The Angiogenic Growth Factors HGF and VEGF in Serum and Plasma from Neuroblastoma Patients

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Abstract. Aim: To determine whether concentrations of the angiogenic growth factors hepatocyte growth factor (HGF) and vascular endothelial growth factor A (VEGF-A) correlate with clinical and genetic markers in samples taken at diagnosis in children with neuroblastoma (NB). Patients and Methods: Heparin plasma (P-) and serum (S-) samples of healthy controls (n=73, mean age ± SD 3.5±2.1; females/males: 23/50) and patients with NB (n=62; 2.2±1.8; 26/36) were collected between 1988 and 1999. Clinical data included age at diagnosis, gender, stage, outcome, amplification of the oncogene MYCN, loss of heterozygosity at the short arm of chromosome 1 (1p LOH) and ploidy. Results: HGF and S-VEGF-A were elevated in NB as compared to controls (38/62 patients, p<0.0001 and p<0.05, Mann-Whitney U test). HGF concentrations were higher in high-stage (stage 3-4) as compared to low-stage (stage 1-2) disease (p<0.01). P-HGF was elevated in patients with 1p LOH (p<0.01), MYCN amplification (p<0.001) and di- or tetraploidy (p<0.001). S-HGF concentration was elevated in patients MYCN-amplified tumors only. Plasma and S-HGF concentrations were higher in the deceased group (p<0.05), but not P or S-VEGF-A. Conclusion: This study showed that concentrations of HGF and S-VEGF-A are elevated in patients with NB. Furthermore, HGF and S-VEGF-A concentrations correlate to higher stage disease and HGF correlates to genetic markers known to indicate a poor outcome. These observations imply that HGF and VEGF-A have biological roles in NB and suggest the possibility of interference with HGF or VEGF-A signaling as a therapeutic strategy.

Neuroblastoma (NB) is a childhood tumor emerging from immature cells in the sympathetic nervous system. NB exhibits unique biological and clinical heterogeneity among human carcinomas in that it ranges from an aggressive tumor with high mortality to a tumor that spontaneously differentiates into a benign ganglioneuroma (GN) and that may even undergo complete regression despite extensive metastases. Important negative prognostic markers are age over one year at onset (1), advanced tumor stage (2), amplification of the MYCN oncogene (3), loss of heterozygosity on the short arm of chromosome one (1p LOH), coding for a putative tumor suppressor gene (3) and diploidy (4). High-risk NB has a long-term survival rate of less than 40% despite intensive treatment protocols involving high-dose chemotherapy, usually with bone marrow rescue, aggressive surgery and radiotherapy (5, 6). Therefore, new treatment strategies are needed for this malignancy.

Angiogenesis, i.e. new blood vessel formation, is a prerequisite for the growth and metastasis of solid tumors (7, 8). Angiogenesis is induced and maintained by angiogenic growth factors, of which more than 20 are known (9). Angiogenic growth factors are secreted or induced by the tumor cells. Vascular endothelial growth factor A (VEGF-A) is present in conditioned media from human NB cell lines.
Healthy controls and patients. Controls below eight years of age with serum and heparin plasma samples available were selected from a more extensive study (17). The controls were children undergoing minor surgical or diagnostic procedures (e.g. inguinal hernia repair, urethrocystoscopy, circumcision, extraction of internal fixation material) and were recruited from our Outpatient Surgery Unit at the University Children’s Hospital, Uppsala between June 1998 and March 1999. Patient samples were obtained from the frozen (−70°C) stock of plasma and serum samples from 62 patients with NB referred to one of the four regional Swedish Pediatric Oncology Centers in Sweden between 1988 and 1999: Stockholm (n=39), Uppsala (n=30), Gothenburg (n=7) and Lund (n=5). Thirty serum and 39 plasma samples, taken at diagnosis, were available. Data from 17 of the serum samples have also been used in a separate study of angiogenic growth factors in childhood malignancies. One 19-year-old girl with a secondary NB following liposarcoma therapy and one newborn girl with stage 4S NB and excessive levels of HGF (>80.000 pg/ml) were excluded from the study. Clinical data extracted from the hospital records included age at diagnosis, gender, stage according to the International Neuroblastoma Staging System (INSS) (18), treatment and outcome. Some, but not all, tumor specimens were analyzed in ancillary studies: MYCN amplification was detected using Southern blot analysis (19) during 1987 to 1992, followed by interphase fluorescent in situ hybridization (FISH) in 1992-1999 (20); 1p LOH was detected using polymerase chain reaction (PCR) (21) and ploidy was determined by image flow cytometry (22). The patients were treated according to the European protocol in use at the time.

Sample handling. Peripheral venous blood samples were collected using Vacutainer® 2 and 5ml serum tubes without additives (BD, Franklin Lakes, NJ, USA) or 2 and 5 ml sodium-heparin tubes (BD). The samples were handled according to the standard protocol in use at the respective hospital, i.e. the samples were sent to the Peripheral venous blood samples were collected using Vacutainer® 2 and 5ml serum tubes without additives (BD, Franklin Lakes, NJ, USA) or 2 and 5 ml sodium-heparin tubes (BD). The samples were handled according to the standard protocol in use at the respective hospital, i.e. the samples were sent to

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**Results**

**Controls and patients.** Seventy-three controls (mean age ± 3.5±2.1 years, range 0.58-7.6; females/males: 23/50) were recruited. The clinical data for all patients are given in Table I. No patient was lost to follow-up.

**Concentrations of HGF and VEGF-A.** Multiple linear regression analysis showed that age (coefficient [SE] –27.0 [9.6], p<0.01) and female sex (90.2 [43.3], p=0.05) correlated
significantly with the P-HGF concentration \((r=0.39, \text{ intercept } \text{524.8 pg/ml \[38.1\], } p<0.0001, n=60)\). No age or gender correlation was found with any of the other variables tested (data not shown). The concentrations of HGF and VEGF-A in the controls and patients are given in Table II and Figure 1A-D. The upper reference values (pg/ml) at the estimated 97.5th percentile were as follows: P-HGF 850.2, S-HGF 1196.8, P-VEGF-A 228.9, and S-VEGF-A 720.6. The plasma and S-HGF concentrations were elevated in NB \((p<0.0001\) and \(p<0.01\)) as compared to controls (Figure 1A-B, Table II). In 44.0\% \((29/66)\) of the NB samples, concentrations above the upper reference value were found. By adding intercepts to the linear regression analysis specified above, it was confirmed that patients with NB had elevated concentrations of P-HGF (data not shown). The serum, but not plasma, VEGF-A concentrations were elevated in NB patients as compared to controls \((p<0.05\), Figure 1C-D; Table II). A total of 13\% \((9/69)\) of the NB samples showed VEGF-A concentrations exceeding the upper reference value.

**Table II. Plasma and serum concentrations of HGF and VEGF-A.**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>NB</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P-HGF</strong></td>
<td>459.0±160.4</td>
<td>1283.9±1357.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(n=60)</td>
<td>50.0% ((18/36))</td>
<td></td>
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</tr>
<tr>
<td><strong>S-HGF</strong></td>
<td>523.5±265.4</td>
<td>1146.6±835.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(n=54)</td>
<td>36.7% ((11/30))</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P-VEGF-A</strong></td>
<td>65.8±58.6</td>
<td>79.5±112.6</td>
<td>N.S.</td>
</tr>
<tr>
<td>(n=69)</td>
<td>5.1% ((2/39))</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S-VEGF-A</strong></td>
<td>248.4±167.5</td>
<td>432.1±353.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(n=56)</td>
<td>23.3% ((7/30))</td>
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</table>

Mean±SD and proportion of elevated samples (over 97.5th percentile, see text for details). *Mann-Whitney \(U\)-test. N.S., not significant; P, plasma; S, serum.

**Correlation with stage and genetic markers.** The mean P- and S-HGF concentrations were higher in high-stage (stage 3-4) disease \((P-HGF 1636.6±1543.5 \text{ pg/ml}; S-HGF 1520.8±986.5 \text{ pg/ml})\) than in low-stage (stage 1-2) \((552.4±262.2 \text{ pg/ml},

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Figure 1. Plasma \((A, C)\) and serum \((B, D)\) concentrations of HGF \((A-B)\) and VEGF-A \((C-D)\) vs. age. All samples taken at diagnosis. Upper reference values (see text for details) for controls are plotted \((- - -\)) . Symbols: Controls \((+\) and neuroblastoma \((\bigcirc\) ).
The concentrations of VEGF-A were not higher in high-stage compared to low-stage disease (data not shown). A trend for increasing P-HGF (r=0.41, p<0.05; Spearman rank order correlation), S-HGF (r=0.46, p<0.05) and S-VEGF-A (r=0.23, p=0.06) with stage was found (Figure 2A-D). Plasma HGF was elevated in patients with the negative prognostic markers 1p LOH (p<0.01), MYCN amplification (p<0.001) and di- or tetraploidy (p<0.001). S-HGF was elevated in tumors with MYCN amplification (Table III). Neither P- nor S-VEGF-A was increased in any of the other groups compared, but the analysis was hampered by the small number of serum samples.

Correlation with outcome. Univariate analysis using a log-rank test of Kaplan-Meier estimates confirmed that age over one year, metastatic disease, MYCN amplification, 1p LOH and di- or tetraploidy were negative prognostic markers (Table IV) compared with the surviving group. Plasma HGF (1752.4±1190.7 vs. 1004.1±1429.5 pg/ml, p<0.01) and S-
**Table III. HGF, VEGF-A and genetic markers in neuroblastoma.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>1p LOH</th>
<th>MYCN amplification</th>
<th>Ploidy</th>
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<tbody>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P-HGF</td>
<td>806.3±483.7</td>
<td>1535.2±1052.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>S-HGF</td>
<td>958.9±543.4</td>
<td>1808.3±1525.2</td>
<td>N.S.</td>
</tr>
<tr>
<td>P-VEGF-A</td>
<td>57.2±60.0</td>
<td>103.0±132.5</td>
<td>N.S.</td>
</tr>
<tr>
<td>S-VEGF-A</td>
<td>462.1±444.7</td>
<td>469.3±403.0</td>
<td>N.S.</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>&lt;3</th>
<th>&gt;10</th>
<th>P-value</th>
<th>2n, 4n</th>
<th>3n, 5n, 6n</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-HGF</td>
<td>710.9±487.6</td>
<td>1593.3±1051.2</td>
<td>&lt;0.001</td>
<td>1416.8±963.5</td>
<td>591.4±196.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>S-HGF</td>
<td>911.1±602.8</td>
<td>1704.5±602.8</td>
<td>&lt;0.05</td>
<td>1201.6±321.8</td>
<td>950.4±484.6</td>
<td>N.S.</td>
</tr>
<tr>
<td>P-VEGF-A</td>
<td>731±109.7</td>
<td>96.4±125.2</td>
<td>N.S.</td>
<td>83.7±108.4</td>
<td>48.0±43.9</td>
<td>N.S.</td>
</tr>
<tr>
<td>S-VEGF-A</td>
<td>410.1±308.7</td>
<td>384.6±354.5</td>
<td>N.S.</td>
<td>504.7±386.4</td>
<td>407.5±249.7</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Mean±SD. Significance tested using Mann-Whitney U-test. N.S., not significant; P, plasma; S, serum; 1p LOH, loss of heterozygosity of the short arm of chromosome one.

**Table IV. Negative prognostic factors in univariate analysis.**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Variable</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Over 1 year of age (n=62)</td>
<td>0.0016</td>
</tr>
<tr>
<td>Gender</td>
<td>Female vs. male (n=62)</td>
<td>0.64</td>
</tr>
<tr>
<td>Stage</td>
<td>1-2 vs. 3-4* (n=55)</td>
<td>0.0001</td>
</tr>
<tr>
<td>MYCN</td>
<td>&lt;3 vs. &gt;10 copies (n=53)</td>
<td>0.0015</td>
</tr>
<tr>
<td>1p LOH</td>
<td>Present vs. absent (n=39)</td>
<td>0.0035</td>
</tr>
<tr>
<td>Ploidy</td>
<td>2n, 4n vs. 3n, 5n, 6n (n=31)</td>
<td>0.0052</td>
</tr>
<tr>
<td>HGF</td>
<td>P-HGF &gt; upper quartile (1150 pg/ml) (n=36)</td>
<td>0.00034</td>
</tr>
<tr>
<td></td>
<td>S-HGF &gt; upper quartile (1465 pg/ml) (n=30)</td>
<td>0.13</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>P-VEGF-A &gt; upper quartile (81 pg/ml) (n=39)</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>S-VEGF-A &gt; upper quartile (631 pg/ml) (n=30)</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Significance tested with the log-rank test of Kaplan-Meier estimates. *Stage 4S excluded. P, plasma; S, serum; 1p LOH, loss of heterozygosity of the short arm of chromosome one.

HGF (1652.6±1040.2 vs. 895.6±439.5 pg/ml, p<0.05) were higher in the deceased group, while no difference was seen in P or S-VEGF-A (data not shown). Splitting the sample by the upper quartile indicated that a high P HGF was a negative prognostic marker (p<0.01, log rank test) (Table Figure 3).

Samples taken during clinical course. In four patients, both samples taken at diagnosis and samples taken preoperatively, after the induction chemotherapy, were available. The concentrations of both HGF and VEGF-A in the serum samples tended to be lower after the induction therapy than at diagnosis (Figure 4, p=0.065, Wilcoxon matched pairs test).

In one patient, plasma samples were taken at surgery, both from the radial artery and from a tumor vein. The concentrations of P-HGF and P-VEGF-A were 3-4 times higher in the tumor vein sample than in the simultaneously drawn arterial blood sample (Figure 5).

**Discussion**

Angiogenic growth factors have been identified and measured in serum, plasma, and urine from adult patients with a variety of cancer types (26). A panel of angiogenic growth factors, including VEGF-A, B, and C, basic fibroblast growth factor, and others have been found to be expressed in a variety of NB cell lines and tumors (11). In a study conducted by Hecht et al., it was also shown that HGF stimulates invasion of NB cells both in vitro and in vivo, a property that leads to rapid tumor progression to advanced stages and consequently to poor prognosis (15). We chose to assay the concentrations of HGF and VEGF-A in a collection of plasma and serum samples from NB patients, since we had observed high concentrations of VEGF-A in conditioned media from human NB cells, as well as expression of VEGF-A protein and VEGF-A mRNA in xenotransplanted human NB tumors (27). We had also noted high concentrations of HGF in sera from...
children with a broad variety of carcinomas (14). HGF is a 92 kDa heterodimer that stimulates cellular proliferation and angiogenesis through activation of the c-Met tyrosine kinase receptor (28). In different studies, 36% of breast cancer patients (29), 40% of multiple myeloma patients (30) and 34% of gastric cancer patients (31) exhibited elevated concentrations of HGF. In our study, 46% of the NB patients had HGF concentrations above the cut-off value, and the P-HGF concentrations correlated with both tumor stage and survival. It would seem that HGF is an important angiogenic growth factor in neuroblastoma. With the advent of HGF antagonists, it is tempting to speculate whether therapeutic interference with the HGF/c-Met pathway might be just as efficient an antitumor strategy in NB as interference with the VEGF-A/KDR pathway seems to be in other types of cancer (27, 32, 33). VEGF-A is perhaps the most extensively studied of the angiogenic growth factors. The originally isolated VEGF-A is part of a ligand family currently consisting of six related proteins (VEGF-A, B, C, D, and E; and PIGF) (34). In adults, circulating VEGF-A is elevated in a variety of malignant diseases, e.g. metastatic nasopharyngeal carcinoma (34), breast, colorectal, ovarian and renal carcinoma (26). The VEGF-A gene is expressed in a hypoxia-inducible fashion in clinical neuroblastoma and in cell lines and the protein is expressed by human neuroblastoma cell lines (10, 35). In homogenized tumor tissue, VEGF-A mRNA and protein are detectable in NB (35), Wilms’ tumor (36) and pediatric brain tumors (37). VEGF-A was found to be elevated in children with solid malignancies (13) and it has also been reported to correlate with a poor outcome in children (38). We have also observed increased serum concentrations of HGF and VEGF-A patients with nephroblastoma (39), and in the present study we have confirmed our previous finding that the concentrations of S-VEGF-A were elevated in 17 patients with NB (14). Since VEGF-A is thought to be transported in platelets, a platelet-free plasma sample might not properly reflect the VEGF-A content in the circulation (40). This may explain why VEGF-A was not higher in plasma samples than in controls. We recommend that serum samples be used to measure circulating VEGF-A in patients. Many prognostic factors are known in NB, e.g. MYCN amplification, 1p LOH and ploidy (41). We found that P-HGF was higher in tumors with MYCN amplification, tumors with 1p LOH and di- or tetraploid tumors. The lack of correlation between S-HGF and genetic markers might be due partly to the small sample size. These results indicate that HGF might be interesting as a prognostic factor, but larger studies are needed to determine whether HGF can serve as an independent prognostic factor. Interference with VEGF-A signaling in experimental NB is associated with a 65% reduction of the tumor growth rate (27). Surprisingly, no correlation was found between VEGF-A and any of the prognostic factors mentioned above. Either VEGF-A is not a key angiogenic growth factor in advanced NB, or its paracrine action in combination with its short half-life in the circulation leads to an underestimation of its biological importance.

It was recently demonstrated that overexpression of HGF and the HGF receptor (c-Met) was associated with a high risk of metastasis and recurrence in children and young adults with papillary thyroid carcinoma (42), and HGF has been found to be expressed in tissue from Wilms’ tumor.
In our study, both P- and S-HGF concentrations were higher in the patients with disseminated disease and a P-HGF concentration above the median was a predictor of tumor-related death. The stage 4S tumors are a special NB subset with initial dissemination followed by spontaneous regression or differentiation to benign GN. High vascularity has been noted (44, 45) in this subset of NB patients. In the current study, only two plasma and two serum samples were available from patient with 4S tumors. We noted, however, that S-VEGF-A was exceptionally high in two patients with disseminated disease (Figure 2D). In one 4S patient who was excluded from the study because of a suspected falsely high HGF, the S-VEGF-A level was also exceptionally high. These observations merit further analysis of samples from the 4S subgroup and such an endeavor is currently underway.

Angiogenesis is not a malignancy-specific process. Indeed, benign tumors also require angiogenesis to grow (46). Alternative explanations for the increased concentrations of HGF and VEGF-A observed must not be overlooked, since HGF is elevated in inflammatory lung disease, for example (47), and VEGF-A in juvenile Crohn’s disease, for instance (48). This means that elevated concentrations of angiogenic growth factors in the circulation cannot distinguish benign from malignant tumors, nor neoplastic from non-neoplastic disease. In order to ascertain whether concentrations of angiogenic growth factors such as HGF and VEGF-A can be used to monitor disease activity or not, multiple longitudinal samples would have to be collected from a large number of patients. HGF has been studied in this respect in multiple myeloma by Seidel et al. (30), who found high concentrations at diagnosis, a reduction in response to treatment, and a further rise at relapse. In our few longitudinal serum samples from patients with NB, HGF and VEGF-A tended to fall during induction therapy. These results must be interpreted with caution, since few samples were available, and this issue should preferably be addressed in a prospective clinical investigation. The concentrations of angiogenic growth factors in the peripheral circulation may not reflect the intratumoral biology. Fuhrmann-Benzakein et al. (49) found that P-VEGF-A concentrations were ten times higher in samples from efferent tumor veins, as compared to the peripheral circulation. We have made a similar observation in one patient in whom samples were taken during surgery. The levels of HGF and VEGF-A were several times higher when a sample was obtained from the efferent tumor vein, as compared to a sample obtained simultaneously from a peripheral artery. This implies that growth factor kinetics are important for the understanding of concentrations in the peripheral blood, and this warrants further studies.

This study has demonstrated elevated plasma and serum concentrations of HGF and VEGF-A in patients with NB. We also observed higher concentrations of HGF and S-VEGF-A in patients with advanced stage disease, and for HGF a correlation with genetic markers. The implications of these observations are that HGF and VEGF-A do indeed have a biological role in NB angiogenesis. Inhibitors of angiogenesis may be useful adjuvants or alternatives in the treatment of NB patients. With the development of specific angiogenesis inhibitors, the patient’s profile of angiogenic growth factors could be used to choose the most suitable inhibitor, e.g. a VEGF-A receptor signaling inhibitor (32), or an HGF antagonist (33).

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References


