Expression of connective tissue growth factor and IGF1 in normal and neoplastic gastrointestinal neuroendocrine cells and their clinico-pathological significance

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*(G A Kaltsas and J L Cunningham contributed equally to this work)

Abstract

Connective tissue growth factor (CTGF) and IGF1 are both expressed in a variety of tumours and are involved in tumorigenesis. However, information about their expression in the gastrointestinal (GI) neuroendocrine (NE) cells and tumours is mainly limited, with the exception of midgut carcinoids where abundant CTGF expression has been demonstrated. Normal mucosa specimens from stomach and ileum, as well as tumour tissue specimens from gastric NE tumours (GNETs; n=58) and midgut NETs (n=38) were included. Immunohistochemical techniques were used to investigate the possible expression of CTGF and IGF1 in GI NE cells and tumours. The latter results were correlated with various clinico-biochemical and histopathological variables. CTGF was expressed in a proportion of NE cells of the normal GI mucosa but not in enterochromaffin-like (ECL) cells, whereas IGF1 was undetectable. CTGF was absent in the foci of ECL cell hyperplasia, and in most of the poorly differentiated carcinomas, but present in some GNETs (mainly in type III ECL cell carcinoids (ECL-CCs)) and in all but one midgut NETs. CTGF correlated with tumour stage in well-differentiated GNETs and with size larger than 1 cm but only in the subgroup of type I ECL-CCs. IGF1 was detected in the foci of ECL cell hyperplasia and in all GI NETs. These findings suggest that both CTGF and IGF1 may be involved in the neoplastic transformation of GI NE cells, whereas IGF1 may play an important role even at early stage.

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Introduction

Neuroendocrine (NE) cells are widely distributed in various organs and can give rise to a variety of neoplasms. In the gastrointestinal (GI) mucosa, more than ten different types of NE cells are characterised with a specific distribution (Rindi et al. 2004). Gastric NE tumours (GNETs) account for about 4% of all types of NE neoplasms (Modlin et al. 2003a,b) and are subdivided into four different types according to the WHO classification (Capella et al. 2000). Recently, a new entity of well-differentiated GNET has been described and termed ‘ghrelinoma’ (Tsolakis et al. 2004). Vesicular monoamine transporter 2 (VMAT-2) has been considered as a specific marker for enterochromaffin-like (ECL) cells and has been used for the diagnosis of GNETs (Erickson et al. 1996, Jakobsen et al. 2001, Tsolakis et al. 2008). Midgut NETs, which are mainly serotonin-producing, are the most common NETs and account for ~25% of all GI NETs (Modlin et al. 2003a,b). In order to precisely
estimate the extent of the tumour disease, a new staging system based on tumor node metastasis (TNM) classification has been introduced for GI NETs by the European Neuroendocrine Tumour Society (Rindi et al. 2006, 2007).

Although, recently established multinational databases have provided significant extent of information regarding the natural history and management of NETs, little is still known about factors involved in regulating cell proliferation during their development. Two potentially interesting proteins in this context are the connective tissue growth factor (CTGF) and insulin-like growth factor 1 (IGF1). Both growth factors are expressed in a variety of tumours and a pathophysiological role for tumourigenesis has been proposed (Bruchim et al. 2009, Dhar & Ray 2010). CTGF exerts biological properties that involve the stimulation of cellular proliferation, migration, adhesion, extracellular matrix formation and also regulation of angiogenesis and tumourigenesis (Bork et al. 1993, Lau & Lam 1999, Chen et al. 2007). Nowadays, CTGF expression in tumours represents a therapeutic target (Aikawa et al. 2006, de Winter et al. 2008). IGF1 is proposed to inhibit apoptosis, induce transformation, promote tumour growth and formation of metastasis in various neoplasms (LeRoith et al. 1995, Khandwala et al. 2000).

Currently, there is limited information regarding the expression of CTGF and IGF1 in human NE GI cells. It has been shown that CTGF is overexpressed in mouse transgenic model of gastric NE carcinoma (Syder et al. 2004), thus it is possible that CTGF may play a central role in regulating ECL cell neoplastic transformation and GNET development. A study evaluating mRNA and protein CTGF expression, in only few patients with GNETs, revealed that although CTGF was present in both hypergastrinaemic (type I) and normogastrinaemic (types III/IV) ECL cell carcinoids (ECL-CCs), it was overexpressed only in the latter group (Kidd et al. 2007a,b). Furthermore, CTGF is abundantly present in serotonin-producing midgut carcinoids (Kidd et al. 2007a,b). Serotonin has been shown to increase expression of both tumour growth factor and CTGF in renal mesangial cells (Hahn et al. 2000, Xu et al. 2007). Serotonin production is occasionally seen in GNETs (Tsolakis et al. 2008) and its relationship to CTGF is not known in these tumours. In a recent study, a correlation was observed between CTGF and serotonin production in NETs even when ileal NETs were excluded from the analysis (Cunningham et al. 2010). IGF1 expression has been reported only in ileal NETs but occasional cases were investigated (Nilsson et al. 1992, Wulbrand et al. 2000).

The aims of this study were 1) to investigate the possible expression of CTGF and IGF1 in human GI NE cells and GI NETs, using immunohistochemical techniques, as well as 2) to correlate the possible expression of CTGF and IGF1 in the above tumours, with various clinico-biochemical and histopathological variables.

Materials and methods

Control tissues

Gastric oxyntic (n = 7) and ileal mucosa (n = 7) from adult patients were included as controls. These sections were obtained from surgical specimens from patients with gastric or ceacal adenocarcinoma. The specimens examined originated from macro- and microscopically normal mucosa located at least 3 cm away from the neoplasm. Peritumoural mucosa from the NETs was also used as an internal control. Tissue specimens from three cases of human mammary carcinomas, exhibiting extensive CTGF immunoreactivity, were used as external controls. Furthermore, surgical biopsy specimens of the liver were used as external control for IGF1.

Patients and tumours

Gastric NE tumours

Primary GNETs (n = 58) and lymph node metastases (n = 9) from 58 patients were collected. The tumours, classified according to the WHO classification scheme (Capella et al. 2000), comprised type I (n = 33), type II (n = 3) and type III (n = 9) ECL-CCs, non-ECL-CC (n = 1), ghrelinoma (n = 1) and poorly differentiated endocrine carcinomas (PDECs; n = 11). Lymph node metastases were derived from the following tumour types: type I (n = 1), type II (n = 1), type III (n = 4) ECL-CCs and PDECs (n = 3); some of these tumours have been included in previous studies (Tsolakis et al. 2008, 2009). The tumours and the extent of the disease were categorised according to the new staging system based on TNM (Rindi et al. 2006).

Type I ECL-CCs (M:F ratio 12:21, median 65 years, age range 29–80 years) consisted of solitary (n = 13) or multiple (n = 20) tumours usually small in size (median size 7.5 mm, range 1–25 mm). The tumour-free oxyntic mucosa showed significant signs of A-CAG, with foci of ECL cell hyperplasia. One patient had local lymph node metastasis. All three patients with type II ECL-CCs (two females and one male of 49, 48 and 47 years of age respectively) had multiple ECL-CCs and suffered from Zollinger–Ellison syndrome in the context of multiple endocrine neoplasia 1 (MEN-1). The oxyntic mucosa showed glandular hyperplasia and
contained foci of ECL cell hyperplasia. One patient had multiple microcarcinoids (about 0.5 mm) and in the remaining cases the largest lesions were 3 and 25 mm in diameter respectively. One of these patients had regional lymph node metastasis at the time of operation. Nine patients were diagnosed with type III ECL-CCs (M:F ratio 3:6, median 60 years, age range 23–77 years). Eight patients had a single tumour and one patient had two (median size 20 mm, range 7–55 mm).

None of the cases exhibited signs of ECL cell hyperplasia or A-CAG in the peritumourous mucosa. Intestinal metaplasia was however seen in two cases. The tumours varied in size (median size 20 mm, range 7–55 mm); all patients, except one, had metastases (in the local lymph nodes and/or liver) at primary diagnosis. The non-ECL-CC (female patient, 71 years old), which was non-functioning (20 mm), contained a subpopulation of ghrelin- (～5%) and gastrin-immunoreactive (IR) (～5%) tumour cells. The ghrelinoma case (male, 63 years old) comprised a solitary lesion (35 mm) with metastatic disease (in the local lymph nodes and liver) at the primary diagnosis. No signs of intestinal metaplasia or A-CAG were detected in the peritumourous mucosa. Eleven patients had PDECs (M:F ratio 8:3, median 58 years, age range 39–77 years); four tumours were of small-cell (SC) type and seven of large-cell (LC) type. All the tumours were solitary (median size 65 mm, range 30–100 mm), and local/distant metastases were detected in nine patients at diagnosis.

**Midgut NETs**

Primary well-differentiated ileal NETs from 36 patients were included in the study, as well as lymph node metastases from seven of these cases and liver metastases from four. One LC- and one SC-PDEC from the hepatic flexure and ceacum respectively, including their lymph node metastases, were also studied. Some of these tumours have been included in an earlier study (Cunningham et al. 2007). The tumours and the extent of the disease were categorised according to the new staging system based on TNM (Rindi et al. 2007).

All the patients with well-differentiated ileal NETs (M:F ratio 26:10, median 59 years, age range 40–78 years) had multiple tumours. At primary diagnosis, eight cases had only liver metastases, seven had lymph node metastases and the remaining 21 cases had metastases in both the organs. The size of the primary tumours examined varied between 3.5 and 29 mm (median 11 mm). The SC- and the LC-PDECs comprised a solitary lesion (both of 40 mm in diameter) with local lymph node metastases at primary diagnosis.

**Double immunofluorescence**

Co-localisation studies were performed using the immunofluorescence technique with the polyclonal rabbit VMAT-2 (AB1767, Chemicon International, Temecula, CA, USA, 1:50), polyclonal goat CTGF (sc-14939, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:100), polyclonal guinea pig peptide tyrosine tyrosine (PYY) (B-GP 520-1, Euro-Diagnostica, Malmö, Sweden, diluted 1:250), polyclonal chicken ghrelin (Y-031-44, Phoenix Pharmaceuticals, Belmont, CA, USA, 1:1600) and monoclonal mouse antibodies versus serotonin (5 HT-H209, M0758, DakoCytomation, Glostrup, Copenhagen, Denmark, 1:30), somatostatin-18 (7360088, NovoClone, Novo BioLabs, Bagsvaerd, Denmark, 1:1600) and glucagon-like peptide-1 (GLP-1) (HYB 147-06, ab26278-100, Abcam, Cambridge, UK, 1:500) as primary antibodies. For double immunofluorescence staining, the sections were initially microwave treated for 2 × 5 min at 750 W in Tris-buffered saline, pH 8.0 and then incubated overnight with a cocktail of the two primary antibodies at 4 ◦C. Before application of the antibody cocktail, the sections were incubated with a non-immune serum from the animal species producing the secondary antibody, diluted 1:10. The secondary antibodies used were tetramethyl rhodamine isothiocyanate (TRITC)-conjugated donkey anti-goat (705-025-147, 1:100), FITC-conjugated donkey anti-rabbit (711-095-152, 1:100), FITC-conjugated donkey anti-guinea pig (706-095-148, 1:100), FITC-conjugated donkey anti-chicken (703-095-155, 1:100) and FITC-conjugated donkey anti-mouse (715-095-150, 1:100) (all Jackson ImmunoResearch Laboratories, West Grove, PA, USA). TRITC gave rise to red fluorescence and FITC gave rise to green. Incubation time of the secondary antiserum was 30 min at room temperature. The sections were examined in a Zeiss Axioplan2 fluorescence microscope and photographed with an AxioCam HRm camera using Axiosvision imaging software and a ×63 plan-apochromat objective. The co-localisation studies were based on sections from the normal gastric oxyntic mucosa (n = 7) and normal ileal mucosa (n = 7).

**Routine staining and immunohistochemistry**

All the tissue samples were fixed in 10% buffered neutral formalin and routinely processed to paraffin wax. Consecutive sections, ~4 μm thick, were attached to positively charged glass slides (Superfrost Plus; Menzel Gläser, Braunschweig, Germany). Sections were routinely stained with haematoxylin–eosin. The majority of the NETs were previously characterised by
immunostaining with general (chromogranin A, synaptophysin) and specific (VMAT-1, -2, serotonin) NE markers (Cunningham et al. 2007, Tsolakis et al. 2008, 2009). For the immunostaining, polyclonal goat antibodies versus CTGF (sc-14939, Santa Cruz, 1:1000) and IGF1 (sc-7144, Santa Cruz, 1:400), as well as monoclonal mouse antibodies versus human Ki-67 (clone MIB-1, M 7240, DakoCytomation, 1:100) and serotonin (M0758, DakoCytomation, 1:100) were applied. The avidin–biotin complex technique (PK-6100, Vectastain ABC, Vector Laboratories Inc, Burlingame, CA, USA) was used with a biotinylated horse anti-goat secondary antibody (BA-9500, Vector Laboratories, 1:100) and a biotinylated horse anti-mouse secondary antibody (BA-2000, Vector Laboratories, 1:100) respectively. Diaminobenzidine was used as final chromogen. Before immunostaining, the sections were microwave treated for 2×5 min at 750 W, using Tris-buffered saline, pH 8.0 as retrieval solution.

**Antibody specificity tests**

Specificity tests were performed in conventional immunohistochemistry analyses and in double immunofluorescence staining. Control immunostainings included omission of the primary antisera, and replacement of the primary antibody by non-immune serum at the same dilution as the primary antibody in question and in the same diluent. A neutralisation test was conducted by a 24 h incubation of primary antiserum with the relevant antigen (10 nmol antigen/ml diluted antibody solution) before application to the sections.

**Calculation of IR cells**

The density of IR cells was estimated by light microscopy at a magnification of ×400 using a square grid in one of the oculars. At least five randomly selected areas were investigated. Ki-67 proliferation index was estimated as the percentage of IR cells among at least 2000 tumour cells in areas of highest nuclear labelling. In smaller lesions, the whole of the neoplastic tissue was evaluated.

**Urinary 5-hydroxylindolacetic acid concentration**

Two 24 h collections of urinary 5-hydroxylindolacetic acid (U-5-HIAA) excretion were measured before treatment initiation in the following cases: 13/33 type I, 2/3 type II, 8/9 type III ECL-CCs, 5/11 gastric PDECs, the ghrelinoma, 31/36 well-differentiated ileal NETs and in the two midgut PDECs, using HPLC and calculated as the mean amount (range <60 µmol/24 h; Wahlund & Edlen 1981).

**Correlations**

All data were analysed using SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). Statistics were calculated on data from the entire collection of tumours as a group and for the following tumour subgroups: well-differentiated NETs, PDECs and types I and III ECL-CCs. The association between CTGF and IGF1 expression in the neoplastic tissue and histopathological and clinico-biochemical variables of each patient (the relative incidence of Ki-67 proliferation index, serotonin immunoreactivity in the tumour, size of the lesion (volume calculated from the measured diameter of tumours), U-5-HIAA concentration, as well as staging according to the TNM system) was tested using Spearman’s rank correlation coefficient. The incidence of CTGF-IR tumour cells was also examined in relation to benchmark variables such as Ki-67 proliferation index >2%, size >1 cm, relative incidence of serotonin-IR tumour cells and tumour stage using Fisher’s exact test. All P values are two sided and a P value <0.05 was considered a statistically significant result.

**Approval by the ethics committee**

The research protocol was reviewed and approved by the local ethics committee at Uppsala University Hospital.

**Results**

**Control tissues**

The control tissues showed strong immunoreactivity for the antibody in question.

**Co-localisation studies**

In the gastric oxyntic mucosa, a proportion of PYY- (Fig. 1A) and somatostatin-IR cells (Fig. 1B) were co-localised with CTGF. The VMAT-2-, ghrelin- and serotonin-IR cells did not exhibit CTGF immunoreactivity. In the ileal mucosa, a proportion of serotonin- (Fig. 1C), PYY-, GLP-1-, serotonin-, somatostatin- and ghrelin-IR cells co-expressed CTGF.

**General tumour features**

The clinico-pathological features of the NETs studied are presented in Tables 1–3.

**Staging of the disease based on TNM classification**

**Gastric NE tumours**

Twenty-one of the 33 patients with type I ECL-CCs had stage I disease, 11 had stage IIa and 1 had IIIb; 2 of
the 3 patients with type II tumours had stage I and 1 had IIIb disease. One of the nine cases with type III ECL-CCs had stage I, three had IIIb and five had stage IV disease. The non-ECL-CC case and the ghrelinoma had both stage IV disease. Two patients with PDEC tumours had stage IIa disease, one had IIIa and eight had stage IV disease.

**Midgut NETs**

Seven of the 36 patients with well-differentiated ileal NETs had IIIb stage disease and 29 had stage IV. The PDECs of the midgut are not included in the stage classification.

**Immunohistochemical expression of CTGF, IGF1, serotonin and Ki-67**

**Gastric and ileal mucosa**

Occasional CTGF-IR cells appeared in the gastric oxyntic mucosa, mainly located in the deeper third. The number of CTGF-IR cells was higher in ileum. No IGF1 immunoreactivity was demonstrated in the deep glands of the gastric and ileal mucosa where the NE cells are localised.

**Gastric NE tumours**

There were no CTGF-IR cells in the foci of endocrine cell hyperplasia seen in types I and II ECL-CCs.

**Table 1** Immunohistochemical results for general/specific NE markers and Ki-67 proliferation index in GNETs. Relative incidence of IR neoplastic cells

<table>
<thead>
<tr>
<th>Type I ECL-CCs (n=33)</th>
<th>Type II ECL-CCs (n=3)</th>
<th>Type III ECL-CCs (n=9)</th>
<th>Non-ECL-CCs (n=1)</th>
<th>Ghrelinoma (n=1)</th>
<th>PDECs (n=11)</th>
<th>Total (n=58)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CgA-IR cells (%)</td>
<td>Synaptophysin-IR cells (%)</td>
<td>VMAT-2-IR cells (%)</td>
<td>Serotonin-IR cells (%)</td>
<td>Ki-67 index (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 (70–100)</td>
<td>90 (60–100)</td>
<td>90 (80–100)</td>
<td>&lt;3 (0–5)</td>
<td>&lt;3 (0.5–5)</td>
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<tr>
<td>95 (90–100)</td>
<td>95 (90–100)</td>
<td>95 (90–100)</td>
<td>&lt;3 (0–1)</td>
<td>&lt;3 (0.5–1)</td>
<td></td>
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<tr>
<td>90 (75–100)</td>
<td>90 (70–100)</td>
<td>90 (75–100)</td>
<td>&lt;3 (0–5)</td>
<td>5 (1–25)</td>
<td></td>
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<tr>
<td>100</td>
<td>100</td>
<td>&lt;3</td>
<td>0</td>
<td>5</td>
<td></td>
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<tr>
<td>90</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td></td>
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<tr>
<td>70 (0–95)</td>
<td>80 (50–100)</td>
<td>10 (0–70)</td>
<td>&lt;3 (0–3)</td>
<td>80 (25–95)</td>
<td></td>
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</tbody>
</table>

NE, neuroendocrine; GNETs, gastric neuroendocrine tumours; CgA, chromogranin A; IR, immunoreactive; VMAT-2, vesicular monoamine transporter 2; ECL-CCs, enterochromaffin-like cell carcinoids; PDECs, poorly differentiated endocrine carcinomas. Within brackets the range (%) is indicated.
CTGF-IR cells were, however, identified in the neoplastic parenchyma of 8/33 (24%) type I ECL-CCs (Fig. 2A) and all except two were larger than 1 cm in diameter. One of the tumour cases exhibited CTGF immunoreactivity in <5% of the neoplastic cells, three between 5 and 50% and the remaining four more than 50%. Only one of the three cases of type II ECL-CCs (diameter 25 mm) contained CTGF-IR cells (80%) in the primaries and in the lymph node metastases (Fig. 2B). Five of nine cases of type III ECL-CCs expressed CTGF-IR in the majority of the tumour cells (Fig. 2C). Only one of 11 cases of PDECs revealed CTGF immunoreactivity in occasional cells. In the metastatic lymph nodes, the neoplastic cells exhibited in general a similar relative incidence and immunostaining pattern for CTGF when compared with the findings in the primary tumours. One case of type III ECL-CC and two cases of PDECs, which were lacking

Table 2 Immunohistochemical results for general/specific NE markers and Ki-67 proliferation index in midgut NETs. Relative incidence of IR neoplastic cells

<table>
<thead>
<tr>
<th></th>
<th>CgA-IR cells (%)</th>
<th>Synaptophysin-IR cells (%)</th>
<th>Serotonin-IR cells (%)</th>
<th>Ki-67 index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileal NETs (n=36)</td>
<td>95 (90–100)</td>
<td>95 (90–100)</td>
<td>95 (90–100)</td>
<td>&lt;3 (0.5–15)</td>
</tr>
<tr>
<td>PDECs (n=2)</td>
<td></td>
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<tr>
<td>Small-cell PDEC</td>
<td>&lt;3</td>
<td>60</td>
<td>0</td>
<td>25</td>
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<tr>
<td>Large-cell PDEC</td>
<td>90</td>
<td>90</td>
<td>0</td>
<td>90</td>
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<tr>
<td>Total (n=38)</td>
<td></td>
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</table>

NE, neuroendocrine; CgA, chromogranin A; IR, immunoreactive; NETs, neuroendocrine tumours; PDECs, poorly differentiated endocrine carcinomas. Within brackets the range (%) is indicated.

CTGF-IR cells were, however, identified in the neoplastic parenchyma of 8/33 (24%) type I ECL-CCs (Fig. 2A) and all except two were larger than 1 cm in diameter. One of the tumour cases exhibited CTGF immunoreactivity in <5% of the neoplastic cells, three between 5 and 50% and the remaining four more than 50%. Only one of the three cases of type II ECL-CCs (diameter 25 mm) contained CTGF-IR cells (80%) in the primaries and in the lymph node metastases (Fig. 2B). Five of nine cases of type III ECL-CCs expressed CTGF-IR in the majority of the tumour cells (Fig. 2C). Only one of 11 cases of PDECs revealed CTGF immunoreactivity in occasional cells. In the metastatic lymph nodes, the neoplastic cells exhibited in general a similar relative incidence and immunostaining pattern for CTGF when compared with the findings in the primary tumours. One case of type III ECL-CC and two cases of PDECs, which were lacking

Table 3 Clinical, biochemical and tumour characteristics in the subgroup of patients with GNETs and CTGF-IR tumour cells

<table>
<thead>
<tr>
<th>Age (years) /gender</th>
<th>CTGF-IR cells in primary tumour (%)</th>
<th>CTGF-IR cells in metastasis (%)</th>
<th>IGF1-IR cells in primary tumour (%)</th>
<th>Serotonin-IR cells in primary tumour (%)</th>
<th>Ki-67 index in primary tumour (%)</th>
<th>U-5-HIAA (µmol/24 h)</th>
<th>Diameter (primary)</th>
<th>TNM/stage</th>
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<tbody>
<tr>
<td>Type I ECL-CCs (n=8)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>55/F</td>
<td>2</td>
<td>No metastasis</td>
<td>30</td>
<td>0</td>
<td>3</td>
<td>ne</td>
<td>23</td>
<td>T2N0M0/IIa</td>
</tr>
<tr>
<td>65/M</td>
<td>10</td>
<td>No metastasis</td>
<td>90</td>
<td>0</td>
<td>0.5</td>
<td>ne</td>
<td>12</td>
<td>T2mN0M0/IIa</td>
</tr>
<tr>
<td>71/F</td>
<td>15</td>
<td>No metastasis</td>
<td>70</td>
<td>2</td>
<td>0.5</td>
<td>20</td>
<td>15</td>
<td>T2N0M0/IIa</td>
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<tr>
<td>52/M</td>
<td>30</td>
<td>No metastasis</td>
<td>80</td>
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<td>1</td>
<td>25</td>
<td>10</td>
<td>T1mN0M0/I</td>
</tr>
<tr>
<td>61/M</td>
<td>60</td>
<td>No metastasis</td>
<td>65</td>
<td>0.5</td>
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<tr>
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<td>90</td>
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<td>2</td>
<td>27</td>
<td>8</td>
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<td>75</td>
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<td>47/M</td>
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<td>5</td>
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<td>61/F&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>ne</td>
<td>40</td>
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GNETs, gastric neuroendocrine tumours; M, male; F, female; CTGF, connective tissue growth factor; IGF1, insulin-like growth factor 1; IR, immunoreactive; U-5-HIAA, urinary 5-hydroxyindolacetic acid (reference range: <60 µmol/24 h); diameter, diameter of primary tumour in millimetres (if multiple neoplasms the diameter of the largest tumour is presented); TNM/stage according to Rindi et al. (2006); m, multiple tumours; ECL-CCs, enterochromaffin-like cell carcinoids; PDECs, poorly differentiated endocrine carcinomas; ne, not evaluated.
<sup>a</sup>Small-cell PDEC.
<sup>b</sup>Large-cell PDEC.
CTGF expression in the primary lesion, displayed IR cells in the metastases in a varying relative incidence. CTGF immunoreactivity was strongest in tumour cells facing the fibrovascular stroma (Fig. 2A and C). Consecutive sections immunostained for VMAT-2 and CTGF indicated co-localisation between the transport protein and CTGF.

In contrast, IGF1-IR cells were present in diffuse, linear and nodular patterns in the foci of endocrine cell hyperplasia seen in types I and II ECL-CCs. All but one the well-differentiated GNETs displayed immunoreactivity for IGF1 in the majority of tumour cells (Fig. 2D); one type I ECL-CCs contained less (about 30%). Nine of the 11 PDECs contained IGF1.
immunoreactivity in between 20 and 40% of the tumour cells (Fig. 2E), in 1 LC- and 1 SC-PDEC in about 60%. The metastases revealed similar relative incidence of IR cells and immunohistochemical pattern for IGF1. Consecutive sections immunostained for VMAT-2 and IGF1 indicated co-localisation between the transport protein and IGF1.

In types I and II ECL-CCs, Ki-67 proliferation index was estimated to be ≤ 2% but in four cases the Ki-67 was between 3 and 5%. Four type III ECL-CCs had a Ki-67 proliferation index ≤ 2%, one had 5% and the remaining had ≥ 10%. All the PDECs exhibited Ki-67 immunoreactivity in the majority of the tumour cells except two cases that showed 25 and 30% respectively.

Occasional (<3%) serotonin-IR cells were observed in all types of GNETs (16/33 type I, 2/3 type II, 3/9 type III ECL-CCs and in 1/11 PDECs) with the exception of the non-ECL tumour and the ghrelinoma, which were non-IR. In the tumours containing both serotonin and CTGF immunoreactivity, serotonin-expressing neoplastic cells were seen among areas with CTGF-IR cells, although there were areas that exhibited CTGF immunoreactivity but not serotonin and the contrary.

**Midgut NETs**

Thirty-three of 36 cases of well-differentiated ileal carcinoids displayed CTGF immunoreactivity in more than 75% of the tumour cells, the remaining 3 cases in about 60%. No CTGF-IR cells were demonstrated in the SC-PDEC, whereas in the LC-PDEC about 75% of the tumour cells were IR. Twenty-three of the 36 cases showed IGF1 immunoreactivity in more than 75% of the tumour cells (Fig. 2F), 10/36 between 50 and 75% and the remaining 3 cases <50%. In the SC- and the LC-PDEC, 60 and 90% respectively of the tumour cells were IR. Virtually all the neoplastic cells of the well-differentiated ileal NETs were IR for serotonin, whereas no immunoreactivity was observed in the two PDECs.

The Ki-67 proliferation index was about 0.5% in 20/36 of the well-differentiated ileal NETs, around 1% in 10 cases, 4–8% in 5, and 1 case showed 15% in hot spots. In the SC- and the LC-PDEC, 25 and 90% of the tumour cells were IR for Ki-67 respectively.

Similar relative incidence of IR cells and immunohistochemical pattern was seen in the metastases for all the antibodies mentioned above. Comparison of the consecutive sections in the well-differentiated tumours indicated co-localisation between serotonin and CTGF, as well as between serotonin and IGF1.

The midgut carcinoids showed constantly strong CTGF immunoreactivity. In the GNETs, the majority of CTGF-IR neoplastic cells showed moderate intensity and were ~30% strong.

**Specificity tests**

Immunostaining with the primary antiserum pre-absorbed with the respective peptide did not reveal any IR cells. The same finding was seen after the omission of the primary antiserum in question or its replacement by non-immune serum. In double immunostaining, the omission of one of the primary antibodies or its replacement by non-immune serum gave an immunostaining pattern corresponding to that obtained with the remaining primary antibody. After the neutralisation test or the omission of both antisera, the controls were non-IR.

**U-5-HIAA concentration**

Among the patients with GNETs, only one patient with type III ECL-CCs and one with LC-PDEC were accompanied by elevated concentrations of U-5-HIAA (100 and 290 μmol/24 h respectively); both tumours expressed serotonin in a subpopulation of their cells (5 and 3% respectively; Table 3). U-5-HIAA concentration was elevated in all but one patients with well-differentiated ileal NETs (range 15–1650 μmol/24 h, median 330 μmol/24 h). The remaining patients had normal U-5-HIAA levels.

**Correlation analysis**

The relationship between CTGF, IGF1 and the histo-pathological and clinico-biochemical variables included differed between tumour types and the correlations depended on how the tumours were grouped. An analysis of the entire population of NETs revealed significant positive correlations for both serotonin and U-5-HIAA concentration to the relative incidence of CTGF-IR tumour cells ($r_s = 0.805$, $P < 0.001$ and $r_s = 0.706$, $P < 0.001$ respectively). Advanced stage was also correlated with CTGF ($r_s = 0.564$, $P < 0.001$). However, a negative correlation was found between CTGF and Ki-67 ($r_s = -0.21$, $P < 0.041$). No correlations were found between CTGF expression, serotonin immunoreactivity and U-5-HIAA concentration when only ECL-CCs were examined. The PDECs ($n = 13$) when compared with the well-differentiated NETs ($n = 83$) expressed less CTGF ($r_s = 0.318$, $P = 0.002$) and IGF1 ($r_s = 0.448$, $P < 0.001$). The correlation between the tumour stage and CTGF in well-differentiated NETs ($n = 83$) is $r_s = 0.8$ and $P < 0.001$, and this correlation exists even when
In this study, we verified abundant expression of CTGF in midgut carcinoids where it is postulated to exert a major role in the fibrotic process (Kidd et al., 2007a, b). CTGF was mainly present in the above neoplasms, and less in ECL-CCs where its expression in PDECs may imply that CTGF is mainly involved in the pathogenesis of well-differentiated NETs, thereby becoming less important in PDECs in the presence of further genetic aberrations. The negative correlation between CTGF expression and Ki-67 proliferative index also favours this view.

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Table 4 Association between relative incidence of CTGF and IGF1 immunoreactive tumour cells, proliferation index and grade of differentiation in gastrointestinal neuroendocrine tumours

<table>
<thead>
<tr>
<th></th>
<th>CTGF</th>
<th>IGF1</th>
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<tr>
<td>Spearman’s r</td>
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<td>WDNET versus PDEC</td>
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<td>Sig. (two-tailed)</td>
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</table>

CTGF, connective tissue growth factor; IGF1, insulin-like growth factor 1; WDNET, well-differentiated neuroendocrine tumour; PDEC, poorly differentiated neuroendocrine carcinoma; Spearman’s rank correlation.

*Percent immunoreactive cells Ki-67.

*Correlation is significant at 0.05 level (two-tailed).

*Correlation is significant at 0.01 level (two-tailed).

well-differentiated ileal NETs are excluded and only well-differentiated GNETs are analysed ($r_s = 0.33$, $P < 0.05$). No correlations were found between the relative incidence of CTGF-IR tumour cells and size of the tumours when the entire population of GNETs ($n = 56$) was analysed. In the type I ECL-CC group, CTGF immunoreactivity was associated with the size of the tumour lesion as a continuous variable ($P = 0.01$) and was significantly more frequent in neoplasms > 1 cm ($P < 0.05$). No significant correlations between CTGF and IGF1-IR were found. Some of the results are presented in Table 4.

Discussion

In this study, a complex relationship between the expression of CTGF and IGF1 in normal GI NE cells and their possible role in the development of gastric and ileal NETs were found. CTGF immunoreactivity was present in a fraction of NE cells in the gastric and ileal mucosa, whereas IGF1 was absent. Although CTGF immunoreactivity was found in a number of well-differentiated ECL-CCs (mainly in type III) and in all ileal NETs, IGF1 had even wider distribution. IGF1 expression was also seen in the foci of NE cell hyperplasia in the mucosa adjacent to types I and II ECL-CCs. Thus, IGF1 seems to be absent in the normal NE cells of the GI mucosa but becomes widely expressed in states of GI NE cell hyperplasia and neoplasia, verifying its significance in NE cell tumourigenesis.

Human GI NE cells appear to constitute a heterogeneous cell population concerning CTGF expression. In the gastric mucosa, only a fraction of L cells (PYY-IR) and D cells (somatostatin-IR) expressed CTGF; the same was observed for EC (serotonin-IR), L (PYY- and GLP-1-IR), D (somatostatin-IR) and P/D1 cells (ghrelin-IR) in the ileal mucosa. It appears that different subpopulations of these cells exist, e.g. one producing CTGF and the other not. It cannot be excluded that the above GI NE cells are in various functional states in which the expression of CTGF is differentiated. Of interest, it is the observation that EC and P/D1 cells in the gastric mucosa showed no CTGF immunoreactivity, but in the ileum they were partially IR.

Additionally, CTGF immunoreactivity, even if absent from the gastric ECL cells and foci of ECL cell hyperplasia, was detected in various types of ECL-CCs. These findings confirm that of a previous small-sized study that also showed expression of CTGF-IR cells in ECL-CCs (Kidd et al., 2007a, b), and also the data reported from the Mastomys model of ECL cell neoplasia, showing that CTGF transcript and CTGF protein are overexpressed in ECL tumour cells compared with normal ECL cells (Tang et al., 1996, Kidd et al., 2007a, b). Furthermore, we demonstrated that CTGF was present in tumours larger than 5 mm, and its expression was correlated with a tumour size > 1 cm but only in type I ECL-CCs. CTGF immunoreactivity in large ECL-CCs may represent a late event in the ECL cell tumourigenesis or that it is present in tumours with higher growth potential. It cannot be excluded that CTGF expression is an epiphenomenon as it is related to hypoxia (Luft 2008), and may occur in ECL-CCs when tumour size exceeds the space that oxygen can diffuse into the tissue. In favour of the above hypotheses is also the finding that CTGF immunoreactivity in tumour cells correlated with tumour stage in well-differentiated ECL-CCs. The almost uniform absence of CTGF expression in PDECs may imply that CTGF is mainly involved in the pathogenesis of well-differentiated NETs, thereby becoming less important in PDECs in the presence of further genetic aberrations. The negative correlation between CTGF expression and Ki-67 proliferative index also favours this view.

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intensity was weaker in the majority of tumour cells than that obtained in midgut NETs. Serotonin-IR cells were less frequently seen in GNETs and when present were limited in number compared to the extensive immunoreactivity in midgut NETs. It is possible that stimulation of CTGF expression by serotonin could have accounted for the difference in CTGF immunoreactivity between these tumour types; however, no correlation between CTGF-IR tumour cells and serotonin expression and/or U-5-HIAA concentrations was noted in ECL-CCs. This is further strengthened by the observation that the gastric EC cells are not IR for CTGF compared to the ileal serotonin-IR cells where a proportion of them express CTGF. It is possible that CTGF immunoreactivity identifies the ileal EC cells that give rise to the CTGF-expressing midgut carcinoids. It would be also interesting to investigate the possible co-existence of CTGF and serotonin in other GI NETs known to express the protein (i.e. rectal NETs). The lack of extensive and intensive CTGF immunoreactivity in GNETs when compared to midgut carcinoid tumours, as well as the absence of correlation with serotonin expression shown in this study may explain why GNETs are not associated with the development of extensive fibrosis. It is possible that mechanisms other than serotonin signalling may be involved in CTGF upregulation in GNETs.

Although many studies in a variety of tumours and tumour cell lines have demonstrated the important role of IGF1 in carcinogenesis (Bruchim et al. 2009), its expression pattern in the human GI tract and GI NETs is largely unknown. Hitherto, only very few cases of ileal NETs have been investigated (Nilsson et al. 1992). Our results showed that, contrary to CTGF, IGF1 was absent from the GI NE cells but became widely expressed in the foci of NE cell hyperplasia in types I and II ECL-CCs, in all types of well-differentiated GI NETs and to a lesser extent in PDECs. This suggests that IGF1 may play an important role in both hyperplastic and neoplastic conditions in GI NETs.

In conclusion, this study shows that CTGF is present in a fraction of GI NE cells, but is absent from the foci of endocrine cell hyperplasia. Its role in the former cells still remains unknown and requires further investigation. CTGF also becomes apparent in a subset of neoplastic lesions larger than 5 mm and metastatic deposits and may be involved in an intermediate neoplastic transformation stage of ECL-CCs. Furthermore, CTGF expression correlates with the extent of the disease in the well-differentiated ECL-CCs. IGF1 is constantly expressed in the foci of NE cell hyperplasia in types I and II ECL-CCs and in the majority of neoplastic cells of the GI NETs. This implies that it may be implicated in the tumourigenesis of GI NETs, even at an early stage.

**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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