RESEARCH

Pituitary tumour fibroblast-derived cytokines influence tumour aggressiveness

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Abstract

Tumour-associated fibroblasts (TAFs) are key elements of the tumour microenvironment, but their role in pituitary neuroendocrine tumours (PitNETs) has been little explored. We hypothesised that TAF-derived cytokines may play a role in tumour aggressiveness and that their release can be inhibited by somatostatin analogues. TAFs were isolated and cultured from 16 PitNETs (11 clinically non-functioning tumours and 5 somatotropinomas). The fibroblast secretome was assessed with a 42-plex cytokine array before and after multiligand somatostatin receptor agonist pasireotide treatment. Angiogenesis and epithelial-to-mesenchymal transition pathway assessment included CD31, E-cadherin and ZEB1 expression. GH3 cells treated with TAF- or skin fibroblast-conditioned medium were assessed for migration, invasion and cell morphology changes. PitNET TAFs secreted significant amounts of cytokines including CCL2, CCL11, VEGF-A, CCL22, IL-6, FGF-2 and IL-8. TAFs from PitNETs with cavernous sinus invasion secreted higher IL-6 levels compared to fibroblasts from non-invasive tumours ($P = 0.027$). Higher CCL2 release from TAFs correlated with more capillaries ($r = 0.672$, $P = 0.004$), and TAFs from PitNETs with a higher Ki-67 tended to secrete more CCL2 ($P = 0.058$). SST1 is the predominant somatostatin receptor in TAFs, and pasireotide decreased TAF-derived IL-6 by 80% ($P < 0.001$) and CCL2 by 35% ($P = 0.038$). GH3 cells treated with TAF-conditioned medium showed increased migration and invasion compared to cells treated with skin fibroblast-conditioned medium, with morphological and E-cadherin and ZEB1 expression changes suggesting epithelial-to-mesenchymal transition. TAF-derived cytokines may increase PitNET aggressiveness, alter angiogenesis and induce epithelial-to-mesenchymal transition changes. Pasireotide's inhibitory effect on TAF-derived cytokines suggest that this effect may play a role in its anti-tumour effects.

Key Words

- ▶ pituitary neuroendocrine tumour
- ▶ tumour microenvironment
- ▶ tumour-associated fibroblasts
- ▶ cytokine
- ▶ pasireotide

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Introduction

The great majority of pituitary neuroendocrine tumours (PitNETs) are benign, although they can cause significant burden to patients due to mass effects, invasion and/or due to excessive or low hormone secretion (Di Ieva *et al.* 2014, Molitch 2017).

Tumour behaviour is greatly influenced by the surrounding extracellular matrix, immune cells, endothelial cells, pericytes and fibroblasts, collectively called the tumour microenvironment, via intensive crosstalk with neoplastic cells (Bissell & Radisky 2001, Balkwill *et al.* 2012). This interaction, mediated by a complex network of cytokines, chemokines and growth factors, plays a crucial role in tumour initiation, progression, angiogenesis, invasion and metastasis (Bissell & Radisky 2001, Balkwill 2012, Balkwill & Mantovani 2012, Balkwill *et al.* 2012, Grizzi *et al.* 2015).

Fibroblasts are the most abundant cells in connective tissues, playing a key role in tissue structure and homeostasis. Quiescent fibroblasts undergo activation in tumours, often then termed as tumour-associated fibroblasts (TAFs) (Cirri & Chiarugi 2012, Shiga *et al.* 2015). TAFs are an important source of cytokines and growth factors which contribute to their pro-tumoural effects, influencing proliferation, invasiveness, angiogenesis, epithelial-to-mesenchymal transition (EMT) and extracellular matrix remodelling by promoting expression of collagen, hyaluronan, fibronectin and matrix proteases (De Boeck *et al.* 2013, Moatassim-Billah *et al.* 2016, Shiga *et al.* 2015). In numerous cancers, such as breast, prostate, lung, gastric and pancreatic cancer (Cirri & Chiarugi 2012, Liao *et al.* 2013, Duluc *et al.* 2015, Shiga *et al.* 2015, Moatassim-Billah *et al.* 2016, Wu *et al.* 2017b), TAFs are associated with increased aggressiveness and poor outcome. The role of PitNET-associated TAFs in the behaviour of pituitary tumours remains unknown.

First-generation (octreotide and lanreotide activating mainly somatostatin receptor subtype 2, SST2) and second-generation (pasireotide activating SST1, SST2, SST3 and SST5) somatostatin analogues have been effectively used in the management of many types of PitNETs (Ibanez-Costa & Korbonits 2017, Gunther *et al.* 2018). In addition to their inhibitory effects on tumour cells (Ibanez-Costa & Korbonits 2017), they may also display an indirect anti-tumoural effect by targeting cells of the tumour microenvironment, as shown in a recent study where the anti-proliferative, anti-invasive and anti-metastatic effects of pasireotide were reported to be mediated through the pharmacological inhibition of stromal pancreas cancer-associated fibroblasts (Moatassim-Billah *et al.* 2016).

We therefore hypothesised that PitNET fibroblast-derived cytokines may play a role in pituitary tumour aggressiveness and that their release could be inhibited by somatostatin analogues.

Materials and methods

Isolation and primary cell culture of fibroblasts from human PitNETs

Fresh human PitNET tissues were obtained at the time of transsphenoidal surgery from patients with clinically non-functioning PitNETs (NF-PitNETs, $n=11$) and acromegaly ($n=5$) (Table 1). This study was approved by the Cambridge East Research Ethics Committee (MREC No. 06/Q0104/133), and samples were obtained after written informed consent. Fresh tumour tissue was collected in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, cat. no. D6429) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco, cat. no. 16000044) and 0.5% gentamicin (Sigma, cat. no. G1397), and cultured on the same operation day. Following rinsing three times in magnesium and calcium-free phosphate buffered saline (PBS) (Sigma, cat. no. D8537), samples were cut into small pieces and incubated in 1:10 diluted Trypsin-EDTA 0.05% Phenol Red (Gibco, cat. no. 25300054) for 45 min at 37°C with frequent pipetting allowing effective cell dispersion. Trypsin digestion was stopped by transfer to complete medium, and after 10 min of sedimentation cell suspensions containing pituitary tumour cells were aspirated to a separate tube. PitNET-derived TAFs were obtained using the so-called outgrowth method (Moatassim-Billah *et al.* 2016): undigested debris pieces were placed in a scratched uncoated six-well plate and incubated at 5% CO₂ at 37°C in 0.5% gentamicin and 10% FBS DMEM. Plates were examined under the microscope daily, and medium was replaced three times a week. After 2–3 weeks, fibroblasts migrated out of the debris and, when confluent (about 4 weeks later), were transferred to uncoated culture flasks. No other cells, including pituitary tumour cells, were seen at this time.

Fibroblast supernatants for cytokine assessment were collected from 5×10^5 early passage fibroblasts seeded in T75 culture flasks and grown in complete medium to 90% of confluence. Following washes, and culture in 6 mL serum-free medium, supernatant was collected after 24 h and stored at –80°C until assay. After 48 h in complete medium, cells were treated with 10^{-7} M pasireotide

Table 1 Baseline clinicopathological features of the 16 patients with PitNETs from whom the tumour-associated fibroblasts were isolated.

Clinicopathological features	n = 16
Gender (n (%))	
Male	11 (68.8%)
Female	5 (31.2%)
Age at first symptoms (years) (mean $\pm$ s.d.)	50.1 ($\pm$ 13.9)
Age at diagnosis (years) (mean $\pm$ s.d.)	51.8 ($\pm$ 13.6)
Clinical diagnosis (n (%))	
Acromegaly	5 (31.2%)
Non-functioning PitNET	11 (68.8%)
Headache at diagnosis (n (%))	8 (50.0%)
Visual impairment at diagnosis (n (%))	9 (56.3%)
Hypopituitarism at diagnosis (n (%))	7 (43.8%)
Number of pituitary deficiencies at diagnosis (mean $\pm$ s.d.)	0.9 ($\pm$ 1.2)
Macroadenoma (n (%))	16 (100.0%)
Suprasellar extension (n (%))	16 (100.0%)
Cavernous sinus invasion (n (%))	6 (37.5%)
Ki-67 $\geq$ 3% (n (%))	3 (18.8%)

(Novartis Pharma) in 6 mL serum-free medium for 24 h, and supernatants collected for cytokine array. Conditioned medium from TAFs and normal skin fibroblasts (isolated from skin biopsies of healthy young subjects, one male and one female) were generated similarly but in complete medium conditions.

Cytokine arrays on TAF supernatants were performed by Eve Technologies (Calgary, Alberta, Canada), according to their protocol using the Multiplexing Analyser BioPlex™ 200 system (Bio-Rad Laboratories, Inc.) and the human cytokine/chemokine array with IL-18 (HD42) kit (Millipore). This array measured 42 different cytokines, chemokines and growth factors in the same sample: G-CSF, GM-CSF, IFN α 2, IFN γ , IL-1 α , IL-1 β , IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, IL-18, CXCL1, CXCL10, CCL2, CCL3, CCL4, CCL5, CCL7, CCL22, CX3CL1, CCL11, sCD40L, Flt-3L, PDGF-AA, PDGF-BB, TGF- α , TNF- α , TNF- β , VEGF-A, EGF, FGF-2.

Cell line culture and *in vitro* functional studies

GH3 cells (rat somatomammotroph cell line, European Collection of Authenticated Cell Cultures) were cultured in high glucose DMEM supplemented with 10% FBS and 0.5% gentamicin at 5% CO₂ at 37°C. Invasion assays were carried out using the BioCoat Matrigel Invasion Chambers with 8 μ m pores (24-well insert; BD Biosciences, CA, USA, cat. no. 354480). Invasion chambers were hydrated for 2 h with 500 μ L of serum-free medium at 37°C. After Matrigel rehydration, 750 μ L of TAF-conditioned medium,

skin fibroblast-conditioned medium or complete medium was added to the lower chamber as chemoattractant and 2.5×10^4 GH3 cells in 500 μ L serum-free medium were added to the upper chambers and incubated at 37°C. After 72 h, invading cells through the Matrigel membrane were fixed in 100% methanol and stained with 2% Giemsa blue (Sigma-Aldrich, cat. no. G5637-5G). The total number of invading cells per chamber were counted, and normalised to invading cells towards complete medium. Migration was evaluated by Transwell BioCoat Migration insert plates with 8 μ m pores (24-well insert; Corning Fisher Scientific, cat. no. 354578) following a similar protocol as described for the invasion assay. Migration and invasion studies were repeated at least three times in duplicate.

Changes in GH3 cell morphology were assessed as previously described (Barry *et al.* 2019). In short, area (area of selection in calibrated square units, μ m²); perimeter (μ m); Feret's diameter (longest distance between any two points along selection boundary); circularity (representing perimeter smoothness, $4\pi \times (\text{Area}) / (\text{Perimeter})^2$, with value of 1 indicating a perfect circle and value close to 0 indicating elongated shape); roundness (representing shape, $4 \times (\text{Area}) / \pi \times (\text{Major axis})^2$, with value of 1 for a circle and 0 for very elongated shapes) and solidity (value of 1 indicating more stiffness and less deformable cells, a parameter indicating cells undergoing EMT (Pasqualato *et al.* 2013)) were measured using ImageJ software (National Institutes of Health, USA). Five images were taken per treatment condition at 40 $\times$, and 15 cells were measured per image; hence, 75 cells were analysed per experiment, with a minimum of three repeat experiments performed.

Immunocytochemistry

Fibroblasts or GH3 cells (5×10^4) were plated on 15 mm coverslips placed in 12-well plates. After overnight attachment, fibroblasts were fixed and stained, while GH3 cells were further treated for 24 h. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, following washes with PBS, cells were permeabilised with 0.1% Triton X-100 in PBS for 5 min at 4°C. Cells were washed and blocked in 1% BSA for 30 min at room temperature, and then incubated with the following primary antibodies: anti-vimentin (1:100; Abcam, cat. no. Ab16700), anti- α -smooth muscle actin (α SMA) (1:500; Sigma-Aldrich, cat. no. A5228), anti-actin (Molecular Probes, 2 drops/mL; cat. no. R37110), E-cadherin (1:50; BD Biosciences, cat. no. 610181) and ZEB1 (1:50; Santa Cruz Biotechnology, cat. no. H-102; sc-25388).

After washing, the cells were incubated for 30 min with secondary antibodies conjugated with fluorochromes (Alexa Fluor 568-conjugated goat anti-mouse IgG, Alexa Fluor 488-conjugated donkey anti-mouse IgG and Alexa Fluor 488-conjugated donkey anti-rabbit IgG; 1:1000; Molecular Probes, Invitrogen). Coverslips with stained cells were mounted with Fluoroshield with DAPI mounting medium (Sigma, cat. no. F6057). Stained slides were visualised on a confocal microscope LSM 880 Zeiss and images taken at 63× magnification. E-cadherin and ZEB1 fluorescent intensities were quantified using the software Carl Zeiss Zen Blue Edition version 2.3.

Immunohistochemical analysis and evaluation

Immunohistochemical staining was performed on 4 µm paraffin-embedded tissue sections using Ventana DAB Map Discovery System (Ventana, Illkirch, France). Slides were deparaffinised in xylene and dehydrated in alcohol to PBS, and then processed for antigen retrieval for 30 min with cell conditioning solution CC1 (Ventana), which is a Tris base buffer (pH~9). After blocking with Blocker D solution (Ventana), the sections were incubated for 60 min with the primary antibodies (anti-vimentin, DAKO M7020, dilution 1:1000; anti-E-cadherin, BD Biosciences 610181, dilution 1:50; anti-ZEB1, Santa Cruz Biotechnology H-102:sc-25388, dilution 1:50; anti-CD31, DAKO M0823, dilution 1:100), and then with the universal secondary antibody (Ventana) for 20 min. Slides were counterstained with haematoxylin. Negative controls with omission of the primary antibody were used. Stained slides were scanned with Panoramic Scanner (3DHISTECH, Budapest, Hungary) and analysed with Panoramic Viewer Software (3DHISTECH, Budapest, Hungary). E-cadherin and ZEB1 immunoreactivities were measured semi-quantitatively by an experienced pathologist, blinded to the diagnosis or clinicopathological features of each case, on the basis of both the extent and intensity of the immunoreactivity. The extent of immunoreactivity was scored according to the percentage of stained cells in relation to the entire section as (0 points for no staining, 1 point for less than 20%, 2 points for 20-50% and 3 points for more than 50% of the cells). Staining intensity was graded on a 0–3 scale 0 (no staining), 1 (weak), 2 (moderate) and 3 (strong). Sum of extent and intensity scores was used as final staining score. Vessels, stained for the endothelial marker CD31, were counted manually in 3–5 different 20× magnification fields, allowing the estimation of microvessel density (number of vessels per high power field (HPF)), and the vessels' contour

was manually traced using the ImageJ software to obtain an estimation of the total microvessel area (µm²/HPF), as previously described (Takano *et al.* 2014).

Quantitative real-time polymerase chain reaction (RT-qPCR)

RNA from TAFs was extracted using Qiagen's RNeasy micro kit (cat. no. 74004) according to the manufacturer's protocol in order to examine the expression of somatostatin receptor subtypes. RNA samples were assessed by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). cDNA was synthesised from 1 µg of RNA using the High-Capacity cDNA RT Kit (ThermoFisher Scientific) following the manufacturer's protocol. RT-qPCR reactions were prepared using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies): 10 ng of cDNA (2 µL) and Master Mix (10 µL MM Brilliant III SYBR Green PCR, 0.3 µL of each forward and reverse primers 10 µM, and 7.4 µL nuclease-free water) per reaction. Loaded qPCR 96-well plates run in the Thermal Cycler with MxPro software (Agilent) using the following two-step programme: pre-incubation 3 min at 95°C, and then 40 cycles of 20 s at 95°C and 20 s at 60°C. Cycle threshold (Ct) values were analysed with standard curve quantification method. Target gene expression was normalised to GAPDH expression used as internal control. Dissociation curves were obtained for each gene, where a single peak was observed. Primers sequence (Sigma-Aldrich) were as follows: SST1 sense 5'-CACATTTCTCATGGGCTTCCT-3', reverse 5'-ACAAACACCATCACCACCATC-3'; SST2 sense 5'-GGCATGTTTGACTTTGTGGTG-3', reverse 5'-GTCTCA TTCAGCCGGGATT-3'; SST3 sense 5'-TGCCTTCTTTGG GCTCTACTT-3', reverse 5'-ATCCTCCTCCTCAGTCTTC TCC-3'; SST4 sense 5'-TGTGCTACCTGCTCATCGTG-3', reverse 5'-GCTGGTCACGAAGAGGTTCA-3'; SST5 sense 5'-CTGGTGTTTGCGGGATGTT-3'; reverse 5'-GAAGCTCT GGCGGAAGTTGT-3'; GAPDH sense 5'-TGCACCACCAA CTGCTTAG-3'; reverse 5'-GGATGCAGGGATGATGTTC-3'.

Statistical analysis

Statistical analyses were carried out using the SPSS statistical software version 20 (IBM) and GraphPad version 6 (Prism). The Mann–Whitney *U* test, one-way or two-way ANOVA tests with *post hoc* comparison tests were applied as appropriate. Correlations between continuous variables were determined by the Pearson correlation coefficient *r*. *P* values <0.05 were considered significant.

Results

Detection and *in vitro* isolation of PitNET-derived tumour-associated fibroblasts

Vimentin-positive TAFs were identified in PitNETs in the intra-tumoural area and in a rim of fibrous connective tissue, probably representing the tumour pseudo-capsule (Jagannathan *et al.* 2009, Taylor *et al.* 2018) (Fig. 1A, B, C and D). Isolated TAFs showed spindle-shaped morphology (Fig. 1E and F), stained for actin and vimentin in all TAFs, with some also expressing α SMA (Fig. 1G and H), suggesting that only some TAFs display an active phenotype. TAF supernatants and conditioned medium was generated from this mixed TAF population (i.e. α SMA-positive and α SMA-negative TAFs). The morphology of TAFs differed from the appearance of skin fibroblasts from healthy individuals (Fig. 1I and J), as they displayed a more prominent spindle-like shape with several cell projections and being more irregularly distributed in the culture flask surface.

TAF secretome may determine increased aggressiveness of PitNETs

We hypothesised that TAFs, as a relevant source of cytokines and growth factors, would influence PitNET invasiveness. To address this, we established cultures of TAFs from 16 human PitNETs (clinicopathological patients features shown in Table 1), and then we assessed their cytokine secretome (Table 2 and Supplementary Table 1, see section on supplementary data given at the end of this article). The most highly secreted cytokines/growth factors by TAFs were CCL2, CCL11, VEGF-A, CCL22, IL-6, FGF-2 and IL-8 (Table 2). TAF secretomes from NF-PitNETs and somatotropinomas did not differ significantly (Table 2). TAF-derived IL-6 levels were higher in the subgroup of PitNETs with cavernous sinus invasion in comparison to non-invasive PitNETs (72.7 ± 10.7 vs 43.9 ± 6.3 pg/mL; $P=0.027$), while there was a trend ($P=0.058$) for TAFs isolated from PitNETs with a higher Ki-67 proliferation index to secrete more CCL2 (Fig. 2). CCL2 secretion was higher in TAFs derived from females than males (6698 ± 1831 vs 3918 ± 220 pg/mL; $P=0.04$), but there were no gender differences regarding the other cytokines. CCL2 secretion was not dependent on age or the females' pre or postmenopausal status. The presence of headache, hypopituitarism or visual impairment was not associated with differences in TAF cytokine release (data not shown). Levels of TAF-derived CCL2, a chemokine with known angiogenic functions (Yadav *et al.* 2010, Yoshimura 2017), were positively correlated with microvessel area

($r=0.672$; $P=0.004$) suggesting a possible role in PitNET angiogenesis (Supplementary Table 2). Platelet-derived growth factor (PDGF)-AA levels were negatively correlated with E-cadherin expression, indicating a possible role for the TAF secretome in promoting EMT by downregulating E-cadherin (Supplementary Table 2).

TAF-conditioned medium increases invasiveness, migration and induces an EMT-like phenotype in GH3 cells

To study the effects of TAF-derived factors in pituitary tumour cells, in the absence of an appropriate human pituitary tumour cell line, we assessed the morphology, migration, invasion and EMT activation of GH3 cells in response to TAF-conditioned medium or skin fibroblast-conditioned medium. GH3 cells showed significantly higher migration and invasion towards TAF-conditioned medium compared to complete medium, but not towards normal skin fibroblast-conditioned medium (Fig. 3). Skin fibroblasts were not able to increase invasion, while TAF-conditioned medium led to an 11-fold increased invasiveness in comparison to complete medium (Fig. 3). TAF-conditioned medium, but not skin fibroblast-conditioned medium, induced EMT-like morphological changes in GH3 cells, leading to a significant increase in cell area, perimeter and Feret's diameter, with decreased solidity, roundness and circularity (Fig. 4). These changes result in larger cells with elongated shape which are more deformable and have better ability to migrate and invade in line with the results in the migration and invasion assays (Fig. 3). These morphological changes were accompanied by granular actin staining with prominent stress fibres and spikes, characteristic of EMT-like cytoskeletal changes (McKay & Simpson 2013), while untreated GH3 cells showed actin distributed in a cortical ring (Fig. 4).

TAF-conditioned medium induced EMT in GH3 cells, significantly decreasing E-cadherin and increasing nuclear ZEB1 expression, while untreated GH3 cells show strong E-cadherin with membranous localisation but also in the cytoplasm as well as low nuclear ZEB1 expression (Fig. 5). EMT induction, in line with increased invasion, migration and altered cell shape, suggest that TAF-derived factors interact with pituitary tumour cells influencing their behaviour and invasiveness.

Pasireotide inhibits cytokine secretion from TAFs

To investigate whether somatostatin analogues affect the TAF cytokine secretome, we first determined SST mRNA

Figure 1

Immunohistochemical detection and *in vitro* isolation of PitNET-derived tumour-associated fibroblasts. (A, B, C and D) Vimentin immunostain of PitNETs shows positive staining in spindle-shaped and long cells with cytoplasmic projections (red arrows), located in the intra-tumoural areas (A and B) but also in a rim of fibrous connective tissue probably representing the tumour pseudo-capsule (C and D). Vimentin staining is also positive in endothelial cells, distinguishable from fibroblasts by their morphology and localisation in vessels lumen (green arrowheads). Representative photographs at different magnifications are shown (A, 20 $\times$; B, 40 $\times$; C, 5 $\times$; D, 5 $\times$). (E and F) TAFs isolated *in vitro* after migrating out from a debris tissue piece (E) and after reaching confluency in cell culture flasks (F). (G and H) Immunofluorescent staining for actin (G) and for vimentin (red) and α SMA (green) with vimentin expression seen in all TAFs, whereas α SMA expression seen in many but not all TAFs (H), suggesting that only some TAFs have an active phenotype (63 $\times$). (I and J) Morphological appearance of dermal fibroblasts from 2 different healthy individuals, having a less prominent spindle-like shape with shorter projections and being more regularly distributed in the culture flask surface.

expression in TAFs. SST1 is the predominant receptor in PitNET-derived TAFs (Fig. 6), similar to pancreas cancer-associated fibroblasts (Duluc *et al.* 2015, Moatassim-Billah *et al.* 2016), while SST5 and SST2 expression was minimal. SST mRNA expression did not differ between

NF-PitNET-derived TAFs and somatotropinoma-derived TAFs (data not shown).

As the TAFs expressed SST1, we selected pasireotide (10^{-7} M) treatment (Duluc *et al.* 2015, Moatassim-Billah *et al.* 2016) to assess TAFs responses. Pasireotide treatment

Table 2 Top 12 highly secreted cytokines/chemokines/growth factors in primary culture supernatants from tumour-associated fibroblasts isolated from PitNETs and healthy skin fibroblasts.

Cytokine/chemokine/ growth factor	Mean concentration (pg/mL) ± s.e.m.				
	TAFs (n = 16)	Healthy skin fibroblasts (n = 2)	Serum-free medium	NF-PitNET-TAFs (n = 11)	Somatotropinoma-TAFs (n = 5)
CCL2	4786.86 ± 642.17	2756.56 ± 1585.22	4.00	4782.87 ± 903.21	4795.62 ± 679.53
CCL11	836.27 ± 328.16	0	0	399.44 ± 168.63	1797.30 ± 894.43
VEGF-A	174.29 ± 80.60	96.09 ± 29.12	0	70.06 ± 46.86	403.59 ± 240.29
CCL22	62.54 ± 21.50	14.87 ± 1.14	20.78	74.17 ± 29.63	36.96 ± 21.92
IL-6	54.76 ± 6.50	68.42 ± 7.82	0	50.60 ± 8.17	63.89 ± 10.45
FGF-2	42.93 ± 5.82	28.40 ± 0	0	45.96 ± 8.24	36.29 ± 4.13
IL-8	42.20 ± 11.11	1.89 ± 0.27	7.06	31.53 ± 7.10	65.69 ± 31.66
CXCL1	28.20 ± 6.56	28.44 ± 28.44	20.78	26.23 ± 6.44	32.54 ± 16.78
CX3CL1	26.86 ± 8.34	9.41 ± 1.55	6.73	29.86 ± 12.09	20.26 ± 3.86
CCL7	13.83 ± 5.97	26.18 ± 15.71	8.20	9.43 ± 3.67	23.51 ± 17.89
PDGF-AA	11.64 ± 3.71	0.20 ± 0	0.12	7.40 ± 3.44	20.98 ± 8.29
IFNα2	8.82 ± 2.40	1.22 ± 1.04	1.79	10.25 ± 3.38	5.68 ± 1.54

PitNET-derived tumour-associated fibroblast (TAF) and healthy skin fibroblast supernatants were collected following 24 h serum-free medium conditions. Results are shown as mean concentration (pg/mL) ± s.e.m. P values were non-significant for comparative analysis per cytokine between TAFs-derived from non-functioning-PitNETs (NF-PitNET-TAFs) and from somatotropinomas (Somatotropinoma-TAFs) (Mann-Whitney U test).

significantly decreased IL-6 release by 80% ($P < 0.001$) and CCL2 by 35% ($P = 0.038$), while the other factors showed a trend for reduction but no statistically significant change (Fig. 7A and Supplementary Table 1). IL-6 secretion was reduced in all the 16 TAFs treated with pasireotide, while CCL2 decreased in 10 out of the 16 cases (62.5%) (Fig. 7B).

Discussion

TAFs determine tumour initiation, proliferation, invasiveness and clinical outcomes for many types of tumour (Cirri & Chiarugi 2012, Shiga *et al.* 2015), but their role in PitNETs remains unknown. Our data suggest that PitNET-derived TAFs are a source of cytokines which may impact on tumour behaviour. Of the cytokines studied, IL-6 and CCL2 emerged as possible mediators of PitNET invasiveness. Furthermore, the effect of pasireotide on TAF cytokine secretion suggest that its beneficial effect may at least partly be mediated via inhibition of TAFs, in addition to any direct effect on pituitary tumour cells (Moatassim-Billah *et al.* 2016, Ibanez-Costa & Korbonits 2017).

TAFs are components of the tumour microenvironment in different tumours, including in PitNETs (Tofrizal *et al.* 2016), and these cells are active sources of cytokines and growth factors (Cirri & Chiarugi 2012, De Boeck *et al.* 2013, Shiga *et al.* 2015). We found highly secreted

Figure 2

Tumour-associated fibroblasts (TAFs) cytokine secretome according to the presence/absence of cavernous sinus invasion (A) and to Ki-67 index (B). Data are shown as mean concentration (pg/mL) ± s.e.m. for the top 12 secreted cytokines/chemokines/growth factors. *, < 0.05 (Mann-Whitney U test).

Figure 3

Matrigel-coated chamber invasion assays (A) and transwell migration assays (B) performed on GH3 cells towards complete medium, tumour-associated fibroblasts-conditioned medium (TAF-CM) and conditioned medium from dermal fibroblasts from healthy individuals (F-CM) after 72 h. Data are represented as a mean ratio of invading or migrated GH3 cells towards TAF-CM or F-CM in relation to invading or migrated GH3 cells in serum-medium conditions $\pm$ S.E.M. *, <0.05 , **, <0.01 (one way-ANOVA with Bonferroni multiple comparison test).

levels of CCL2, CCL11, VEGF-A, CCL22, IL-6, FGF-2 and IL-8 in TAF supernatants. CCL2 levels were higher in supernatants from fibroblasts originating from tumours with more proliferation and more capillaries, suggesting a role for TAF-derived CCL2 in PitNET aggressiveness and angiogenesis. CCL2 has a number of roles, including in immune cell chemotaxis, angiogenesis, tumour proliferation and invasion (Yadav *et al.* 2010). While CCL2 has not previously been described in PitNETs, cell culture supernatants and lysates of human craniopharyngiomas show CCL2 release (Nie *et al.* 2017). The reason for the gender difference in CCL2 secretion is unclear as no gender-specific effect has previously been described for CCL2 release (Arakelyan *et al.* 2005, Wu *et al.* 2017a).

Chemokines and growth factors, such as PDGF (Heldin 2013, Wu *et al.* 2013), often secreted by TAFs or other

non-tumoural cells of the tumour microenvironment, are able to induce EMT, a process whereby tumour cells are reprogrammed to a mesenchymal phenotype acquiring a migratory and invasive characteristics by losing epithelial polarity and adhesion molecules, in particular E-cadherin (Lee *et al.* 2006, Thiery *et al.* 2009, De Craene & Berx 2013). In our study, TAF-derived PDGF-AA levels were negatively correlated with E-cadherin expression, suggesting a possible role for the TAF secretome in promoting EMT in PitNETs, in line with our *in vitro* experiments findings.

IL-6 plays a role in progression and aggressiveness of PitNETs (Jones *et al.* 1994, Velkeniers *et al.* 1994, Arzt *et al.* 1999, Kurotani *et al.* 2001, Haedo *et al.* 2009). Invasive PitNETs have a high proportion of IL-6 expression (67.5%), while non-invasive PitNETs expressed IL-6 only in 22.5% of cases (Wu *et al.* 2016). Suppression of

Figure 4

Morphology of GH3 cells after treatment for 72 h with serum-medium ($n = 3$), tumour-associated fibroblasts-conditioned medium (TAF-CM) ($n = 4$) and with conditioned medium from dermal fibroblasts from healthy individuals (F-CM) ($n = 3$). GH3 cell morphology was evaluated for six parameters: cell area, Feret's diameter, solidity, perimeter, roundness and circularity. Data are shown as mean $\pm$ S.E.M. for the morphological parameters evaluated. Seventy-five cells were analysed per experiment, with a minimum of three experiments per treatment condition. Scale bar: 25 μm . ***, <0.001 (two-way ANOVA with Bonferroni multiple comparison test). On the right side is shown alterations on actin fibers in GH3 cells after treatment with TAF-CM for 72 h in comparison to complete medium (63 $\times$) magnification; DAPI was used to stain the nuclei.

Figure 5

Alterations in the E-cadherin and ZEB1 expression by GH3 cells after treatment for 72 h with tumour-associated fibroblasts-conditioned medium (TAF-CM) or in complete medium. Pictures were taken on confocal microscope at 63 $\times$ magnification. DAPI was used to stain the nuclei. E-cadherin and ZEB1 fluorescent intensities were quantified in 30 different cells per treatment condition using the Carl Zeiss Zen Blue Edition, version 2.3 software. Data are shown as mean fluorescent intensity $\pm$ s.e.m. ***, <0.001 (Mann-Whitney *U* test).

the cytokine transducer gp130, which usually results in inhibition of IL-6 secretion, impaired the development of transplanted GH3 cell tumours in nude mice (Castro *et al.* 2003). In GH3 cells, IL-6 stimulates cell proliferation and DNA synthesis, as well as GH and prolactin release (Arzt *et al.* 1993). IL-6 can also be secreted by non-tumoural folliculo-stellate cells, which can have paracrine effect on pituitary tumour cells causing increased proliferation and aggressiveness (Renner *et al.* 1998, Haedo *et al.* 2009, Sapochnik *et al.* 2017a,b). Our study shows that TAF-derived IL-6 levels were higher in PitNETs with cavernous sinus invasion, supporting a possible role for the paracrine effects of IL-6 on pituitary tumour invasiveness. Thus, IL-6 may represent a drug target for PitNETs to reduce the paracrine effects of TAFs.

Our *in vitro* data showed that TAF-derived factors, but not normal skin fibroblasts factors, are able to induce numerous effects on GH3 cells. Direct induction of EMT, in line with increased invasion, migration and altered cell shape, suggest that TAF-derived factors interact with pituitary tumour cells to influence their behaviour and invasiveness. We noted a non-significant trend for increased GH3 cell migration towards skin fibroblast-CM, less marked than in the presence of TAF-CM, which is not surprising considering that skin fibroblasts are also a source of cytokines and chemokines (Kubo & Kuroyanagi 2005, Nolte *et al.* 2008), suggesting that fibroblast factors in general may alter tumour cell migration. However, invasion requires not only the capacity for cells to migrate, but also their ability to secrete enzymes to degrade Matrigel (Kramer *et al.* 2013, Benton *et al.* 2014); this seems to be induced only by TAF-derived factors

and not by factors derived from normal skin fibroblasts. In fact, skin fibroblast-CM was not able to increase invasion, whereas TAF-CM remarkably increased GH3 cell invasion in comparison to complete medium, and almost significantly in comparison to skin fibroblast-CM. The EMT induction by TAF-derived factors, a crucial process for migration and invasion of neoplastic cells (De Craene & Berx 2013), support the human data linking cavernous sinus invasion and TAF-derived cytokines, particularly IL-6, an interleukin of high importance for fibroblasts biology (Kinoshita *et al.* 2013, Nagasaki *et al.* 2014).

Somatostatin controls hormone secretion and proliferation in normal and neoplastic pituitary and reduced IL-6 and IL-8 in human somatotropinoma cultures (Thiele *et al.* 2003, Vindelov *et al.* 2011). Somatostatin's inhibitory effect on IL-6 secretion was also shown in non-pituitary cells (Grimaldi *et al.* 1997, Andoh *et al.* 2002,

Figure 6

SST expression profile in human PitNET-derived tumour-associated fibroblasts determined by RT-qPCR. Data are shown as relative SSTx mRNA fold change expression to GAPDH $\pm$ s.e.m., determined by RT-qPCR using the standard curve method. $n = 16$. ***, <0.001 (one way-ANOVA with Bonferroni multiple comparison test).

Figure 7

Cytokine secretome from human PitNET-derived tumour-associated fibroblasts at baseline (untreated) and after treatment with pasireotide (10^{-7} M). Data are shown in concentration (pg/mL) $\pm$ S.E.M. for the top 12 highly secreted cytokines/chemokines/growth factors in PitNET-derived TAF supernatants collected following 24-h serum-free medium conditions with pasireotide (10^{-7} M) or without (untreated) (A). IL-6 and CCL2 levels before (left side, square mark) and after pasireotide treatment (right side, triangle mark) are shown per case individually (B). *, <0.05 , ***, <0.001 (Mann-Whitney *U* test).

Spangelo *et al.* 2004). In a study using human NF-PitNET primary cultures, it was demonstrated that pasireotide can inhibit tumour cell viability by inhibiting VEGF secretion (Zatelli *et al.* 2007). Pasireotide, by activating SST1 expressed in pancreas cancer-associated fibroblasts, inhibits various cytokines including IL-6, with abrogation of metastasis and prevention of EMT (Duluc *et al.* 2015, Moatassim-Billah *et al.* 2016). The inhibitory effect of pasireotide on IL-6 secretion from PitNET-derived TAFs that we observed suggests that this effect may play a role in the clinical effectiveness of pasireotide. Furthermore, the benefits of targeting TAFs with pasireotide probably extends beyond its role in inhibiting cytokine release. Fibroblasts are mediators of fibrosis due to their ability to secrete collagen, hyaluronan, proteoglycans and other extracellular matrix-related proteins (Kalluri & Zeisberg 2006, Cirri & Chiarugi 2012). A correlation between collagen-producing cell number and fibrous deposition degree was seen in PitNETs, with thyrotrophinomas displaying the highest number of collagen-producing cells and fibrous matrix (Tofrizal *et al.* 2016), in line with their recognised firm consistency (Yamada *et al.* 2014) which may hinder surgical resection (Chentli & Safer-Tabi 2015). Thus, a drug able to target TAFs and reduce the fibrotic process may be valuable in improving outcomes in patients with PitNETs. Emerging data support the anti-fibrotic properties of somatostatin analogues, mainly through the inhibition of fibroblast proliferation and induction of fibroblast apoptosis (Priestley *et al.* 1994, Pasquali *et al.* 2000, Borie *et al.* 2008). Pasireotide has been recently shown to be as effective as methylprednisolone in improving clinical outcomes in patients with Graves' orbitopathy (Le Moli *et al.* 2018), a condition in which SST-expressing orbital fibroblasts are key pathophysiological elements (Priestley *et al.* 1994, Pasquali *et al.* 2000).

The anti-secretory and anti-proliferative effects of a specific somatostatin analogue in a certain PitNET

depends on its SST expression pattern and the SST binding profile of that somatostatin analogue (Iacovazzo *et al.* 2016, Ibanez-Costa *et al.* 2016, Ibanez-Costa & Korbonits 2017). However, the mere abundance of a given SST does not necessarily correlate with the level of response to a somatostatin analogue with strong affinity for that SST (Ibanez-Costa *et al.* 2016). In fact, some studies found no correlation between inhibitory effects of octreotide or pasireotide and a particular SST expression pattern or even less prominent responses of pasireotide in pituitary neoplastic cells expressing high levels of SST5 (Ibanez-Costa *et al.* 2016, Gatto *et al.* 2017). The reasons for such discrepancies are still unknown, but may well be related to extrapituitary effects, such as the modulatory effect of somatostatin analogues directly on non-tumoural cells present in the tumour microenvironment including TAFs. The pharmacological effect on TAFs might also explain why pasireotide efficacy *in vivo* is superior to octreotide in patients with acromegaly (Colao *et al.* 2014, Gadelha *et al.* 2014), while *in vitro* pasireotide and octreotide similarly inhibit pituitary tumour cells (Ibanez-Costa *et al.* 2016, Gatto *et al.* 2017), or the contradictory observations in NF-PitNETs in which octreotide was able to stabilise tumour size in most patients (Fusco *et al.* 2012), whereas *in vitro* there was a poor response or even a paradoxical increase in cell viability after treatment with both octreotide and pasireotide (Ibanez-Costa *et al.* 2016).

The limitations of our study include the fact that we studied only a small cohort of cases with a relatively short postoperative follow-up, as this study is based on fresh primary cell culture, rendering data on longer term clinical outcomes and recurrence unavailable. Our cytokine array experiments lack fresh fibroblasts derived from normal pituitary as controls, we used an alternative suitable control – normal skin fibroblasts. In our *in vitro* experiments we used a rat pituitary tumour cell line rather than a human cell line, as a human pituitary tumour cell line does not exist.

In summary, our data suggest that TAFs, as part of the PitNET tumour microenvironment, represent a source of cytokines influencing tumour proliferation, invasiveness, and neovascularisation, with IL-6 and CCL2 emerging as possible key mediators. Dedicated functional studies are needed to confirm the direct involvement of TAF-derived IL-6 and CCL2 in PitNET invasiveness. We have shown that TAF-conditioned medium, but not normal skin fibroblast medium, induces EMT-like morphological changes, downregulates E-cadherin and upregulates ZEB1 expression, and increases the migration and invasion of GH3 cells. We suggest that the inhibitory effects of pasireotide on cytokine release from TAFs may play a key role in its anti-tumoural effects.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-19-0327>.

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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