Molecular Angiogenic Signaling in Angiofibromas After Embolization

Implications for Therapy

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Objectives: To examine (1) the molecular angiogenic relationship between endothelial and stromal cells of angiofibromas and how this may elucidate the pathogenesis of angiofibromas and (2) the effects of embolization on the expression of angiotrophic factors and proapoptotic and antiapoptotic factors within the tumor.

Design: The expression of mesenchymal and endothelial stem/progenitor cell–associated proteins (MECAPs) such as proangiogenic cytokine vascular endothelial growth factor (VEGF), VEGF receptors (VEGFR1, VEGFR2, and VEGFR3), angiopoietin receptors (Tie-1 and Tie-2), and stem cell subset marker CD133 was assessed by immunohistological staining in 7 embolized angiofibroma specimens. Expression of proapoptotic Bax, antiapoptotic Bcl-2 and Bcl-xL, nuclear proliferation protein MiB-1, and hypoxia-inducible factor 1α (Hif-1α) in peri-ischemic areas of the embolized angiofibromas was also assessed.

Setting: A single pediatric institution.

Patients: Seven patients (identified from medical records, January 1, 2001, through December 31, 2005) who were diagnosed as having juvenile angiofibroma and who underwent surgical treatment. Archival tissues were retrieved for immunostaining.

Main Outcome Measures: The immunostaining results were evaluated by microscopy and the staining intensities were also recorded.

Results: All angiofibroma specimens expressed the stem cell subset marker CD133 and MECAPs except VEGFR3 (a few cases). In the only case tested, we found evidence of VEGF-induced angiogenic signaling as the expression of phosphorylated VEGFR2 (Tyr951). Endothelial cells expressed VEGFR1 and VEGFR2 and angiopoietin receptors Tie-1 and Tie-2 but not VEGF. In contrast, VEGF was expressed within stromal cells. Viable tumor adjacent to the ischemic areas demonstrated increased staining intensities to VEGFR2, Tie-1, Tie-2 (all cases), and VEGFR3 (2 cases) and increased nuclear proliferation (5%-20%). All cases expressed proapoptotic and antiapoptotic factors, and the expression of Hif-1α was unaffected by ischemia.

Conclusions: Stromal cells appear to be similar to mesenchymal stem cells with endothelial differentiation potential in umbilical cord blood cells. Stromal cells support endothelial growth by providing VEGF as a paracrine factor. Under ischemic stress, the embolized tumor tissues show upregulation of angiogenic receptors, retention of Hif-1α, and increased nuclear proliferation rates. Specific angiogenesis blockers may represent a novel treatment strategy for angiofibromas.
angiobromas was from mesenchymal progenitor or stem cells. We used immunohistochemical stains to demonstrate that angiobroma stromal cells were similar to stem/progenitor mesenchymal cells with endothelial cell potential and interacted with their endothelial cells through similar angiogenic patterns. We also examined whether embolization of angiobromas would alter angiogenic factor expression in the tumor. We showed that elucidating this relationship might provide a rationale for novel medical management strategies.

**METHODS**

We studied the medical records of 7 patients diagnosed as having juvenile angiobroma who underwent surgical treatment for juvenile angiobroma from January 1, 2001, through December 31, 2005. All were adolescent boys ranging in age from 10 to 15 (average, 13) years old. Location of the tumors included the nasal sinuses (in most of the patients), retromaxillary area, nasopharynx, foramen rotundum, and nasal antrum. All had undergone embolization before surgical excision. Representative tissue sections of angiobroma that also contained a portion of embolized blood vessels were used for this study. We deparaffinized 5-µm-thick sections of formalin-fixed paraffin-embedded tissue from each of these lesions. The sections were stained with hematoxylin-eosin and then underwent immunohistochemical staining with the use of commercially available biotinylated monoclonal antibodies against 6 different groups of antigens. Their functional properties, dilutions, and sources were as follows:

1. Angiogenesis and MECAPs: CD133, a stem cell marker (1/100 dilution; Abcam, Cambridge, Massachusetts) and VEGF (1/300), VEGFR1 (1/50), VEGFR2 (2/20), VEGFR3 (1/20), Tie-1 (1/100), and Tie-2 (1/200) (all from Santa Cruz Biotechnology, Santa Cruz, California).
2. VEGF-induced VEGFR2 signaling–associated phosphorylation and activation: phosphorylated VEGFR2 (Tyr951) (1/20) (Cell Signalling, Beverly, Massachusetts).
5. Cell proliferation: MiB-1 (1/20) (Dakocytomation).

Immunostaining was performed using an automated immunostainer (Ventana Medical Systems Inc, Tucson, Arizona). Antigen retrieval procedures (30-minute heating in a decloacing chamber with citric acid [pH 6]) were applied when necessary. Antibody staining was detected using the avidin-biotin complex procedure, and the staining reagents and procedures were applied according to the recommendations by the manufacturer (Ventana Medical Systems Inc). For the immunostaining of Tyr951, cryo–snap frozen tissue was required. Results in only 1 of the 7 cases were available. Acetone-fixed cryostat sections were prepared and immunostaining was performed with some modifications to the procedures by Maharaj et al.4 Antibody incubation was performed at room temperature for 30 minutes.

The immunostaining results were assessed by means of microscopy by one of us (B.-Y.N.) and were scored as weak, moderate, strong, or negative according to the brown staining intensities of the vascular endothelium or the stromal cells. For the angiobroma tissues that were embolized, the staining intensities of the viable cells near the embolized vessels (which represented cells under ischemic stress) were also scored. The nuclear proliferation index was determined by MiB-1 nuclear staining, and the results were expressed as a percentage of stained nuclei per 100 unstained nuclei of the endothelium or the stroma of the angiobroma.

**RESULTS**

The hematoxylin-eosin–stained sections of the excised tumors of all 7 patients showed unequivocal features of angiobroma (data not shown). There were limited areas of focal coagulative necrosis as a result of embolization. Embolic materials could be seen in some of the arterioles. These ischemic areas were often sharply demarcated from the viable tumor parenchyma (data not shown).

Immunostains with stem cell marker CD133 showed strong cytoplasmic staining of the stromal spindle cells (Figure 1A). Cytoplasmic staining of the endothelial cells was present but weaker (Figure 1A), and CD34 stained all of the endothelial cells that lined the vascular and capillary spaces in the tumor (Figure 1B, labeled by a letter C). It only stained a small subset of stromal spindle cells (Figure 1B).

Immunostains with a panel of MECAPs showed that the endothelial cells of angiobromas did not express VEGF (Figure 1C). Rather, VEGF was expressed in the cytoplasm of the stromal spindle cells (Figure 1C). The stromal cells showed stronger expression of VEGFR1 than VEGFR2 (Figure 1D and Figure 2A). In focal areas deep in the tumor that contained higher vascular densities, some of the stromal spindle cells and tumor vascular endothelial cells expressed Tyr951, the marker of VEGF-induced VEGFR2 signaling that is associated with phosphorylation and activation of VEGFR2 (Figure 2A [inset]). In the viable tumor tissue adjacent to the postembolization ischemic tissue, there was an induction of VEGFR2 expression in the stromal spindle cells (Figure 2A and B). The angiobroma stromal cells also demonstrated an induction of expression of angiopoietin receptors Tie-2 and Tie-1 after embolization (Figure 2C and D and Figure 3A and B, respectively). These staining results are summarized in the Table.

Staining of angiobroma tissues for the nuclear proliferative protein MiB-1 showed that less than 2% of cells yielded positive results (Figure 3C). The viable tumor tissue that was located at the margin of ischemic areas (Figure 3D) showed increased numbers of MiB-1–positive cells (both stromal and endothelial cells).

The endothelial and stromal cells both expressed proapoptotic and antiapoptotic regulatory proteins Bax, Bcl-2, and Bcl-xL, respectively (data not shown).

Immunostains for the nuclear expression of the hypoxic stress response protein Hif-1α showed that the stromal cells and vascular endothelial cells in the vicinity of the embolic material continued to yield positive results (Figure 4).
Juvenile angiofibromas are benign but locally aggressive tumors that occur more frequently in male patients from 7 years of age to young adulthood. The tumor may involve the posterior nasal cavities and nasopharynx and not infrequently extend to adjacent structures. Radio graphically, it is a mass lesion in the nose or nasopharynx that frequently causes anterior bowing of the posterior wall of the maxillary sinus and erodes the base of the medial pterygoid plate.

Juvenile angiofibromas are unusual tumors that contain endothelial and stromal spindle cells, both proliferating tissues. An angiogenic relationship between these 2 major components has been suggested previously by others. In this immunostaining study, we demonstrated that the stromal spindle cells express proangiogenic cytokine VEGF protein, some related members of the VEGFR family (Flt-1/VEGFR1, Flk-1/VEGFR2, and VEGFR3 to a lesser extent), and members of the angiopoietin receptor family (Tie-1 and Tie-2). Stromal spindle cells were also found to express stem cell marker CD133. A small subset of single spindle cells had also acquired the potential to become endothelial cells, as evidenced by the expression of the endothelial marker CD34. These observations were strikingly similar to the progenitor cells of human mesenchymal stem cells previously identified in human umbilical cord blood. This stem cell–like characteristic distinguished most of the stromal cells of the studied angiofibromas from terminally differentiated fibroblasts. Thus, our results raised the question that part of the term fibroma that was used in angiofibroma may be a misnomer. In light of these findings, we propose that angiofibromas most likely arise from developmentally misplaced remnants of primitive mesenchymal and endothelial stem/progenitor cells (cell rests). The growth and resurgence of these misplaced cell rests eventually lead to the formation of angiofibromas.

Vascular endothelial growth factor and its receptors (Flt-1/VEGFR-1 and Flk-1/VEGFR-2), one of the recep-

Figure 1. Immunohistological staining for some of the mesenchymal and endothelial stem/progenitor cell–associated proteins in angiofibromas. A, Stem cell subset marker CD133 shows cytoplasmic staining of stromal cells (S) and endothelial cells (E). B, Progenitor and endothelial cell marker CD34 was detected in a small subset of stromal cells (arrow). The marker is expressed in differentiated endothelial cells that line the capillary lumen (C). C, Vascular endothelial growth factor (VEGF) expression is seen in the cytoplasm of stromal spindle cells (S) only and not in the endothelium (E) that lines the tumor vascular space. D, Expression of VEGF receptor 1 is found in the stromal spindle cells (S) and endothelium (E) of tumor vascular spaces (A, CD133; B, CD34; C, VEGF, and D, VEGF receptor 1).
tors for angiopoietin (Tie-2), and an orphan receptor (Tie-1) represent key signal transduction systems involved in the regulation of embryonic vascular development. The interactions between VEGF and angiopoietins with their corresponding receptors play a critical role in initiating angiogenesis. In our study, we showed that VEGF induced angiogenic signaling after phosphorylation, and activation of VEGFR2 by VEGF binding occurred in some stromal and endothelial cells as evidenced by the positive staining for phosphorylated VEGFR2. Vascular endothelial growth factor is known to induce angiogenesis through binding to the VEGFR2 tyrosine kinase.

Although other investigators had previously reported the presence of VEGF or of VEGF, VEGFR1, VEGFR2, Tie-1, and Tie-2 in angiofibromas, our results further demonstrate that VEGF is expressed in stromal spindle cells only and not in endothelial cells. The endothelial cells, on the other hand, expressed VEGFRs. This finding implied that there may be a paracrine growth relationship between the vascular endothelium and the spindle cells in the stroma of angiofibromas.

Arterial embolization of an angiofibroma is often performed before surgery to reduce operative bleeding. In this study, we assessed the impact of embolization on angiogenesis in the angiofibroma tissues. We specifically endeavored to determine whether embolization would induce cell death by increasing the expression of proapoptotic factors. We were also interested in knowing whether embolization would suppress angiogenesis by reducing the expression of angiogenic factors. In the present study, the hematoxylin-eosin–stained tissue sections showed that embolic materials were present intravascularly and were often associated with fibrin thrombi. Only limited localized ischemia of the stroma could be found. In the ischemic areas and the tissues that were immediately adjacent to the ischemic areas, we found that the expression of proapoptotic factor Bax was balanced by the expression of antiapoptotic factors Bcl-2 and Bcl-xL. When we examined the expression of MECAPs in the
viable stromal tissues under ischemic stress (Table), the expression of the angiogenic factors and receptors persisted. In addition, the expression of VEGFR2, Tie-1, and Tie-2 in the stromal cells was induced, and 2 cases showed induction or elevation of VEGF expression. These findings demonstrate unequivocally that embolization does not suppress angiogenesis and that the induced cell death is counteracted by antiapoptotic factors.

Previous reports on vascular angiogenesis showed that the activation of VEGFRs (which are tyrosine kinase receptors) on endothelial cells plays a critical role in signal transduction that regulates vasculogenesis, angiogenesis, and vascular permeability in vertebrates.\(^3\) Vascular endothelial growth factor bound and activated VEGFR1 and VEGFR2; VEGFR1-transduced signals mediated endothelial cell migration, whereas activated VEGFR2 mediated endothelial cell proliferation.\(^4\) In studies of normal tissues, the receptor tyrosine kinase Tie-1 is expressed in endothelial cells and is physically associated with the related receptor Tie-2. Positive signaling through Tie-2 is associated with microvessel stability, and the suppression of this signal is required for VEGF-induced microvessel remodeling or growth.\(^17\) Experimental animal embryos that were deficient in Tie-1 failed to establish structural integrity of vascular endothelial cells, and Tie-1 was required for vascular development and maintenance.\(^18\) Studies of embryos deficient in Tie-2 showed that the angiopoietin receptor was also important in vascular network formation in endothelial cells.\(^17,19\) In our study, the tumor stromal cells that were under ischemic stress showed an induction of Tie-1 and Tie-2 expression. This finding suggests that Tie-1 and Tie-2 in the stromal cells may also be important in mediating the growth and maintenance of the vascular component of angiofibromas.

Hypoxia-inducible factor 1 is known to stimulate angiogenesis by activating transcription of the gene encoding VEGF, and its \(\alpha\) subunit is reported to be an oxygen- and growth factor–regulated subunit.\(^20,21\) In a previous study by Schuon et al,\(^7\) Hif-1\(\alpha\) expression was noted in areas of angiofibroma with high vessel density. We observed that, in the ischemic areas of embolized angiofibromas, the viable cells maintained high levels of Hif-1\(\alpha\) expression. This finding and that of increased VEGFR2

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**Figure 3.** Immunohistological expression of Tie-2 and nuclear proliferative activity in nonembolized (A and C, respectively) and embolized (B and D, respectively) angiofibroma tissues. A, Tumor vascular components (V) are lined by positively stained endothelium. B, Only sporadic spindle cells in the stroma express Tie-2. These cells, however, show upregulation of Tie-2 expression in embolized tissues. C, Tumor tissue normally has low numbers of MiB-1–positive cells. D, In contrast, viable tumor cells (labeled as nonischemic) show increased numbers of MiB-1–positive cells (A and C, Tie-2; B and D, MiB-1).
examples of anti-VEGF monoclonal antibodies. The other sodium (Macugen), and ranibizumab (Lucentis) are ex-
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maintenance of tumor cells.

The results of this study give some insights into the 
causes of the ineffectiveness of embolization as the sole 
therapeutic intervention for angiofibroma. When it is used 
in conjunction with surgery, embolization is extremely 
useful. The observed retention and/or induction of re-
cceptor tyrosine kinases in ischemic angiofibroma tissue 
have stimulated the rationale for investigating the use of 
angiogenesis inhibitors in the treatment of angiofibro-
mas. This may be especially applicable for unresectable 
and recurrent tumors. Two groups of antiangiogenic 
agents currently target VEGF specifically. One group of 
agents consists of monoclonal antibodies that specifi-
cally bind to VEGF and prevent downstream interaction 
with VEGFRs. Bevacizumab (Avastin), pegaptanib 
sodium (Macugen), and ranibizumab (Lucentis) are ex-
amples of anti-VEGF monoclonal antibodies. The other 
group consists of small-molecule VEGF-signaling inhibi-
tors that bind to all 3 VEGFRs at the intracellular 
domain and prevent them from binding to VEGF. 
Examples of VEGFR inhibitors include vatalanib, AE-941 
(Neovastat), sorafenib, and sunitinib malate. Recently, 
nelfinavir (Viracept), an anti–human immunodeficiency 
 virus protease inhibitor, was reported to down-
regulate Hif-1α and VEGF expression in lung carci-
noma cell lines in vitro. This downregulation resulted in 
growth delays that were detected in tumor xenograft re-
growth assays in nude mice. Although these novel agents 
are promising, appropriate testing in angiofibroma xeno-
graft animal models and vigorous clinical trials are re-
quired to establish their effectiveness and potential tox-
icity before being used in the treatment of angiofibromas.

Submitted for Publication: January 1, 2007; final revi-
sion received January 2, 2008; accepted February 4, 2008. 
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Author Contributions: Drs Ngan and Forte had full ac-
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Ngan and Campisi. Critical revision of the manuscript for 
important intellectual content: Ngan, Forte, and Campisi. 
Administrative, technical, and material support: Ngan and 
Campisi. Study supervision: Ngan and Campisi.

Financial Disclosure: None reported.

Previous Presentation: This article was presented at the 
American Society of Pediatric Otolaryngology Annual 
Meeting; April 28, 2007; San Diego, California.

Additional Contributions: Michael R. Ho, MLT, and Erika 
Takarich, MLT, BSc, assisted in the immunohistochem-
istry.

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