene-
ity of staining within tumors, especially for VEGF, where there was greatest positivity toward the tumor margins in most sections. Expression of VEGF-C tended to be more homogeneous. An example of immunostaining with PECAM to measure MVD is shown in Figure 3.

The expression of VEGF-A, VEGF-C, and MVD was found to be normally distributed. Table 1 demonstrates that tumor stage and grade, 2 potentially confounding variables, were similar in both groups. The cN+ group was significantly younger, however.

The VEGF-C was expressed significantly more in patients with nodal metastases ($P<.001$). There was no difference in the expression of VEGF-A and MVD between patients with and without nodal metastases (Table 2). However the expression of VEGF-A did correlate with MVD ($r=0.62$, $P<.001$). No other significant correlations were observed (Table 3).

**COMMENT**

This study shows that there is a significant association of VEGF-C expression with lymph node metastasis in piriform fossa hypopharyngeal squamous cell carcinoma. While this was shown only by univariate analysis, the selection of the study group was narrow so as to eliminate many confounding variables. Of those confounding factors, tumor site is perhaps the most important to control for in HNSCC. Squamous cell carcinomas of the head and neck are known to behave very differently clinically depending on site, particularly with respect to lymphatic metastasis, and this is obviously pertinent when the effect on this clinicopathologic variable is studied. In addition, subsite selection is equally important when tumors of the larynx or hypopharynx are studied, yet this is rarely taken into account. Other confounding variables, T stage and tumor grade in particular, were similar in tumors with and without metastases. The patients with lymph node metastases were, however, significantly younger. Interestingly, it has been noted before that older patients with HNSCC tended to have less lymph node metastasis as an independent phenomenon. Hence, age is a theoretical confounding factor, i.e., VEGF-C is expressed more in the cN+ group because VEGF-C is expressed more in younger patients. However there is no obvious biological rationale to support such a theory.

The association between VEGF-C expression and lymph node metastasis is similar to those reported recently in carcinomas of the breast, prostate, and stomach. Moreover, the lack of association between VEGF-C and MVD is in keeping with studies in mesothelioma and prostate cancer and suggests that the association with lymphatic metastasis is not secondary to any effects on vascular angiogenesis. This is reinforced by the lack of association between MVD and lymphatic metastasis. In view of the known biological functions of VEGF-C, its effect on lymphatic metastasis in HNSCC is probably brought about by effects on tumor lymphatic endothelial cells. Supporting evidence arises from the findings that (1) the expression of VEGFR3 is largely restricted to lymphatic endothelial cells in adults; (2) VEGF-C is expressed in embryogenesis at sites of lymphatic development, and (3) overexpression of VEGF-C in transgenic mice causes lymphatic dilation and hyperplasia. If such effects on lymphatic endothelial cells are stimulated by tumors themselves, this would facilitate invasion and spread via lymphatics and would contradict the more traditional view of lymphatic metastasis.
sis being a passive process.\textsuperscript{28} Recent work has shown that VEGF-C overexpression promotes lymphatic metastasis by increasing the surface area of lymphatics on the tumor margin rather than inducing intratumoral lymphangiogenesis per se.\textsuperscript{29}

The expression of VEGF-A was significantly associated with MVD, in keeping with its being the prime inducer of angiogenesis in HNSCC. This is in agreement with the majority of previous studies in HNSCC that have analyzed the relationship between these 2 factors.\textsuperscript{18} However, neither the expression of VEGF-A nor MVD was associated with lymph node metastasis. This is in keeping with most other studies looking at these relationships in HNSCC, although there is controversy.\textsuperscript{18} This study does not necessarily dispute the importance of angiogenesis for tumor growth in HNSCC but implies that specific effects on tumor lymphatic endothelial cells brought about by the expression of VEGF-C may be more important in the process of lymphatic metastasis.

While the specificity of the tumors selected in this study has advantages alluded to in the preceding paragraphs, there are drawbacks. These findings apply to the piriform fossa subsite of the hypopharynx, and it cannot be automatically assumed that the conclusions are relevant to other subsites within the hypopharynx, or other HNSCC sites. For example, in sites where there is poor lymphatic drainage and a lower propensity toward lymphatic metastasis, these findings may not apply. A possible reason for this could be the need for preexisting lymphatic vessels if the effect of VEGF-C is increasing lymphatic surface area. The study used clinicoradiologic detection of lymph node metastasis as an end point, rather than using pathologic staging. Further studies could elicit whether these findings apply also to micrometastases. However, the prognostic relevance of micrometastases in HNSCC is questionable.\textsuperscript{30}

This study did not analyze any relationships with clinical outcome, eg, survival or locoregional recurrence, because of varied treatment regimens and inadequate follow-up interval. Further work could ascertain whether the expression of VEGF-C or its lymphatic receptor, VEGFR3, will predict the risk of clinically undetectable micrometastases or future lymphatic metastases in HNSCC. This would be of significant clinical benefit in selecting patients who require elective treatment of cervical lymph nodes when clinically staged as node negative. The identification of a possible mediator of lymphangiogenesis is of great relevance to the process of metastasis, as HNSCC, like other carcinomas, primarily spreads via lymphatics to lymph nodes. Hence, future clinical applications of this type of research include the prediction of lymphatic metastasis and the therapeutic targeting of the VEGF-C/VEGFR3 pathway, recently reviewed by Toi et al.\textsuperscript{31}

In summary, this study suggests that the expression of VEGF-C is biologically important in the process of lymph node metastasis in squamous cell carcinoma of the piriform sinus, probably via an effect on lymphatic endothelial cells. We believe that these endothelial cells play an active role in the process of lymphatic metastasis.

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| Table 1. Comparison of Piriform Sinus Tumors With and Without Lymphatic Metastases |
|-----------------|--------|--------|---|
| **Characteristic** | **cNO Group** | **cN+ Group** | **P Value** |
| Age, y | 76 (13) | 67 (21) | .005* |
| Grade, No. | | | |
| Well | 4 (3) | 3 (8) | .29 |
| Moderate | 6 (8) | 8 (2) | .29 |
| Poor | 3 (10) | 10 (4) | .29 |
| T stage, No. | | | |
| 2 | 2 (3) | 3 (2) | .27 |
| 3 | 4 (2) | 2 (2) | .27 |
| 4 | 7 (16) | 16 (4) | .27 |

| Table 2. MVD and Expression of VEGF-A and VEGF-C in Piriform Sinus SCC With and Without Nodal Metastasis |
|-----------------|--------|--------|---|
| **Expression of Cytokine, Mean (SD)** | **cNO** | **cN+** | **P Value** |
| VEGF-A | 49.1 (19.9) | 43.6 (19.9) | .44 |
| VEGF-C | 23.1 (11.8) | 56.9 (20.8) | <.001† |
| MVD | 26.5 (12.4) | 25.7 (10.4) | .85 |

| Table 3. Correlation Between MVD and Expression of VEGF-A and VEGF-C in Piriform Sinus SCC |
|-----------------|--------|---|
| **Correlation** | **r Value** | **P Value** |
| VEGF-A/VEGF-C | 0.008 | .96 |
| VEGF-A/MVD | 0.625 | <.001* |
| VEGF-C/MVD | 0.027 | .88 |

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Corresponding author: John Greenman, PhD, Medical Research Laboratory, University of Hull, Cottingham Road, Hull HU6 7RX, England (e-mail: j.greenman@hull.ac.uk).
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