Angiopoietin-2 Promotes Disease Progression of Neuroendocrine Tumors
Katharina M. Detjen1, Svenja Rieke1, Antje Deters1, Petra Schulz1, Annett Rezin1, Sonja Vollmer2, Peter Hauff2, Bertram Wiedenmann1, Marianne Pavel1, and Arne Scholz1

Abstract

Purpose: Inhibition of angiogenesis represents a promising therapeutic strategy in neuroendocrine tumors. Angiopoietin-2 (Ang-2), a ligand of the endothelial tyrosine kinase Tie-2, is emerging as a key regulator of vascular remodeling during tumorangiogenesis. We therefore addressed the expression and biological significance of Ang-2 in human neuroendocrine tumors.

Experimental Design: Surgical specimens and serum from neuroendocrine tumor patients were used to determine Ang-2 expression by in situ hybridization or ELISA (circulating Ang-2). Ang-2 biological effects were evaluated following stable transfection into BON human pancreatic neuroendocrine tumor cells. BON clones were grown as orthotopic xenografts in nude mice to determine tumor growth and abdominal metastatic spread. Further analyses included microvessel density, lymphatic vessel density, and nodal invasion.

Results: Specimens from pancreatic neuroendocrine tumors and nontransformed pancreatic tissue revealed uniform expression of Ang-2 mRNA in endothelial cells. In contrast, epithelial expression of Ang-2 mRNA occurred exclusively in neuroendocrine tumors. Overexpression of Ang-2 in BON orthotopic xenografts did not affect primary tumor growth, although successful Ang-2 induction was confirmed from elevated serum levels. However, increased microvessel density and enhanced lymphatic metastasis were evident in Ang-2–expressing tumors, indicating a functional role of Ang-2 in experimental neuroendocrine tumors. Consistent with this notion, circulating Ang-2 was significantly elevated in neuroendocrine tumor patients compared with healthy controls. Circulating Ang-2 furthermore correlated with metastatic versus localized disease. The highest Ang-2 concentrations occurred in patients with liver metastasis, and concentrations ≥75th percentile predicted shorter survival (P = 0.0003).

Conclusion: Induction of Ang-2 in neuroendocrine tumors represents a clinically relevant pathomechanism of disease progression and constitutes an adverse prognostic marker. Clin Cancer Res; 16(2); 420–9. ©2010 AACR.

Neuroendocrine tumors of the gastroenteropancreatic system represent a diverse group of tumors with unique features and a highly variable prognosis (1). They are derived from the diffuse neuroendocrine system of the gastrointestinal tract and pancreas. Although considered rare, they are diagnosed with steadily increasing frequency, creating an urgent need for improved clinical management (2).

Antiangiogenic treatment of neuroendocrine tumors is currently being evaluated in several clinical trials (1–3).

This therapeutic approach is based on the success of antiangiogenic treatments drugs in other tumor entities such as colorectal, breast, and renal cancer, which provided proof of principle for the clinical benefit (4) and on the dense vascularization observed in a substantial portion of neuroendocrine tumors (5). However, in contrast to other solid malignancies such as breast and colon cancer, dense vascularization per se does not necessarily correlate to an unfavorable prognosis in neuroendocrine tumor disease (5–7). Rather, it was proposed to reflect a unique combination of physiologic endocrine and adaptive angiogenesis. Unfortunately, the exact mechanisms that control angiogenesis during malignant transformation and spread of neuroendocrine tumor are poorly characterized (1), although data on angiogenesis regulators such as vascular endothelial growth factor (VEGF), platelet-derived growth factor, and cKIT have recently been emerging (8, 9). Understanding these angioregulatory mechanisms is expected to yield important insights into neuroendocrine tumor biology. More importantly, it may be mandatory to

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maximally exploit the potential of antiangiogenic therapies for neuroendocrine tumors and/or correctly assign patients to such treatment modalities.

In this context, the angiopoietin family of angiogenic growth factors has received increasing attention as potent regulators of blood- and lymphangiogenesis (10–12). The two major and best characterized members, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), both bind to their shared membrane receptor, Tie-2, which is expressed on endothelial and lymphendothelial cells as well as on a subset of monocytes. Whereas Ang-1 functions as an agonist, Ang-2 acts context- and concentration-dependent as either antagonist or as a partial agonist on the Tie-2 receptor. Ang-2 was shown to be of central importance for postnatal angiogenic remodeling. In blood vessels, Ang-2 promotes vessel destabilization and regression via disruption of Tie-2 signaling, unless VEGF is present, which turns Ang-2 into an inducer of angiogenesis. On lymphendothelial cells, both Ang-1 and Ang-2 apparently promote Tie-2 activation (13). Thus, the relative abundance of Ang-1 and Ang-2 and the cellular context of Tie-2 activation all contribute to the outcome of Tie-2 activation, which may account for the diverse and occasionally conflicting biological effects that have been attributed to Ang-1 and Ang-2 (see ref. 12 for recent review).

Within the context of tumor biology, strong evidence supports a key contribution of angiopoietsins, notably of Ang-2, to the control of tumorangiogenesis in experimental settings (14–16). Furthermore, overexpression of Ang-2 is associated with advanced disease and poor prognosis in several tumor entities such as breast cancer, gastric cancer, melanoma, lung cancer, colorectal cancer, and hepatocellular cancer (15, 17–21).

Overexpression of Ang-2 in human pancreatic neuroendocrine tumors has been suggested from microarray expression profiling (22), but its specific biological role has not yet been explored.

Here we study the expression and localization of Ang-2 in neuroendocrine tumor tissue and determine circulating Ang-2 in serum samples of patients with neuroendocrine tumors. We furthermore use an orthotopic mouse model of BON pancreatic neuroendocrine tumors (23) to overexpress Ang-2 and study the functional consequences in the context of neuroendocrine tumor disease.

Materials and Methods

Antibodies were from following sources: Tie-2 from Santa Cruz Corporation, CD31 and CD34 from BD Pharmingen, and LYVE-1 from Relia Tech. Secondary antibodies were from Dianova. Rompun was from Bayer GmbH, Ketavet from Pharmacia GmbH.

Neuroendocrine tumor specimens and serum samples. A total of 35 neuroendocrine tumor tissue samples were obtained from individuals who underwent surgical resection at the Department of Surgery at Charité University Hospital during the period from 1998 through 2005. Serum samples (n = 90) were from neuroendocrine tumor patients treated at the Charité, Department of Internal Medicine, Division of Gastroenterology during the period from 1998 to 2005. The study was approved by the local ethics committee and all patients gave written informed consent prior to surgery. Tumor specimens were classified retrospectively according to the tumor-node-metastasis classification proposed by the European Neuroendocrine Tumor Society (24, 25). Survival data were obtained from a systematic review of the medical records. Healthy controls were employees at the Charité research facilities of the department or blood donors without medical history of a malignant disease.

Cell culture. BON cells were a generous gift from CM Townsend (Galveston, TX; ref. 26) and were cultured as described (27). Early passage cultures were used for generation of stable transfectants.

Angiopoietin-2 in situ hybridization. Cryosections of tissues from 10 pancreatic neuroendocrine tumors and from 8 specimens of nontransformed pancreas were analyzed. digoxigenin-UTP–labeled sense and antisense RNA-probes were generated by in vitro transcription (DIG-RNA Labeling Kit, Roche) of a 1,519-bp human Ang-2 cDNA fragment subcloned into the pBluescript II SK vector. The in situ hybridization procedure was done as previously described (19). A grading system for semiquantitative evaluation of Ang-2 mRNA expression was applied (grade 0, ≤5% of epithelial cells; grade 1, 5–25% grade 2, 25–75%; grade 3, ≥75%).

Immunohistochemical analyses. Cryostat sections (7–10 μm) of human and murine tissues were fixed in 4% paraformaldehyde, pH 7. Immunoperoxidase staining was done with an avidin-biotin-complex method (Vectastain Elite ABC
kit, Vector Laboratories) using 3-amino-9-ethylcarbazole (DAKO) substrate. Antibody dilutions were: Tie-2, 1:1000; CD31, 1:100; CD34 1:200; LYVE-1, 1:200. Primary antibodies were omitted in negative controls.

Cloning and generation of stable cell lines with expression of Ang-2. The Ang-2 full-length cDNA was a kind gift from F. Bussolino (Division of Molecular Angiogenesis, Institute for Cancer Research and Treatment, University of Torino, Torino, Italy; ref. 17) and was subcloned into the mammalian expression vector pSecTag2/Hygro A (pSecTag2-Ang-2). BON cells were transfected using Effectene transfection reagent (Quiagen) and stable clones were selected and expanded in culture medium supplemented with 400 μg/mL hygromycin B.

Determination of Ang-2 and soluble fragments of Tie-2 concentration in serum and culture supernatants. Levels of Ang-2 and soluble fragments of its receptor Tie-2 (sTie-2) were determined from frozen serum samples and tissue culture supernatants using a human Ang-2 and sTie-2 ELISA (both R&D Systems GmbH) according to the manufacturer's instructions.

Growth assays. Cells were plated in a 24-well dish at a concentration of 20,000 cells/well. Cells were allowed to attach overnight. Alamar blue dye (10% v/v; Biosource International Inc.) was added to the cultures for 3 h every 24 h, and fluorescence was determined at 560 nm excitation and 590 nm emission wavelength.

Tumor implantation. Female NMR1nu/nu mice (21-25 g) were from Bomholtgard. Animal care followed institutional guidelines, and experiments were approved by local animal research authorities. Mice were anesthetized by i.p. administration of Ketavet (100 mg/kg) and Rompun (10 mg/kg). For tumor induction, the pancreas was exposed and 1 × 10⁶ BON cells were injected into the head of the pancreas. After 8 wk, the mice were sacrificed, blood samples were collected, and primary tumors were removed and weighed. Enlarged lymph nodes and macroscopically evident metastases were collected for histology. Macroscopic metastases were defined as visible intra-abdominal suspect lesions with a diameter ≥1 mm with subsequent histologic confirmation of tumor cells. Moreover, liver tissue and liver hilus lymph nodes were routinely obtained.

Determination of microvessel density and lymphatic microvessel density. For quantification of microvessel density (MVD) and lymphatic vessel density (LVD), respectively, the average number of CD31-, CD34-, and LYVE-1-positive vessels in a 0.6 mm² measurement area was determined from three regions of maximal vascular density (vascular hotspots; ref. 28). Observers were blinded to the experimental conditions when evaluating vessel densities.

Detection of BON cells in mouse lymph nodes. Genomic DNA of mouse mesenteric, liver hilus, and all suspect abdominal lymph nodes was extracted using the QiaAmp tissue kit (Qiagen). Routinely, two lymph nodes per mouse were analyzed (one from the liver hilus, one mesenterial lymph node). The presence of human, BON cell-specific DNA was detected based on PCR amplification of an
850-bp fragment of the -satellite region of the human chromosome 17, exactly as described (29). DNA from BON cell \textit{in vitro} cultures and from non–tumor-bearing mice were used as positive and negative controls.

\textbf{Statistical analysis.} Data analysis was done by ANOVA utilizing the Kruskal-Wallis test, Mann Whitney or Fisher’s exact test (Graph Pad Software) unless stated otherwise. Differences were considered significant at \( P < 0.05 \). Receiver operating characteristic and Kaplan-Meier curves were calculated using Graph Pad Prism.

\textbf{Results}

\textit{Ang-2 is elevated in the serum of patients with neuroendocrine tumors of the gastroenteropancreatic system.} Given that Ang-2 is secreted and released into systemic circulation, we initially determined the Ang-2 content of serum samples obtained from neuroendocrine tumor patients (\( n = 90 \)) and from healthy controls (\( n = 40 \); Fig. 1A). The median Ang-2 concentration was 2,650 pg/mL in control sera from healthy individuals versus 4,048 pg/mL in sera from neuroendocrine tumor patients (\( P < 0.001 \)), strongly suggesting an involvement of Ang-2 in the pathobiology of neuroendocrine tumors. This led us to further specify the source of Ang-2 induction. We utilized \textit{in situ} hybridization to identify the neuroendocrine tumor compartments where Ang-2 mRNA is expressed (Fig. 1B). As expected, we could detect a distinct hybridization signal in endothelial cells surrounding vascular structures. However, we also found specific signals in a high fraction of tumors cells (middle) but not in the corresponding nontransformed pancreatic tissue (left). Of the 10 neuroendocrine tumor tissues, 6 revealed Ang-2 mRNA expression in >75% of tumor cells, 2 in 25% to 50%, and 2 in 5% to 25%, suggesting that \textit{de novo} expression of Ang-2 in neuroendocrine tumor cells contributed to the elevated levels of circulating Ang-2 in neuroendocrine tumor patients.

\begin{itemize}
  \item Next, we determined the expression of the Tie-2 receptor in neuroendocrine tumor samples using immunohistochemistry (right). Distinct reactivity was observed in endothelial structures, but not in the neuroendocrine tumor cells. The endothelial identity of Tie-2 immunoreactive cells was subsequently confirmed via staining of serial sections with the endothelial marker CD31.
\end{itemize}
Combined, the presence of Ang-2 and its receptor in neuroendocrine tumor tissues suggested that an autocrine or paracrine action of the angioregulatory growth factor occurred in neuroendocrine tumors.

**Ang-2 overexpression in orthotopic pancreatic neuroendocrine tumor xenografts stimulates neangiogenesis.** To experimentally address the functional relevance of Ang-2 induction in neuroendocrine tumors, we chose to study the consequences of Ang-2 overexpression in BON orthotopic pancreatic neuroendocrine tumors in vivo. This approach was feasible because BON wild-type cells did not produce detectable levels of Ang-2 in vitro and thus could be transfected with a full-length Ang-2 expression construct to generate stable clones with abundant Ang-2 production. Three clones were selected based on the Ang-2 levels present in their culture supernatants and three mock transfected clones were chosen as controls (Fig. 2A). In vitro growth of all six clones was followed over a period of 6 days without revealing differences in proliferation or apoptosis, suggesting that Ang-2 did not directly affect BON cell growth in an autocrine fashion (Fig. 2B). Ang-2–expressing clones and mock controls were then implanted orthotopically in the pancreas of nude mice and were allowed to form tumors over a course of 8 weeks. Mice were sacrificed, blood was collected, and primary tumor growth and metastatic spread were evaluated (Fig. 2C). Serum samples were used to determine the level of BON cell–derived, human Ang-2. Human Ang-2 was readily detected in mice bearing tumors from Ang-2–overexpressing BON clones, but not from control cells, indicating that in vivo expression of Ang-2 had been successfully achieved (Fig. 2C, middle). However, Ang-2 induction had not affected primary tumor growth, as neither tumor weight nor tumor volume differed from control tumors (Fig. 2C, right, and data not shown). Nonetheless, a trend towards more frequent macroscopic metastases (histologically confirmed suspect lesions of ≥1 mm diameter liver) was noted in animals bearing Ang-2–expressing tumors (25% of animals versus 8% of control animals; Fig. 3A), and liver metastasis occurred exclusively in this group (7% versus none). This prompted us to test whether Ang-2 expression had effects on tumor vascularization. Indeed, Ang-2–expressing tumors displayed a significant increase in MVD, as measured via immunodetection of CD34 on endothelial cells (Fig. 3B). Thus, tumor cell–derived Ang-2 promoted tumor vascularization in experimental pancreatic neuroendocrine tumors, which was reflected in a concomitant trend towards more frequent metastasis formation.

**Ang-2 increases nodal spread of orthotopic pancreatic neuroendocrine tumor xenografts.** Although hematogeneous

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**Fig. 3.** Ang-2 expression promotes lymph node metastasis of orthotopic BON pancreatic neuroendocrine tumors. A, at autopsy, the abdominal situs was inspected, macroscopically suspect lesions were recorded, and the presence of human metastatic cells was subsequently confirmed by immunohistochemistry with a human specific pan-cytokeratin antibody (left); metastatic cells stain red. Graph shows a comparison of histologically confirmed abdominal metastatic lesions in animals bearing Ang-2–expressing tumors versus control tumors (8 versus 2 lesions). B, determination of MVD in primary tumors. Immunostaining was conducted with an endothelial-specific anti-CD34 antibody. MVD was calculated based on the CD34 staining. Shown are the mean values of mock- and Ang-2 tumor-bearing animals. **P** < 0.05, Fisher’s exact test. C, analysis of lymph node metastasis by human microsatellite PCR (left) and summary graph depicting the percentage of lymph nodes that tested positive for metastatic cells. **P** < 0.05, Fisher’s exact test.
metastasis to the liver represents the primary site of metastasis for neuroendocrine tumors, lymphatic spread of tumor cells significantly contributes to disease progression. Accordingly, we examined whether Ang-2 expression had affected the capacity of neuroendocrine tumor cells to invade draining lymph nodes (Fig. 3C). The presence of tumor cells in lymph nodes was assessed via amplification of human microsatellite DNA, which yielded specific amplification products in almost 100% of the animals bearing Ang-2–expressing tumors as compared with 75% of animals with control tumors (P < 0.05). Thus, Ang-2 had significantly increased lymph node metastasis. Because lymphangiogenesis has been identified as one important determinant of lymphatic metastasis we also measured LVD in the primary tumors. However, LVDs revealed considerable variability within groups, such that no significant differences were obtained (data not shown).

Taken together, animals bearing Ang-2–expressing tumors more frequently suffered from nonlocalized disease, possibly due to increased tumor vascularization.

**Levels of circulating Ang-2 correlate with metastatic disease in neuroendocrine tumor patients.** These experimental observations raised the question of whether the elevated Ang-2 serum levels observed in patients with neuroendocrine tumors might conversely reflect nonlocalized, metastatic disease in the clinical situation. Of the 90 neuroendocrine tumor patients, for whom Ang-2 serum levels had been determined, a subset of 42 patients was identified, for whom blood sampling prior to surgery, disease staging according to the recently proposed tumor-node-metastasis classification, and long-term follow-up were available. This subgroup was well matched with respect to gender and tumor functionality. Pancreatic neuroendocrine tumors were strongly represented and constituted 60% of cases, followed by ileal tumors (29%). More details on patients’ characteristics are given in Table 1. In sera from this subgroup, a median Ang-2 concentration of 3,833 pg/mL was calculated (Fig. 4A), which was significantly elevated when compared with healthy controls (P = 0.0085), although slightly lower than the value obtained from the larger group of neuroendocrine tumor patients. Furthermore, a correlation of Ang-2 and CgA concentrations was evident (n = 41, P = 0.0043, r = 0.437, data not shown). We then attempted to correlate the level of circulating Ang-2 to several clinicopathologic parameters (Table 2). No correlation was obtained for localization of the primary or functionality. However, circulating Ang-2 was significantly increased in grade 3 neuroendocrine tumors. Elevated Ang-2 levels also correlated with metastatic disease (Fig. 4B), a finding that was further accentuated in patients with liver metastases. Because Ang-2 may be sequestered and inactivated by sTie-2, we also determined the abundance of this endogenous inhibitor. However, use of the Ang-2/sTie-2 ratio did not improve the correlation to clinicopathologic parameters (not shown). In a subset of 31 neuroendocrine tumor patients, in which corresponding serum and tissue samples were available, we also determined MVDs (median, 42/0.6 mm²). High concentrations of circulating Ang-2 were not associated with higher MVDs, but rather revealed a borderline significant inverse correlation (r = -0.3634, P = 0.045).

**High levels of circulating Ang-2 represent an adverse prognostic factor in neuroendocrine tumor disease.** Taken together, high circulating Ang-2 levels were present in patients with advanced disease. Based on these findings we hypothesized that Ang-2 determination in serum samples might serve as a prognostic indicator. Accordingly, patients were allocated to a group with high (>75th percentile of overall group) or low and intermediate (<75th percentile) levels of circulating Ang-2, and Kaplan-Meier curves were generated (Fig. 4C). The median follow-up period was 9 months from the time of blood sampling. Survival was significantly different in the two groups, with a median survival time of 13 months in patients with high Ang-2 serum levels as compared with undefined survival time in the group with medium or low Ang-2 serum concentration (P = 0.0003). This difference persisted during prolonged follow-up of 59 months (P = 0.0101). Thus, determination of circulating Ang-2 enabled the identification of patients with very advanced disease and/or high risk of rapid disease progression.

**Discussion**

Neuroendocrine tumors have long been known to be highly vascularized. With the advent of antiangiogenic cancer treatment modalities, the dense vascularization has received increasing attention as a putative therapeutic target. Surprisingly, however, concepts of tumor neoangiogenesis that have been emerging from other tumor entities do not readily apply to neuroendocrine tumors. In contrast to the widely applicable concept that high VEGF production and dense vascularization constitute adverse prognostic parameters, their significance has remained controversial in neuroendocrine tumors, most notably in

<table>
<thead>
<tr>
<th>Table 1. Demographic information and tumor characteristics of patients included in the analysis of circulating Ang-2</th>
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<tbody>
<tr>
<td><strong>Patient characteristics (n = 42)</strong></td>
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<tr>
<td><strong>Median age, y</strong></td>
</tr>
<tr>
<td><strong>Male</strong></td>
</tr>
<tr>
<td><strong>Female</strong></td>
</tr>
<tr>
<td><strong>Localization of the primary tumor</strong></td>
</tr>
<tr>
<td>Pancreas</td>
</tr>
<tr>
<td>Ileum</td>
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<tr>
<td>Duodenum</td>
</tr>
<tr>
<td>Cecum</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Functionality</strong></td>
</tr>
<tr>
<td>No</td>
</tr>
<tr>
<td>Yes</td>
</tr>
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<td>Unknown</td>
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</table>

Published OnlineFirst January 12, 2010; DOI: 10.1158/1078-0432.CCR-09-1924
pancreatic neuroendocrine tumors (5, 9). For instance, the dense vasculature of low-grade pancreatic neuroendocrine tumors with high VEGF expression was found quiescent, and the regular vascular architecture of these tumors was reminiscent of the situation in nontransformed endocrine tissues (30). By comparison, high-grade pancreatic neuroendocrine tumors (30). By comparison, high-grade pancreatic neuroendocrine tumors with high VEGF expression was found quiescent, and the regular vascular architecture of these tumors was reminiscent of the situation in nontransformed endocrine tissues (30). By comparison, high-grade pancreatic neuroendocrine tumors provided functional evidence that Ang-2 participates in the control of neuroendocrine tumor angiogenesis and/or remodeling. Indeed, the phenotypes of mice with genetic gain or loss of function alterations in the angiopoietin/Tie-2 system have firmly implicated Tie-2 signaling in the control of endothelial cell activation and vessel maturation (reviewed in ref. 12). Also, reduced pericyte coverage, endothelial cell apoptosis, and dysfunctional vessels were reported in a murine breast cancer model using a cell line with overexpression of Ang-2, although overall vessel abundance was increased (31). In human tumor samples, reduced pericyte coverage of tumor vessels was noted in renal cell carcinomas with high Ang-2 expression (32). Thus, it seems conceivable that high-level Ang-2 expression supports a more immature vessel phenotype in malignant neuroendocrine tumors. In consequence, circulating Ang-2 levels might provide a biomarker that reflects the adaptive component of neuroendocrine tumor angiogenesis.

The data obtained from BON orthotopic pancreatic neuroendocrine tumors provided functional evidence that Ang-2 induction has the capacity to regulate selected aspects of tumor growth. In this well-defined xenograft model, Ang-2 increased the MVD of primary tumors, consistent with the increased vessel abundance associated with Ang-2 in none-endoendocrine carcinomas (31). Such proangiogenic effects of Ang-2 were previously shown to depend on the concurrent expression of Ang-2 in neuroendocrine tumor patients; we found (a) de novo expression of Ang-2 in neuroendocrine epithelial tumor cells and expression of both Ang-2 and its cognate receptor in neuroendocrine tumor vasculature. (b) increased vascularization and lymphatic metastasis following overexpression of Ang-2 in a mouse model of pancreatic neuroendocrine tumors, and (c) elevated levels of circulating Ang-2 in the serum of neuroendocrine tumor patients; which are clearly associated with adverse prognosis, although possibly with lower MVDs.

These observations raise the intriguing possibility that circulating Ang-2 concentration in neuroendocrine tumor patients prior to or without surgery. Lines in scatter dot plot, median and interquartile range; n = 40 for healthy controls and n = 42 for neuroendocrine tumor patients, P = 0.0085. B, serum Ang-2 levels in nonmetastatic (n = 9) versus metastatic (n = 28) disease. Lines in scatter dot plot, median with interquartile range. C, survival of neuroendocrine tumor patients with high versus low levels of circulating Ang-2. Patients were allocated to a group with low (<75% percentile, n = 11) or high (≥75% percentile, n = 31) serum levels of Ang-2 levels, and Kaplan-Meier analyses were conducted. Median survival was 13 mo in patients with high Ang-2 levels versus undefined. Hazard ratio, 0.04838; 95% confidence interval, 0.009203-0.2544; P = 0.0085. Median survival of neuroendocrine tumor patients with high Ang-2 levels and axillary lymph node invasion has previously been reported in breast cancer (17). Interestingly, Ang-2 expression in these breast cancer samples was present not only in the endothelial compartment, but also on the transformed epithelial cells, similar to the experimental situation created in the BON xenograft tumors. An
Ang-2 Promotes Progression of Neuroendocrine Tumors

The clinical relevance of results obtained from in vivo studies using manipulated neuroendocrine tumor cell lines has been challenged (1, 40). This is a valid concern with Ang-2, as previously published effects of Ang-2 over-expression in experimental in vivo models have been highly variable, likely reflecting the unique and complex functional network of Ang-1, Ang-2, Tie-2, sTie-2, and VEGF in the tumor microenvironment in each of these experimental model systems (reviewed in ref. 12). In addition, results from the BON cell model may only be applicable to the subgroup of pancreatic neuroendocrine tumors (41). In the current study, however, the proposed relevance of Ang-2 for the tumor biology of neuroendocrine tumors is firmly supported by the analysis of circulating Ang-2 in the serum of neuroendocrine tumor patients.

In line with data from a recent study (42), we find that Ang-2 serum levels are elevated in neuroendocrine tumor patients with metastatic disease, in particular with presence of liver metastasis. Furthermore, when using the 75th percentile of Ang-2 measurements as cutoff, circulating Ang-2 was a highly significant prognostic factor with respect to patient survival both after 9- and 59-month follow-up periods. In comparison, the absence or presence of metastases did not predict better or worse survival, suggesting a better prognostic performance for the biomarker Ang-2. Although a recent report by Srirajaskanthan et al. (42) associated above-median Ang-2 serum concentration with shorter time-to-disease progression, the median Ang-2 serum concentration was less suitable as a cutoff to identify subgroups with different survival in our study ($P = 0.0409$ and $P = 0.31$ after short and prolonged follow-up, respectively). Also, MVD was not per se associated with patients’ survival, underscoring the potential prognostic benefit obtained by measuring circulating Ang-2. We also noted particularly high levels of circulating Ang-2 in patients with poorly differentiated neuroendocrine tumors. Given the limited value of CgA in this subgroup of patients, Ang-2 might prove useful for tumor follow-up under these conditions.

### Table 2. Correlation of circulating Ang-2 with clinico-pathologic parameters

<table>
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<tr>
<th>Category</th>
<th>$n$</th>
<th>Median Ang-2 (pg/mL)</th>
<th>$P$</th>
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<td>Localization of the primary tumor</td>
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<tr>
<td>Pancreas</td>
<td>25</td>
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<td>n.s.</td>
</tr>
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<td>Ileum</td>
<td>12</td>
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<tr>
<td>Cecum</td>
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<tr>
<td>Nonfunctional</td>
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<td>Tumor-node-metastasis classification</td>
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<tr>
<td>$T_1$</td>
<td>4</td>
<td>2,308</td>
<td>n.s.</td>
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<tr>
<td>$T_2$</td>
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<tr>
<td>$M_1$</td>
<td>29</td>
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<td>2,096</td>
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<tr>
<td>With liver metastases</td>
<td>28</td>
<td>4,546</td>
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<tr>
<td>Grading</td>
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<tr>
<td>$G_1$</td>
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<td>0.04 ($G_2$ vs $G_3$)</td>
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<tr>
<td>$G_3$</td>
<td>5</td>
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</table>

Abbreviation: n.s., not significant.

association of Ang-2 expression and presence of lymph node metastasis was furthermore reported for non–small cell lung cancer specimens (36). Moreover, Tie-2 activation was recently shown to facilitate the access of tumor cells into the vascular system and their dissemination (37). Given that Ang-2 acts as an agonist of Tie-2 receptors on lymphatic endothelial cells, but as an antagonist on blood endothelial cells, this mechanism could provide an explanation for the enhancement of lymphatic metastasis without alteration of LVD in BON tumors. Finally, Ang-2 stimulation of lymph node metastasis may occur in a Tie-2–independent fashion via interaction with the fibronectin receptor α5β1 integrin, as shown in a breast cancer model (38). As BON cells do express the fibronectin receptor in vitro (39), Tie-2–independent, direct effects of Ang-2 on the tumor cells could have contributed to enhanced metastasis formation.

For the interpretation of our in vivo experimental results it should be kept in mind that Ang-2 derived from the host vasculature was likely present in control and Ang-2–expressing tumors. This is important when considering Ang-2 as a therapeutic target, as functional inactivation of both endothelial cell and tumor cell–derived Ang-2 might permit more efficacy than anticipated from the current data. An elegant experimental study used Ang-2–deficient mice as hosts, to dissect the impact of host-derived Ang-2 in a melanoma model (14). Tumor growth was found impaired during early, but not later stages of tumor development when compared with Ang-2–competent wild-type mice. Within the limitations of the end point analysis inherent to the orthotopic approach used in the current study, Ang-2 expression in tumor cells apparently affected later stages of tumor development. Whether such differences reflect discrete functions of Ang-2 in the endothelial as compared with the tumor cell compartment, or differences in the overall abundance of Ang-2 is currently unclear. Our analysis of clinical pancreatic neuroendocrine tumor specimen clearly documented mRNA expression in tumor cells, suggesting that Ang-2 directed therapies could act both at the level of the transformed epithelial and tumor endothelial cells.
Similar observations of potential prognostic use of Ang-2 have been reported in selected tumor entities such as hepatocellular carcinoma (19), melanoma (15), or lung cancer (20), although elevated serum levels are not necessarily prognostic indicators (12).

Taken together, our clinical and experimental data strongly link Ang-2 to disease progression of neuroendocrine tumors. Importantly the current study provides functional evidence for the capacity of Ang-2 to advance systemic disease. In view of Ang-2 directed experimental therapeutics that have entered phase II clinical trials (43), future work to experimentally address the benefit of targeting Ang-2 in preclinical neuroendocrine tumor models is urgently required. In addition, our data advocate further validation of Ang-2 as a much needed prognostic marker in advanced neuroendocrine tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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