The association of the angiopoietin/Tie-2 system with the development of metastasis and leukocyte migration in neuroendocrine tumors

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Abstract

The aim of this study was to explore the possible involvement of the angiopoietin (Ang)-1, -2/Tie-2 system in the development, growth, and metastases evolution of gastroenteropancreatic-neuroendocrine tumors (GEP-NETs). We prospectively examined the serum levels of Tie-2, Ang-1, and Ang-2 by ELISA in 42 patients with proven GEP-NETs and 27 controls. We also determined the expression of the Ang/Tie-2 system in freshly isolated peripheral blood monocytes and in tumor cells from malignant primary tumors and/or liver metastases samples from GEP-NET patients by flow cytometry and/or RT-PCR. Furthermore, the function of the Ang/Tie-2 system in monocytes from controls and patients was assessed by a chemotaxis assay. GEP-NET patients showed enhanced serum levels of soluble form of Tie-2 (sTie-2), Ang-1, and Ang-2 ($P < 0.05$ in all cases), compared to controls. sTie-2 and Ang-2 levels were significantly higher in GEP-NETs with metastases compared to those with no metastases. In addition, a significant correlation was detected between Ang-2 levels and chromogranin A or sTie-2 concentrations or 5-hydroxy-indole acetic acid excretion ($r = 0.71$, $r = 0.60$, and $r = 0.81$ respectively, $P < 0.01$ in all cases). Furthermore, we observed an enhanced expression of Ang-1, Ang-2, and Tie-2 in freshly isolated tumor cells from GEP-NET both by immunohistochemistry and by RT-PCR. Interestingly, an enhanced expression and function of Tie-2 was detected in monocytes from GEP-NET patients. Our data suggest that the Ang/Tie-2 system is involved in the growth and development of metastases of GEP-NETs, and that favors the recruitment of Tie-2⁺ monocytes to the tumor site, where they can promote inflammation and angiogenesis.

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Introduction

Neuroendocrine tumors (NETs) comprise a heterogeneous group of uncommon neoplasms that include the gastroenteropancreatic (GEP)-NETs, which

constitute 70% of all NETs and 2% of all digestive tract tumors (Modlin *et al.* 2008, Oberg 2009). These GEP-NETs are originated from enterochromaffin epithelial cells, which retain many structural and

functional features of normal endocrine cells, including the synthesis of chromogranin A (CgA), synaptophysin, peptidic hormones, and neuropeptides (Lubensky & Zhuang 2007, Modlin *et al.* 2008). According to the WHO (Solcia *et al.* 2000), these tumors are classified into well-differentiated NETs and carcinomas (wdNET and wdNEC respectively), and poorly differentiated neuroendocrine carcinomas (pdNEC). Moreover, pancreatic endocrine tumors (PETs) are classified into functioning (insulinoma, gastrinoma, glucagonoma, somatostatinoma, and VIPoma) or nonfunctioning neoplasias (Ehehalt *et al.* 2009). Carcinoid tumors are the most frequent gastrointestinal NETs and are able to secrete different molecules, including CgA and 5-hydroxy-indole acetic acid (5-HIAA) that are used as markers for the diagnosis and follow-up of these patients (Haverback *et al.* 1956, Rindi *et al.* 1996, von Wichert *et al.* 2008).

Angiogenesis, the growth of new blood vessels from pre-existing normal vasculature, occurs in different tissues under physiological (embryogenesis and wound repair) and pathological (cancer and inflammatory diseases) conditions (Carmeliet 2003, Szekanecz & Koch 2007). Among the molecules that exert an important regulatory effect on angiogenesis are included the vascular endothelial growth factor (VEGF), angiopoietins (Ang)-1 and -2, and the tyrosine kinase receptor Tie-2 (or Tek) family (Ferrara & Davis-Smyth 1997, Jones 2003, Eklund & Olsen 2006). VEGF acts as a pro-angiogenic factor on vascular endothelium, inducing their proliferation and new microvessel formation (Ferrara & Davis-Smyth 1997, Chang *et al.* 2009). On the other hand, Angs comprise four soluble proteins (Ang-1, Ang-2, Ang-3, and Ang-4), which bind to the unique receptor Tie-2 (Eklund & Olsen 2006). These molecules are secreted by endothelial and epithelial cells, and their synthesis is induced by pro-inflammatory cytokines, hypoxia, and stress (Jones 2003, Eklund & Olsen 2006, Fiedler & Augustin 2006). Interaction of Ang-1 with Tie-2 promotes endothelial cell (EC) survival (Eklund & Olsen 2006, Fiedler & Augustin 2006). In addition, it has been reported that Ang-1 exerts an anti-inflammatory and anti-permeable effect. In contrast, Ang-2 is expressed at sites of vascular remodeling, and its binding to Tie-2 causes vasculature regression (Maisonpierre *et al.* 1997). However, this cytokine exerts a marked pro-angiogenic effect when VEGF is present (Lobov *et al.* 2002). Thus, Ang-2 seems to be closely related to physiological or pathological processes that involve vascular network remodeling (Lobov *et al.* 2002, Fiedler *et al.* 2006).

The tyrosine kinase Tie-2 receptor is mainly expressed by EC, and its synthesis is stimulated by hypoxia and pro-inflammatory cytokines. There is a soluble form of Tie-2 (sTie-2), which is generated by proteolytical cleavage at the cell surface. Although little is known about the physiological role of this soluble receptor, it has described an increase of sTie-2 in several conditions such as hepatitis C, autoimmune thyroid diseases, and cancer (Salcedo *et al.* 2005, Niedźwiecki *et al.* 2006, Figueroa-Vega *et al.* 2009). In addition, some hematopoietic cells such as CD34⁺ stem cells and CD14⁺ monocytes express this receptor (De Palma *et al.* 2005, Murdoch *et al.* 2007). In this regard, Tie-2⁺ monocytes have been detected in the peripheral blood from healthy subjects or patients with different tumors, and it has been reported that these cells are recruited into inflamed or neoplastic tissues, where they can promote angiogenesis, favoring the growth of tumor cells (Venneri *et al.* 2007). Thus, it has been shown that the selective depletion of Tie-2⁺ monocytes inhibits angiogenesis and tumor growth in animal models (De Palma *et al.* 2007). However, the expression and possible role of Tie-2 in monocytes from GEP-NET patients on the development of metastases have not been previously addressed.

Since increased serum levels of Ang-2 have been recently reported in GEP-NET patients (Srirajaskanthan *et al.* 2009), we decided to further study the possible role of the Ang/Tie-2 system in the pathogenesis of GEP-NETs. For this purpose, we analyzed the serum levels of Tie-2, Ang-1, and Ang-2 in GEP-NET patients as well as their expression in freshly isolated tumor cells. In addition, we explored the number and function of Tie-2⁺ monocytes in patients with GEP-NETs. Our results suggest a role of this angiogenic system in the growth and the development of metastases of GEP-NETs.

Materials and methods

Individuals

Forty-two patients with GEP-NETs were studied (19 with carcinoid tumor and 23 with PETs). All patients were carefully screened for the presence of other malignancies, and special attention was paid to an association with neurofibromatosis, multiple endocrine neoplasia type 1, and the von Hippel–Lindau syndrome (Table 1). One patient was carrier of a *MEN-1* gene mutation, but no other apparent genetic abnormalities were found. According to the WHO criteria (tumor site and size, angioinvasion, infiltration level, cell proliferation index, immunohistochemical phenotype, and

Table 1 Clinical and pathological features of gastroenteropancreatic-neuroendocrine tumors' (GEP-NETs) patients. Values correspond to the mean $\pm$ s.d.

Gender (M/F)	15/27
Age (years, mean $\pm$ s.d.)	57.64 $\pm$ 14.76
CgA (nmol/l)	20.84 $\pm$ 27.79
5-HIAA (mg/24 h)	10.64 $\pm$ 9.94
Carcinoids	19
Appendix	1
Colon	2
Duodenum	1
Intestine	11
Rectum	2
Stomach	2
PETs	23
Gastrinoma	3
Insulinoma	1
Nonfunctioning pancreatic tumor	19
Hormone secretion (yes/no)	15/27
Distant metastases (yes/no)	28/14
Localization of metastases	
Liver	23
Lymph nodes	20
Bones	3
Angioinvasion (yes/no) ^a	17/17
Tumor stage (wdNET/wdNEC) ^b	14/28
Survival	
Progression	13
Stabilization	12
Curation	15
Exitus	1
Previous treatment	
Carcinoid	
SA	7
SA + IFN- α	3
Functional	
SA	1
C	1
Nonfunctional	
SA	7
C	1
SA + IFN- α	2
SA + C	1
Surgery (yes/no)	
Carcinoids	10/6
Functional	2/2
Nonfunctional	11/8

M, male; F, female; CgA, chromogranin A; 5-HIAA, 5-hydroxy-indole acetic acid; PETs, pancreatic endocrine tumors; NFPT, nonfunctioning pancreatic tumor; wdNET, well-differentiated neuroendocrine tumors; wdNEC, well-differentiated neuroendocrine carcinoma; SA, somatostatin analogs; IFN- α , interferon α ; C, chemotherapy. Serum CgA levels: < 4.0 nmol/l; urinary output of 5-hydroxy-indole acetic acid: 2–14 mg/24 h.
^aUnknown in eight patients.
^bWHO classification.

metastases) and histopathological findings, GEP-NETs were classified as wdNETs, wdNECs, and pdNECs (Table 1; Solcia *et al.* 2000). Cell proliferation activity was determined by counting Ki-67⁺ cells, as described

(Rindi *et al.* 2006). In all cases, blood samples were obtained at least 4 weeks after the last dose of somatostatin analogs and 3 days after the last dose of interferon.

Complete work-up including history, physical examination, and hormone levels was performed in all cases. Patients were classified according to the localization of the primary tumor (foregut, midgut, hindgut, and unknown), hormone secretion (functional or nonfunctional), presence or absence of metastases, and presence or absence of angioinvasion. Controls included 27 age-matched healthy subjects who also had complete hormonal work-up. In all cases, a written informed consent was obtained. This study was approved by the local Hospital Bioethical Committee.

Samples

Peripheral blood samples were obtained from all individuals. Freshly isolated tumor cells were obtained during surgical resection in three primary GEP-NET tumors and in three liver metastases. Paraffin-embedded formalin-fixed tissues had been obtained by surgical resection from 12 patients (4 wdNETs and 8 wdNECs), out of whom 5 were carcinoids and 7 were PETs. Normal control tissues were obtained from three patients undergoing pancreatectomy or liver resection, and were used as negative controls. All samples were taken in accordance with the regulations and approval of the Institutional Review Board of Hospital Universitario de la Princesa.

Laboratory evaluation

Serum CgA levels were measured by radioimmuno-metric assay (Euro-Diagnostica AB, Malmö, Sweden), with a reference range of < 4.0 nmol/l. In addition, urinary excretion of 5-HIAA was determined by ELISA (IBL International GmbH, Hamburg, Germany) with a reference range 2–14 mg/24 h.

Cell isolation

Tumor cells were isolated from three primary and three metastatic tumors as follows: briefly, tissue specimens were minced and digested with collagenase (1 mg/ml; Boehringer Mannheim, Roche) during 1 h and passed through a steel mesh. Then, cells were washed in HBSS (Lonza, Verviers, Belgium), and cell viability was assessed by trypan blue dye exclusion, and it was always higher than 95%.

Angiogenesis serum markers measurement

Levels of sTie-2, Ang-1, Ang-2, and VEGF were determined by ELISA (Quantikine, R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) following the manufacturer's instructions and employing a Tecan Sunrise ELISA reader and Magellan software for windows (Tecan, Barcelona, Spain). The lower detection limits for sTie-2, Ang-1, Ang-2, and VEGF were 1.0, 1.36, 1.20, and 9.0 pg/ml respectively. All analyses were performed by duplicate.

Immunohistochemistry

Tissue sections were dewaxed, rehydrated, and washed in Tris-buffered saline (Dako Cytomation, Copenhagen, Denmark). Antigen retrieval was done by incubation in 10 mM citrate buffer (pH 6.0; Master Diagnostica, Granada, Spain) in a pressure cooker. Next, endogenous peroxidase was inhibited with a peroxidase-blocking solution (Dako Cytomation) during 10 min, and then Fc receptors were blocked with goat or rabbit serum, as appropriate. Then, sections were immunostained with the indicated antibodies (Santa Cruz Biotechnology Inc., Heidelberg, Germany) and the proper HRP-conjugated secondary Abs (Envision Dako Cytomation). Finally, sections were developed with 3,3'-diaminobenzidine (Envision Dako Cytomation), counterstained with Carazzi's hematoxylin, dehydrated in alcohol, cleared with xylene, and mounted.

Flow cytometry analysis

Cell surface expression of Tie-2 was assessed on freshly isolated tumor cells or peripheral blood monocytes by staining with a phycoerythrin (PE)-conjugated anti-human Tie-2 (R&D Systems) mAb or an isotype-matched irrelevant mAb. For detection of intracellular expression of Ang-1 and Ang-2, cells were permeabilized, fixed, and then incubated with goat polyclonal anti-human Ang-1 or Ang-2 Abs (R&D Systems), followed by a donkey anti-goat IgG F(ab')₂ secondary Ab labeled with Alexa Fluor 488 (Invitrogen). As positive controls for neuroendocrine cell staining, anti-synaptophysin and anti-CgA mAbs were employed (Master Diagnostica). Cell analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Migration assays

Chemotaxis assays were performed on Transwell chambers with 5.0 µm pore size inserts (Corning, Corning, NY, USA). Lower chambers were filled with

RPMI-1640 culture media supplemented with 0.2% human serum albumin, and with or without recombinant human Ang-1 or Ang-2 at 100 ng/ml (R&D Systems). Monocytes (5×10^5 in 100 µl) were poured into the upper chamber, and after 2 h of incubation at 37 °C, filters were removed, fixed, and stained with 4'-6-diamidino-2-phenylindole. Cells that had migrated and were attached to the lower side of the membrane were counted per field with an epifluorescence microscope. Control conditions were included to cells pretreated with 5.0 µg/ml of neutralizing anti-Tie-2 mAb (Santa Cruz Biotechnology Inc.) or the addition of MCP-1 (CCL2) at 100 ng/ml (R&D Systems) to the lower chamber. Results were expressed as the percent of increase of cell migration referred to the baseline condition (medium alone = 0%).

Quantitative real-time RT-PCR

Total RNA was extracted from snap-frozen GEP-NETs or normal control tissues using the Ultraspec reagent, according to the manufacturer's protocol (Biotec Laboratories, Inc., Houston, TX, USA), and isolated RNA was quantified with a Nanodrop ND-100 u.v. Spectrophotometer (NanoDrop Technologies). Then, mRNA was reverse transcribed using random hexamers and the Moloney murine leukemia virus reverse transcriptase (Promega Biotech Ibérica, S.L.). Real-time quantitative RT-PCR was performed by duplicate employing the LightCycler Detection System (Roche Diagnostics GmbH), and using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH). Design of PCR primers was performed by using the Primer3 software. Results were normalized according to the value of the histone H3 housekeeping gene. All analyses were performed by duplicate.

Statistical analysis

Data are showed as the arithmetic mean $\pm$ S.D., mean $\pm$ S.E.M., or the median and interquartile range, as appropriate. For continuous variables, normality was assessed by the Kolmogorov–Smirnov test. Data were analyzed using both parametric and nonparametric tests. Mean group values were compared by one-way ANOVA or nonparametric ANOVA (Kruskal–Wallis test), and *post hoc* comparisons were carried out using the Bonferroni's and Dunn's test respectively. Pearson's and Spearman's correlation coefficients were determined for variables with a bivariate normal distribution or not. Partial correlation analysis controlling for confounding factors was performed. Student's *t*-test and Mann–Whitney *U* test were used

for comparing two independent groups, and paired *t*-test and Wilcoxon sum rank test were employed to analyze two related samples. Analyses were performed using SPSS v. 16.0 for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software v. 5.0 (San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Serum levels of sTie-2 and their ligands Ang-1 and Ang-2 are increased in patients with carcinoid and pancreatic tumors

We observed a significant increase in serum levels of sTie-2 in patients with GEP-NETs (25.9 ± 9.5 ng/ml) compared to healthy controls (17.1 ± 4.1 ng/ml; $P < 0.001$; Fig. 1A). When the outlier values (arithmetic mean plus 3 s.d.) of sTie-2 observed in GEP-NET patients were deleted, a similar significant difference was detected (data not shown). Likewise, serum levels of Ang-1 and Ang-2 were increased significantly in patients with GEP-NETs (41.1 ± 25.9 and 3.6 ± 2.4 ng/ml respectively) compared to controls (8.7 ± 9.9 and 1.7 ± 0.9 ng/ml respectively, $P < 0.001$ in both cases; Fig. 1B and C). As in the case of sTie-2, when the outlier values of Ang-2 were deleted, a similar significant difference was observed between patients and controls (data not shown). When patients were divided into those with carcinoid and pancreatic

Figure 1 Serum concentrations of sTie-2, Ang-2, Ang-1, and VEGF in patients with GEP-NETs. Levels of sTie-2 (A), Ang-1 (B), Ang-2 (C), and VEGF (D) were determined by ELISA in sera from 42 patients with GEP-NETs and 27 healthy individuals, as described in Materials and methods. Horizontal lines correspond to the arithmetic mean. Mean group values were compared by Student's *t*-test and Mann–Whitney *U* test as appropriate. *** $P < 0.001$.

Figure 2 Serum concentrations of sTie-2, Ang-2, Ang-1, and VEGF in GEP-NET patients with and without metastases. Levels of sTie-2 (A), Ang-1 (B), Ang-2 (C), and VEGF (D) were determined by ELISA in sera from 42 GEP-NETs' patients with and without evidence of metastases as well as in sera from 27 healthy controls, as described in Materials and methods. Horizontal lines correspond to the arithmetic mean. Mean group values were compared by one-way ANOVA and nonparametric ANOVA (Kruskal–Wallis test), and *post hoc* comparisons were carried out using the Bonferroni's and Dunn's tests as appropriate. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

tumors, we found that Tie-2, Ang-1, and Ang-2 levels were significantly higher in both groups compared to healthy volunteers ($P < 0.01$, $P < 0.001$, and $P < 0.05$ respectively), with no significant differences between both types of tumors ($P > 0.05$). No significant differences were detected between the serum concentrations of VEGF in patients with carcinoid or PET and healthy controls (Fig. 1D).

sTie-2 and their ligands are elevated in different subsets of patients with GEP-NETs

GEP-NET patients with tumor metastases showed significantly higher sTie-2 levels (28.1 ± 9.1 ng/ml) compared to patients with no evidence of metastases (20.9 ± 8.9 ng/ml; $P < 0.01$; Fig. 2A) or with healthy controls (17.1 ± 4.1 ng/ml, $P < 0.05$). No differences were found in sTie-2 levels between patients with no tumor metastases and controls (Fig. 2A). Ang-1 levels were significantly higher in patients with or without tumor metastases (51.6 ± 27.1 and 35.8 ± 24.1 ng/ml respectively) than in healthy controls (8.7 ± 9.9 ng/ml; $P < 0.001$ in both cases; Fig. 2B). No differences were found between patients with and without metastases. Ang-2 levels were significantly higher in patients with metastases (3.6 ± 2.1 ng/ml) when compared to healthy controls (1.7 ± 0.9 ng/ml; $P < 0.001$; Fig. 2C). In contrast, no differences were found between patients

with no metastases (2.6 ± 1.1 ng/ml) and healthy subjects or patients with metastases (Fig. 2C). Likewise, no differences were detected in VEGF levels among the groups studied (Fig. 2D). Accordingly, when we attempted to define the risk of metastases according to the levels of Ang/sTie-2, no significant associations were detected when patients were divided into those with normal or high values of Ang/sTie-2 and with or without metastases ($P > 0.05$ in all cases, 2×2 contingency tables, Fisher exact test, data not shown). In addition, when patients were divided into responsive and nonresponsive to somatostatin analogs, no significant association was detected with metastases ($P > 0.05$, data not shown).

Patients with both hormone- and no hormone-secreting tumors had significantly elevated levels of Tie-2 ($P < 0.001$ and $P < 0.01$ respectively), Ang-1 ($P < 0.01$ and $P < 0.0001$ respectively), and Ang-2 ($P < 0.05$ and $P < 0.01$ respectively), but not VEGF, when compared to controls. However, no significant differences were found between patients with both types of tumors. In addition, both wdNETs and wdNECs had elevated levels of sTie-2 ($P < 0.01$ and $P < 0.001$ respectively), Ang-1 ($P < 0.001$ in both cases), and Ang-2 ($P < 0.05$ and $P < 0.001$ respectively), but not VEGF ($P > 0.05$), when compared to healthy controls. However, no differences were found between patients with wdNETs and wdNECs.

When GEP-NET patients were classified according to the presence or absence of tumor angioinvasion, we observed significantly higher levels of sTie-2 ($P < 0.01$ and $P < 0.05$ respectively), Ang-1 ($P < 0.01$ and $P < 0.05$ respectively), and Ang-2 ($P < 0.05$ and $P < 0.005$ respectively) in both groups compared to healthy subjects (data not shown). In contrast, no significant differences in VEGF levels were observed among the three groups studied (data not shown). Likewise, we did not find significant differences in sTie-2, Ang-1, or Ang-2 levels between patients with and without tumor angioinvasion. However, Ang-1 levels tended to be higher in patients with angioinvasion.

Serum levels of Ang-2 correlate with CgA concentration and 5-HIAA excretion in patients with GEP-NETs

We then analyzed the possible association of serum levels of sTie-2 and Ang-2 with different biochemical parameters, including CgA concentrations and urinary 5-HIAA. As shown in Fig. 3A, in patients with carcinoid tumors, a significant positive correlation was detected between Ang-2 and CgA levels

Figure 3 Correlation analysis of laboratory parameters and levels of serum pro-angiogenic molecules in patients with GEP-NETs. Serum levels of the indicated molecules as well as the urinary excretion of 5-HIAA were determined in patients with GEP-NETs. Then, the association between the indicated parameters was determined by calculating the corresponding Pearson's or Spearman's correlation coefficients, as appropriated and as described in Materials and methods. Panels A, B, and C correspond to patients with carcinoid tumors, and panels D, E, and F correspond to GEP-NET patients with metastases. r and P values are indicated.

($r = 0.712$; $P = 0.003$; Fig. 3A) or 5-HIAA excretion ($r = 0.810$; $P = 0.015$; Fig. 3B). A significant association was also detected in these patients between Ang-2 and VEGF levels ($r = 0.528$; $P = 0.029$; Fig. 3C). On the other hand, in the case of patients with PETs, we found a significant association between serum sTie-2 and Ang-2 levels ($r = 0.482$; $P = 0.043$, data not shown). Finally, in those patients with GEP-NETs and metastases, significant associations were detected between Ang-2 and CgA concentrations ($r = 0.634$; $P = 0.001$; Fig. 3D), Ang-2 and sTie-2 levels ($r = 0.603$; $P = 0.002$; Fig. 3E), and Ang-2 and VEGF concentrations ($r = 0.509$; $P = 0.011$; Fig. 3F).

Tie-2 and their ligands Ang-1 and Ang-2 are overexpressed in GEP-NETs

Immunohistochemical analysis showed the expression of Tie-2, Ang-1, and Ang-2 in tumor cells from PETs (Fig. 4A). In addition, we also found a positive immunostaining of Tie-2, Ang-1, and Ang-2 in tumor

Figure 4 Analysis of expression of angiogenic molecules in primary tumors and metastatic liver from patients with GEP-NETs. Serial paraffin sections from pancreatic GEP-NETs (A) and a digestive GEP-NET (B) were stained with H&E (top left panel) or immunostained for Tie-2 (top right panel), Ang-1 (bottom left panel), or Ang-2 (bottom right panel), as stated in Materials and methods. Positive tumor cells are highlighted in the insets and indicated with asterisks (bottom right of each panel). Positive staining of endothelial cells is indicated by arrows. Original magnification $\times 125$ and $\times 500$ (insets). N, normal tissue; T, tumor tissue. (C) Serial paraffin specimens from metastatic liver tumors were immunostained for Tie-2 (upper panel), Ang-1 (middle panel), and Ang-2 (lower panel). Positive staining for tumoral cells is indicated with asterisks. Original magnification $\times 500$. T, tumoral tissue.

cells from digestive GEP-NET tumors (Fig. 4B) and in GEP-NETs from metastatic liver tissue (Fig. 4C). These results were corroborated by flow cytometry analysis (FACS) of freshly isolated tumor cells from primary and metastatic tumors, which expressed both Tie-2 and Ang-2 (Fig. 5). Accordingly, real-time RT-PCR analysis confirmed the presence of *Tie-2*, *Ang-1*, and *Ang-2* mRNA in tissue samples from three GEP-NETs. Although the levels of expression of these three genes were higher in tumor tissue, no significant differences were detected when they were compared with those observed in control samples, likely by the small number of cases analyzed (Fig. 5).

Monocytes from patients with GEP-NETs show an enhanced expression of Tie-2 and are able to migrate in response to Ang-2

We also examined the expression of Tie-2 by peripheral blood monocytes from 14 patients with GEP-NETs and 10 healthy subjects. FACS showed that the percentage of Tie-2⁺ CD14⁺ monocytes was significantly augmented in patients with GEP-NETs compared to healthy subjects (27.3 and 7.9% respectively, $P < 0.05$; Fig. 6A). Since Angs are able to induce the migration of monocytes (Murdoch *et al.* 2007), we also examined *in vitro* the chemotactic effect of Ang-2 and Ang-1 on cells from patients with GEP-NETs and healthy controls. As shown in Fig. 6B and C,

monocytes from patients with GEP-NETs showed a significantly enhanced chemotactic response to recombinant Ang-2 compared to cells from healthy controls. However, although the monocytes from GEP-NET patients tended to show a higher response to Ang-1 compared to controls, no significant difference was reached. As expected, the chemotactic effect of Ang-1 and Ang-2 was abolished when the assays were performed in the presence of a blocking anti-Tie-2 antibody, but not with an irrelevant IgG (Fig. 6B and C, and data not shown). Finally, the chemokine CCL2/MCP-1 induced a similar chemotactic response in cells from patients and controls (Fig. 6B and C).

Discussion

NETs are an apparently heterogeneous group of malignancies with common biological features and cellular origin (Modlin *et al.* 2008, Oberg 2009). As in other malignant conditions, it is feasible that angiogenesis may also have a relevant role in the pathogenesis of these tumors since it has been demonstrated in different experimental models that blood vessel neoformation is involved in tumor growth and development of metastases (Shojaei *et al.* 2008). In this work, we have hypothesized that the Ang-1, Ang-2/Tie-2 system could be involved in the pathogenesis of GEP-NETs. Our results indicate enhanced

Figure 5 Neuroendocrine tumor cells express Tie-2 and Ang-2. (A–C) Freshly isolated tumor cells from metastatic liver from patients with carcinoid tumors were stained with anti-Ang-2 (b) tagged with Alexa Fluor 488 or anti-Tie-2 labeled with PE (c) antibodies, and analyzed by flow cytometry, as stated in Materials and methods. Thin line histograms correspond to the negative control, and thick line histograms correspond to specific staining. Size and complexity characteristics of the cells analyzed are shown in A. (D–F) Expression of *Ang/Tie-2* mRNA by GEP-NETs. Total RNA was isolated from tissue samples from GEP-NETs. Then, RNA was reverse transcribed, and the level of *Ang-1* (D), *Ang-2* (E), and *Tie-2* (F) mRNA was determined by real-time PCR, as stated in Materials and methods. Data correspond to the arithmetic mean $\pm$ S.E.M. of gene expression (fold induction) and were referred to the level of expression of the constitutive gene histone H3. Data from three tumor samples and three normal control tissues are shown.

levels of sTie-2, Ang-1, and Ang-2 in 42 patients with GEP-NETs as well as evidence of local up-regulation of these angiogenic factors in metastatic tissue of these tumors. Data on Ang-2 are in agreement with previous reports showing an enhanced expression of Ang-2 at protein and mRNA levels in a small number of GEP-NETs (Detjen *et al.* 2010). In addition, a recent work (Srirajaskanthan *et al.* 2009) reported enhanced serum levels of Ang-2 in patients with NETs. However, in that report, Ang-1 levels were similar in

GEP-NET patients and healthy controls, and the sTie-2 concentrations were not determined (Srirajaskanthan *et al.* 2009).

We consider that our analysis of patient subsets has provided interesting data. We found higher serum levels of sTie-2 in patients with GEP-NET and metastases compared with those without evidence of metastases. In addition, Ang-2 concentrations were only elevated in GEP-NET patients with metastases. Moreover, Ang-2 and Tie-2 expression was detected in GEP-NET metastatic tissue. We consider that all these data suggest that the Ang/Tie-2 system could be involved in the dissemination of GEP-NETs. This point is in agreement with a previous report that found a relationship between Ang-2 concentrations and the volume of metastases in GEP-NET patients (Srirajaskanthan *et al.* 2009). On the other hand, our data also suggest that, at least in the patients included in this study, VEGF alone does not seem to play an important role in tumor development. However, up-regulation of the VEGF angiogenic system has been demonstrated in different conditions, mainly neoplastic, in digestive endocrine tumors (La Rosa *et al.* 2003). In addition, it has reported the over-expression of VEGF by NET metastatic tissue (Zhang *et al.* 2007). In this regard, therapeutic biological agents such as mAbs directed against VEGF (bevacizumab) (Yao *et al.* 2008) or drugs that are able to block the downstream signaling pathways induced through its receptor (everolimus) (Grozinsky-Glasberg *et al.* 2008) decrease tumor blood flow and have been successfully used in the treatment of these patients. However, Oxboel *et al.* (2009) recently reported that VEGF is not overexpressed by NETs, and that the expression of this gene in this condition is highly variable. Therefore, it is very likely that the combined activity of VEGF with the high concentrations of Ang-2 observed by us could exert an important angiogenic effect in GEP-NETs, promoting thus their growth. This possibility is further supported by the significant correlation that we found between Ang-2 and VEGF levels, mainly in those patients with metastases. However, our data show that in patients with NETs, Ang-1 serum levels are higher than those of Ang-2, suggesting the predominance of the anti-angiogenic effect exerted by Ang-1. Although this is a commendable possibility, we consider that there are alternative interpretations for this phenomenon. In this regard, it is well known that in healthy individuals, the levels of Ang-1 are four- to five fold higher than those of Ang-2. Therefore, it is feasible that under physiological and pathological conditions, very high concentrations of Ang-1 are required for an effective

Figure 6 Analysis of Tie-2 expression and function by monocytes from GEP-NET patients. (A) Peripheral blood mononuclear cells from patients with GEP-NETs were immunostained for Tie-2, and monocytes were analyzed by flow cytometry, as stated in Materials and methods. Data correspond to the median and interquartile range, and were compared by the Mann–Whitney *U* test. (B) Chemotactic effect of Ang-2 and Ang-1 on monocytes from patients with GEP-NETs. Monocytes were seeded onto the upper chamber of a 5 μ m pore size membrane transwell plates, and in the lower chamber, MCP-1, Ang-1, or Ang-2 was added to the medium at 100 ng/ml. When indicated, a blocking anti-Tie-2 mAb was added. At the end of incubation, cell migration was assessed by immunofluorescence microscopy, as stated in Materials and methods. Immunofluorescence images from a representative experiment are shown. (C) Data of monocyte migration experiments are shown as the arithmetic mean $\pm$ s.d. of the percent of increase of cell migration (baseline migration=0%) of five patients and seven controls. All experiments were performed by duplicate. Mean group values were compared by Mann–Whitney *U* test sum rank test. *P* values are indicated. NS, nonsignificant.

negative regulation of the pro-angiogenic effect of Ang-2/VEGF. Thus, it is possible that in patients with NETs, the ongoing angiogenic effect of Ang-2 (induced by tumor hypoxia and/or the tumor infiltrating monocytes) triggers the release of both Ang-1 and sTie-2 as a homeostatic mechanism during blood vessel neoformation (Thomas & Augustin 2009). In this regard, it has shown the lethal effect of systemic overexpression of Ang-2 in mice (Cao *et al.* 2007) and the significant association of very high levels of this cytokine with mortality during sepsis in humans (Thomas & Augustin 2009). On the other hand, although the anti-angiogenic effect of Ang-1 has been widely demonstrated in different experimental models, the role of this cytokine in tumor-associated angiogenesis remains controversial (Thomas & Augustin 2009). In this regard, it has been

shown that Ang-1 is able to promote and to inhibit tumor growth (Shim *et al.* 2001, Machein *et al.* 2004). Therefore, the precise functional role of Ang-1 in GEP-NET development and metastases remains as an important point to be determined. Likewise, it will be interesting to define the possible relationship between the Ang/Tie-2 system and somatostatin receptor expression.

It is worth mentioning that our work is the first report describing an enhanced expression and function of Tie-2 in peripheral blood monocytes from GEP-NETs' patients. Monocytes are recruited from the bloodstream into tumors where they can promote both tumor angiogenesis and metastases (Lewis *et al.* 2007). Thus, it has been shown that Tie-2-expressing monocytes (TEMs) exert an important pro-angiogenic activity in malignant conditions. Accordingly, in mice,

the selective elimination of TEMs by a suicide gene dramatically impairs angiogenesis and induces substantial tumor regression (De Palma *et al.* 2003, 2008). In addition, TEMs have been detected in Rip-Tag2 transgenic mice that develop spontaneous PETs (Shojaei *et al.* 2008). Tie-2⁺ monocytes can be detected not only in tumors, but also at low frequency in peripheral blood mononuclear cells (De Palma *et al.* 2005, Murdoch *et al.* 2007, Venneri *et al.* 2007). Therefore, it is feasible that monocytes have an active role in tumor angiogenesis, favoring their growth and metastases. Similar to murine cells, human TEMs also have pro-angiogenic activity and promote tumor growth when coinjected with human glioma cells in nude mice (Venneri *et al.* 2007). In this regard, Ang-2 exerts an important chemoattractant effect on monocytes, promoting their recruitment to tumor tissues (Lewis *et al.* 2007), favoring their angiogenic effect, and enhancing the blood vessel neof ormation induced by the combined activity of Ang-2 and VEGF (Maisonpierre *et al.* 1997). Furthermore, Ang-2 is able to inhibit the synthesis of TNF- α and the anti-tumor activity of monocytes (Cao *et al.* 2007, Lewis *et al.* 2007). As TNF- α promotes apoptosis of both malignant and ECs, its down-regulation by Ang-2 would further favor the survival and proliferation of tumor cells. Although the cause of the enhanced expression of Tie-2 by monocytes from GEP-NET patients remains to be determined, it is very likely that hypoxia, which usually occurs in the tumor microenvironment, may contribute to this phenomenon (De Palma *et al.* 2005). In addition, it is feasible that the enhanced levels of Ang-2 contribute to the induction of expression of Tie-2 by monocytes. We could thus speculate that an increased number of Tie-2⁺ monocytes present in GEP-NETs would be attracted by Ang-2 present in tumor sites, where they could cooperate in tumor growth and in the development of metastases. We consider that this is an interesting possibility to be studied in the near future.

In summary, our data strongly suggest that the Ang/Tie-2 system is involved in the pathogenesis of NETs, and that monocytes could play an active role in this phenomenon. Therefore, Angs and its receptor seem to be interesting molecular targets for new biological therapies for NETs.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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