Altered Pigment Epithelium–Derived Factor and Vascular Endothelial Growth Factor Levels in Lymphangioma Pathogenesis and Clinical Recurrence

Douglas M. Sidle, MD; John Maddalozzo, MD; Jason D. Meier, MD; Mona Cornwell, HT(ASCP); Veronica Stellmach, PhD; Susan E. Crawford, MD

Objective: To determine the role of angiogenesis in the clinical behavior and pathogenesis of lymphangioma tumors.

Design: A retrospective study. Median follow-up period was 44.5 months.

Setting: Children’s Memorial Hospital, Chicago, Ill.

Patients: Tumor specimens from 12 pediatric patients who underwent surgical excision of cervicofacial lymphangioma were examined for expression of angiogenic inducer vascular endothelial growth factor (VEGF) and angiogenic inhibitor pigment epithelium–derived factor (PEDF) using immunohistochemical analysis. Specimens were divided into recurrent and nonrecurrent tumors based on clinical information.

Main Outcome Measures: Staining patterns of VEGF and PEDF were evaluated in lymphangioma specimens. Staining patterns were then compared in both recurrent and nonrecurrent groups and graded in a blinded fashion. Histological evidence of increased angiogenesis including microvascular density, stromal fibrosis, and inflammation were graded in each group and correlated with recurrence.

Results: Lymphangioma specimens demonstrated histological evidence of increased angiogenic activity including multiple areas of increased VEGF staining combined with little PEDF staining. Sex, age at onset, or tumor location did not correlate with recurrence. Furthermore, recurrent specimens had increased histological evidence of angiogenesis as well as increased VEGF and decreased PEDF activity compared with nonrecurrent lesions.

Conclusions: Lymphangiomas exhibit tumorlike pathogenesis owing to the high expression of angiogenic inducers compared with the low expression of inhibitors. Recurrence may be influenced by this imbalance of angiogenic mediators. Further research with antiangiogenic therapy using agents such as PEDF analogues or anti-VEGF receptor antibodies is indicated because they may stabilize or suppress the growth of these neoplasms.


The pathogenesis and classification of lymphatic malformations has been debated since they were first described by Redenbacher in 1828. Landing and Farber in 1956 initially classified them into the following 3 entities: lymphangioma simplex, cavernous lymphangioma, and cystic lymphangioma (cystic hygroma). However, these categories tend to overlap. A unified concept then developed that declared that the previously described lymphangiomas are variations of a single entity, and their classification is determined by their location in the head and neck. Current classification by Williams separates lymphatic malformations into the following 4 categories: (1) lymphangioma, subdivided into cavernous and simplex; (2) cystic hygroma; (3) lymphedema (Milroy disease); and (4) lymphangiectasia. Furthermore, Smith et al clinically describe the lymphatic malformations as macrocystic or microcystic depending on their appearance with contrast cystography. Because there is no significant histological difference between the types of lymphatic malformations, in the present study we describe both cystic hygromas and lymphangiomas and make no distinction between them.

The classification of lymphangiomas is controversial and has been difficult owing to their rare occurrence and incomplete knowledge about their pathogenesis. Approximately 90% of lymph-
Lymphangiomas occur in the head and neck region. As they slowly grow and progress, they can cause symptoms of dysphagia, airway obstruction, and gross disfigurement. Infection and inflammation also commonly occur. Surgical excision is the most widely accepted primary therapy for treatment of lymphangiomas. However, complete excision is often not possible and recurrence is frequent. Other forms of therapy have been investigated including OK-432 (a hemolytic streptococcal preparation), interferon alfa, bleomycin, laser therapy, and radiation with variable results.

Currently, most researchers believe lymphangiomas to be congenital malformations of the lymphatic system. They are characterized histologically by a proliferation of blood vessels and lymphatics with intervening fibrous tissue and lymphoid aggregates. Some researchers have argued that they are true neoplasms resulting from transformed lymphatic endothelial and/or stromal cells. Likely, lymphangiomas have characteristics of both in their pathogenesis. In the tumor model of lymphangioma pathogenesis, a dysregulation of angiogenesis may contribute to lymphangioma growth and progression. This is supported histologically by the increase in microvascular density (MVD) exhibited in lymphangiomas, which is the hallmark of angiogenesis.

Angiogenesis, the induction of new capillaries from preexisting vessels, is a key event in several biological processes ranging from wound healing to the formation of benign and malignant neoplasms. Angiogenesis is regulated by a delicate balance between angiogenic inducers and naturally occurring inhibitors. Tumor growth and progression is favored when inducers of angiogenesis, such as basic fibroblast growth factor and vascular endothelial growth factor (VEGF), predominate locally or systemically. Expression of VEGF and its receptors (VEGFR-2 and VEGFR-3) have extensively been correlated with malignant behavior, metastasis, and worsened prognosis in many different tumor types including colorectal cancer, prostate cancer, head and neck squamous cell cancer, breast cancer, ovarian cancer, and lung cancer. In addition, anti-VEGF receptor antibodies have been demonstrated to decrease tumor growth, decrease angiogenesis, and increase hypoxia in preclinical studies. Clinically, they also have been shown to increase patient survival when combined with chemotherapy.

Much less is known about the role of angiogenic inhibitors in disease processes. Pigment epithelium–derived factor (PEDF) has been discovered to be a potent inhibitor of angiogenesis. Also, PEDF expression has been isolated in many different tumor types. In contrast to VEGF, PEDF expression has been correlated with an inhibition of tumor growth, a decrease in metastasis, and a favorable prognosis. Previous studies have suggested the presence of a PEDF receptor on retinoblastoma cells; however, the identity of this receptor remains elusive.

In lymphangioma, angiogenic activity has been shown to be, in part, due to elevated levels of potent angiogenic inducer, basic fibroblastic growth factor. Although VEGF has been implicated in the regulation of lymphangioma growth, PEDF has not been previously studied in lymphangioma. A proposed model for the pathogenesis of lymphangiomas suggests that they are in part neoplastic, and growth or recurrence of these lesions depends on an imbalance of angiogenic mediators.

METHODS

After obtaining institutional review board approval, tumor specimens from 12 pediatric patients who underwent surgical excision of cervicofacial lymphangioma at the Children’s Memorial Hospital in Chicago, Ill, were examined for expression of the angiogenic factors VEGF and PEDF using immunohistochemical analysis. The clinical records of the 12 patients were also retrospectively reviewed for demographic data, site of lesion, sex of the patient, age at onset, and presence of recurrence. Only tumor specimens from patients in whom curative resections were attempted were used in the study. Specimens were divided into recurrent and nonrecurrent tumors. Paraffin blocks were available for examination for the 12 tumors. All of the paraffin blocks examined in this study were from the primary resection of tumor. Sections were examined for histological markers that might distinguish recurrent from nonrecurrent lesions. Histological factors evaluated included vessel density, fibrosis, and inflammation (lymphocyte aggregation). Microvascular density, stromal fibrosis, and lymphocyte aggregation were graded on a 0 to 3 scale (0 = none; 1 = focal; 2 = multifocal; and 3 = diffuse) in 4 high-power fields per slide.

Expression of the angiogenic factors was analyzed by immunohistochemical analysis using slides that were incubated with monoclonal human IgG antibodies to VEGF-C (SC-507; Santa Cruz Biotechnology, Santa Cruz, Calif) at a dilution of 1:200 or PEDF (generated by the authors) at a dilution of 1:75 through an immunoperoxidase staining method. In brief, 3-μm sections were deparaffinized in xylene and rehydrated in graded ethanol solutions. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol. Sections were blocked with 10% normal equine serum for 20 minutes, rinsed with phosphate-buffered saline as a negative control. The sections were next rinsed and incubated for 30 minutes with biotin-conjugated equine anti-mouse and anti-rabbit antibody (Vector Laboratories, Burlingame, Calif) at a dilution of 1:50. An avidin-biotin complex (ABC elite; Vector Laboratories) was applied for 30 minutes. All incubations took place in a humidified chamber at 37°C. Sections were rinsed a final time with phosphate-buffered saline and neutralized with buffered sodium acetate. Then 3,3′-diaminobenzidine (DAB) reagent was applied to produce a permanent color change. The slides were counterstained with hematoxylin-eosin, mounted, and evaluated by light microscopy. The Vectastain Universal Elite ABC Kit (Vector Laboratories) and the Biogenex (San Ramon, Calif) Liquid DAB Kit were used for staining. Our pathologist (S.E.C.) and senior author (D.M.S.) examined each slide to determine the intensity and pattern of VEGF and PEDF expression. This was done in a blinded fashion with negligible variability between the 2 observers. Staining intensity was scored on a similar 0 to 3 scale as described for histological characteristics (0 = none; 1 = light; 2 = moderate; and 3 = heavy). Because the pattern of staining was focal, specific areas within the tissue specimen were graded based on the staining intensity in that particular area. In particular, vascular smooth muscle, endothelial cells, inflammatory cells, and surrounding matrix were graded. By adding the scores of each of the 4 graded areas, a total score of VEGF and PEDF staining pattern for each specimen was established. Endothelial cell–positive staining for VEGF and PEDF...
was the internal positive control within tumor specimens. Because of the differing tissues surrounding the tumor in each specimen, no uniform negative control specimen could be evaluated. These data were then correlated with clinical recurrence of disease.

Statistical analysis was performed using the Mann-Whitney rank sum test with statistical significance defined as $P < .05$. Data, unless otherwise specified, are reported as mean ± SEM.

### RESULTS

The demographics of each patient, primary site of tumor, recurrence, and age at first resection are given in Table 1. The study group included 7 boys and 5 girls with a mean age of 2.6 years. There were 10 cervical and 2 facial lymphangiomas. At a median follow up of 44.5 months, 5 patients were disease free, whereas 7 patients had recurrence of disease.

All tumor specimens displayed histological characteristics of lymphangiomas. Histopathologic data for each tumor specimen are presented in Table 2. Tumor specimens demonstrated increased MVD, stromal fibrosis, and lymphoid aggregates, whereas surrounding tissue did not exhibit these characteristics. In total, 9 tumor specimens showed high MVD ($>2.0$ or $>3.0$), including all of the recurrent tumors. Recurrent tumors demonstrated significantly higher MVD than did nonrecurrent tumors ($2.7 ± 0.18$ vs $1.4 ± 0.25$; $P = .01$). Also, recurrent tumor specimens displayed a significant increase in lymphoid aggregation ($2.4 ± 0.20$ vs $0.8 ± 0.20$; $P = .003$) and more fibrosis; however, fibrosis did not reach statistical significance ($2.0 ± 0.38$ vs $1.6 ± 0.25$; $P = .53$). Figure 1 shows hematoxylin-eosin-stained slides where recurrent tumor (Figure 1A) has higher MVD, interstitial fibrosis, and inflammatory reaction than does nonrecurrent tumor (Figure 1B).

The results of immunohistochemical staining and examination are presented in Table 3. Because VEGF and PEDF localized differently to various tissues within each specimen, the slides were graded based on the following 4 different areas: vascular smooth muscle, inflammatory cells, endothelial cells, and surrounding matrix. The scores from each of these areas were then added for each specimen for a possible total score of 12. As a group, the recurrent tumor specimens showed significantly more intense VEGF staining than did nonrecurrent specimens ($9.6 ± 0.72$ vs $6.4 ± 0.81$; $P = .048$). Also, nonrecurrent tumor specimens significantly stained more intensely to PEDF than did recurrent tumors ($5.8 ± 1.74$ vs $1.9 ± 0.34$; $P = .03$). Specimen 5, a nonrecurrent lesion, demonstrated the highest PEDF score, but no unique clinical or pathologic aspects were determined. Figure 2 demonstrates that recurrent tumors (Figure 2C and D) have increased VEGF and decreased PEDF staining compared with nonrecurrent tumors (Figure 2A and B). Figure 3 demonstrates that salivary ductal tissue stains intensely for VEGF but little for PEDF. Sex of the patient, location of the primary tumor, and age at presentation did not correlate with recurrence ($P > .05$).

### COMMENT

Lymphangiomas of the cervical and facial regions pose a very difficult problem to the pediatric head and neck
surgeon. They present at birth or within the first 2 years of life in 80% to 90% of patients. The current treatment of choice for head and neck lymphangiomas remains surgical resection with preservation of all important neurovascular structures. It is often difficult to obtain surgical margins, and the subsequent high recurrence rates seen with these tumors has led some clinicians to pursue other treatment modalities with variable results.

The etiology of lymphangiomas is not well understood. They are defined histologically by a benign proliferation of blood vessels and dilated lymphatic channels intervened with areas of fibrosis and lymphoid aggregates. The channels are lined by flattened, elongated endothelial cells creating “staghorn vessels,” which are characteristic of lymphatic spaces (Figure 2). High angiogenic activity has been demonstrated in cells isolated from lymphangiomas. The senior author’s group previously has shown high levels of angiogenic inducer, basic fibroblast growth factor, and lower levels of naturally occurring angiogenic inhibitor, thrombospondin-1. Not all the angiogenic activity could be relieved by a blocking antibody to basic fibroblast growth factor. Therefore, other angiogenic mediators such as VEGF were thought to play a role.

Investigators have suggested that VEGF induces lymphangiogenesis and VEGF-C appears to be the first angiogenic mediator that is specific for the lymphatic system. VEGFR-3, the receptor for VEGF-C, is localized primarily to lymphatics in normal tissue but can also be localized to the proliferating endothelium of vascular tumors and anywhere neovascularization is occurring. Not all the angiogenic activity could be relieved by a blocking antibody to basic fibroblast growth factor. Therefore, other angiogenic mediators such as VEGF were thought to play a role.

Our data also demonstrate the novel finding of PEDF expression in lymphangiomas. In many tumor types, PEDF expression has been studied and, opposite to that of VEGF, has been correlated with an inhibition of tumor growth, a decrease in metastasis, and a favorable prognosis. In the present study, PEDF expression in lymphangiomas correlated with a decrease in recurrence. This study also demonstrated a higher rate of recurrence in tumors that had a high MVD, the hallmark of angiogenesis, and increased lymphocytic infiltration. A known potent inhibitor of angiogenesis, PEDF likely plays a part in the delicate balance that keeps these tumors in check because lymphangioma specimens with the highest MVD had the lowest PEDF staining.

An interesting finding was that the endothelium in salivary tissue stained intensely to VEGF, an angiogenic inducer (Figure 3). These same structures did not appear

---

**Figure 1.** Recurrent tumors (A) show significantly more inflammatory cell infiltration and higher microvascular density than nonrecurrent tumors (B) (hematoxylin-eosin, original magnification ×20).

**Table 3. Immunohistochemical Staining Results for Lymphangioma Specimens**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>VEGF Total Score</th>
<th>PEDF Total Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3, 2, 2, 1</td>
<td>1, 0, 1, 1</td>
</tr>
<tr>
<td>2</td>
<td>3, 2, 2, 1</td>
<td>3, 0, 2, 1</td>
</tr>
<tr>
<td>3</td>
<td>2, 0, 2, 1</td>
<td>5, 2, 0, 2</td>
</tr>
<tr>
<td>4</td>
<td>2, 0, 2, 0</td>
<td>4, 0, 0, 1</td>
</tr>
<tr>
<td>5</td>
<td>3, 0, 2, 2</td>
<td>7, 3, 3, 3</td>
</tr>
<tr>
<td>6</td>
<td>3, 0, 3, 1</td>
<td>7, 1, 0, 1</td>
</tr>
<tr>
<td>7</td>
<td>3, 3, 3, 1</td>
<td>10, 0, 0, 0</td>
</tr>
<tr>
<td>8</td>
<td>3, 3, 3, 3</td>
<td>12, 1, 0, 0</td>
</tr>
<tr>
<td>9</td>
<td>3, 3, 3, 2</td>
<td>10, 1, 0, 1, 1</td>
</tr>
<tr>
<td>10</td>
<td>3, 0, 3, 1</td>
<td>7, 0, 0, 1, 1</td>
</tr>
<tr>
<td>11</td>
<td>3, 3, 3, 2</td>
<td>11, 1, 0, 0, 0</td>
</tr>
<tr>
<td>12</td>
<td>3, 3, 3, 1</td>
<td>10, 1, 0, 0, 1</td>
</tr>
</tbody>
</table>

**Abbreviations:** PEDF, pigment epithelium–derived factor; VEGF, vascular endothelial growth factor.

*Immunohistochemical staining of vascular smooth muscle, inflammatory cells, endothelial cells, and surrounding matrix was evaluated for all specimens. Staining intensity was scored on a 0 to 3 scale (0 = none; 1 = light; 2 = moderate; and 3 = heavy). A total score for each specimen was assigned based on staining patterns.
to produce any significant amount of PEDF, an angiogenic inhibitor. In addition, stratified squamous epithelium also stains strongly for VEGF. The higher recurrence rates noted by other authors in lesions of the suprathyroid areas are likely in close proximity to both salivary tissue and oral/pharyngeal mucosa.\textsuperscript{31,36} Local recurrence in these locations may be facilitated by high local levels of VEGF, which appears to be secreted by these tissues.

Figure 2. Under immunoperoxidase staining, recurrent tumors (A) stain more intensely for vascular endothelial growth factor C than do nonrecurrent tumors (B) (original magnification ×20); recurrent tumors (C) stain less intensely for pigment epithelium–derived factor than do nonrecurrent tumors (D) (original magnification ×10). Brown discoloration is considered positive staining.

Figure 3. Salivary glands show positive staining with vascular endothelial growth factor primarily in endothelial cells (A) but no significant staining for pigment epithelium–derived factor (B) (vascular endothelial growth factor and pigment epithelium–derived factor immunoperoxidase staining, original magnification ×20, where brown discoloration is considered positive staining).
These data support our conclusion that lymphangiomatosis has a tumoralike pathogenesis and is dependent on angiogenesis to support its growth. As demonstrated in other tumors, the dysregulation of angiogenesis by loss of naturally occurring inhibitors may be the underlying mechanisms of growth and proliferation. An imbalance of VEGF and PEDF may also increase the risk of recurrence after surgical resection.

Identification of the mechanism of action for these and other angiogenesis markers in lymphangiomatosis would aid in our understanding of the pathophysiologic features of these tumors. In turn, this knowledge may open new avenues of therapy using antiangiogenic agents. If an imbalance between angiogenic inducers and inhibitors is the underlying mechanisms of their growth and proliferation, then restoring the balance through therapy with angiogenic inhibitors such as PEDF analogues or anti-VEGF receptor antibodies may be of some use in treating these tumors, and further investigation is warranted.

Submitted for Publication: February 23, 2005; final revision received June 29, 2005; accepted July 7, 2005.

Correspondence: John Maddalozzo, MD, Pediatric Otolaryngology, Children’s Memorial Hospital, 2300 Children’s Plaza, Box 25, Chicago, IL 60614 (jpmaddalozzo@childrensmemorial.org).

Financial Disclosure: None.

Previous Presentation: This study was presented at the American Society of Pediatric Otolaryngology section of the 2003 Combined Otolaryngological Spring Meetings; May 5, 2003; Nashville, Tenn.

REFERENCES