Vessel Density, Proliferation, and Immunolocalization of Vascular Endothelial Growth Factor in Juvenile Nasopharyngeal Angiofibromas

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Background: Juvenile nasopharyngeal angiofibroma (JNA) is a rare, highly vascularized neoplasm of the nasopharynx that affects boys and young men. The underlying dysregulated molecular mechanisms remain unclear. The participation of angiogenic growth factors has been suggested, but few studies have been published.

Objectives: To evaluate the expression and localization of vascular endothelial growth factor (VEGF), proliferating cells, and vessel density in JNA.

Study Design: Immunohistochemical examination of 10 consecutive JNAs (8 primary tumors and 2 recurrent tumors).

Methods: Paraffin-embedded and cryopreserved JNA samples were included. VEGF-, CD31-, and Ki67-specific antibodies were applied and visualized using light microscopy. Vascularization was determined by counting CD31-positive vessels. Proliferating and VEGF-expressing vessels as well as stromal cells were quantified by the same method. Patients' age at the time of surgery and tumor stage were correlated with the immunohistochemical data.

Results: All tumors were heavily vascularized, but major differences were noted between the samples. About half of the vessels were proliferating (Ki67 positive) and half of the Ki67-positive cells were also VEGF positive. The tumor stroma was VEGF positive in 8 of 10 samples and proliferating in 5 of these 8. The 5 samples with both VEGF- and Ki67-positive stroma showed high vessel densities. No correlation was observed between age or tumor stage and vessel density, VEGF expression, or Ki67 expression.

Conclusions: In JNA, VEGF is frequently expressed by stromal cells and vessels and is associated with proliferation and increased vessel density. We suggest the promotion of vascularization by VEGF, but the involvement of androgens in JNA angiogenesis still needs to be analyzed.

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In the present study, we analyzed the vessel density and the expression and localization of VEGF and the proliferation marker Ki67 in JNA. We found high vessel densities along with strong proliferating and VEGF-expressing vessel endothelium cells associated with VEGF-expressing and proliferating stromal cells.

**METHODS**

**PATIENTS AND TISSUE SAMPLES**

The study included 10 samples of 9 consecutive patients who underwent surgery between August 1999 and April 2003 in Mainz, Germany; Poznan, Poland; and Holon, Israel. The age of the patients at the time of surgery ranged from 15 to 33 years (mean age, 21 years). One patient underwent surgery again after 12 months because of a relapse; this patient’s primary and recurrent JNAs were labeled case 3 and case 10, respectively. Case 4 was also a recurrence, but no sample of the primary tumor was available for analysis. Tumor stages were classified according to Fisch16 (Table 1).

**IMMUNOHISTOCHEMICAL STAINING AND MICROSCOPIC ANALYSIS**

Tissues were either snap frozen in liquid nitrogen immediately after resection and stored until further use at −80°C or paraffin embedded after fixation. For immunohistochemical staining, cryopreserved samples were sectioned, equilibrated to room temperature, fixed in acetone at −20°C for 10 minutes, air dried, and washed in 0.05% polysorbate (Tween) solution with Tris-buffered saline (TBS-Tween) for 5 minutes. Paraffin-embedded samples, after dewaxing and rehydration, were treated by microwave for antigen retrieval (three 5-minute cycles at 600 W in 10mM citrate buffer, pH 6.0). Endogenous peroxidase was inhibited by immersing slides in 3% hydrogen peroxide methanol solution for 20 minutes. Slides were washed for 5 minutes in distilled water and for 5 minutes in TBS-Tween.

After preincubation with 10% normal serum in 1% bovine albumin phosphate-buffered saline solution for 1 hour to avoid unspecific binding, the primary antibodies (Table 2) were stored overnight at 4°C. Dilutions of antibodies were prepared in 1% bovine albumin phosphate-buffered saline solution at room temperature. Slides were washed twice with TBS-Tween and consecutively incubated with biotinylated secondary antibody for 30 minutes; again washed twice with TBS-Tween and incubated with streptavidin horseradish peroxidase conjugate (DAKO, Hamburg, Germany) for 30 minutes; washed twice again with TBS-Tween and finally incubated with 1.85mM diaminobenzidine/hydrogen peroxide (Sigma, St Louis, Mo) for 1 minute.

Immediately after the staining developed, slides were washed with distilled water for 5 minutes and counterstained with hematoxylin (1:5 in phosphate-buffered saline; Merck, Darmstadt, Germany). Slides were rinsed for 3 minutes with distilled water and dehydrated for 3 to 3 minutes each with 80% → 100% isopropanol. Finally, samples were immersed twice in xylol (5 minutes each time), closed with a coverslip, and embedded with Enthelan (Merck).

Vascular endothelial growth factor stainings were analyzed and documented using an inverted microscope (Zeiss, Jena, Germany), and images were saved as jpg files. For microvessel density, the vessels marked by CD31 were counted in 3 “hot spot” fields of view (areas of high vessel density) at ×100 magnification, and the expression and localization of VEGF and the proliferation marker Ki67 in JNA. We found high vessel densities along with strong proliferating and VEGF-expressing vessel endothelium cells associated with VEGF-expressing and proliferating stromal cells.
nification (1 mm²), and the mean counts were calculated. Ki67-positive vessels and VEGF-positive vessels were counted by the same method. Expression of VEGF and Ki67 by tumor stroma cells was graded as positive or negative at ×400 magnification. Sections of normal kidney and squamous cell carcinoma served as positive controls. Sections incubated without the primary antibody served as negative controls (data not shown). Countings were performed by the same person to achieve minimum variability.

RESULTS

We analyzed 10 samples, corresponding to 9 patients, including 2 samples from recurrent tumors. Vessel densities were quantified by CD31 staining. Localization and quantification of proliferating or VEGF-expressing vessels and stromal cells were determined by Ki67 and VEGF staining (Figure). Patient age at the time of surgery and
In this series of JNA specimens, we observed high vessel densities associated with stromal VEGF and Ki67 expression. The participation of androgens and angiogenic growth factors has been suggested in the growth of JNA because of the sex-dependent occurrence and the high vessel densities observed. However, the results of antiandrogen therapies and of immunohistochemical analyses of androgen receptors remain conflicting. One study reported the regression of JNA after antiandrogen therapy in 4 of 5 patients. Another comprehensive study demonstrated the expression of androgen receptors in most of the samples in the vessel endothelium as well as in the tumor stroma cells, which might indicate a role for these receptors in the pathophysiology of JNA. In contrast, Gatalica compared androgen receptors in JNA samples with normal turbinat e tissue and found similar immunoreactivity in both groups in stromal and endothelial cells.

A mechanism of androgen action might be the induction of angiogenic growth factors that leads to the observed high vessel densities in JNA. Lissbrant and colleagues reported strong endothelial proliferation in rat reproductive organs during testosterone treatment and decreased proliferative activity after testosterone treatment was stopped. In a recent ex vivo study conducted on lung vascular endothelial cells, the induction of endothelial cell proliferation by testosterone could be demonstrated in endothelial cells isolated only from male rats. Female rats were completely unresponsive to testosterone, which indicates a sex-dependent responsiveness of endothelial cells to androgen stimulation. In another study, Haggstrom and colleagues reported that testosterone stimulates rat prostate endothelial cell proliferation and vascular growth and that this effect is probably mediated through the induction of VEGF synthesis by testosterone.

The secretion of proangiogenic growth factors by tumors is well established and in fact is a prerequisite for progression and metastasis in many tumors. However, several tumor types—frequently benign ones—secrete proangiogenic factors, which leads to vessel growth but has little or no impact on tumor growth. We observed strong expression of VEGF predominantly in the vessel endothelium of JNA and lower levels in the tumor stroma. This observation is in accordance with the findings of Schiff, who described the expression of basic fibroblast growth factor, the second most prominent factor, in the endothelium. Dillard and colleagues reported the expression of transforming growth factor β1, another growth factor with proangiogenic and proliferation-promoting activity in both JNA tumor stroma and in vessel endothelium. We found a high degree of congruence of VEGF-expressing and proliferating endothelial cells. Interestingly, the tumors with high vessel densities (and high proportions of proliferating vessels) showed VEGF-expressing and proliferating stromal cells. This finding might be indicative of a proliferation-promoting activity of the tumor stroma cells. One might speculate that stromal VEGF leads to the stimulation of endothelial cell growth and VEGF expression by an autocrine loop as has been reported in human placenta vessels.

We found no correlation between tumor stage and vessel density. This is in line with observations in other benign tumors where a correlation of proangiogenic factors with angiogenesis, but not with tumor size, has been shown (unpublished data, 2003). Therefore, in JNA the expression of VEGF might also lead to high vessel densities but not necessarily to large or aggressive tumors.

Another frequent observation was that of vessel-free spaces surrounding large, mostly round vessels. The average distance from large vessel to large vessel was 100 to 200 μm. That is equal to the distance that 2 vessels are able to support their interadjacent tissues with nu-
trients and oxygen. Conversely, this might suggest that the small, densely arranged, and irregularly shaped vessels might have restricted functionality, maybe because of dysregulated vessel growth as a consequence of overproduction of proangiogenic growth factors.

Collectively, the published animal data concerning the sex hormone dependence of endothelial cell growth, together with our findings and the data of others, support the view that JNA vascularization might be promoted by the action of angiogenic growth factors secreted by stromal cells, perhaps via the stimulation of androgens like testosterone. An autocrine loop of vessel endothelium expression of epidermal growth factor and therefore growth might be feasible. Other angiogenic factors like the reported basic fibroblast growth factor and transforming growth factor β1 may also be considered, given our observation that 2 of 10 tumors (cases 2 and 7, Table 1) showed high vessel densities despite low Ki67 and VEGF vessel counts and negative or rare Ki67 and VEGF stromal staining.

The results of the present study increase our understanding of the pathophysiology of JNA. We conclude that VEGF is secreted by JNA and suggest that VEGF contributes to the strong vascularization of this benign tumor. Therefore, antiangiogenic therapy might be considered. However, the impact and action of androgens on JNA vascularization requires analysis.

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REFERENCES