ENDOGLIN AS A MARKER IN CERVICAL PARAGANGLIOMAS

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Abstract: Background. Endoglin is expressed on endothelium and is implicated in the control of angiogenesis. This study compares the expression of endoglin with vascular endothelial growth factor (VEGF), commonly used as a marker for neoangiogenesis in cervical paragangliomas (CPG).

Methods. The CPG were surgically obtained from 5 patients and compared with nontumoral lung obtained from patients subjected to pulmonary resection. Detection with specific antibodies was used to determine the expression of the proteins VEGF and endoglin. The expressions of hypoxia-inducible factor (HIF) and vascular cell adhesion molecule-1 (VCAM-1) were used to determine the degree of hypoxia and capillarization, respectively.

Results. Endoglin is located at the plasma membrane of endothelial cells. The relative expression of endoglin is significantly higher in CPG respect to lung (p < .02), whereas that of VEGF is similar.

Conclusion. Endoglin expression in CPG is significantly superior to that of VEGF and correlates with tumor vascularization. © 2009 Wiley Periodicals, Inc. Head Neck 32: 737–743, 2010

Keywords: paraganglioma; endoglin; vascular endothelial growth factor (VEGF); angiogenesis; endothelium

Paragangliomas are infrequent and usually benign neoplasias that derive from the neural crest. They are rare and can be found between the base of the cranium and the pelvic floor, most commonly affecting the cervical-cephalic area. Paragangliomas of the head and neck represent 0.6% of all tumors in the area and around 0.03% of all tumors. They are most commonly found in the carotid body, jugular bulb, and vagus nerve, in that order. Hypoxia, estrogens, and genetic factors contribute to the development of paragangliomas. It is known the presence of carotid body neoplasia in patients with chronic respiratory dysfunction/failure, as well as in normal people living at high altitude; in both cases, the frequency is higher in women. Moreover, the presence of a genetic autosomal dominant defect could trigger tumor development resulting in a 10% to 15% of cases in familiar paragangliomas. They are highly vascularized and the risk of their surgical extirpation frequently obligates to perform a preoperative embolization.

Endoglin (CD105) is a homodimeric transmembrane glycoprotein (180 kDa) predominantly expressed in endothelial cells. Endoglin is an
accessory protein operating as nonsignaling receptor in the transforming growth factor (TGF)-β receptor complex. It is necessary for signaling transduction and modulates cellular responses to TGF-β in different types of cells. Endoglin plays a critical role in vascular development and function and participates in normal angiogenesis in adult. Endoglin is the mutated gene for hereditary hemorrhagic telangiectasia type I (HHT-1), an autosomal dominant disorder characterized by multisystemic vascular dysplasia, and multiple arteriovenous shunts.

Endoglin could have a key function in the regulation of neoangiogenesis crucial for tumor growth and progression. Studies of cultured endothelial cells propose that endoglin acts as a proangiogenic molecule. It is upregulated in proliferating endothelial cells and is strongly expressed in the neovascularure of a wide range of solid tumors. Besides, endoglin expression is weaker in nonmalignant adult tissue vessels, including preneoplastic lesions, than in tumor vessels.

We assess the expression of endoglin, with diagnostic and prognostic purposes, in cervical paragangliomas (CPG) surgically obtained from 5 patients, and we compare with the expression of vascular endothelial growth factor (VEGF), an angiogenic factor upregulated in paragangliomas. Recent reports suggest that elevated plasma levels of endoglin in patients with colorectal cancer or breast carcinoma correlate with poor prognosis. Moreover, we study the expression of hypoxia-inducible factor (HIF) as the primary angiogenic mediator liberated by the lack of oxygen occurring in the initiation of the neoangiogenesis pathway. The expression of vascular cell adhesion molecule 1 (VCAM-1) indicated as tumor vascularization. As a control, we used 5 surgically removed nontumorous parts of the lung, as this tissue shows the highest rate of endothelial cell abundance.

**MATERIALS AND METHODS**

**Tissue Samples.** Five tumors were collected from patients of the Unit of Cardiovascular Surgery of the Hospital Clínico Universitario (Salamanca, Spain) from 2002 to 2005. Tumors were numbered from 1 to 5 as indicated in Table 1.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>Case 4</th>
<th>Case 5</th>
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<tr>
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<td>69/female</td>
<td>38/female</td>
<td>56/male</td>
<td>70/male</td>
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<td>Carotid</td>
<td>Carotid</td>
<td>Carotid</td>
<td>Vagal</td>
<td>Carotid</td>
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<tr>
<td>Tumor</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>+</td>
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<td>ND</td>
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<td>+</td>
<td>ND</td>
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<td>ND</td>
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<td>+</td>
<td>+</td>
<td>ND</td>
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<td>+</td>
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<td>–</td>
<td>+</td>
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<tr>
<td>Multicentric</td>
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<td>–</td>
<td>+1‡</td>
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<tr>
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<td>–</td>
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<tr>
<td>Functional</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Size, cm</td>
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<td>3.5</td>
<td>3.6</td>
<td>4.3</td>
<td>5.2</td>
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<td>ND</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Years</td>
<td>1997/02*</td>
<td>2002</td>
<td>2003</td>
<td>2003</td>
<td>2005</td>
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<td>–</td>
<td>–</td>
<td>+§</td>
<td>–</td>
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<tr>
<td>CVA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Death</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviations: DSA, digital subtraction angiography; CVA, cerebrovascular accident; ND, not done.

*Carotid body tumor contralateral <1 cm.
†The tumor tissue of the case number 1 was collected in the second operation carried out in 2002.
‡Vagal paraganglioma contralateral.
§X and XII par.
Also, 5 samples from lung tissue were obtained from men (aged 50–60 years) subjected to pulmonary resection of lung tumors in the Respiratory Surgery Unit of the same hospital. These samples were obtained distally from tumors and were macroscopically and microscopically free of tumor. In each sample, 1 piece was immediately frozen and kept in liquid nitrogen until use, and another piece was fixed in buffered formaldehyde for histologic studies.

In all cases, informed consent with approval was obtained from patients in accordance with the international and national institutions guidelines for the care and use of samples for experimental investigation with human subjects.

Western Blot Analysis. HIF, VCAM-1, VEGF, endoglin, and actin contents in tumoral tissues were measured by Western blot analysis, as previously described. Tissue samples frozen in liquid nitrogen were pulverized on dry ice to obtain homogeneous samples and then stored at −80°C until use. Protein-enriched extracts were prepared from 100 mg of tissue in lysis buffer. Protein concentrations were determined using a kit assay (Bio-Rad, Madrid, Spain).

Primary antibodies were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Their references and dilutions used were: anti-HIF (SC-10790, 1/200); anti-VCAM-1 (SC-1504, 1/1000); anti-endoglin (SC-H300, 1/400); anti-VEGF (SC-50, 1/5000); and anti-actin (SC-1616, 1/2000). Secondary antibodies used were horse-radish peroxidase-labeled anti-rabbit immunoglobulin G (IgG) (Santa Cruz Biotechnology; 1/10000 for HIF, endoglin, and VEGF detection), anti-goat (Bio-Rad; 1/2000 for VCAM-1 and actin detection). Western blots where developed using a chemiluminescence assay (Amersham, Buckinghamshire, UK). Actin was used as loading control.

In order to understand the relative expressions of VEGF and endoglin in lung and CPG, it is necessary to consider a limitation imposed by the quantitative method in immunoblots. Preparation of protein samples do not permit to charge the same proportional amount of parenchymal and endothelial cell extracts in the lanes of either CPG or lung immunoblots. For this reason, quantifications obtained should consider: (1) that CPG have proportionally more parenchymal cells, whereas lungs contain more endothelial cells, and (2) that VEGF is mainly present in parenchymal cells and endoglin (and VCAM-1) are mainly expressed by endothelial cells.

Histologic Studies. Sections (3 μm thick) were cut, mounted on glass slides, and counterstained with hematoxylin-eosin for light microscopy analysis. Immunohistochemistry was performed as previously described with the following primary antibodies: rabbit polyclonal antibody directed against human VEGF amino terminal peptides 1-20 that cross-reacts with murine VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-human CD105 endoglin, clone SN6h (DAKO, Carpinteria, CA) and rabbit monoclonal antibody against human Ki67 (MD, Granada, Spain).

Statistics. Data are expressed as mean ± SEM. Statistical significance has been assessed using 1-way analysis of variance (ANOVA) or t test, as adequate.

RESULTS

Protein Expression in Immunoblots. The expression of HIF was higher in CPG compared with the expression in a similar amount of pulmonary tissue; however, these differences did not reach statistical significance (Figure 1A and 1B). Individual data showed dispersion between values in the 5 tumors, whereas the expression in lung is homogeneous.

VCAM-1 expression was significantly higher in CPG (p < .02) compared with lung tissue (Figure 1A and 1C). No dispersion was observed between individual values in both tissues.

Endoglin expression was significantly higher in CPG (p < .02) than in lung tissue (Figure 1A and 1D), whereas expression of VEGF was similar in both tissues (Figure 1A and 1E). Individual values for VEGF and endoglin were homogeneous in both tissues.

Histologic Studies. Microscopically, tumors are formed by nests of polygonal cells with a clear and eosinophilic cytoplasm and ovoid nucleus. These cells present scarce pleomorphism and a low degree of mitosis. The nests are separated by capillaries (Figure 2A).

Nest cells show intense staining for VEGF, whereas capillary endothelial cells shows moderate staining for VEGF (Figure 2B). Endoglin was exclusively restricted to endothelial cells without
any staining in tumor parenchymal cells (Figure 2C). We expected an active endothelial proliferation in CPG, but Ki immunostaining showed proliferating nuclei in some parenchymal cells but not in endothelial cells (Figure 2D).

**DISCUSSION**

It has long been established that tumor progression is strictly dependent on angiogenesis for both primary tumor growth and metastatic spreading. Some recent experimental
evidences suggest the involvement of endoglin in the regulation of angiogenesis and tumor development in vivo. We demonstrated a decreased growth and vascularization in a model of rapidly developing subcutaneous tumor\textsuperscript{25} and a reduced number of benign papillomas in a model of chemically induced skin carcinogenesis\textsuperscript{27} in endoglin haploinsufficient mice (Eng\textsuperscript{\textasciitilde}). Moreover, data from our laboratory demonstrating reduced angiogenesis after hind limb ischemia in Eng\textsuperscript{\textasciitilde} mice.\textsuperscript{12}

In our experiments, endoglin appeared at the plasma membrane of endothelial cells and was highly expressed in the 5 cases of CPG studied. A similar increase in VCAM-1 expression was observed in CPG compared with lung, suggesting a relationship between the increase in endoglin expression and tumor neoangiogenesis. A significant correlation between levels of endoglin and markers of cell proliferation in tumor endothelia was already found.\textsuperscript{28} However, we demonstrated that development of CPG is not associated with increased expression of VEGF, although this cytokine has been long considered the best marker of neoangiogenesis in paragangliomas.\textsuperscript{21,29} Minhajat et al\textsuperscript{30} recently described that endoglin, but not other markers as VEGF, specifically mark tumor angiogenesis of brain, lung, breast, stomach, and colon cancers.

Angiogenesis is tightly regulated by hypoxia. Hypoxia activates the transcription of genes that mediate adaptive responses of the organisms to counteract the low oxygen availability.\textsuperscript{31} Transcription of a large variety of hypoxia-regulated genes—including VEGF and endoglin—is activated via HIF-1, a heterodimeric transcriptional complex.\textsuperscript{32,33} In our data, the levels of HIF found in CPG suggest a certain degree of hypoxic stress in tumoral cells. Although there are no reports about HIF in CPG, 1 of the best known genetic defects associated with paragangliomas are mutations of subunits of succinate dehydrogenase; these defects result in a dysfunction of the enzymatic activity in the complex II of the mitochondrial respiratory chain with activation of the hypoxic routes and neoangiogenesis induced by HIF, VEGF, and hypoxic-angiogenic responsive genes.\textsuperscript{21,29,34,35} Although there is not a purpose of the present work to investigate the mechanism by which hypoxia induces the transcription of angiogenic factors,
our results suggest a stronger relation between hypoxia and endoglin expression than between hypoxia and VEGF.

In our understanding, differences in endoglin should be even bigger because quantification of protein bands exhibited in immunoblots is limited and can hide some interesting considerations. Thus, the relative protein expression of VEGF and endoglin in lung and CPG is performed upon a detected band corresponding to a similar amount of protein charged in each lane, independently of the proportion of parenchyma (expressing more VEGF) and capillaries (expressing more endoglin) present in CPG (with more tumoral cells) or lungs (with more endothelial cells). We emphasize that elevation in endoglin and VCAM-1 expression in CPG respect to lung are underestimated considering that both proteins are present in endothelium, and the proportion of endothelium/parenchyma is lower in CPG than in lungs. On the other hand, the relative amount of VEGF appeared similar in both tissues. Reasoning, in a similar manner, if VEGF is a soluble cytokine mainly present in parenchyma, its relative expression in CPG is even lower as tumors have a higher proportion of parenchyma than lungs. Summarizing, we can infer that the amounts of endoglin and VEGF in CPG relative to the type of cells expressing both molecules (endothelial for endoglin and parenchymal for VEGF) are higher and lower, respectively, than shown in the histograms of Figure 1.

These results give additional experimental support to the concept that endoglin is essential for tumoral neoangiogenesis. However, the main finding of the present work is that endoglin and VCAM-1, but not VEGF, are highly expressed in human CPG, suggesting that endoglin may have a remarkable diagnostic, prognostic, and therapeutic relevance in tumor development and malignancy of CPG.

REFERENCES