Presence of Vascular Endothelial Growth Factor in Progressive Equine Ethmoid Hematoma

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ABSTRACT

Tissue samples from four progressive ethmoid hematomas were positive for expression of vascular endothelial growth factor (VEGF), also known as vascular permeability factor. VEGF is known to have an important role in tumor biology, including but not limited to angiogenesis, malignant ascites, and the proliferative retinopathies. Further study of its activity in ethmoid hematoma could provide new insight into the natural history of this uncommon lesion, and perhaps into medical approaches to prevent recurrence.

INTRODUCTION

Progressive ethmoid hematoma was first described in 1973 by Cook and Littlewort[1] in an analysis of 16 cases. It is a rare vascular lesion of the upper respiratory tract and is found only in horses.[2] A reported incidence of one in 2,500 horses is based on cases presenting to large referral or teaching hospitals.[2] In most reported cases of ethmoid hematoma, the first clinical sign is an intermittent serosanguineous nasal discharge that is usually unilateral and unassociated with exercise, having a history of months to years duration.[1–4] Other clinical signs include respiratory noise or distress, coughing, facial deformity, malodorous breath, and head-shaking or shyness.[1,2,4] The diagnosis is based on clinical signs, history, endoscopic examination, or if endoscopy is inconclusive, radiographic evidence of soft tissue densities in the ethmoid region.[2] Histologic examination to confirm the diagnosis is advisable, since ethmoid hematoma may be confused with other nasal masses or lesions that can cause epistaxis.[2]

Usually originating in the ethmoid region, progressive ethmoid hematoma may be located in the paranasal sinuses and/or the nasal cavity.[3,5] Endoscopically, the lesion is viewed as a protruding rounded red or greenish mass; radiographic examination is useful for determining the extent of the lesion.[2–5] Bilateral involvement is present in about 16% of cases and is believed to result from extension "along the lines of least resistance."[1,3–5] In the two horses with bilateral lesions described by Cook and Littlewort,[1] physical signs had been evident for 2 years in one case and 7 years in the other.

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The mucosa of the ethmoid labyrinth is highly vascular.[1] Based on the pleomorphic histologic appearance of the tissue, progressive ethmoid hematoma is presumed to form through repeated bleeding.[2,3] The lesion characteristically consists of material from old and fresh hemorrhage, hemosiderin deposits, or hemosiderin-filled macrophages, irregular multinucleated cells, and fibroblastic tissue.[2,4,6] Concretions containing ferric iron, bilirubin, cholesterol, and lipid may be found within giant cells or free.[6] The hemorrhagic mass is surrounded by an irregular zone of submucosal fibrous tissue, which underlies a capsule of respiratory epithelium that may be discontinuous or ulcerated.[2,4] Lesions are extensively vascularized, with many small vessels showing calcareous deposits in their walls.[6]

Complete extirpation of the hematoma is curative, but recurrence is common, occurring in an estimated 30% to 50% of cases from 3 months to 3.5 years after surgery.[1,5] Cryosurgery reportedly yields better outcomes, and laser surgery has been recommended as providing greater accuracy in excision and reducing intraoperative blood loss.[1,3,5]

The etiology of progressive ethmoid hematoma is unknown.[1,5] Epidemiologic data gathered by Bell and colleagues[3] indicate the incidence increases with age, with an average age of 9.9 years in 67 cases of diagnosed or suspected ethmoid hematoma. Although benign, the lesion is locally destructive and progressive with pressure erosion of bone, eventually resulting in severe facial deformity and partial asphyxia.[1–3]

Progressive neoplastic growth can exist only with adequate nutrition and cellular support systems. Such growths tend to rapidly exceed the existing blood supply. To maintain growth and invasiveness, the vascular supply also must grow and keep pace with cellular demands. When the relative hypoxia of a rapidly growing tissue is not met with an angiogenic response adequate to support tissue demands, growth is not maintained, and necrosis may result. Thus, it is suggestive that ethmoid hematomas are sites of intense cytokine activity. In particular, it is quite plausible to consider the presence of vascular endothelial growth factor (VEGF), known to be produced in response to hypoxia.[7–9] The correlation of angiogenesis and neoplasia has been previously reviewed in the literature.[10–12] In the study described here, the presence of VEGF in conjunction with a marker of cellular proliferation was examined to test the hypothesis that new blood vessel growth is occurring in clinically significant ethmoid hematomas.

MATERIALS AND METHODS

Tissue samples from four ethmoid hematomas obtained from equine patients treated at the George D. Widener Hospital for Large Animals at the University of Pennsylvania, Kennett Square, PA were immersed in 10% neutral buffered formalin for 4 to 6 hours and processed routinely in paraffin. Serial sections (5 µm) were taken from blocks representative of different sections of the ethmoid hematoma. Slides were either stained with hematoxylin and eosin or used for immunohistochemical studies.

Serial sections were used to assess immunoreactivity to proliferating cell nuclear antigen (PCNA) and to VEGF. A rabbit polyclonal antibody to VEGF (Santa Cruz Biotechnology) was tested for specificity by Western blot analysis, and then used in the immunochemical studies at a dilution of 1:300. After deparaffinization and rehydration, tissue samples were treated in 1 mM levamisole for 5 minutes to decrease endogenous alkaline phosphatase activity, followed by a trypsin antigen-retrieval step. Samples were blocked with diluted serum from the species in which the secondary antibody was raised. Phosphate-buffered saline was the diluent, with final concentrations of bovine serum albumin 1% and goat serum 20%. Samples were incubated with the primary antibodies at room temperature for 30 or 60 minutes. After washing and reblocking, the samples were incubated with the secondary antibody for 30 minutes at room temperature. Color was

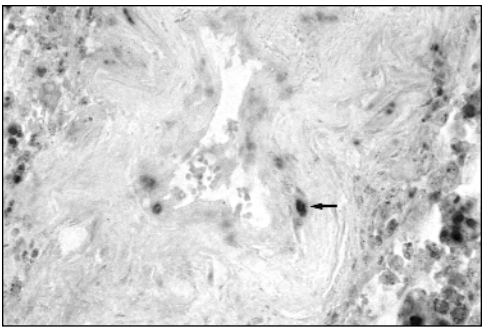

Figure 1. Proliferating cell nuclear antigen (PCNA). *Arrow* denotes positive immunoreactivity to PCNA (magnification oil × 400).

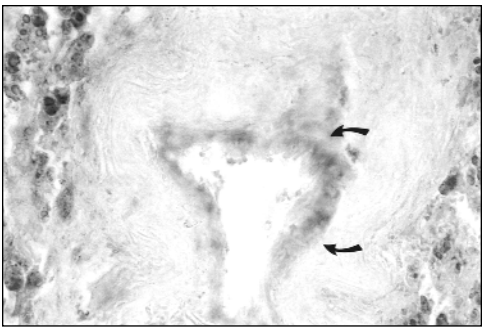

Figure 2. Vascular endothelial growth factor (VEGF). Area between *curved arrows* is intimal layer positive to VEGF (magnification oil × 400).

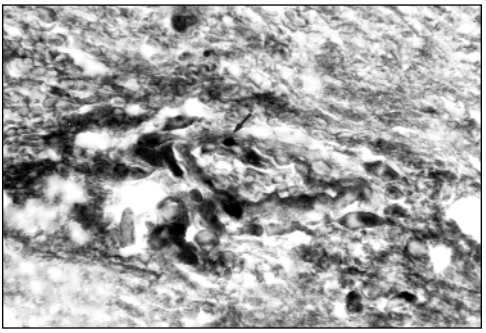

Figure 3. Double label for PCNA and VEGF. *Arrow* denotes double label for PCNA and VEGF (magnification × 200).

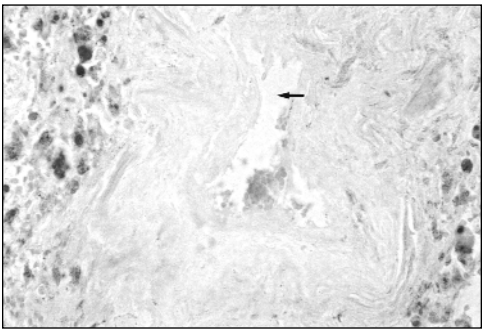

Figure 4. Intimal layer control for VEGF and PCNA. *Arrow* denotes vessel lumen.

developed using the AP-(alkaline phosphatase) Substrate Vector Kit (Vector Laboratories). A coverslip was placed on the sections. Controls were antibody isotype from a non-immune rabbit, omission of the primary antibody, omission of primary and secondary antibodies, and an adsorbed antibody control.

Similar procedures were followed for the mouse monoclonal antibody to PCNA (Dako) and the smooth muscle cell actin antibody (Sigma), with the exception that no antigen retrieval step was used, and the PCNA antibody was tested at a dilution of 1:50 and the smooth muscle cell actin antibody was tested at 1:50,000. Lymph node tissue was examined as a positive tissue control in addition to four controls used with the rabbit polyclonal antibody.

RESULTS

Samples from one ethmoid hematoma showed relatively mild positive staining for VEGF that was limited to blood vessels within the core and a relatively low level of PCNA staining. The other three specimens showed more extensive PCNA staining as well as more extensive and intensive staining for VEGF (Figures 1–4). In these samples, positive immunoreactivity for VEGF was observed on the capsule, adjacent blood vessels, blood vessels within the mass, and on some of the connective tissue (Figure 5). Labeling for PCNA was correspondingly distributed.

DISCUSSION

This report demonstrates immunohistochemical evidence of VEGF co-localized with PCNA in cases of progressive equine ethmoid hematoma. Findings are suggestive of new blood vessel growth in the presence of a hypoxia-responsive cytokine. Results also raise questions as to the relative importance of this growth factor in the developing vasculature of the neoplasia and thus in the

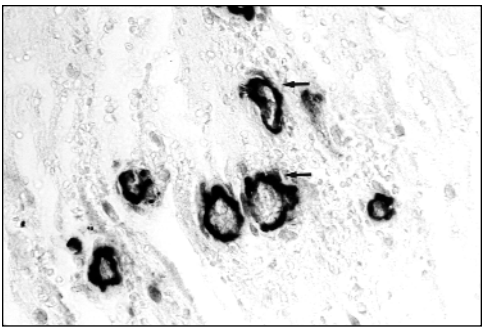

Figure 5. Smooth muscle cells actin. *Arrows* denote blood vessel (magnification × 200).

potential role for VEGF in supporting and promoting the growth of progressive ethmoid hematoma.

PCNA, one of the other proteins for which we assayed, is a cofactor for DNA polymerase, expressed primarily during the S-phase of the cell cycle and is generally considered to be indicative of cellular replication. PCNA appears to be a highly conserved mammalian protein.[13,14] The presence of immunochemical reactivity to PCNA in the tissue samples indicates a degree of cellular activity and division. It is not known whether ethmoid hematomas grow at a steady, continuous rate, or whether activity is cyclical in nature. Serial samples taken over a period of time would be necessary to address this question. However, results of this preliminary study are supportive of the hypothesis of active angiogenesis occurring in progressive, locally invasive ethmoid hematomas. This finding is consistent with previously described correlations between neoplasia and new blood vessel growth.

VEGF, also called vascular permeability factor, is an angiogenic cytokine, highly specific for endothelial cells. VEGF has been shown to play a crucial role in the vasculogenesis required for normal growth and development as well for wound healing.[10] Expressed in response to hypoxia or activated oncogenes, VEGF has also been implicated in the vasculogenesis of pathologic processes such as tumor growth and proliferative retinopathies.[10,11,15,16] Additionally, VEGF appears to have a significant role in delayed hypersensitivity reactions, psoriasis, hemangiomas, rheumatoid arthritis, and numerous other disease states.[17–20]

VEGF is a glycosylated heparin-binding protein that may exist in several different isoforms due to alternative splicing from a single gene.[10,21] The isoforms differ in mass as well as in properties, such as binding to cell-surface receptors. Two isoforms, $VEGF_{121}$ and $VEGF_{165}$, are secreted and soluble. Two other species, $VEGF_{189}$ and $VEGF_{206}$, are bound to the cell surface or the extracellular matrix. Other isoforms have been identified, some of which may predominate in certain species, tissues, or conditions.[22]

In addition to angiogenic properties, VEGF is also a potent inducer of microvascular hyperpermeability.[17] Extravasation of plasma proteins, induced by VEGF and/or other factors, has been hypothesized to be an important step in the development of new blood vessels.[17] The endothelial cell proteins induced by VEGF and related cytokines and the extravasated plasma proteins are thought to interact in activating the extrinsic coagulation pathway, resulting in the generation of active thrombin from prothrombin and conversion of soluble extravasated fibrinogen to insoluble fibrin, thereby affecting the composition of the extracellular matrix.[10,17]

VEGF has been studied in humans, rhesus monkeys, rats, mice, sheep, pigs and quail.[7,10,23–30] The molecule appears to be well conserved across species.[10] Nonetheless, a question may be raised regarding the use of antihuman antibodies for analysis of equine tissue. Although there was a good signal with the immune serum relative to the isotype control, it is possible that there was not optimum epitope recognition and that the presence of VEGF has in fact been underestimated. The more intensive staining seen in three of four samples could possibly support the role of VEGF in the development and progression of ethmoid hematomas.

The causes of progressive ethmoid hematoma have remained obscure over the 30 years since it was first reported. Further

investigation of the role of VEGF in its splice variant forms and the associated receptors could yield some insights into its origins and perhaps into the functioning of this important and pervasive cytokine as well. Finally, if VEGF and other angiogenic factors do play a role in the development of ethmoid hematoma, then angiogenesis inhibitors might have a useful role in treatment, perhaps serving to reduce the potential for recurrence following surgery.

REFERENCES

1. Cook WR, Littlewort MCG: Progressive hematoma of the ethmoid region in the horse. *Equine Vet J* 1974; 6:101–108.
2. Bell BTL, Baker GJ, Foreman JH: Progressive ethmoid hematoma: Background, clinical signs, and diagnosis. *Compend Contin Educ Pract Vet* 1993; 15:1101–1111.
3. Etherington WG, Vasey JR, Horney FD: Ethmoid hematoma of the equine. *Can Vet J* 1982; 23:231–234.
4. Behrens E: Ethmoid hematoma in a stallion. *Equine Pract* 1988; 10:24–27.
5. Bell BTL, Baker GJ, Foreman JH: Progressive ethmoid hematoma: Characteristics, cause, and treatment. *Compend Contin Educ Pract Vet* 1993; 15:1391–1398.
6. Platt H: Haemorrhagic nasal polyps of the horse. *J Pathol* 1975; 115:51–55.
7. Mukhopadhyay D, Tsiokas L, Zhou X, Foster D, Brugge JS, Sukhatme VP: Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. *Nature* 1995; 375(6532):577–581.
8. Levy AP, Levy NS, Goldberg MA: Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. *J Biol Chem* 1996 2; 271(5):2746–2753.
9. Forsythe JA, Jiang BH, Iyer NV, et al: Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol* 1996; 16(9):4604–4613.
10. Senger DR, Van De Water L, Brown LF, et al: Vascular permeability factor (VPF), (VEGF) in tumor biology. *Cancer Metastasis Rev* 1993; 12:303–324.
11. Folkman J: What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990; 82:4–6.
12. Marme D: Tumor angiogenesis: The pivotal role of vascular endothelial growth factor. *World J Urol* 1996; 14(3):166–174.
13. Bravo R, Frank R, Blundell PA, Macdonald-Bravo H: Cyclin/PCNA is the auxillary protein of DNA polymerase-delta. *Nature* 1987; 326(6112):515–517.
14. Hall, PA, Levision DA, Woods AL, et al: Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: An index of cell proliferation with evidence of deregulated expression in some neoplasms. *J Pathol* 1990; 162(4):285–294.
15. Aiello LP, Avery RL, Arrigg PG, et al: Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *New Engl J Med* 1994; 331:1480–1487.
16. Feldkamp MM, Lau N, Rak J, Kerbel RS, Guha A: Normoxic and hypoxic regulation of vascular endothelial growth factor (VEGF) by astrocytoma cells is mediated by Ras. *Intl J Cancer* 1999; 81(1):118–124.
17. Senger DR: Molecular framework for angiogenesis. *Am J Pathol* 1996; 149:1–7.
18. Arbiser JL: Angiogenesis and the skin: A primer. *J Am Acad Dermatol* 1996; 34:486–497.
19. Koch AE, Harlow LA, Haines GK, et al: Vascular endothelial growth factor. A cytokine modulating endothelial cell function in rheumatoid arthritis. *J Immunol* 1994;152(8):4149–4156.
20. Nagashima M, Yoshino S, Aono H, Takai M, Sasano M: Inhibitory effects of anti-rheumatic drugs on vascular endothelial growth factor in culture rheumatoid synovial cells. *Clin Exp Immunol* 1999; 116(2):360–365.
21. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW: The vascular endothelial growth factor family; identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 1991; 5(12):1806–1814.
22. Jingjing L, Xue Y, Agarwal N, Roque RS: Human Muller cells express VEGF 183, a novel splice variant of vascular endothelial growth factor. *Invest Ophthalmol Vis Sci* 1999; 40(3): 752–759.
23. Wiltin J, Eichmann A, Christ B: Expression of the avian VEGF receptor homologues Quek1 and Quek2 in blood-vascular and lymphatic endothelial and non-endothelial cells during quail embryonic development. *Cell Tissue Res* 1997; 288(2)207–223.
24. Ravindranath N, Little-Ihrig L, Phillips HS, Ferrara N, Zeleznik AJ: Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary. *Endocrinology* 1992; 131(1):254–260.
25. Sharma HS, Tang ZH, Gho BC, Verdouw PD: Nucleotide sequence and expression of the porcine vascular endothelial growth factor. *Biochem Biophys Acta* 1995; 1260(2):235–238.

26. Bacic M, Edwards NA, Merrill MJ: Differential expression of vascular endothelial growth factor (vascular permeability factor) forms in rat tissues. *Growth Factors* 1995; 12(1):11–15.

27. Cheung CY, and Brace RA: Ovine vascular endothelial growth factor: Nucleotide sequence and expression in fetal tissues. *Growth Factors* 1998; 16(1):11–22.

28. Shima DT, Kuroki M, Deutsch U, Ng YS, Adamis AP, D'Amore PA: The mouse gene for vascular endothelial growth factor. Genomic structure, definition of the transcriptional unit, and characterization of transcriptional and post-transcriptional regulatory sequences. *J Biol Chem* 1996; 271(7):3877–3883.

29. Schmidt M, Flamme I: The in vivo activity of vascular endothelial growth factor isoforms in the avian embryo. *Growth Factors* 1998; 15(3):183–197.

30. Flamme I, Breier G, Risau W: Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (flk-1) are expressed during vasculogenesis and vascular differentiation in the quail embryo. *Dev Biol* 1995; 169(2):699–712.