Hypoxia stimulates CXCR4 signalling in ileal carcinoids

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Abstract

Tumour hypoxia is associated with increased metastatic potential and resistance to radiotherapy and chemotherapy. Ileal carcinoids are usually metastatic at the time of diagnosis and respond poorly to chemotherapy. The aim of this study was to investigate the extent of hypoxia in ileal carcinoids and the response of tumour cells to induced hypoxia. Vascular endothelial growth factor (VEGF), carbonic anhydrase (CA-IX), hypoxia-inducible factor (HIF)-1α and HIF-2α were studied by immunohistochemistry in biopsies from 24 patients with ileal carcinoids. All hypoxic markers were shown to be highly expressed in localized areas of the tumours irrespective of tumour location or stage. However, HIF-2α expression was significantly higher in distant metastases compared to primary tumours in the same patient. Global gene expression profiling of GOT1 carcinoid cells revealed a marked response to hypoxia. Expression of genes related to epithelial-to-mesenchymal transition and development was altered including increased expression of the C-X-C chemokine receptor type 4 (CXCR4), an important regulator of invasive growth and metastasis formation. High expression of CXCR4 was confirmed by immunohistochemistry in tumour biopsies. Stimulation of GOT1 cells by the CXCR4 ligand, CXCL12 (stromal cell-derived factor 1 (SDF-1)), activated the mitogen-activated protein kinase (MAPK) p42/44 signalling pathway and increased tumour cell migration. We conclude that ileal carcinoids contain hypoxic areas expressing HIF-1α, HIF-2α and CXCR4. Signalling through the CXCL12–CXCR4 axis may contribute to the metastatic potential of ileal carcinoids. Targeting of HIFs and/or the CXCR4 signalling pathway may offer new therapeutic strategies for carcinoid tumour disease.

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Introduction

Metastases, i.e. growth of tumour cells in lymph nodes and distant organs, represent the most serious consequence of malignancy, and account for more than 90% of tumour-related deaths. Most patients with ileal carcinoids have metastatic disease at the time of diagnosis, with localized spread to lymph nodes in 38–44% and distant metastases in 26–30% of patients (Modlin et al. 2007). The only curative treatment is surgical removal of all lesions, but in widely disseminated disease, complete surgical resection is not possible. Chemotherapy has been attempted in patients with disseminated disease but is usually less effective (Plöckinger 2008). Ileal carcinoids often metastasize to the liver via the portal system, which transports detached tumour cells from the primary tumour to the liver. The microenvironment in the liver is of major importance for tumour lodgement and metastasis formation.

The metastatic process consists of a series of complex steps that are highly organized, non-random and organ-specific (Chambers et al. 2002). Tumour hypoxia (O₂ deprivation) initiates the metastatic cascade by changing the microenvironment of the tumour, resulting in prolonged tumour cell survival and resistance to chemotherapy. As solid tumours have
an inadequate blood supply, hypoxia is common in both primaries and metastases (Brizel et al. 1995). Low O2 tension in solid tumours (as compared to the surrounding normal tissue) has been extensively studied, but not in ileal carcinoids (reviewed in Brown & Wilson (2004)). Many of the cellular responses to hypoxia are mediated by the hypoxia-inducible factors (HIFs). HIFs are members of the bHLH–PAS family of transcription factors. They consist of an O2-sensitive α-subunit (HIF-1α, HIF-2α or HIF-3α) that heterodimerizes with a β-subunit (ARNT) and binds to the hypoxia-regulated element in the promoter or enhancer regions of target genes. Target genes encode proteins that regulate glucose metabolism, survival, motility, basement membrane integrity, angiogenesis and haematopoiesis (Semenza 2003).

The chemokine CXCL12 (stromal cell-derived factor 1 (SDF-1)) and its receptor C-X-C chemokine receptor type 4 (CXCR4) are known to regulate the homing process of haematopoietic cells as well as developmental processes of the brain, heart and blood vessels (Bleul et al. 1996, Nagasawa et al. 1996, Baggioiini 1998, Tachibana et al. 1998, Zou et al. 1998, Burger & Kipps 2006). CXCR4 is expressed in a wide range of tumours, and signalling through the CXCL12–CXCR4 axis has been shown to be crucial for the metastatic process and tissue-specific spread of breast cancer and neuroblastoma (Burger & Kipps 2006). CXCR4 is known to be expressed in two types of aggressive neuroendocrine tumours, small-cell lung cancer and neuroblastoma (Burger et al. 2003, Nevo et al. 2004). Furthermore, CXCR4 has emerged as a marker of cancer stem cells, a population of tumour cells that is crucial for tumour growth, invasion and recurrence. CXCR4 is known to be expressed in two types of aggressive neuroendocrine tumours, small-cell lung cancer and neuroblastoma (Burger et al. 2003, Nevo et al. 2004). To date, the role of CXCR4 signalling has not been evaluated in well-differentiated neuroendocrine tumours such as carcinoids.

The aim of this study was to examine the extent of hypoxia in ileal carcinoids and the role of CXCR4 signalling during hypoxia. We provide the first evidence for stabilization of HIF-1α and HIF-2α in ileal carcinoid tumours. Gene expression profiling and immunohistochemical analysis suggest that HIF-2α is a key regulator of the hypoxic response in ileal carcinoids. Furthermore, CXCR4 was found to be highly expressed in the same tumours. In an experimental setting, CXCR4 expression and tumour cell migration were shown to be enhanced in response to hypoxia, which indicates that CXCL12–CXCR4 signalling may have a role in carcinoid tumour spread.

Materials and methods

Patient material

Formalin-fixed, paraffin-embedded tumour biopsies from 24 patients with ileal carcinoids were retrieved from the Department of Pathology at Sahlgrenska University Hospital. A total of 58 biopsies, including primary tumours and their respective lymph node and distant metastases, were analysed. The clinico-pathological data in patients are given in Table 1.

Immunohistochemistry

Immunohistochemistry was performed on tumour biopsies using a DAKO Autostainer and Envision FLEX+ detection system. Briefly, deparaffinized sections were subjected to antigen retrieval by boiling at high pH for 20 min, followed by blocking with hydrogen peroxide and incubation with primary antibodies against HIF-1α (cat. no. NB-100-131; Novus Biologicals, Littleton, CO, USA), HIF-2α (cat. no. NB-100-132; Novus Biologicals), CXCR4 (cat. no. 1949.00.02; Strategic Diagnostic Inc., Newark, DE, USA), vascular endothelial growth factor (VEGF) (VG1; cat. no. 18-7328, Zymed Laboratories Inc., San Francisco, CA, USA), carbonic anhydrase (CA-IX) (cat. no. NB100-417, Novus Biologicals), CD34 (cat. no. M7165, Dako, Glostrup, Denmark) and phospho-mammalian target of rapamycin (mTOR; Ser2448) (cat. no. 2976, Cell Signalling Technology Inc., Danvers, MA, USA). Positive and negative controls were included in each run. The significance of the differences in protein expression between metastasis and primary tumour was tested using a permutation test. For each permutation and each patient, the values for the metastasis and primary tumour were interchanged independently with probability 0.5. The P value was then calculated as the number of permutations with a mean difference higher or equal to the observed mean difference divided by the total number of permutations (one million).

Cell culture and hypoxia treatment

The ileal carcinoid cell line GOT1 established in our laboratory (Kölby et al. 2001) was cultured on uncoated plastic ware (Nunclone; Nuncbrand, Roskilde, Denmark) and maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 5 μg/ml transferrin, 5 μg/ml insulin, 200 IU/ml penicillin and 200 μg/ml streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 21% O2 and 5% CO2 (normoxia). For hypoxia experiments,
the cells were exposed to the same growth conditions but with 1% O\textsubscript{2} in an Innova CO-14 incubator (New Brunswick Scientific, Edison, NJ, USA) for 4–72 h. Primary cell cultures of ileal carcinoids were established from biopsies collected at the time of surgery from three patients with ileal carcinoids (cases no. 8 and 14 involved lymph node metastases and case no. 15 involved a liver metastasis). Tumour tissue was removed by treatment with lysis buffer (155 mM NH\textsubscript{4}Cl, 10 mM KHCO\textsubscript{3} and 0.1 mM EDTA) for 1 min. The cells were seeded onto collagen I-coated plastic ware (Biocoat; Becton Dickinson Labware, Bedford, MA, USA) and cultured in the same medium as GOT1 cells except for the addition of 4% FBS. The primary cells were allowed to recover overnight at 21% O\textsubscript{2} (normoxia) before further experiments.

Quantitative real-time PCR

Four replicate experiments of GOT1 cells and primary cell cultures from three patients were exposed to 21% O\textsubscript{2} (normoxia) or 1% O\textsubscript{2} (hypoxia) for 4 or 72 h. The cells were lysed in TRizol reagent (Invitrogen), and total RNA was purified using RNeasy Mini spin columns (Qiagen). Total RNA was reverse-transcribed and total RNA was purified using RNeasy Mini spin columns (Qiagen). Total RNA was reverse-transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Samples in triplicate were subjected to PCR cycling conditions recommended by Applied Biosystems in a 7500 Fast Real-Time PCR System (Applied Biosystems). Primer and probe specific for \textit{CXCL12} (Hs00171022_ml), \textit{CXCR4} (Hs00237052_m1), HIF-1\textalpha (Hs00936 368_ml), \textit{HIF-2\alpha} (Hs00936 365_ml), \textit{VEGF} (Hs01026142_ml), \textit{CA-IX} (Hs00900054_m1) and endogenous control \textit{ACTB} (Hs99999903_m1) were purchased from Applied Biosystems. The cycle threshold (C\textsubscript{T}) of target genes and \textit{ACTB} was determined using the 7500 Fast System Software version 1.3.1. \textit{ACTB} was used as endogenous

### Table 1

Clinical and histo-pathological data for 24 patients with ileal carcinoids analysed by immunohistochemistry

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TNM, grade and stage according to ENETS proposal (Rindi et al. 2007). No, number of primary tumours; MI, mitotic index (number of mitoses per 10 high-power fields). Immunohistochemical staining of HIF-1\textalpha, HIF-2\textalpha, VEGF, CA9 and CXCR4 is recorded as follows: 1, 1–25% of tumour cells were positive; 2, 25–75% of tumour cells were positive; 3, 75–100% of tumour cells were positive. –, specimen not available. T (primary tumour)/N (lymph node metastasis)/M (distant metastasis).

\textsuperscript{a}This case also analysed in primary cell cultures.
control since ACTB in GOT1 cells showed low variability in expression levels (Ct value) under normoxic and hypoxic conditions. Values were expressed as mean 2^(-ΔΔCt) ± s.d. Statistical significance between experimental conditions was calculated by the two-tailed unpaired t-test using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA), and a P value of <0.05 was considered significant.

**Gene expression microarray**

Gene expression microarray experiments were performed on four replicate experiments of GOT1 cells exposed to 21% O2 (normoxia) or 1% O2 (hypoxia) for 3 days. RNA extraction was performed as described above. cDNA synthesis and cyanine 5 (Cy5) labelling were carried out using the low RNA input linear amplification kit PLUS (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer’s instructions. Universal human reference RNA (URR; Stratagene, La Jolla, CA, USA) was labelled with cyanin 3 (Cy3). Cy5-labelled sample cRNA (825 ng) and Cy3-labelled URR cRNA (825 ng) were then hybridized to an expression microarray containing ~44,000 unique oligonucleotide probes (cat. no. G4112F; Agilent Technologies). The microarray slides were read in an Agilent G2505B microarray scanner (Agilent Technologies), and the fluorescence.

Figure 1 Immunohistochemical demonstration of CXCR4 and markers of hypoxia in ileal carcinoids. (A–C) Consecutive sections from an ileal carcinoid (liver metastasis, case no. 7) showing co-expression of CXCR4, HIF-1α and HIF-2α in tumour cells. (D and E) Expression of CXCR4 in ileal carcinoids (primary tumours, case no. 7 (D) and case no. 5 (E)). CXCR4 was localized to tumour cell membranes. No labelling was observed in surrounding stroma. (F) Nuclear expression of HIF-2α in an ileal carcinoid (lymph node metastasis, case no. 7). (G) Expression of CA-IX in an ileal carcinoid (lymph node metastasis, case no. 21). The labelling was localized to tumour cell membranes. (H and I) Consecutive sections of an ileal carcinoid (liver metastasis, case no. 7) showing expression of activated mTOR in the vicinity of CD34-positive microvessels.
intensities were converted to numeric data using Feature Extraction software version 7.5 (Agilent Technologies). Statistical analyses were performed using Genespring 7.3.1 software (Agilent Technologies). For each hybridization, fluorescence ratios (Cy5: Cy3) were normalized with the LOWESS algorithm. Significant difference in gene expression levels between normoxic and hypoxic tumour cells was determined by Welch’s t-test, and multiple testing correction was determined using Benjamin and Hochberg false discovery rate (FDR). A fold change of 2 and a FDR of 0.05 were used as a cut-off to identify differentially expressed genes. Test of functional enrichment for gene ontology using the GOstat package for the statistical language R 2.8.1 (Ashburner et al. 2000, Falcon & Gentleman 2007) was made.

Western blot analysis

Western blot was performed on GOT1 cells subjected to normoxic or hypoxic treatment. Whole-cell lysates were prepared by adding ice-cold lysis buffer (10 mM Tris–HCl (pH 7.2), 160 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA and Protease Inhibitor Cocktail Set III (cat. no.: 539134; EMD Biosciences, La Jolla, CA, USA)). A total of 20 μg cell lysate was run on 10% or 4–12% NuPAGE Bis–Tris polyacrylamide gels (Invitrogen) and transferred to PVDF membranes (Invitrogen). The membranes were probed using antibodies against HIF-1α (cat. no. 610958; BD Biosciences, San José, CA, USA), HIF-2α (cat. no. ab 199; Abcam, Cambridge, UK), CXCR4 (cat. no. 1949.00.02; Strategic Diagnostic Inc.) and VEGF (cat. no. GF25; Oncogene, Cambridge, MA, USA).

Figure 2 Expression of HIF-1α, HIF-2α, VEGF and CA-IX in ileal carcinoid. Immunohistochemical analysis of HIF-1α, HIF-2α, CD34 and the hypoxia markers VEGF and CA-IX in consecutive sections of a liver metastasis (case no. 10). HIF-1α, HIF-2α and VEGF showed a strong cytoplasmic expression pattern in tumour areas distant from CD34-positive blood vessels. CA-IX was homogenously expressed in all tumour cells, while stromal cells were negative. Haematoxylin and eosin (H&E)-stained sections showed no apoptotic tumour cells or necrosis in HIF-1α-, HIF-2α- and VEGF-positive areas.
Membranes were stripped with ReBlot Plus Strong Antibody Stripping Solution (Millipore, Temecula, CA, USA) and reprobed with antibody against β-actin (cat. no. MAB 8226; Abcam) to estimate the amount of protein transferred. Blotted proteins were visualized using HRP-conjugated secondary antibodies and chemiluminescence detection (Super Signal West Dura Extended Duration Substrate; Thermo, Rockford, IL, USA). The chemiluminescence signals were detected with an image reader (LAS 4000; Fujifilm, Tokyo, Japan) and quantified using MultiGauge version 3.1 software (Fujifilm).

**Immunocytochemistry**

GOT1 cells were grown on chamber slides for 3 days under normoxia or hypoxia and then fixed with 4% paraformaldehyde. Immunocytochemistry was performed as previously described (Arvidsson et al. 2008). Mouse anti-CXCR4 (MAB172; R&D Systems, Minneapolis, MN, USA) and rabbit anti-E-cadherin (cat. no. 3195; Cell Signalling Technology) were used as primary antibodies. Goat anti-mouse Alexa Fluor 594 (cat. no. A11032; Molecular Probes, Eugene, OR, USA) and goat anti-rabbit Alexa Fluor 488 (cat. no. A11034; Molecular Probes) were used as secondary antibodies. The primary antibody was omitted in the negative controls. Coverslips were mounted using ProLong Gold anti-fade reagent with DAPI (Molecular Probes). Cells were analysed by confocal microscopy using a Zeiss LSM 510 META system with LSM-5 software.

**Phosphorylation of MAPK p42/44 and of the serine–threonine kinase Akt**

GOT1 cells were seeded in 12-well plates and incubated in 1% O₂ for 3 days. Tumour cells were grown under hypoxic conditions in serum-free, insulin-free and transferrin-free medium for 17 h before stimulation with 100 ng/ml CXCL12 ((CXCL12)β cat. no. 300-28B; Peprotech, London, UK) for 0, 2, 10 or 30 min. Western blot analysis was carried out, and the degree of phosphorylation of the intracellular kinases 44/42 mitogen-activated protein kinase (MAPK) (Thr 202/Tyr 204) and Akt was determined using phospho-specific monoclonal antibodies against phospho-p44/42 MAPK and phospho-Akt (cat. no. 9101, 9271; Cell Signalling Technology, Beverly, MA, USA). Equal loading of the lanes was confirmed by stripping the blots and reprobing with rabbit polyclonal antibodies against p44/42 MAPK and Akt (cat. no. 4912, 9272; Cell Signalling Technology). Western blots were performed twice with similar results.

**In vitro scratch assay**

GOT1 cells were seeded in triplicates at a density of 0.75 million cells per cm² on collagen I-coated 12-well plates. When the cells had grown into a confluent layer, they were washed once with PBS, and the medium supplemented with 2% FBS was added; then, one plate was placed in hypoxic conditions and one plate in normoxic conditions. The next day, each well was scratched with a toothpick to create a thin line. Cells were incubated in the medium supplemented with 2% FBS in the presence or absence of 100 ng/ml...
CXCL12 (CXCL12β, cat. no. 300-28B; Peprotech). Digital images were taken with a camera attached to a phase contrast microscope after 0 and 2 days of CXCL12 stimulation, and the sizes of scratched areas were measured by calculating the number of pixels per \( \mu \text{m}^2 \) using AxioVision software release 4.4 (Zeiss).

**Ethics**

These studies were approved by the Regional Ethical Review Board in Göteborg, Sweden.

**Results**

**Ileal carcinoids express markers associated with hypoxia: increased expression of HIF-2α in tumour metastases**

To investigate the extent of hypoxia present in biopsies of ileal carcinoids, 24 cases were analysed for the expression of HIF-1α, HIF-2α, VEGF and CA-IX by immunohistochemistry (Table 1). Primary tumours from patients with non-metastatic disease (\( n=4 \)) and primary tumours with associated metastases from patients with disseminated disease (\( n=20 \)) were analysed. VEGF and CA-IX were expressed by all tumours independent of tumour location or stage. The labelling was cytoplasmic for VEGF, but localized to cell membranes for CA-IX (Fig. 1G). The majority of tumour cells were positive for both VEGF and CA-IX. HIF-1α and HIF-2α were also expressed in all tumours, irrespective of location and stage. HIF-2α was expressed by a smaller population of tumour cells than HIF-1α, usually located in the centre of tumour cell nests. Using permutation test, HIF-2α expression was shown to be significantly higher (\( P=0.0034 \)) in distant metastasis compared to primary tumour in the same patient. Co-expression of HIF-1α, HIF-2α, VEGF and CA-IX was observed in defined tumour areas, usually distant from CD34-positive blood vessels (Fig. 2). Expression of HIF-1α, HIF-2α, CA-IX and VEGF was lower in the stromal tissue surrounding tumour cell nests. Routine histology of the 58 biopsies showed two tumours with areas of focal necrosis and eight tumours with areas of apoptotic tumour cells.

**HIF-1α and HIF-2α gradually accumulate in hypoxic GOT1 cells**

To investigate the HIF-1α and HIF-2α expression in response to hypoxia, the ileal carcinoid cell line, GOT1, was cultivated under hypoxic growth conditions (1% \( \text{O}_2 \)) for 72 h. **HIF-1α mRNA** was constitutively expressed under normoxia with reduced expression in cells exposed to hypoxia for 72 h, while **HIF-2α mRNA** accumulated over time (Fig. 3A). The ratio of **HIF-1α mRNA** to **HIF-2α mRNA** was 6:1 in
normoxic cells, as compared to 1.4:1 after 72 h of hypoxia. The HIF-1\(\alpha\) protein was rapidly stabilized after 4 h of hypoxia; thereafter, it decreased (Fig. 3B). HIF-2\(\alpha\) protein showed a more delayed response, with highest levels in cells exposed to hypoxia for 24 h (Fig. 3C).

**Hypoxic GOT1 cells upregulate genes associated with invasiveness and metastasis formation**

To analyse the transcriptional changes in ileal carcinoids during hypoxia, GOT1 cells were exposed to 21 or 1% \(O_2\) for 72 h followed by analysis of global gene expression. Out of 2260 significantly regulated genes \(P<0.05\), 574 genes had at least a twofold change in expression level. Four hundred and twenty-four genes were upregulated and 150 genes were downregulated. The ten most upregulated and downregulated genes are listed in Table 2. Upregulated genes were associated with a shift from oxidative metabolism to anaerobic metabolism and glycolysis \((PYGL\) and \(HK2\)), metastatic behaviour \((CXCR4\) and \(LOX\)), retinoic acid metabolism \((CYP26A1)\) and protection of neuronal cells from hypoxia \((MT3)\). Downregulated genes were associated with tumour suppressor activity \((ITIH5\) and \(INHBB)\), actin regulation \((AVIL\) and \(SEMA3D)\) and \(\beta\)-catenin signalling \((DACT1)\). In summary, several of the ten most upregulated and downregulated genes control tumour invasiveness and metastasis formation. Grouping of the significantly regulated genes according to biological processes highlighted several mechanisms related to altered metabolism as a result of hypoxia, and also mechanisms involved in epithelial-to-mesenchymal transition \((EMT)\) and development (Table 3).

**The chemokine receptor CXCR4 is upregulated and its ligand CXCL12 is downregulated in hypoxic GOT1 cells**

Global gene expression profiling showed upregulation of CXCR4 (22.5-fold) and downregulation of its ligand CXCL12 (0.47-fold) in hypoxic GOT1 cells. Quantitative real-time PCR analysis verified that CXCR4 gene expression in GOT1 cells was highly upregulated (49-fold) after prolonged exposure to hypoxia (72 h), with even higher expression in cells exposed to acute (4 h) hypoxia (Fig. 4C). CXCR4 was also upregulated in primary tumour cell cultures subjected to hypoxia, but the response was not as marked as in the carcinoid cell line GOT1 (Fig. 4A). CXCL12 showed the reverse expression pattern with downregulation in GOT1 cells exposed to hypoxia (Fig. 4G). Western blot analysis confirmed that there were increased CXCR4 protein levels in hypoxia-exposed GOT1 cells (Fig. 4E). Interestingly, CXCR4 protein expression was also detected in GOT1 cells grown under normoxic conditions. The expression of VEGF was analysed in parallel in order to monitor the hypoxic response. Both the RNA and the protein levels of VEGF were upregulated in GOT1 cells and primary tumour cells subjected to hypoxia (Fig. 4B, D, and F).

**CXCR4 is expressed in ileal carcinoids**

In order to analyse the expression of CXCR4, we performed immunohistochemical analysis of biopsies from ileal carcinoids. CXCR4 protein was present in all tumours (Table 1). A majority of tumour cells were positive for CXCR4, with labelling accumulating over tumour cell membranes (Fig. 1). Stromal tissue was devoid of CXCR4 labelling. There was no difference in CXCR4 labelling of tumours depending on location or stage. In order to determine the subcellular localization of CXCR4 in carcinoid tumour cells, we performed confocal laser scanning microscopy on GOT1 cells (Fig. 5). The localization of CXCR4 was similar in tumour cells grown under either normoxia or hypoxia, with accumulation in the cytoplasm and the free plasma membrane. Only minor amounts of CXCR4 were localized to the plasma membrane between adjacent tumour cells. In contrast, the cell adhesion protein E-cadherin showed the opposite localization.
pattern with strongest expression in the plasma membranes between tumour cells. In hypoxia, E-cadherin was downregulated in cells located at the outer edge of tumour cell colonies.

**CXCL12 activates MAPK p42/44 signalling and regulates migration of GOT1 cells**

To identify the signalling pathways that are activated upon CXCR4 stimulation, the MAPK p42/44 and phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathways were analysed in GOT1 cells (Fig. 6A).

Tumour cells were incubated for 3 days in 1% O₂ and then stimulated with 100 ng/ml CXCL12 for 0, 2, 10 and 30 min. To minimize basal activity of the kinases being investigated, the cells were starved (in medium free of serum, insulin and transferrin) overnight before stimulation with CXCL12. Western blots showed increased and stable phosphorylation of MAPK p44/42 (relative to total MAPK p44/42) after 2–30 min of stimulation with CXCL12. However, Akt showed only minor, transient phosphorylation after CXCL12 stimulation. The activation of the

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**Figure 4** Hypoxia causes upregulation of CXCR4 and VEGF in carcinoid cells. (A and B) mRNA levels of CXCR4 and VEGFA increased in primary carcinoid cells maintained under normoxia (21% O₂) (white bars) or hypoxia (1% O₂) for 4 h (grey bars) or 72 h (black bars). (C and D) mRNA levels of CXCR4 and VEGFA increased in GOT1 cells subjected to hypoxia. CXCR4 and VEGF mRNA levels are relative to ACTB, and represented as fold change (mean ± s.d.) compared to cells kept under normoxia. (E and F) Western blot showing expression of CXCR4 and VEGF in GOT1 cells kept under normoxia (21% O₂) or hypoxia (1% O₂). In (E), the 47 kDa CXCR4 protein demonstrated in cells grown under normoxia with two to three times increased levels in hypoxic cells. In (F), VEGF protein was found to increase with time under hypoxia. The densities of CXCR4 and VEGF are relative to β-actin. (G) qRT-PCR analysis of CXCL12 mRNA expression in GOT1 cells kept under normoxia or hypoxia for 4 or 72 h. CXCL12 was significantly downregulated in hypoxic GOT1 cells. CXCL12 mRNA levels are relative to ACTB, and represented as fold change (mean ± s.d.) compared to ACTB cells kept under normoxia (1.0). Asterisks indicate statistically significant differences between cells kept under normoxia and under hypoxia (unpaired t-test). ***P<0.0001.
MAPK pathway shows that the CXCR4 receptor is fully functional in GOT1 cells and respond to chemokine stimulation.

To investigate the effect of CXCL12 stimulation on GOT1 cell migration, scratch assays were performed (Fig. 6B and C). Cells were removed from a confluent layer of cells, and the scratched area was measured after 0 and 2 days of stimulation with CXCL12 (100 ng/ml). Addition of the ligand for 2 days under hypoxia induced migration of GOT1 cells into the scratched area, which decreased significantly in size. CXCL12 had no effect on migration under normoxia.

Taken together, these results indicate that the CXCL12–CXCR4 receptor interaction stimulates migration of hypoxic carcinoid cells, which may be due to activation of MAPK p42/44.

Discussion

This study shows for the first time that HIF-1α and HIF-2α are present in ileal carcinoids, suggesting that tumour cell hypoxia is a characteristic feature of these tumours. Additional evidence for tumour cell hypoxia was obtained from immunohistochemical analysis demonstrating expression of VEGF and CA-IX in ileal carcinoids. Expression profiling of hypoxic carcinoid cells demonstrated significant regulation of a large number of genes, including a marked upregulation of the chemokine receptor CXCR4. We therefore investigated the expression of CXCR4 in a group of ileal carcinoids and further analysed the functional importance of CXCR4 in carcinoid cells. We demonstrated that stimulation of CXCR4 by its ligand during hypoxia activated the MAPK p42/44 signalling pathway and increased tumour cell migration. The expression of CXCR4 in hypoxic tumour cells may therefore contribute to the local invasiveness of ileal carcinoids.

The microenvironment in malignant tumours is frequently hypoxic and characterized by shortage of nutrients, low pH and deprivation of oxygen. The importance of tumour cell hypoxia for invasiveness and metastatic behaviour has been extensively studied in common cancers, e.g. breast and prostate cancer (Kimbro & Simons 2006). However, little is known about tumour cell hypoxia in endocrine tumours, except for pancreatic endocrine tumours arising in patients with von Hippel–Lindau (VHL) syndrome. A strong co-expression of HIF-1α, CA-IX and VEGF in endocrine microadenomas in patients with VHL has been demonstrated as well as involvement of HIF pathway in pancreatic endocrine tumourigenesis (Perigny et al. 2009). Kaelin suggests that HIF-2α protein contributes to the pathological development in
tissues lacking the VHL suppressor protein (Kaelin 2008). In biopsies of ileal carcinoids, we showed that both HIF-1α and HIF-2α are expressed by tumour cells. Compared to HIF-1α which was homogenously expressed across the whole tumour, HIF-2α displayed a more restricted expression pattern, with higher expression in distant metastases compared to primary tumours. It is therefore conceivable that HIF-2α plays a major role in the metastatic process of carcinoid tumours.

Regulation of HIFα is complex, and mechanisms other than hypoxia may contribute to HIFα stabilization. One such mechanism is the PI3K/Akt signalling pathway including the mTOR. mTOR, in turn, is regulated by hypoxia (Toschi et al. 2008, Wouters & Koritzinsky 2008). We analysed ileal carcinoids for the expression of activated mTOR by immunohistochemistry and found a heterogeneous expression pattern of phosphorylated mTOR within tumours. Activated mTOR was often localized to the peripheral parts of tumour cell nests, whereas HIF-1α, HIF-2α and VEGF showed stronger staining in central parts, further away from microvessels i.e. areas of low oxygen tension (Fig. 1). This observation suggests that the upregulation of HIF-1α, HIF-2α and VEGF is driven by hypoxia rather than mTOR. Further analysis of mTOR signalling in ileal carcinoids is warranted to elucidate the importance of this signalling pathway in stabilizing HIFα.

Our analysis of ileal carcinoid cells revealed that HIF-1α is transiently expressed and stabilized as an acute response to hypoxia, whereas HIF-2α mRNA and protein gradually accumulate with time as a sustained response to hypoxia. Similar responses have previously been demonstrated in neuroblastoma and breast cancer (Holmquist-Mengelbier et al. 2006, Helczynska et al. 2008). Hypoxia can induce a Notch downstream response in several tumour cell lines. Accumulation of HIF-2α promotes an aggressive phenotype with dedifferentiation and activation of Notch signalling in neuroblastoma cells (Jögi et al. 2004, Holmquist-Mengelbier et al. 2006, Sahlgren et al. 2008). Expression data of hypoxic GOT1 cells did not reveal expression patterns indicative of activation of Notch downstream target genes or dedifferentiation. However, grouping of significantly regulated genes according to gene ontology biological processes demonstrated that genes related to EMT and developmental processes were activated in hypoxic GOT1 cells, i.e. mechanisms that are relevant for tumour invasiveness and metastasis formation.

HIF-1α and HIF-2α bind to the same DNA consensus sequence within the hypoxia-responsive

Figure 6 CXCL12 induces MAPK p42/44 activation and migration of hypoxic GOT1 cells. (A) Phosphorylation of MAPK p42/44 and Akt in GOT1 cells by CXCL12 stimulation is demonstrated by western blot analysis. GOT1 cells were stimulated with CXCL12 (100 ng/ml), and probed with an anti-phospho-MAPK p42/44 antibody or an anti-phospho-Akt antibody. To confirm equal loading, membranes were stripped and reprobed with an anti-MAPK p42/44 antibody or an anti-Akt antibody. (B) Scratch assay of GOT1 cells cultured under normoxia or hypoxia for 48 h on a collagen I-coated surface in the absence (white bars) or presence (black bars) of 100 ng/ml CXCL12. Quantification of relative closure of the scratched area was determined. CXCL12 induced migration of GOT1 cells and significantly covered 18.4% of the scratched area after 48 h CXCL12 stimulation of hypoxic cells, but did not influence cells grown under normoxia. Migration is expressed as mean ± s.d. Asterisk indicates statistically significant difference (unpaired t-test). *P < 0.05. (C) Phase contrast images of scratch assay of hypoxic GOT1 cells. Photographs were taken from the same area after 0 and 48 h of stimulation with CXCL12.
elements of their target genes. Heterodimers of HIF-1β and HIF-1α or HIF-2α, can activate the same set of genes, but experiments on knockout mice have shown that HIF-1α and HIF-2α do not compensate for each other during development (Iyer et al. 1998, Tian et al. 1998). The majority of hypoxia-responsive genes are dependent on HIF-1α but there is a small group of genes that are preferentially dependent on HIF-2α in combination with ETS transcription factors (Aprelikova et al. 2006). After sustained exposure to hypoxia, ETS2 expression was upregulated 2.9-fold in carcinoid cells, as were the HIF-2α/ETS-specific genes IGFBP3, CITED2 and FAM13A1. Insulin-like growth factor-binding protein 3 (IGFBP3) has been shown to either inhibit or stimulate growth-promoting effects of IGF1 signalling (Pollak 2008). The increase of IGFBP3 during hypoxia is of interest since activation of the IGF1 receptor has been shown to be an important growth-stimulating mechanism in carcinoid tumours. Targeting the IGF1 signalling in carcinoid tumours should therefore be explored (Bowen et al. 2009). The transcriptional co-activator CITED2 is known to modulate transforming growth factor β (TGFβ)-mediated upregulation of matrix metalloprotease 9, which may affect tumour cell invasion mediated by TGFβ (Chou et al. 2006). Together these data suggest that stabilizing of HIF-2α in ileal carcinoid tumours causes upregulation of genes promoting tumour growth and invasiveness.

In this study, we found that hypoxia rapidly increased the expression of the chemokine receptor CXCR4 in ileal carcinoids, which was accompanied by decreased expression of CXCL12. Autocrine stimulation by the chemotactically attracting ligand CXCL12 in normoxic tumour tissue may inhibit tumour migration and invasiveness. As the tumour increases in size, tumour cells become hypoxic with relative deficiency of CXCL12, promoting migration of tumour cells towards higher concentration of ligand in surrounding tissues. The expression of CXCL12 in the lymph nodes, liver and bone may contribute to the homing of carcinoid cells to these organs. Metastatic dissemination of cancer cells is traditionally viewed as a late-stage event (Hanahan & Weinberg 2000). We have shown that hypoxia within ileal carcinoids increases the expression of several genes associated with tumour invasiveness and metastasis formation, and that CXCR4 is one of those regulated genes. CXCR4 is a Gβ protein-coupled receptor that can activate multiple signalling pathways in response to ligand binding. Gi binding of CXCR4 is only dependent on the third intracellular loop of the receptor (Roland et al. 2003). We found that CXCL12 stimulated carcinoid cells via the Gγ-dependent MAPK p42/44 signalling transduction pathway and induced migration in vitro. MAPK p42/44 activation is a well-known regulator of migration, but CXCR4-dependent migration appears to involve different regulators (Roland et al. 2003). There is some evidence to suggest that the CXCL12–CXCR4 axis is associated with tissue-specific tumour spread; for example, tissue-specific CXCR4-mediated growth of bone metastases has been demonstrated in neuroblastoma, prostate cancer and breast cancer (Muller et al. 2001, Taichman et al. 2002, Kang et al. 2003, Russell et al. 2004, Sun et al. 2005). By expression profiling of breast cancer cell lines, Kang et al. (2003) discovered a signature of 102 genes, including upregulation of CXCR4 and connective tissue growth factor (CTGF), which were strongly associated with metastatic dissemination to bone. Elevated expression levels of CTGF and TGFβ1 have been documented previously in ileal carcinoids (Chaudhry et al. 1994, Kidd et al. 2007). In our experiments, we also found significant upregulation of CTGF and TGFβ1 during hypoxia in GOT1 cells. TGFβ1 is critical for EMT and also downregulates CXCL12 expression (Wright et al. 2003, Heldin et al. 2009). These results indicate that increased expression of CXCR4, CTGF and TGFβ1 is a characteristic feature of ileal carcinoid tumours, and it may be due to tumour hypoxia.

In conclusion, our studies have provided evidence for expression of HIF-1α, HIF-2α and CXCR4 in ileal carcinoids, and suggest a role of these factors in promoting tumour invasiveness and metastases. Our findings suggest that novel treatment strategies should be explored in ileal carcinoids. Targeting the HIFα pathway can be achieved by the HIF-1α inhibitor PX478 (Palayoor et al. 2008). An other strategy would be to specifically target the CXCR4–CXCL12 pathway by antagonistic peptides, small molecules or monoclonal antibodies (Murakami et al. 2002, Smith et al. 2004, Sun et al. 2005, Yoon et al. 2007, Kajiyama et al. 2008, Kim et al. 2008).

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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