Expression of Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor in Sporadic Vestibular Schwannomas Correlates to Growth Characteristics

Dimitrios Koutsimpelas, Tobias Stripf, Ulf R. Heinrich, Wolf J. Mann, and Juergen Brieger

Department of Otorhinolaryngology, University Hospital of Mainz, Mainz, Germany

Hypothesis: Expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) may have an impact on the growth characteristics of sporadic vestibular schwannomas (VSs).

Background: Vestibular schwannoma is a benign, slow-growing neoplasm that accounts for 6% of all intracranial tumors. The biological backgrounds for neoplastic growth and especially for the various growth patterns of VS remain largely unknown. Because several angiogenic and cytotrophic factors have been described to be involved in the growth of malignant tumors, we initiated this study to examine 2 major representatives of such growth factors in VS and their possible correlation to the growth characteristics of sporadic VSs.

Methods: Surgical specimens from 17 patients with sporadic VS were examined, and the expression of 2 major angiogenic and neurotrophic factors, bFGF and VEGF, was quantitatively analyzed at the mRNA and protein levels. The microvessel density (MVD) was defined by CD31 staining.

Results: All tumors showed expression of bFGF and VEGF at both the mRNA and protein levels. The mRNA expression and the protein expression of both growth factors correlated positive to tumor volume, to tumor growth index, and to MVD.

Conclusion: The bFGF and VEGF mRNA expression and the bFGF and VEGF protein expression in sporadic VS correlates to the tumour volume, to the tumor growth index, and to the MVD. This might indicate an angiogenic and neurotrophic influence of these factors and a possible involvement in the growth of sporadic VS.

Key Words: bFGF—CD31—Growth index—Microvessel density—Tumor volume—VEGF—Vestibular schwannoma.

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Vestibular schwannomas (VSs) are rare, slow-growing neoplasms that occur as a result of increased proliferation of the Schwann cells of the vestibular branch of the VIIIth cranial nerve. The tumors usually arise near the myelin-glial junction near the porus acusticus internus. They are typically well circumscribed and encapsulated by epi-neurium. They may grow continuously or only to a certain size, followed by stagnation or even shrinkage (1). Progressive growth will eventually lead to displacing neighboring neural structures without direct invasion. The biological background and reasons for the diverse growth patterns of VSs are, however, largely unknown.

Mutations may be the early genetic event that initiates tumor genesis (2). Although further neoplastic growth seems to depend on cytokines with angiogenic and mitogenic properties (3,4), only very limited data concerning the expression and relevance of growth factors for VS growth are available. Among the numerous proangiogenic growth factors, the vascular endothelial growth factor (VEGF) (5) and basic fibroblast growth factor (bFGF) (6) are the most prominent. In a previous immunohistochemical study, we reported that VSs only occasionally express VEGF (7). These results have been contradicted by Cayé-Thomasen et al. (8). However, the reason for this difference remained unclear. The second most important angiogenic growth factor, bFGF, is frequently expressed in the nervous system and is known to have a mitogenic function in relation to Schwann cells (9). But again, beside 1 study reporting mRNA expression of bFGF in VS without further analysis of protein expression or vessel density analysis (10), no further analyses of bFGF have been published. To
clarify the impact of these 2 major proangiogenic growth factors for VS vascularization and growth, we performed a quantitative comprehensive analysis of VEGF and bFGF expression at the mRNA and protein level and correlated our data to vessel density, tumor growth index, and tumor volume.

**MATERIALS AND METHODS**

**Patients/Specimens**

Seventeen consecutive adult patients (11 men, 6 women) with unilateral VSs operated at the Department of Otorhinolaryngology, University Hospital of Mainz, during the year 2004 were included in this study. Magnetic resonance imaging scanning images were used for estimating the diameters (anteroposterior, lateromedial, and rostrocaudal) of the tumors. For calculation of the tumor volumes, we used the following equation assuming an ellipsoid tumour shape: volume = \( \frac{1}{6} \pi \times d_1 \times d_2 \times d_3 \). The specimens obtained in the operating room were equally divided into 2 parts. One part of each tumor was fixed in formalin for 24 hours, paraffin-embedded, and used for immunohistochemical analysis. The other part of the tumor was immediately subjected to mRNA isolation.

**Immunohistochemistry**

Immunohistochemical analysis was performed using standard procedures. In brief, formalin-fixed, paraffin-embedded tissues were used. Heat-induced antigen retrieval was performed using microwave treatment (3 times for 5 min each, 600 W in 10 mmol/L citrate buffer, pH 6.0) of all slides after dewaxing and rehydration followed by blocking of endogenous peroxidase with 3% H\(_2\)O\(_2\)/methanol. After preincubation with 10% normal serum in 2% bovine serum albumin/phosphate-buffered saline (PBS) for 20 minutes to avoid unspecific binding, primary antibodies (CD31, 1:100; Santa Cruz Biotechnology, CA, USA; bFGF, 1:100; Santa Cruz Biotechnology; VEGF, 1:100) were overlaid overnight at 4°C. Because of the discrepancy between data published previously (8,9), we performed the immunohistochemical analysis twice using as primary antibodies the polyclonal goat antihuman VEGF antibody (R&D, Wiesbaden, Germany) that we had used in our former study (8) and the polyclonal rabbit antihuman VEGF antibody (Santa Cruz Biotechnology) that had been used by Cayé-Thomasen et al. (8). Slides were then incubated with biotinylated secondary antibody (1:100; DAKO, Hamburg, Germany) and streptavidin peroxidase (1:100; Dianova, Hamburg, Germany). All washing procedures were performed in PBS. Slides were counterstained with hematoxylin. Negative controls were performed, substituting the primary antibodies with PBS. Tumor tissue from angiofibromas served as positive control for VEGF, bFGF, and CD31 immunostaining. A second control of the specificity of the VEGF antibody (Santa Cruz Biotechnology) was performed by absorption of the primary antibody with the specific blocking peptide (VEGF [A20]-P;sc-152-P, Lot H222; Santa Cruz Biotechnology). The blocking peptide was combined with the primary antibody with a 10-fold excess of the peptide in BSA/Tris-buffered saline buffer overnight at 4°C and then used like described before (data not shown).

We analyzed the microvessel density (MVD) by means of CD31 staining of endothelial cells. Analysis was performed in areas of the tumor sample without signs of necrosis or inflammation. We counted the microvessels in 3 “hot spots” of each section. Single CD31-positive cells or cell clusters were regarded as microvessels. Microvessel density was analyzed by counting all CD31-positive cells in 1 field of view at 400-fold magnification, each corresponding to 25 × 25 µm. Results were expressed as the highest number of microvessels in any single ×400 field. Counts were performed by 1 of the authors (T.S.) and later confirmed by a second blinded author (J.B.).

**FIG. 1.** Immunohistochemical expression of CD31 (A, ×100), VEGF (B, ×100), and bFGF (C, ×400).
TABLE 1. Spearman correlation coefficient and statistical significance

<table>
<thead>
<tr>
<th></th>
<th>MVD</th>
<th>VEGF mRNA</th>
<th>bFGF mRNA</th>
<th>Growth index</th>
<th>Tumor volume</th>
<th>VEGF (protein)</th>
<th>bFGF (protein)</th>
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</thead>
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<tr>
<td><strong>Correlation</strong></td>
<td>0.574</td>
<td>0.553</td>
<td>0.616</td>
<td>0.571</td>
<td>0.654</td>
<td>0.628</td>
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<tr>
<td><strong>Significance (2-tailed)</strong></td>
<td>$p &lt; 0.02$</td>
<td>$p &lt; 0.02$</td>
<td>$p &lt; 0.01$</td>
<td>$p &lt; 0.02$</td>
<td>$p &lt; 0.004$</td>
<td>$p &lt; 0.007$</td>
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<tr>
<td><strong>Correlation</strong></td>
<td>0.574</td>
<td>0.667</td>
<td>0.632</td>
<td>0.551</td>
<td>0.608</td>
<td>0.333</td>
<td></td>
</tr>
<tr>
<td><strong>Significance (2-tailed)</strong></td>
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<td>—</td>
<td>$p &lt; 0.003$</td>
<td>$p &lt; 0.007$</td>
<td>$p &lt; 0.03$</td>
<td>$p &lt; 0.01$</td>
<td>NS</td>
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<tr>
<td><strong>Correlation</strong></td>
<td>0.616</td>
<td>0.632</td>
<td>0.766</td>
<td>—</td>
<td>0.766</td>
<td>0.509</td>
<td>0.640</td>
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<tr>
<td><strong>Significance (2-tailed)</strong></td>
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<td>$p &lt; 0.007$</td>
<td>$p &lt; 0.0001$</td>
<td>—</td>
<td>$p &lt; 0.0001$</td>
<td>$p &lt; 0.04$</td>
<td>$p &lt; 0.006$</td>
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<tr>
<td><strong>Correlation</strong></td>
<td>0.571</td>
<td>0.551</td>
<td>0.676</td>
<td>0.869</td>
<td>—</td>
<td>0.228</td>
<td>0.559</td>
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<tr>
<td><strong>Significance (2-tailed)</strong></td>
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<td>$p &lt; 0.03$</td>
<td>$p &lt; 0.003$</td>
<td>$p &lt; 0.0001$</td>
<td>—</td>
<td>NS</td>
<td>$p &lt; 0.03$</td>
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<tr>
<td><strong>Correlation</strong></td>
<td>0.654</td>
<td>0.608</td>
<td>0.468</td>
<td>0.509</td>
<td>0.228</td>
<td>—</td>
<td>0.316</td>
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<td><strong>Significance (2-tailed)</strong></td>
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<td>$p &lt; 0.01$</td>
<td>NS</td>
<td>$p &lt; 0.04$</td>
<td>NS</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Correlation</strong></td>
<td>0.628</td>
<td>0.333</td>
<td>0.507</td>
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<td>$p &lt; 0.006$</td>
<td>$p &lt; 0.03$</td>
<td>NS</td>
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Spearman coefficient and statistical significance of the correlations between MVD, VEGF mRNA, and bFGF mRNA, tumor growth index, tumor volume, and VEGF and bFGF immunohistochemical expression.

bFGF indicates basic fibroblast growth factor; MVD, microvessel density; VEGF, vascular endothelial growth factor.

For evaluation of the VEGF, and bFGF expression, we measured the stained area and intensity of each section in at least 3 fields by a computer-based image analysis method, previously described in detail (11). In brief, stainings were quantified by multiplication of the stained area by the staining intensity and expressed as arbitrary units (AU).

Quantitative Polymerase Chain Reaction Analysis of VEGF and bFGF mRNA Levels

Total cellular RNA was prepared of each specimen using AGS Tristar reagent (AGS, Heidelberg, Germany) as described by the manufacturer. RNA concentrations were analyzed by measurement of optic density (260 nm). Two micrograms of total RNA was used each for cDNA synthesis, including 2.5 μl Moloney murine leukemia virus reverse transcriptase (Roche, Mannheim, Germany), 10 μl reverse-transcriptase buffer, 22 μl 25mmol/L MgCl₂, 20 μl 25 mmol/L deoxynucleoside triphosphate, 2 μl RNase inhibitor, and 5 μl random hexamers in a total volume of 100 μl. The mixture was incubated at 48°C for 30 minutes, and the enzyme was inactivated at 95°C for 5 minutes. All cDNAs were stored at –20°C. Each sample was diluted 1:5 with distilled water before polymerase chain reaction (PCR) analysis.

For quantitative PCR analysis, we used the Taq-Man PCR technology (GeneAmp 5700 Sequence Detection System; ABI, Weiterstadt, Germany). The method enables quantification of the transcript of interest (Sequence Detector User Bulletin; ABI). For VEGF and bFGF assays on demand were used (Applied Biosystems, Weiterstadt, Germany). Polymerase chain reactions were performed in duplicate and according to the standard protocol suggested by the manufacturer. For each PCR analysis, 5 μl cDNA was subjected. The huPO gene was used as endogenous reference, and the quantification of the transcripts was performed by the ΔΔCₚ method. Levels are relative levels compared with an internal calibrator.

For all PCR analyses a no-template negative control was included. Precautions suggested by Kwok and Higuchi were strictly followed (12).

Tumor Growth Index

We assumed that the onset of tumor genesis should vary randomly and theoretically could be even zero. The tumor growth index was therefore calculated by dividing the maximal tumor diameter by the patient’s age. This method has been described and discussed previously (13).

Statistics

The Spearman rank correlation test was used for statistical data evaluation, with $p < 0.05$ as the level of significance. Data shown are mean values ± SD. All calculations were performed using the SAS software, version 6.12 (Statistical Analysis Systems, SAS Institute Inc., Cary, NC, USA). Data were not adjusted for multiple testing because of the explorative nature of the study. Calculated significances have to be handled cautiously because of the relatively small number of samples.

RESULTS

Seventeen patients (age: range, 28–71 yr; mean, 51.5 ± 12.2) were studied. The tumor volumes varied between 36 and 2,556 mm³ (mean, 424 ± 658), and the tumor growth index varied between 0.09 and 0.57 mm²/hr (mean, 0.26 ± 0.14).

We found MVDs varying between 4.6 and 27.4 (mean, 9.7 ± 5.3) (Fig. 1A). Using the VEGF antibody from Santa Cruz Biotechnology, the cytoplasm of the tumor cells was stained. Furthermore, staining also occurred in...
FIG. 2. Scattergrams of positive correlation between MVD and tumor growth index (A), MVD and tumor volume (B), VEGF mRNA and MVD (C), bFGF mRNA and MVD (D), VEGF protein expression and MVD (E), bFGF protein expression and MVD (F), bFGF mRNA and tumor growth index (G), and between bFGF mRNA and tumor volume (H).

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the cytoplasm of endothelial cells (Fig. 1B). The VEGF expression varied from 203 to 5,190 AU (mean, 2,359 ± 1,483 AU). Using as primary antibody the VEGF antibody from R&D, none of the VS stained positive.

Basic fibroblast growth factor immunoreactivity was observed in the tumor cell cytoplasm and in the nuclei of the tumor cells as in previous studies of neuronal cells described by others (14). The bFGF expression varied from 147 to 2,521 AU (mean, 1,457 ± 625 AU) (Fig. 1C).

The VEGF mRNA expression varied from 0.13 to 7.06 (mean, 1.9 ± 1.6) and the bFGF mRNA level was from 2.5 to 71.3 (mean, 13.6 ± 19.6). The Spearman rank correlation test revealed a significant positive correlation between the expression of VEGF and of bFGF at the protein level and the respective expression levels of VEGF and bFGF mRNA. The MVD correlated to the growth index and to the tumor volume. Protein expression levels of VEGF and bFGF as well as VEGF and bFGF mRNA correlated positively to MVD, growth index, and, with the exception of the protein expression of VEGF, also to the tumor volume (all \( p < 0.05 \) and \( 0.507 < r < 0.869 \) (Table 1 and Fig. 2).

**DISCUSSION**

We investigated the MVD of sporadic VSs, the VEGF and bFGF mRNA expression, and the VEGF and bFGF protein expression. We found that MVD correlates to the growth index and to the volume of the tumors. Moreover, we showed that VEGF and bFGF are expressed in the tumor cells of VSs, and that the expression of these angiogenic factors correlates to the MVD, the volume, and the growth index of the tumors.

Angiogenesis is a major prerequisite for the proliferation and progression of several neoplasms (15). Despite the fact that VS are generally slow-growing tumours and therefore may not need excessive vascularization, the necessity for a functional vascular system still remains important for further growth of the tumor. Our results, showing a positive correlation of MVD with tumor growth rate index and tumor volume, are consequently consistent with a previous study (1) and suggest that angiogenesis might play a role in the growth of VS.

Two of the most potent mediators of neoangiogenesis are VEGF (6) and bFGF (7). In addition to its role as a key angiogenic factor, VEGF also possesses neurotrophic and neuroprotective properties both in the peripheral and in the central nervous system, exerting a direct action not only on neurons but also on Schwann cells, astrocytes, neural stem cells, and microglia (16). Pietsch et al. (17) and Nishikawa et al. (18) analyzed several types of brain tumors for VEGF expression and demonstrated this cytokine not only in strong vascularized brain tumors such as glioblastoma but also in less vascular astrocytoma and meningioma. It has recently also been reported that Schwann cells are the major source of bFGF and that bFGF expression after trauma increases in Schwann cells (19). Increased bFGF expression in proliferating Schwann cells stimulates mitosis and acts as differentiation and proliferation factor for a variety of neuron populations (9,20).

If VSs express VEGF and bFGF, and the possible impact of these 2 growth factors on neoangiogenesis and growth of VS have not been investigated in detail yet. The available data concerning VEGF expression are contradictory. In a previous study, we reported only rarely expressed VEGF in VS (7). However, Cayé-Thomasen et al. (8) showed frequent VEGF expression in VS, an observation that we are able to confirm now. We learned that the detection of VEGF largely depends on the used antibody, that is, using an antibody, probably with higher sensitivity, results in frequent VEGF-positive VS.

Except for 1 study reporting increased bFGF mRNA levels in VS (10), there are no further studies in the literature. In our study now, we demonstrate the frequent expression of bFGF in VS and its correlation to MVD, tumor growth index, and tumor volume.

In the present study, there is a significant correlation of mRNA and protein expression of both examined growth factors to MVD, to tumor growth index, and, with the exception of VEGF protein, also to tumor volume in VS, suggesting that both factors might affect neoangiogenesis in VS. However, because bFGF is additionally correlated to tumor volume, this growth factor might be of special relevance for VS growth. We reported in association with an earlier study that large tumors tend to proliferate faster (13). We hypothesize that accelerated growth is due to higher bFGF levels, resulting in larger tumors. It is to debate if antiangiogenic therapies targeting VEGF and/or bFGF can therefore be beneficial for limiting tumor growth rates.

**REFERENCES**


