ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR–A IN RECURRENT RESPIRATORY PAPILLOMATOSIS

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Vascular endothelial growth factor–A (VEGF-A) is known to play an important role in the angiogenic response essential for tumor growth in a variety of human and experimental tumors. This study was designed to investigate whether VEGF-A may play a role in the pathogenesis of recurrent respiratory papillomatosis (RRP). A retrospective study with institutional review board approval was performed at a tertiary care medical center on 12 patients with a history of laryngeal RRP. Their ages at the time of initial diagnosis ranged from 19 to 96 months (mean, 56 months). All patients had involvement of right and left true vocal cords. All patients required multiple endoscopic procedures (range, 4 to 66; mean, 12). Normal pediatric larynx samples from 5 autopsy patients were used as controls. Formalin-fixed, paraffin-embedded sections of laryngeal squamous papillomas from the 12 patients with a diagnosis of RRP and the 5 control patients were examined by in situ hybridization for the presence of messenger RNA (mRNA) for VEGF-A and vascular endothelial growth factor receptor 1 (VEGFR-1) and vascular endothelial growth factor receptor 2 (VEGFR-2). The biopsy specimens were from the true vocal cord (N = 10) or subglottis (N = 2) in the patients with RRP and consisted of large sections of larynx including the true vocal cord in the control patients (N = 5). Strong expression of VEGF-A mRNA was noted in the squamous epithelium of papillomas of all 12 patients. Strong expression of VEGFR-1 and VEGFR-2 was noted in the endothelial cells of the underlying vessels in all 12 patients. Neither strong labeling of VEGF-A mRNA nor labeling of its receptors was noted in the control patients. We conclude that the angiogenic growth factor VEGF-A is strongly expressed in the epithelium of squamous papillomas in RRP. Also, VEGFR-1 and VEGFR-2 mRNAs are strongly expressed by underlying vascular endothelial cells, suggesting an important role in the pathogenesis of RRP.

KEY WORDS — angiogenesis, recurrent respiratory papillomatosis, vascular endothelial growth factor–A.

INTRODUCTION

The squamous papilloma is the most common benign neoplasm of the larynx in the pediatric population.[1] This is a disease of viral causation, most commonly presenting as recurrent respiratory papillomatosis (RRP). Despite its benign histologic appearance, it has significant morbidity and occasional mortality due to airway obstruction. Currently, no single or combined treatment modalities reliably cure this disease. The management is often difficult and frustrating because of a high rate of local recurrence and the potential for spread of the papillomas to different parts of the respiratory tract.

In the past decade, there has been a surge of interest in the role of angiogenesis and different angiogenic growth factors in the pathogenesis and management of a number of tumors.[2-5] Vascular endothelial growth factor–A (VEGF-A) is known to play an important role in the angiogenic response essential for tumor growth in a variety of human and experimental tumors.[2,6-8] The goal of this study was to investigate whether VEGF-A may play a role in the pathogenesis of RRP.

MATERIALS AND METHODS

The patients included 12 consecutive children who underwent biopsy of RRP during 2000 and 2001 at Children's Hospital in Boston. All data with respect to age, gender, initial presentation, location of RRP, number of surgical procedures, and complications were reviewed. The biopsy sites were in the true vocal cord (N = 10) and subglottis (N = 2).

Tissue sections of normal larynges including both respiratory and squamous (true vocal cord) mucosa from 5 autopsy patients (7 months to 20 years of age; mean, 5 years) without a history of RRP were obtained as a control. All specimens were examined grossly, fixed in 10% formalin, and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin and eosin. Immunohistochemical staining for the endothelial marker CD31 (BioGenex Laboratories, San Ramon, California) was performed

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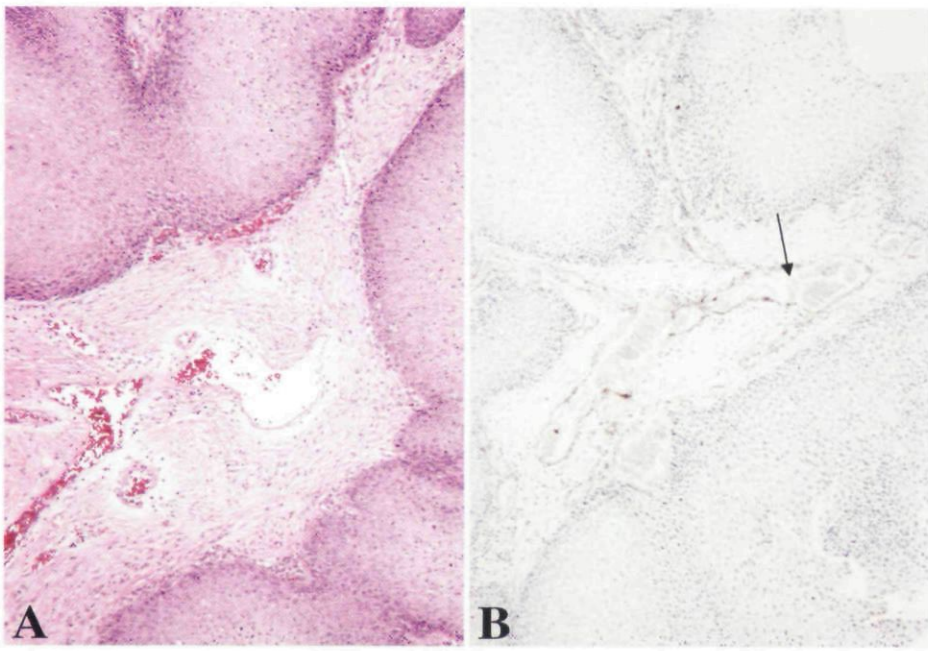

Fig 1. Squamous papilloma from larynx shows squamous epithelium overlying core of fibroconnective tissue with prominent vasculature. **A)** H & E, original ×20. **B)** CD31 immunostain highlights endothelium (arrow; original ×20).

on paraffin-embedded sections by means of a streptavidin-biotin–based alkaline phosphatase detection kit (Universal Multi-Species USA Horseradish Peroxidase Kit, Signet Laboratories, Dedham, Massachusetts) with liquid DAB-Plus (Zymed, San Francisco, California) as the chromogen.

In situ hybridization was performed on 5-μm paraffin sections with antisense probes for VEGF-A, VEGF-A receptors 1 (flt-1) and 2 (KDR), and control sense probes. The details of in situ hybridization have been published previously.[9] Briefly, slides were passed through xylene, graded alcohols, 0.2 mol/L hydrochloric acid; Tris–ethylenediaminetetraacetic acid (EDTA) with 3 μg/mL proteinase K; 0.2% glycine; 4% paraformaldehyde in phosphate-buffered saline solution, pH 7.4; 0.1 mol/L triethanolamine containing 1/200 (vol/vol) acetic anhydride; and 2X SSC. The slides were hybridized overnight at 50°C with ^{35}S-labeled riboprobes in the following mixture: 0.3 mol/L sodium chloride, 0.01 mol/L Tris pH 7.6, 5 mmol/L EDTA, 50% formamide, 10% dextran sulfate, 0.1 mg/mL yeast tRNA, and 0.01 mol/L dithiothreitol. Post-hybridization washes included 2X SSC/50% formamide/10 mmol/L dithiothreitol at 50°C; 4X SSC/10 mmol/L Tris/1 mmol/L EDTA with 20 μg/mL ribonuclease A at 37°C; and 2X SSC/50% formamide/10 mmol/L dithiothreitol at 65°C and 2X SSC. The slides were then dehydrated through graded alcohols containing 0.3 mol/L ammonium acetate, dried, coated with Kodak NTB 2 emulsion, and stored in the dark at 4°C for 2 weeks. The emulsion was developed with Kodak D19 developer, and the slides were counterstained with hematoxylin. The antisense 204 base pair (bp) single-stranded ^{35}S-labeled VEGF-A RNA probe and its sense control have been described previously.[10] The antisense probe hybridizes specifically with a region of VEGF-A messenger RNA (mRNA) common to all known splicing variants. ^{35}S-labeled single-stranded antisense probes targeted to VEGF-A receptors 1 and 2 have been described previously.[7] This study was performed under an Institutional Review Board–approved protocol.

RESULTS

In all 12 patients, the diagnosis of RRP was confirmed on the basis of clinical and histopathologic findings. There were 5 boys and 7 girls. Their ages at the time of initial diagnosis ranged from 19 to 96 months (mean, 56 months). All patients presented with hoarseness. The RRP involved the right and left vocal cords in all 12 patients, 2 of whom also had subglottic involvement. There was no tracheal or distal airway involvement in any patient. All 12 patients required multiple endoscopic procedures (range, 4 to 66; mean, 12) for debulking the RRP. There were no complications.

The diagnosis of squamous papilloma was histologically confirmed in all 12 patients. Microscopic examination showed polypoid fragments of tissue lined by well-differentiated nonkeratinizing squamous mucosa and supported by a fibrovascular stromal core (Fig 1A). The CD31 stains highlighted endothelium, confirming the presence of frequent stromal blood vessels in all cases (Fig 1B). In situ hybridization studies were performed on paraffin sections from 12 patients with respiratory papillomas for mRNAs of VEGF-A and its receptors, VEGFR-1 and VEGFR-2. Focal strong expression of VEGF-A

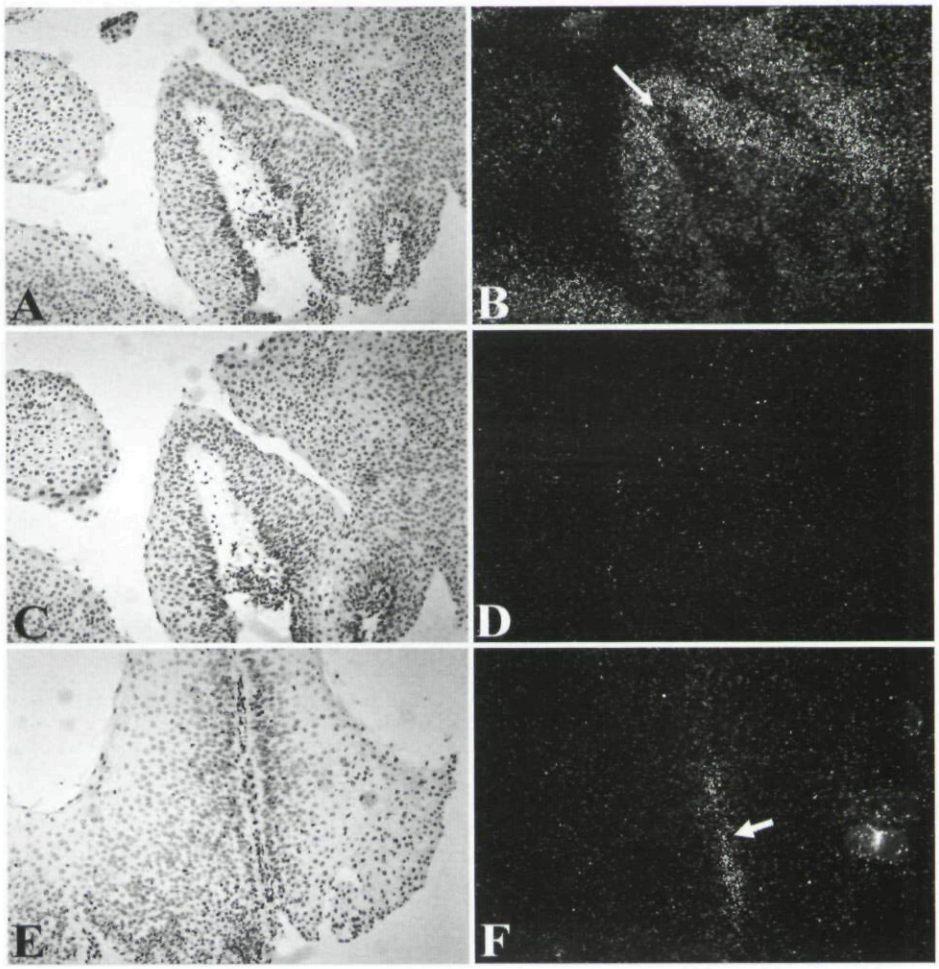

Fig 2. In situ hybridization, bright-field and corresponding dark-field photomicrographs. **A,B)** Vascular endothelial growth factor–A (VEGF-A) antisense probe shows epithelial expression, strongest in suprabasal levels (arrow). **C,D)** VEGF-A control sense probe shows no staining. **E,F)** VEGF-A receptor is seen in endothelial cells (arrow).

mRNA was detected in the squamous epithelium of the papillomas. Expression of VEGF-A was strongest in the suprabasal levels of the epithelium (Fig 2A,B). No specific labeling was seen with the control sense probe (Fig 2C,D). Strong expression of mRNAs for the VEGF-A receptors VEGFR-1 and VEGFR-2 was detected in endothelial cells of the prominent blood vessels in the fibrovascular cores of the papillomas (Fig 2E,F).

Gross histologic examination of 5 control patients did not show any evidence of squamous papilloma. Immunohistochemical analysis and in situ hybridization studies were performed on the 5 control laryngeal biopsy specimens. Neither expression of VEGF-A mRNA nor expression of its receptors was noted in any of the control specimens (Fig 3).

DISCUSSION

The incidence of RRP in children is estimated at 0.6 to 4.3 per 100,000 children; approximately 2,350 new cases occur in the United States per year.[11,12]

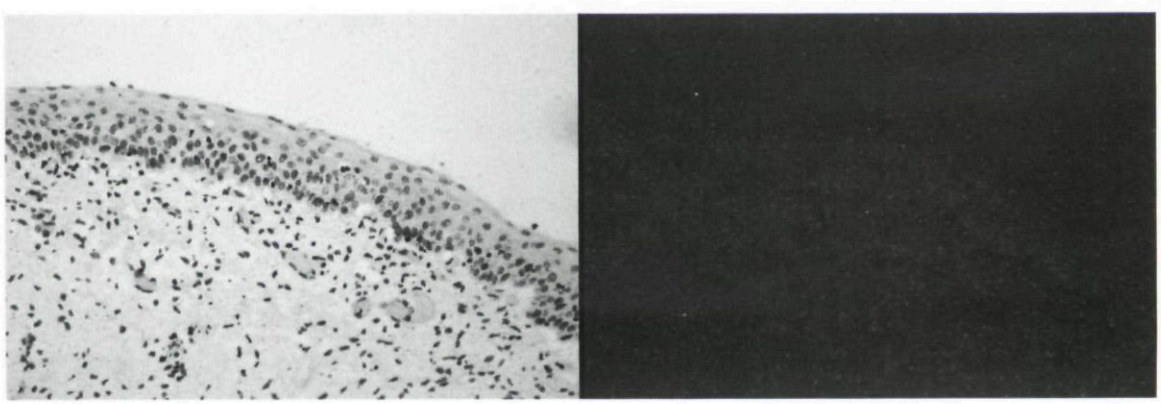

Fig 3. Control specimen. No evidence of VEGF-A messenger RNA or its receptors.

Human papillomavirus (HPV), subtypes 6 and 11, is most commonly associated with RRP. The HPV DNA has been identified in virtually all lesions studied.[13] An association between maternal cervical HPV infection and development of RRP has been established.[14,15] However, the mode of transmission remains controversial, and no study has clearly shown that children born to mothers with HPV are more likely to develop RRP.[16] The larynx is affected in almost all children with RRP. Papillomas most commonly appear as multiple irregular and pedunculated masses. Distal spread to the trachea and bronchi is of major concern because of the difficulty of surgical removal to provide a safe and patent airway.

Currently, there is no consensus about the best and most effective single or combined management of RRP. The most common treatment modality is surgery (carbon dioxide, KTP, or neodymium:yttrium-aluminum-garnet laser; cold technique; or tracheotomy).[17] Regardless of the surgical method, it is essential not only to provide a safe airway but to prevent complications of scarring, including stenosis and voice disorders. Because of the aggressiveness of the disease process, some patients may require adjuvant medical therapy. The commonly recommended adjuvant therapies are interferon, photodynamic therapy, indole-3-carbinol, and cidofovir.[18,19] Despite combined surgical and medical treatments, some patients continue to have an aggressive disease process that requires frequent endoscopic evaluation and debulking of the papillomas to maintain a safe airway.

In the past 2 decades, there has been a surge of interest in the role of angiogenesis and angiogenic growth factors in the presentation and management of solid tumors. The purpose of our study was to evaluate the role of VEGF-A and its receptors (VEGFR-1, VEGFR-2) in RRP in the hope of providing insight into the pathogenesis and progression of the disease.

Historical Perspective. In the late 1970s, Dvorak et al[20-22] discovered a potent vascular permeabilizing protein with a capacity to increase the permeability of microvessels to plasma and plasma proteins. This protein was subsequently purified and given the name vascular permeability factor (VPF).[23,24] In 1989, Connolly et al[25] reported that VPF not only enhances vascular permeability, but also acts as a selective mitogen for cultured vascular endothelial cells. In the late 1980s, several investigators isolated an endothelial cell mitogen from pituitary cell cultures and named this protein vascular endothelial growth factor–A (VEGF-A).[26,27] Further independent experiments determined that the vascular permeabilizing and the endothelial cell mitogenic activities were mediated by the same protein, and the term VPF/VEGF was coined.[28,29] However, VEGF-A is the term most commonly used today.

Mechanism of Action of VEGF-A. Vascular endothelial growth factor–A exerts a variety of effects on vascular endothelium. It is among the most potent vascular permeabilizing agents known, with a potency that is 50,000 times that of histamine.[20] After exposure to VEGF-A, the earliest biological effect is increased vascular permeability, which is noted within seconds to minutes.[20] An increase in vascular permeability to plasma and plasma protein is noted within minutes of VEGF-A injection into normal skin or other tissues.[20] Other, delayed reactions include changes in endothelial cell shape, migration, adhesion, and altered mRNA and protein expression that occur hours to weeks after exposure.[20]

All of VEGF-A's actions are mediated by interaction with two high-affinity receptors, VEGF-A receptor–1 (VEGFR-1) and VEGF-A receptor–2 (VEGFR-2). The fact that both of these receptors are expressed predominantly on vascular endothelium accounts for the high selectivity of VEGF-A on endothelial cells.[20] Vascular endothelial growth factor–A induces microvascular permeability, leading to the extravasation of plasma proteins with consequent proangiogenic stromal changes.[26,30,31] Vascular endothelial growth factor–A is also an endothelial cell mitogen, and it alters the synthetic profile of endothelial cells in a manner that may promote angiogenesis.[31]

VEGF-A and Angiogenesis. Vascular endothelial growth factor–A and its endothelial receptors have been shown to play a role in a number of normal physiologic and non-neoplastic pathologic processes that involve angiogenesis. Overexpression of VEGF-A mRNA and its receptors is noted in delayed hypersensitivity reactions of both the tuberculin and the contact allergy type.[32] It has also been shown that VEGF-A expression increases dramatically during the wound healing phase.[33] Brown et al[33] have shown that VEGF-A overexpression reaches a peak at 2 days and persists at an elevated level for 1 week — the time required for granulation tissue to form. Overexpression of VEGF-A and its receptors has also been shown in retinopathies,[34] rheumatoid arthritis,[35] psoriasis, and inflammatory skin disorders.[36]

The effect of VEGF-A in the pathogenesis of human and animal tumors has also been of interest in the past 2 decades.[21,23] Many investigators have shown overexpression of VEGF-A, both the mRNA and its protein products, in kidney and bladder carcinomas,[6] adenocarcinomas of the gastrointestinal tract,[7] breast cancer,[2] Kaposi's sarcoma, and cutaneous angiosarcoma.[8] It is also known that the endothelial cells lin-

ing microvessels that supply VEGF-A–secreting tumors themselves show an increased expression of VEGFR-1 and VEGFR-2.

Because of the overexpression of VEGF-A, there is an increased microvascular hyperpermeability to plasma proteins and other circulating macromolecules in certain physiologic and neoplastic disorders. Unlike blood vessels supplying normal tissue, tumor blood vessels are undifferentiated, are distributed unevenly, and do not correspond closely to arterioles, capillaries, and venules.[20,37,38] Many investigators have also shown that vessels supplying different solid tumors are approximately 4 to 10 times more permeable to macromolecules than are the microvessels of normal tissue.[21,39,40] These findings strongly suggest that tumor cell expression and secretion of VEGF-A is responsible for the microvascular hyperpermeability characteristic of tumor microvessels.[20]

It has been well documented that the generation of a vascular stroma is essential for solid tumor growth.[3,41] The formation of a vascular stroma in a solid tumor involves interactions among tumor cells, endothelial cells, and angiogenic growth factors.[42,43] Weidner et al[42,43] have shown a correlation between microvascular density and the prognosis of breast cancer. Brown et al[30] studied the role of stimulatory and inhibitory growth factors in the vascular stroma formation in breast cancer. They noted strong expression of VEGF-A mRNA by tumor cells and strong expression of VEGF-A receptors in the endothelial cells of small vessels in breast cancer.[30] They concluded that the formation of the vascular stroma preceded invasion, thus raising the possibility that tumor cells do not invade the normal breast stroma but rather invade a richly vascular stroma that has been induced.[30] They also concluded that the formation of a vascular stroma might play a role not only in growth of the primary tumor, but also in invasion and metastasis.[30]

It is also important to state that many investigators have shown the effect of other growth factors in a number of solid tumors. Myers et al[44] have reported on the effects of androgen manipulation on epidermal growth factor (EGF) receptor and transforming growth factor–α (TGF-α) in prostatic adenocarcinoma. De Luca et al[45] have investigated the role of EGF in human breast carcinoma and concluded that EGF-related peptides are involved in the proliferation and survival of breast carcinoma cells. Bovee et al[5] have reported on the expression of fibroblast growth factor type 2 (FGF-2), EGF, and their respective receptors FGFR-1 and EGFR in adamantinoma of long bone.

This study is the first to evaluate the role of VEGF-A and its receptors in RRP. Histologically, respiratory tract papillomas are projections of extremely vascular connective tissue covered by stratified squamous epithelium.[46] The formation of a vascular stroma in RRP is a process that involves complex interactions among tumor cells, stromal cells, and endothelial cells. We have found a marked expression of VEGF-A mRNA and its receptors, which have a direct effect on vascular stroma formation. Our study provides insight into the process of vascular stroma formation in RRP and the possible role that it may play in its progression. A number of investigators have shown tumor growth suppression by inhibition of VEGF-A or its receptors in several experimental models.[4,47] It is our belief that better understanding of the role of VEGF-A and its receptors in RRP may provide valuable insight into the pathobiology of this disease and may lead to novel therapies.

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