Expression and function of the chemokine receptor CCR7 in thyroid carcinomas

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Abstract

The chemokine receptor CCR7 plays a critical role in lymphocyte and dendritic cell trafficking into and within lymph nodes, the preferential metastatic site for papillary (PTC) and medullary (MTC) thyroid carcinomas. In order to determine a possible role for CCR7 in mediating the metastatic behaviour of thyroid carcinomas, we analysed its expression in normal and tumoral thyroid tissues of different histotypes and studied the *in vitro* effects of its activation by the CCR7 ligand, CCL21. Using real-time quantitative-PCR, we observed that CCR7 expression was higher in PTCs and MTCs than in follicular and poorly differentiated thyroid carcinomas. CCR7 expression was ninefold higher in classic compared with follicular variants of PTCs, and its expression in MTCs was significantly correlated with lymph node

metastases. Immunohistochemical staining for CCR7 showed protein expression in neoplastic thyroid cells, with higher intensity in PTCs, MTCs and their lymph node metastases (LNMs). We further showed that CCL21 stimulation of a CCR7-expressing thyroid tumour cell line (TPC-1) promotes cell proliferation and migration, and the chemotactic effect of CCL21 in these cells involves actin polymerization, increased β 1-integrin expression and increased matrix metalloproteinase secretion. Taken together, our results demonstrate that CCR7 activation on thyroid carcinoma cells by CCL21 – a chemokine abundantly expressed in lymph nodes – favours tissue invasion and cell proliferation, and therefore may promote thyroid carcinoma growth and LNM.

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Introduction

Metastasis, the leading cause of death in cancer patients, is a complex, non-random and organ-specific process, which depends on the successful accomplishment of several sequential steps by the tumour cells, from the primary tumour to the secondary organs (Chambers *et al.* 2002). Two main theories have been proposed to explain the organ specificity seen for many tumours. According to the ‘seed and soil’ concept, different organs provide growth conditions optimized for specific cancers (Chambers *et al.* 2002, Fidler 2003); while the ‘homing theory’ states that different organs produce chemotactic factors, which preferentially attract specific types of metastatic tumour cells (Liotta 2001, Murphy 2001). Nevertheless, our knowledge of the molecular mechanisms underlying the metastatic process is still limited.

Papillary (PTC) and medullary (MTC) thyroid carcinomas usually metastasize to the lymph nodes, whereas follicular (FTC) and poorly differentiated (PDTC) thyroid carcinomas, albeit originated from the same cell type as the PTCs,

preferentially form bone, liver, lung and brain metastases (DeLellis *et al.* 2004). PTCs can be further subdivided into classic and follicular variants, which also exhibit different metastatic behaviours (classic PTCs have higher propensity to lymph node metastasis (LNM)). Therefore, thyroid carcinomas provide a good model to study the mechanisms underlying the metastatic process.

Chemokines are a family of small chemoattractant cytokines that mediate their effects by binding to G-protein-coupled receptors. Their main biological function is leukocyte activation and homing to specific anatomical sites (Zlotnik & Yoshie 2000). Because both metastasis and normal migration of leukocytes involve site-directed movement across vascular barriers, it was hypothesized that tumour cells may also use chemokine-mediated mechanisms during the metastatic process (Müller *et al.* 2001).

The chemokine receptor CCR7 is mainly involved in lymphocyte and dendritic cell trafficking into and within lymphoid tissues (Förster *et al.* 1999, Zlotnik & Yoshie 2000, Horuk 2001). In fact, the chemokines CCL19 (ELC) and

CCL21 (SLC), the two CCR7 ligands, were reported to be constitutively expressed in the lymph nodes, being essential for the migration of lymphocytes to those tissues (Gunn *et al.* 1998, 1999, Ngo *et al.* 1998). CCR7 expression has also been associated with LNM in gastric carcinoma (Mashino *et al.* 2002), oesophageal squamous cell carcinoma (Ding *et al.* 2003), non-small cell lung cancer (Takanami 2003), squamous cell carcinoma of the head and neck (Wang *et al.* 2004) and colorectal carcinoma (Gunther *et al.* 2005).

In order to clarify the role of CCR7 in thyroid carcinomas, we analysed CCR7 expression in thyroid tumoral and normal tissues, and observed the *in vitro* effects of CCL21 in the proliferative, migratory and invasive characteristics of a human thyroid carcinoma cell line.

Materials and Methods

Tissue samples and cell lines

Tissue samples were obtained from normal thyroid gland and thyroid carcinomas of patients who underwent thyroidectomy at our Institute between 2000 and 2003. After surgery, samples were immediately frozen in liquid nitrogen or formalin-fixed and embedded in paraffin. Haematoxylin- and eosin-stained sections from each case were evaluated histologically to classify the tumours according to the 1988 WHO histological classification of thyroid tumours. For the present investigation, 46 samples were studied, including 19 papillary carcinomas (14 classic variants and five follicular variants), six follicular carcinomas, nine medullary carcinomas, four poorly differentiated carcinomas and eight normal tissues. Cases with lymphocytic thyroiditis or significant lymphocytic infiltration were excluded from this study, because lymphocytes express both chemokines and their receptors.

RNAs from the following cell lines were kindly provided by Dr Paula Soares: kat-10, B-CPAP, FB-2, K1 and TPC-1 (derived from PTCs), HTh74, 8505C, C643 and kat4 (from ATCs), kak-1 (from follicular adenoma) and XTC-1 (from Hürthle cell carcinoma). TPC-1 cell line, derived from PTC, was cultured in RPMI medium (Gibco, Invitrogen, Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml) and L-glutamine (2 mM) at 37 °C and in 5% CO₂.

RNA extraction, reverse transcriptase (RT)-PCR and real-time quantitative-PCR analysis

Total RNA from tissue samples and cell lines was extracted and purified with TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Total RNA (1 µg) was reverse transcribed at 37 °C for 90 min, using oligo (dT) primers and Superscript II reverse transcriptase (both from Life Technologies). PCR was performed in 35 cycles of amplification (95 °C for 1 min, 56 °C (CCR7) or 57 °C (CCL19 and CCL21) for 1 min and 72 °C for 1 min) using

specific primers for CCR7 (forward, 5'-CAGCCTTCCTGTGTGGTT-3'; reverse, 5'-AGGAACCAGGCTTTA-AAGT-3'; PCR product, 218 bp), CCL19 (forward, 5'-ATGGCCCTGCTACTGGCC-3'; reverse, 5'-CAATGCTTGACTCGGACT-3'; PCR product, 341 bp) and CCL21 (forward, 5'-ATGGCTCAGTCACTGGCT-3'; reverse, 5'-GGCCCTTTAGGGGTCTGT-3'; PCR product, 401 bp). Lymphocytic thyroiditis cDNA was used as a positive control. RNA integrity and cDNA synthesis efficiency were confirmed by PCR amplification for the housekeeping gene phosphoglycerate kinase-1 (*PGK-1*). PCR products were analysed by electrophoresis in 2% agarose gel stained with ethidium bromide.

Real-time quantitative (RQ)-PCR was performed according to the manufacturer's instructions using TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA), and CCR7- and CD45-specific primers and probes designed by Pre-Developed TaqMan Assay Reagents (Gene Expression TaqMan assays; Applied Biosystems). The reaction was performed in a 96-well reaction plate on an ABI-Prism sequence detector (model 7900 HT; Applied Biosystems). CCR7 expression in each sample was normalized relatively to an endogenous control (GAPDH, primers and probe by assays-on-demand; Applied Biosystems) and a calibrator (a pool of normal thyroid tissues). Each sample was analysed in triplicate.

Immunohistochemical staining

Paraffin-embedded thyroid carcinoma sections were immunohistochemically stained for CCR7 using conventional horse-radish peroxidase immunohistochemical staining methods. Briefly, 2 µm sections were deparaffinized and treated with 0.6% H₂O₂ in methanol to inhibit endogenous peroxidase. After microwave antigen retrieval, tissues were incubated with 10% normal goat serum (Dako X907; DAKO Corp., Glostrup, Denmark) for 10 min and endogenous avidin and biotin were blocked (Vector SP-2001, Vector Laboratories, Burlingame, CA, USA). The tissue sections were incubated with 1:500 purified mouse anti-human CCR7 monoclonal antibody (2H4; BD PharMingen, San Diego, CA, USA), overnight at 4 °C. This was followed by sequential incubations with biotin-conjugated secondary antibody, streptavidin-peroxidase and 3,3'-diaminobenzidine (DAB; Dako K5001, Glostrup, Denmark) for visualization. The sections were counterstained with Mayer's haematoxylin. Negative controls included omission of the primary antibody.

CCR7 cell surface expression analysis

For *in vitro* studies of CCR7 expression regulation, TPC-1 cells were cultured in 10% FBS, 1% FBS or serum-free RPMI. After 72 h, the cells were fixed in a 4% paraformaldehyde (PFA) solution for 10 min, incubated with 0.5 mg/ml anti-CCR7 mouse monoclonal antibody (2H4; BD PharMingen) for 30 min at 4 °C, and sequentially incubated with 1:500 FITC-conjugated goat anti-mouse IgM (Alexa-fluor 488; Molecular Probes, Invitrogen, Barcelona, Spain) for 1 h. To

determine the percentage of CCR7-positive cells, 5000 cells were collected for each sample by flow cytometry (FACScan; Becton, Dickinson and company, Franklin Lakes, NJ, USA). Lymphocytes were used as positive control and negative controls included omission of the primary antibody. The percentages of CCR7-positive cells were normalized relative to the normal growth conditions (RPMI/10% FBS). These experiments were repeated three times.

Immunofluorescence staining

TPC-1 cells grown on glass coverslips until 70% confluent were left untreated or stimulated with 200 nM CCL21 (6Ckine; R&D Systems, Minneapolis, MN, USA) for 20 h. The cells were then fixed in 4% PFA for 5 min, blocked in 5% normal serum and incubated overnight at 4 °C with the primary antibodies: mouse anti-human β 1-integrin (P5D2) at 1:1000 dilution, rabbit anti-human E-cadherin (H-108) at 1:75 dilution and goat anti-human fibronectin (N-20) at 1:100 dilution (all from Santa Cruz Biotechnology, Heidelberg, Germany). The cells were then incubated with conjugated secondary antibodies (goat anti-mouse Alexa 488, goat anti-rabbit Alexa 594 and donkey anti-goat 488, all from Molecular Probes) for 2 h at room temperature. The samples were mounted in Vectashield mounting medium (Vector-H-1000, Vector Laboratories) and analysed by confocal microscopy. For negative controls, primary or secondary antibodies were omitted. Sets of optical sections with 0.5 μ m intervals along the *z*-axis were obtained using a True Confocal scanner microscope (Leica TCSISP2), with 63 $\times$ 1.4 oil objectives. Acquisition and image treatment were performed with the LSC software (Leica) and with ImageJ 1.33u software (USA).

Actin polymerization assay

TPC-1 cells, grown until 70% confluent in glass coverslips, were incubated in the presence or absence of 200 nM CCL21 for 30 min and 1 h. For neutralizing experiments, CCR7 neutralizing antibody (5 μ g/ml, monoclonal anti-human CCR7 antibody, R&D Systems) was added 1 h before stimulation with CCL21. The cells were fixed in 4% PFA for 5 min, permeabilized in 0.1% (v/v) Triton X-100 for 30 min, labelled with 1 μ g/ml FITC-phalloidin (Sigma-Aldrich) for 30 min, and analysed by confocal microscopy as described before.

Gelatinolytic zymography

Supernatants from 4 $\times$ 10⁵ viable TPC-1 cells were collected after 12- and 24-h incubation in serum-free medium, with or without CCL21 (200 nM), and metalloproteinase activity was measured by gelatinolytic zymography, as previously described (Leber & Balkwill 1997, Dias *et al.* 2000). Briefly, cell culture supernatants were processed through SDS-PAGE gels containing 1% gelatin. The gels were subsequently incubated in 2.5% Triton X-100 for 1 h at room temperature, rinsed in distilled water and placed in low-salt collagenase buffer (50 mM Tris (pH 7.6),

0.2 M NaCl, 5 mM CaCl₂ and 0.2% Brij-35) at 37 °C for 16 h. Bands of gelatinolytic activity were visualized after staining the gels with 10 ml of a 0.2% Coomassie blue solution and 190 ml destain (distilled water, methanol and glacial acetic acid, 6:3:1) for 30 min at room temperature. Image acquisition and densitometry analysis were performed with an Epson Perfection 1200 Photo scanner and with ImageJ 1.33u software.

Migration experiments

TPC-1 cells were resuspended in serum-free RPMI medium and aliquots (100 μ l) of 5 $\times$ 10⁵ cells/ml with or without CCR7 neutralizing antibody (5 μ g/ml, monoclonal anti-human CCR7 antibody; R&D Systems) were added to 8 μ m pore Transwell inserts (Corning Incorporated Life Science, Corning, NY, USA) placed into the wells of a 24-well plate. The lower compartment contained serum-free RPMI medium with or without CCL21 (42 nM). The migration was carried out at 37 °C and 5% CO₂ for 12–20 h, and cells that had migrated to the bottom of the well were counted (ten high-power fields/well). Each experiment was repeated five times.

Cell proliferation assay

TPC-1 cells were cultured in 12-well plates at a cell density of 8 $\times$ 10⁴ cells per well, in serum-free medium with or without CCL21 (200 nM), and in the presence of a CCR7 neutralizing antibody (5 μ g/ml, monoclonal anti-human CCR7 antibody; R&D Systems) as a control condition. After 24, 48 and 72 h, the number of viable cells was determined by Trypan blue exclusion in a haemocytometer. Each experimental condition was performed in duplicate and the experiments were repeated three times.

Apoptosis analysis

TPC-1 cells were incubated in the presence or absence of 200 nM CCL21 for 48 h. The cells were washed with incubation buffer (10 mM HEPES (pH 7.4), 10 mM NaCl, 5 mM CaCl₂), and incubated for 30 min at room temperature with 0.5 mg/ml propidium iodide (Sigma-Aldrich) and annexin V-FITC (BD Biosciences, Pharmingen, CA, USA). Five thousand cells were collected for each sample by flow cytometry (FACScan; Becton-Dickinson).

Statistical analysis

Statistical significance of differences in CCR7 expression was determined by Mann-Whitney test. Correlation between CCR7 and CD45 expressions was analysed by Spearman's test. Statistical analysis of functional assays was performed by one-way ANOVA or unpaired *t*-test as appropriate. Values are given as average $\pm$ S.E.M. All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). *P* < 0.05 were considered significant.

Results

CCR7 is expressed in thyroid carcinoma cell lines and human thyroid tissues

In order to determine if thyroid carcinoma cell lines and human thyroid tissues express CCR7 and CCL21, and if this expression is dependent on the tumour histotype, we performed RT- and RQ-PCR.

CCR7 mRNA expression was detected in 8 out of 11 human thyroid carcinoma cell lines (Fig. 1A), including TPC-1 cells, whereas CCL19 and CCL21 mRNAs were present in only two and three cell lines respectively (data not shown).

As determined by RQ-PCR, CCR7 mRNA expression in normal thyroid tissues (2.45 ± 0.87 ; Fig. 1B) was not significantly different from PTCs (4.58 ± 1.02) or MTCs (5.37 ± 3.35), but it is statistically different from FTCs (0.46 ± 0.21 ; $P < 0.05$) and PDTCs (0.0004 ± 0.0002 ; $P < 0.01$). Interestingly, CCR7 expression was tenfold higher in PTCs than in FTCs ($P < 0.05$). Furthermore, when PTCs were subdivided into classic and follicular variants, the CCR7

expression level was ninefold higher in the former (6.0 ± 1.2 vs 0.64 ± 0.23 ; $P < 0.05$; Fig. 1C). PTCs with or without metastases had similar CCR7 mRNA levels (Fig. 1D), whereas CCR7 expression in MTCs was significantly correlated with the presence of lymph node metastases (LNM+, 7.96 ± 5.4 ; LNM-, 0.17 ± 0.08 ; $P < 0.05$; Fig. 1E).

Even though cases with lymphocytic thyroiditis or significant lymphocytic infiltration were excluded from this study, the presence of leukocytes could be influencing CCR7 expression levels. To test this hypothesis, RQ-PCR for CD45 (leukocyte common antigen) was performed. A moderate correlation was observed between CCR7 and CD45 expression (Spearman $r = 0.3448$, $P = 0.046$), but there was no significant correlation between CD45 expression and LNM in both PTC and MTC. Therefore, even though CCR7 expression levels must be analysed carefully, they do not seem to be directly due to lymphocytic infiltrates.

Thyroid tissues (normal tissues and primary tumours) were also analysed by RT-PCR for the presence of CCL21 expression, and this was detected in four out of four normal thyroid tissues, three out of four classic PTCs, zero out of four follicular PTCs, three out of four MTCs, one out of four FTCs and four out of four PDTCs. Although the series was small, no apparent correlation was observed between CCL21 expression and the presence of LNM.

Immunohistochemical staining with a CCR7-specific antibody showed the presence of CCR7 protein in the cell membrane and cytoplasm of tumour cells of different histologies (Fig. 2), mainly in the tumour periphery. CCR7 immunoreactivity was detected with high intensity in most of the neoplastic cells of PTCs, MTCs and their LNMs, and lower expression was seen in FTCs, FTAs, goitres and PDTCs (Fig. 2; Table 1). We observed that in LNM, there was consistently higher percentage of CCR7-positive cells compared with the corresponding primary tumours. Moreover, primary tumours showed a higher percentage of staining cells or cells with higher intensity than matching peritumoral normal tissues.

Analysing the sections of CCR7 staining, we could also observe that the number of infiltrating lymphocytes in the cases studied is low and that they do not seem to express CCR7, as shown in Fig. 2J. This observation further reduces the possibility that the presence of leukocytes could be influencing CCR7 expression levels.

CCR7 is regulated by extracellular conditions

Next, we sought to determine whether extracellular conditions that may be present inside tumours, such as decreased nutrient content, regulate CCR7 expression at the cell surface. For this purpose, TPC-1 cells were cultured in 10% FBS, 1% FBS or serum-free medium and, after 72 h, CCR7 cell surface expression was analysed by flow cytometry. We observed a 17% increase in CCR7 expression in 1% FBS ($P > 0.05$) and a 53% increase in serum-free conditions ($P < 0.01$), compared with normal growth

Figure 1 CCR7 is expressed in human thyroid carcinomas and cell lines. (A) Reverse transcriptase (RT)-PCR analysis on RNA isolated from human thyroid carcinoma cell lines. PGK-1 amplification was used for normalization. L, CD3⁺ lymphocytes (positive control); CCR7 expression was assessed by real-time quantitative RT-PCR in (B) normal and tumoral thyroid tissues of different histological types, (C) classic and follicular variants of PTCs, (D) classic PTCs with (N1) and without (N0) metastases and (E) MTCs with (N1) and without (N0) metastases. CCR7 expression in each sample was normalized relative to an endogenous control (GAPDH) and to a calibrator (pool of normal thyroid tissues). CCR7 was demonstrated to be highly expressed in classic PTCs and MTCs with lymph node metastases. Results are means $\pm$ s.e.m. (Mann-Whitney t -test; * $P < 0.05$; † $P < 0.01$).

Figure 2 Human thyroid tissues and metastatic lymph nodes show immunoreactivity for CCR7. CCR7 immunoreactivity was observed mainly in the cell membrane and cytoplasm of tumour cells of (A) classic PTCs (original magnification (o.m.), $\times 200$), (B) MTCs (o.m., $\times 200$) and (C) metastatic lymph node of MTC (o.m., $\times 200$). Staining of (D) follicular PTCs (o.m., $\times 100$), (E) FTCs (o.m., $\times 100$), (F) PDTCs (o.m., $\times 100$), (G) FTAs (o.m., $\times 100$), (H) goitre (o.m., $\times 100$), (I) peritumoral normal thyroid tissue (o.m., $\times 100$) and (J) infiltrating lymphocytes (o.m., $\times 100$) was weak or absent.

Table 1 CCR7 immunostaining of thyroid neoplasias

	Stage	Primary tumour		Normal thyroid		LNM	
		%	Intensity ^a	%	Intensity ^a	%	Intensity ^a
Diagnosis							
MTC	T2N1M1	30	+	20	+	70	+ / + +
MTC	T3N1M0	100	+ +			100	+ +
MTC	T4N1M0	100	+ + +	60	+ +	100	+ + +
MTC	T4N1M1	90	+ +	10	+	100	+ + +
Class PTC	T2N0M0	0		0			
Class PTC	T4N1M0	10	+	0		90	+ +
Class PTC	T4N1M1	100	+ +			100	+ +
Class PTC	T4N1M1	100	+ + +			100	+ + +
Class PTC	T4N1M0	100	+ +			100	+ +
Class PTC	T4N1M0	60	+	10	+	60	+ +
FVPTC	T2N0M0	100	+ + +	50	+		
FVPTC	T2bN0M0	90	+ +	60	+		
FVPTC	T3N0M0	90	+	30	+		
FVPTC	T3N0M0	80	+				
FTC	T4N1M1	80	+ +	80	+ +		
FTC	T3N0M0	80	+	10	+		
FTC	T2N0M0	80	+	20	+ / + +		
FTC	T2N0M0	50	+				
PDTC	T4N0M1	20	+				
PDTC	T2N0M0	0		40	+		
PDTC	T4N0M0	0					
PDTC	T4N0M0	90	+	0			
FTA		50	+	0			
FTA		0		10	+		
FTA		100	+				
FTA		80	+	30	+		
FTA		80	+ / + +				
Goiter		0					
Goiter		50	+				
Goiter		50	+				
Goiter		40	+				
Goiter		60	+				

MTC, medullary thyroid carcinoma; Class PTC, classic papillary thyroid carcinoma; FVTC, follicular variant of papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; FTA, follicular thyroid adenoma; LNM, lymph node metastases.

^aImmunostaining was classified according to the percentage of stained cells (%) and the intensity of staining: weak (+), moderate (++) or strong (+++).

conditions (10% FBS; data not shown). This suggests that *in vitro* conditions that mimic nutrient depletion, such as serum deprivation, modulate CCR7 expression on thyroid carcinoma cells.

CCL21 modulates tumour cell migration, invasion and proliferation

Subsequently, we investigated the phenotypic and molecular changes induced by CCL21 on thyroid carcinoma cells. For this purpose, TPC-1 cells were stimulated with CCL21, and its effect in promoting cell migration, invasion and proliferation was evaluated.

Reorganization of the actin cytoskeleton is an early event in the migratory response to chemokines (Van Haastert & Devreotes 2004). To investigate whether actin polymerization could be observed in response to CCL21, we examined FITC-conjugated phalloidin staining by confocal microscopy. After 30 min (data not shown) and 1 h of CCL21 stimulation (Fig. 3A), increased F-actin polymerization and the formation

of cell extensions resembling filopodia (Fig. 3A(b), arrowheads) were observed in TPC-1 cells. These changes were inhibited in the presence of CCR7-neutralizing antibody (Fig. 3A(c)).

We also investigated whether there were alterations in the expression of cell adhesion molecules, particularly β1-integrin, E-cadherin and fibronectin in CCL21-stimulated cells. Compared with control TPC-1 cells (Fig. 3B(a)), the cells treated with CCL21 presented β1-integrin expressing cell extensions (Fig. 3B(b); arrowheads) and increased β1-integrin expression and polarization at the membrane level (Fig. 3B(b); arrows). No significant differences were observed in fibronectin and E-cadherin expression in the presence of CCL21 (data not shown).

To determine if CCL21 induced migration of CCR7-positive thyroid tumour cells, we performed transwell migration assays. As shown in Fig. 4A, TPC-1 cells migrated significantly more in response to CCL21 than in control conditions (55% increase in CCL21-induced migration, *P* < 0.01). This increase in CCL21-induced cell migration

Figure 3 CCR7 activation leads to actin polymerization and $\beta 1$ -integrin expression in thyroid tumour cells. (A) TPC-1 cells were (a) left untreated, (b) treated for 1 h with 200 nM CCL21 or (c) treated for 1 h with 200 nM CCL21 in the presence of CCR7-neutralizing antibody. (b) The cells were stained for phalloidin, and show a considerable increase in F-actin polymerization and cell extensions resembling filopodia; (c) these effects were blocked by the CCR7-neutralizing antibody. (B) TPC-1 cells were (a) left untreated or (b) exposed to 200 nM CCL21 for 20 h, and subsequently immunostained for $\beta 1$ -integrin. Basal optical sections obtained by confocal microscopy (original magnification, $63\times$) are shown. In the presence of CCL21, TPC-1 cells presented increased $\beta 1$ -integrin expression and polarization at the membrane level (B(b); arrows) and $\beta 1$ -integrin expressing cell extensions (B(b); arrowheads).

was blocked in the presence of the CCR7-neutralizing antibody ($P<0.05$).

Next, we analysed the supernatants of cells grown in the presence or absence of CCL21 by gelatinolytic zymography, in order to determine the differences in matrix metalloproteinases (MMPs) secretion. Regarding MMP-2 (72 kDa), a 1.6-fold ($P<0.05$) increase was observed after 12 h in the presence of chemokine, and a 1.2-fold increase after 24-h stimulation (Fig. 4B). Similarly, the levels of MMP-9 (92 kDa) exhibited a twofold ($P<0.05$) and a 1.7-fold increase after 12 and 24 h respectively, in the presence of CCL21 (Fig. 4B).

Next, we examined the growth and viability of TPC-1 cells for 24, 48 and 72 h in the presence and absence of CCL21. As shown in Fig. 4C, upon CCL21 stimulation, TPC-1 cells proliferated significantly more than in control conditions after 48 ($P<0.01$) and 72 h ($P<0.05$). As above, the proliferative effect of CCL21 was blocked by the CCR7-neutralizing antibody. No difference in serum-free-induced apoptosis was detected in cells grown with or without CCL21 for up to 48 h, as observed by the annexin V and

propidium iodide binding assay (data not shown). Therefore, CCL21 activation of its receptor on thyroid carcinoma cells results in cell proliferation, but may not exert an anti-apoptotic effect.

Taken together, these findings suggest that CCL21 may promote cell migration and invasion through the modulation of cell adhesion molecules, actin polymerization and MMP production, and uphold thyroid carcinoma cell proliferation.

Discussion

Chemokines and their receptors have been suggested to play a key role in regulating the metastatic destination of tumour cells (Balkwill 2004). In fact, the recent demonstration of specific chemokine receptors on tumour cells and their response to the respective ligands has provided some insight into how tumour cells may home to specific organs to form metastases (Müller *et al.* 2001).

Figure 4 CCL21 induces MMP secretion, migration and proliferation of CCR7-positive thyroid carcinoma cells. (A) The migratory capacity of TPC-1 cells in response to CCL21 was evaluated by a Transwell migration assay. A 55% increase in the number of migrated cells was observed after 20 h in the presence of CCL21 compared with the control. This effect was inhibited by the CCR7-neutralizing antibody (mAbCCR7). (B) MMP secretion by TPC-1 cells after 12- and 24-h incubation with or without 200 nM CCL21, was analysed and quantified by gelatinolytic zymography (a). A significant increase in MMP activity after CCL21 stimulation was observed for both (b) MMP-2 and (c) MMP-9. (C) To assess the effects of CCL21 in thyroid tumour cells proliferation, TPC-1 cells were cultured in serum-free medium with or without CCL21 and in the presence of the CCR7-neutralizing antibody (mAbCCR7). The number of viable cells was determined after 24, 48 and 72 h. A significant increase in cell proliferation was observed at 48 and 72 h (* $P < 0.01$ and † $P < 0.05$).

It has also been demonstrated that antigen-presenting cells home to secondary lymphoid organs, through lymphatic vessels, by a CCR7-dependent mechanism (Gunn *et al.* 1998, 1999). Moreover, the homeostatic chemokine CCL21, a high-affinity ligand for CCR7, is abundantly expressed by lymphatic endothelial cells and T-cell paracortical regions in the lymph nodes and was shown to be responsible for the attraction of CCR7-positive cells (Gunn *et al.* 1998, Saeki *et al.* 1999). CCR7 expression has also been associated with LNM in some types of human tumours (Mashino *et al.* 2002, Ding *et al.* 2003, Takamami 2003, Wang *et al.* 2004, Gunther *et al.* 2005).

In the present study, we used RQ-PCR to quantify CCR7 expression in thyroid carcinomas and normal thyroid tissues. CCR7 expression was observed to be higher in tumours

prone to LNMs, namely classic PTCs and MTCs, whereas it was low or absent in tumours that usually do not metastasize to the lymph nodes (follicular variants of PTCs, FTCs and PDTs). Immunohistochemical studies confirmed the presence of CCR7 protein in the cytoplasm and cell membrane of neoplastic cells of PTCs, MTCs and their LNMs, and weak or no expression in normal thyroid epithelium and FTCs. Moreover, we have shown that CCR7 expression is regulated *in vitro* by extracellular conditions, namely nutrient deprivation, which may mimic areas inside tumours, particularly those with high proliferation rates. This finding also indicates that this receptor may have an important role in thyroid tumour biology. The regulation of chemokine receptors expression (other than CCR7) by extracellular signals has also been described in

response to other conditions, such as growth factors and hypoxia (Scotton *et al.* 2001, Chen *et al.* 2005).

High levels of actin polymerization are required for the formation of pseudopodia, which are needed for chemokine-mediated cell migration and invasion into surrounding tissues and efficient metastasis formation (Pokorna *et al.* 1994, Van Haastert & Devreotes 2004). In order to determine cell motility in stimulated cells, we performed phalloidin staining and observed that actin polymerization was enhanced in response to CCL21, with the formation of filopodia. Furthermore, CCL21 stimulation increased β 1-integrin expression. Since β 1-integrin has been suggested to play a crucial role in mediating the adhesion and arrest of other cell types at the endothelial vessels of lymph nodes (de la Rosa *et al.* 2003), this change also supports a putative invasive phenotype induced by CCR7 activation on thyroid carcinoma cells. Other adhesion molecules may also be involved in these processes.

The secretion and activation of proteolytic enzymes, namely matrix metalloproteinases, are also believed to be essential for tumour cell invasion through and across extracellular barriers and, consequently, to form metastases (Yu & Stamenkovic 2000, Egeblad & Werb 2002). TPC-1 cells stimulated with CCL21 showed an increase in MMP-2 and MMP-9 secretion, as analysed by gelatinolytic zymography, which suggests a more invasive phenotype.

We have also shown that TPC-1 cells migrate in response to CCL21 using the Transwell migration assay, thus supporting the hypothesis that CCL21 concentrations in the lymph nodes probably induce thyroid tumour cells migration into these organs through a CCR7-mediated mechanism. Therefore, we suggest that CCR7 activation by CCL21 may promote thyroid carcinoma LNM via an increase in actin polymerization, cell adhesion molecules modulation, cell migration and MMPs secretion.

After tumour cells have reached a certain organ, their ability to grow and effectively form metastases is dictated by molecular interactions of the cells with the environment on that specific organ. The effect of CCL21, which is highly expressed in the lymph nodes, in cell proliferation, has previously been reported in haematopoietic (Ploix *et al.* 2001) and mesangial cells (Banas *et al.* 2002), but it has never been described in tumour cells. We examined the effect of CCR7 activation and demonstrated that cell proliferation is significantly increased after 48- and 72-h stimulation with CCL21. This effect suggests that the lymph nodes, where CCL21 is abundantly expressed, provide conditions that favour the proliferation of metastatic thyroid tumour cells, once migrated and arrested at these secondary sites.

In conclusion, this study demonstrates that functional CCR7 is expressed in specific subsets of thyroid carcinoma cells, and that its ligand, CCL21, promotes thyroid tumour cells migration, invasion and proliferation, leading to a more invasive phenotype and contributing to lymph node-specific metastatic growth. Taken together, these findings support the

use of chemokine receptor antagonists (Robinson *et al.* 2003, Howard & Galligan 2004) for the treatment of specific subsets of thyroid tumours.

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References

- Balkwill F 2004 Cancer and the chemokine network. *Nature Reviews. Cancer* **4** 540–550.
- Banas B, Wormle M, Berger T, Nelson PJ, Cohen CD, Kretzler M, Pfistering J, Mack M, Lipp M, Grone HJ *et al.* 2002 Roles of SLC/CCL21 and CCR7 in human kidney for mesangial proliferation, migration, apoptosis, and tissue homeostasis. *Journal of Immunology* **168** 4301–4307.
- Chambers AF, Groom AC & MacDonald IC 2002 Dissemination and growth of cancer cells in metastatic sites. *Nature Reviews. Cancer* **2** 563–572.
- Chen S, Tuttle DL, Oshier JT, Knot HJ, Streit WJ, Goodenow MM & Harrison JK 2005 Transforming growth factor-beta1 increases CXCR4 expression, stromal-derived factor-1alpha-stimulated signalling and human immunodeficiency virus-1 entry in human monocyte-derived macrophages. *Immunology* **114** 565–574.
- DeLellis RA, Lloyd RV, Heitz PU & Eng C (Eds). *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Endocrine Organs*. Ch. 2, pp. 49–124. Lyon: IARC Press, 2004.
- Dias S, Hattori K, Zhu Z, Heissig B, Choy M, Lane W, Wu Y, Chadburn A, Hyjek E, Gill M *et al.* 2000 Autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration. *Journal of Clinical Investigation* **106** 511–521.
- Ding Y, Shimada Y, Maeda M, Kawabe A, Kaganai J, Komoto I, Hashimoto Y, Miyake M, Hashida H & Imamura M 2003 Association of CC chemokine receptor 7 with lymph node metastasis of esophageal squamous cell carcinoma. *Clinical Cancer Research* **9** 3406–3412.
- Egeblad M & Werb Z 2002 New functions for the matrix metalloproteinases in cancer progression. *Nature Reviews. Cancer* **2** 161–174.
- Fidler IJ 2003 The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nature Reviews. Cancer* **3** 453–458.
- Förster R, Schubel A, Breitfeld D, Kremmer E, Renner-Müller I, Wolf E & Lipp M 1999 CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* **99** 23–33.
- Gunn MD, Tangemann K, Tam C, Cyster JG, Rosen SD & Williams LT 1998 A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *PNAS* **95** 7118–7122.
- Gunn MD, Kyuwa S, Tam C, Kakiuchi T, Matsuzawa A, Williams LT & Nakano H 1999 Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *Journal of Experimental Medicine* **189** 451–460.
- Gunther K, Leier J, Henning G, Dimmler A, Weissbach R, Hohenberger W & Förster R 2005 Prediction of lymph node metastasis in colorectal carcinoma by expression of chemokine receptor CCR7. *International Journal of Cancer* **116** 726–733.
- Horuk R 2001 Chemokine receptors. *Cytokine and Growth Factor Reviews* **2** 313–335.

- Howard OM & Galligan CL 2004 An expanding appreciation of the role chemokine receptors play in cancer progression. *Current Pharmaceutical Design* **10** 2377–2389.
- Leber TM & Balkwill FR 1997 Zymography: a single-step staining method for quantitation of proteolytic activity on substrate gels. *Analytical Biochemistry* **249** 24–28.
- Liotta LA 2001 An attractive force in metastasis. *Nature* **410** 24–25.
- Mashino K, Sadanaga N, Yamaguchi H, Tanaka F, Ohta M, Shibuta K, Inoue H & Mori M 2002 Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric cancer. *Cancer Research* **62** 2937–2941.
- Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN *et al.* 2001 Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410** 50–56.
- Murphy PM 2001 Chemokines and the molecular basis of cancer metastasis. *New England Journal of Medicine* **345** 833–835.
- Ngo VN, Tang HL & Cyster JG 1998 Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naïve T cells and activated B cells. *Journal of Experimental Medicine* **188** 181–191.
- Ploix C, Lo D & Carson MJ 2001 A ligand for the chemokine receptor CCR7 can influence the homeostatic proliferation of CD4 T cells and progression of autoimmunity. *Journal of Immunology* **167** 6724–6730.
- Pokorna E, Jordan PW, O'Neill CH, Zicha D, Gilbert CS & Vesely P 1994 Actin cytoskeleton and motility in rat sarcoma cell populations with different metastatic potential. *Cell Motility and the Cytoskeleton* **28** 25–33.
- Robinson SC, Scott KA, Wilson JL, Thompson RG, Proudfoot AE & Balkwill FR 2003 A chemokine receptor antagonist inhibits experimental breast tumour growth. *Cancer Research* **63** 8360–8365.
- de la Rosa G, Longo N, Rodriguez-Fernandez JL, Puig-Kroger A, Pineda A, Corbi AL & Sanchez-Mateos P 2003 Migration of human blood dendritic cells across endothelial cell monolayers: adhesion molecules and chemokines involved in subset-specific transmigration. *Journal of Leukocyte Biology* **73** 639–649.
- Saeki H, Moore AM, Brown MJ & Hwang ST 1999 Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *Journal of Immunology* **162** 2472–2475.
- Scotton C, Milliken D, Wilson J, Raju S & Balkwill F 2001 Analysis of CC chemokine and chemokine receptor expression in solid ovarian tumours. *British Journal of Cancer* **85** 891–897.
- Takanami I 2003 Overexpression of CCR7 mRNA in nonsmall cell lung cancer: correlation with lymph node metastasis. *International Journal of Cancer* **105** 186–189.
- Van Haastert PJ & Devreotes PN 2004 Chemotaxis: signalling the way forward. *Nature Reviews. Molecular Cell Biology* **5** 626–634.
- Wang J, Xi L, Hunt JL, Gooding W, Whiteside TL, Chen Z, Godfrey TE & Ferris RL 2004 Expression pattern of chemokine receptor 6 (CCR6) and CCR7 in squamous cell carcinoma of the head and neck identifies a novel metastatic phenotype. *Cancer Research* **64** 1861–1866.
- Yu Q & Stamenkovic I 2000 Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF- β and promotes tumour invasion and angiogenesis. *Genes and Development* **14** 163–176.
- Zlotnik A & Yoshie O 2000 Chemokines: a new classification system and their role in immunity. *Immunity* **12** 121–127.

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