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Iodine regulates G_2/M progression induced by CCL21/CCR7 interaction in primary cultures of papillary thyroid cancer cells with RET/PTC expression

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Abstract. Treatment with high iodine concentrations can delay oncogenic activation effects, reduce cell growth and return thyroid-specific gene and protein expression levels to normal. During rearranged during transfection (RET)/papillary thyroid carcinoma (PTC) 3 activation, excess iodine can act as a protective agent in thyroid follicular cells. The chemokine receptor CCR7 serves a critical role in lymphocyte trafficking into and within lymph nodes, the preferential metastatic site for PTC. However, the potential associations between chemokine (C-C motif) ligand 21 (CCL21)/C-C chemokine receptor type 7 (CCR7) interaction and iodine concentrations in primary cultures of PTC with RET/PTC expression remain unclear. Proliferation assays of primary cultures of PTC cells with RET/PTC1 and RET/PTC3 expression indicated that CCR7 activation by its specific ligand, CCL21, was associated with significantly increased cell proliferation. Flow cytometry data indicated that CCL21/CCR7 interaction significantly increased the fraction of cells in the G_2/M phase of the cell cycle. Western blotting indicated that CCL21/CCR7 interaction significantly upregulated cyclin A, cyclin B1 and cyclin-dependent kinase 1 (CDK1) expression. Western blotting determined that CCL21/CCR7 interaction significantly enhanced the levels of phosphorylated extracellular signal-regulated kinase (P-ERK). Co-immunoprecipitation confirmed that there was interaction between P-ERK and cyclin A, cyclin B1 or CDK1, particularly in the presence of CCL21. Sodium iodide (NaI, 10^{-5} M) significantly abolished the effects of exogenous CCL21. These results suggest that CCL21/CCR7 interaction contributes to G_2/M progression of RET/PTC-expressing cells via the ERK pathway in association with 10^{-5} M NaI.

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Introduction

Papillary thyroid carcinoma (PTC) is the most common type of thyroid gland cancer, and it is commonly well differentiated with abilities for iodine uptake, thyroglobulin secretion and responsivity to thyroid-stimulating hormone (TSH) (1). Rearranged during transfection (RET)/PTC rearrangement and the BRAF V600E point mutation are the two most common genetic alterations associated with PTC (2). PTC is frequently associated with a RET gene rearrangement that generates a RET/PTC oncogene. This fusion results in a constitutively active mitogen-activated protein kinase (MAPK) pathway, which serves a key role in PTC development. PTC accounts for approximately 80% of thyroid malignancies (3,4), and ionizing radiation has been described as an important etiological factor in PTC development (5), with it widely known that following the Chernobyl disaster, 1,000s of people developed thyroid cancer (6,7). PTC is characterized by gene rearrangements affecting the RET proto-oncogene located on chromosome 10q11.2 and coding for a cell membrane tyrosine kinase receptor (8). RET serves a regulatory role in cell survival, growth, differentiation and migration (9). In PTC, RET fuses with different ubiquitous genes on the same or alternate chromosomes to yield various RET/PTC fusion rearrangements, leading to the abnormal expression of a chimeric RET protein that is constitutively activated in thyroid follicular cells (10). Among the 13 fusion patterns of RET with 12 different genes reported at present (11), RET/PTC1 and RET/PTC3 are the major variants, while the others are rare and hypothesized to be of little clinical significance.

CCR7 is expressed on all naïve T-cells and on certain memory T-cells, B-cells and mature dendritic cells (12). Chemokine (C-C motif) ligand 21 (CCL21)/C-C chemokine receptor type 7 (CCR7) interaction drives cell cycle progression involving the G_2/M phase. CCR7 is highly expressed in PTC, non-small cell lung cancer, breast cancer and head and neck squamous cell carcinoma, and it mediates metastasis in certain cancer cell lines (13,14).

Iodine serves an important role in the normal thyroid follicular cell, regulating differentiation and proliferation, whereas excess iodine serves an anti-oncogenic role during thyroid oncogenic activation. RET/PTC3 fusion is primarily associated with radiation-associated PTC. Epidemiological studies have reported a lower incidence of

PTC in radiation-exposed regions that are associated with an iodine-rich diet (6). At present, the association between iodine concentrations and CCL21/CCR7 interaction and the cell cycle in PTC remain to be fully elucidated.

In the current study, it was demonstrated that CCL21/CCR7 interaction contributes to the time-dependent proliferation of PTC cells by upregulating cyclin A, cyclin B1 and cyclin-dependent kinase 1 (CDK1) expression via the extracellular signal-regulated kinase (ERK) pathway associated with iodine [10^{-5} M sodium iodide (NaI)]. The current study aimed to provide insight into the mechanisms of survival of CCL21/CCR7-mediated cancer cells in association with iodine and elucidate the implications for treatment targets in PTC.

Materials and methods

Cell culture. Primary cultures of thyrocytes derived from PTC thyroid tissue from six patients aged 25-57 years (two men and four women) from Jinshan Hospital, Fudan University, Shanghai, China between March 2013 and May 2013, obtained immediately subsequent to thyroidectomy, were tested in accordance with a previously described method (15). Patient consent was obtained, and the institutional review board had approved the project. Thyroid cells were dissociated using a discontinuous trypsin-ethylene glycol-O-O´-bis(2-amino-ethyl)-N,N,N',N'-tetra-acetic acid (EGTA) treatment. Aliquots of freshly isolated cell suspension (5 ml; $3x10^6$ cells per ml) in Eagle's minimum essential medium (pH 7.4), containing 10% (v/v) foetal calf serum and 1 mU/ml TSH, were seeded onto polystyrene flasks treated for tissue culture (Nunc®; Nalge Nunc International, Copenhagen, Denmark) and incubated at 37°C in a 95% air/5% CO_2, water-saturated atmosphere. Under these conditions, cells organized themselves into follicle-like structures adhering to the plastic-treated surface. Thyroid cells were observed using phase-contrast microscopy (Olympus IMT-2; Olympus Corp., Tokyo, Japan). Subsequent to dissociation, the primary culture cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with TSH. Fibroblast contamination was controlled by pathologists; potential fibroblast contamination was detected with a rabbit anti-vimentin monoclonal antibody (cat. no. ab92547; 1:200; Abcam, Cambridge, UK), and no immunostaining was observed. Subsequently, all cultured cells were immunostained using antibodies against a mouse mesothelioma monoclonal antibody (HBME-1; cat. no. ab101139, 1:100; Abcam) and/or a mouse cytokeratin 19 monoclonal antibody (1:200; cat. no. ab7754, Abcam). The PTC1 and PTC3 cell lines derived from the six primary PTC cell cultures were cultured in RPMI-1640 or Dulbecco's modified Eagle's medium-F12 supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in an atmosphere of 5% CO_2 at 37˚C. Cells were grown in culture flasks and harvested in trypsin-ethylenediaminetetraacetic acid solution during the logarithmic growth phase. The antibodies used were: Anti-cyclin A2 (rabbit monoclonal antibody; cat. no. ab32498, 1:500, Abcam), anti-cyclin B1 (mouse monoclonal antibody, cat. no. ab72, 1:800, Abcam), anti-CDK1 (rabbit monoclonal antibody; cat. no. ab32384, 1:2,000, Abcam), anti-phosphorylated ERK (P-ERK; (rabbit monoclonal antibody; cat. no. ab76299, 1:8,000, Abcam), anti-ERK (rabbit monoclonal antibody; cat. no. ab184699, 1:10,000, Abcam), anti-immunoglobulin G (IgG; rabbit, cat. no. sc-2027, dilution 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-β-actin [rabbit monoclonal antibody (13E5); cat. no. #4970, 1:1,000, Cell Signalling Technology, Inc., Beverly, MA, USA). Recombinant human CCL21 and Cell Counting Kit-8 (CCK-8) were obtained from the State Key Laboratory of Molecular Oncology, Chinese Academy of Medical Sciences (Beijing, China).

For the incubations, all cultured cells were washed with phosphate-buffered saline (PBS; pH 7.0) three times and blocked with 10% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA) for 30 min at room temperature; subsequently, the cells were incubated overnight at 4°C with the first antibodies. After washing with PBS, cells were incubated with the secondary antibody for 1 h at room temperature. Finally, cells were stained with 3,3'-diaminobenzidine.

Cell proliferation assay. Cell proliferative activities were examined using CCK-8. PTC1 and PTC3 cells were seeded in 96-well plates and treated with CCL21 (100 ng/ml) for 0, 24, 48 or 72 h. Following treatment, CCK-8 was added to each well according to the manufacturer's instructions and incubated for 4 h at 37˚C. The optical density was measured using a microplate reader (Guava Technologies, Inc., Hayward, CA, USA) at 450 nm.

Cell cycle analysis. Subsequent to 24-h treatment with CCL21, cells were harvested and washed twice with PBS and fixed in 75% ethanol for 2 h at 4˚C. The fixed cells were washed twice with 500 μl cold PBS, and then were stained with 500 μl propidium iodide staining solution for 30 min at room temperature in the dark. A total of 10,000 events per sample were acquired using a FACScan flow cytometer (Guava Technologies, Inc.), and the percentage of cells in the G_0/G_1, S and G_2/M phases of the cell cycle were determined using ModFit LT software, version 3.0 (Guava Technologies, Inc.).

Western blot analysis. Western blot analyses were performed as previously described (16). Briefly, proteins were electrophoresed on 12% polyacrylamide gels and transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare Life Sciences, Chalfont, UK). Western blot analysis was conducted with specific primary antibodies (against cyclin A2, cyclin B1, CDK1, P-ERK and ERK; see the previous section for further details) diluted in 1% BSA (Sigma Chemical Co.) in Tris-buffered saline with Tween-20 (TBST), followed by the peroxidase-conjugated secondary antibody [goat anti-mouse polyclonal antibody; cat. no. L3032, dilution 1:6,000; Signalway Antibody (SAB), College Park, MD, USA]. After blocking with 5% non-fat milk in TBST for 1 h at room temperature, Western blot analysis was performed with the specific primary antibodies in 1% BSA in TBST for an incubation overnight at 4°C, followed by an incubation with the peroxidase-conjugated secondary antibody for 1 h at room temperature. TBST was used for the washing steps. Target proteins were observed using the enhanced chemiluminescence detection system

(GE Healthcare Life Sciences) and autoradiography on Fuji Super RX film (Fujifilm Corporation, Tokyo, Japan), with 1-2 min exposure.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. PCR was used to quantify mRNA expression, and phosphoglycerate kinase (PGK-1) was used as the internal control. For mRNA, RT-PCR was performed using a One-Step RT-PCR kit (cat. no. RR057A; Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. Aliquots of 20 μl cDNA (150 ng) were used for the amplification reaction. The primer sequences for CCR7, RET/PTC1, RET/PTC3 and PGK-1 used were as described previously (11,17). The cycling conditions were as follows: denaturation at 95˚C for 15 sec, followed by 40 cycles of annealing at 56˚C for 60 sec and extension at 72˚C for 60 sec

Co-immunoprecipitation. Cells were extracted with lysis buffer and homogenized for 30 min at 4˚C subsequent to 24-h treatment with CCL21. The extracts were centrifuged at 12,000 x g for 15 min at 4˚C, and the supernatants containing total protein were harvested. Equal amounts of protein were exposed to the antibodies against cyclin A, cyclin B1, CDK1, P-ERK or IgG. The beads were washed extensively with lysis buffer, boiled, and microcentrifuged at 3,000 rpm (1,007 g) for 3 min at 4˚C following a 3 h incubation at 4˚C. Proteins were detected with antibodies against cyclin A, cyclin B1, CDK1, P-ERK or IgG by western blotting.

Statistical analysis. Differences between the groups were analyzed by one-way analysis of variance using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate statistically significant difference.

Results

NaI (10^{-5} M) abolishes the effects of CCL21/CCR7 interaction on proliferation. The current study identified two cell lines from six primary PTC cultures, RET/PTC1 and RET/PTC3, which both have high CCR7 expression (Figs. 1 and 2). According to the results of a previous study (18), 100 ng/ml CCL21 markedly promoted cell proliferation as compared with 50 ng/ml CCL21, while there was no clear difference observed between the effects of 100 and 200 ng/ml CCL21 on cell proliferation. Therefore, 100 ng/ml CCL21 was selected for use in the current study.

The experiments determined that 10^{-5} M NaI significantly abrogated the effects of CCL21/CCR7 interaction on cell proliferation; the intermediate concentration of 10^{-5} M NaI alone did not affect cell proliferation significantly, and the physiological concentration of 10^{-7} M NaI could not abrogate the effects of CCL21/CCR7 interaction on cell proliferation. The high concentration of 10^{-3} M NaI alone had significant effects on cell proliferation. The 10^{-5} M NaI concentration was used to verify whether it could affect the effects of CCL21/CCR7 interaction on PTC1 and PTC3 cell proliferation. Cell viability and cell cycle distribution were examined using the CCK-8 assay and flow cytometry, respectively. It was identified that

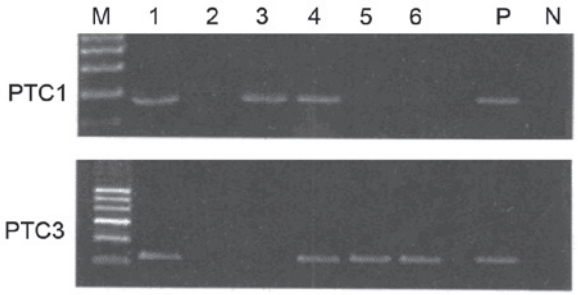

Figure 1. PTC1 and PTC3 cell lines derived from six primary PTC cell cultures as measured by reverse transcription-polymerase chain reaction. PTC, papillary thyroid carcinoma; M, marker; P, positive control; N, negative control.

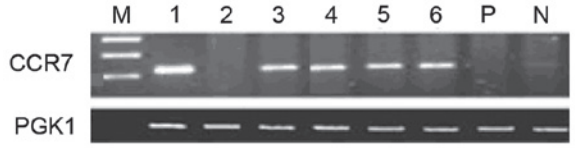

Figure 2. CCR7 expressed in six primary papillary thyroid carcinoma cell cultures as measured by reverse transcription-polymerase chain reaction. CCR7, C-C chemokine receptor type 7; M, marker; P, positive control; N, negative control; PGK1, phosphoglycerate kinase.

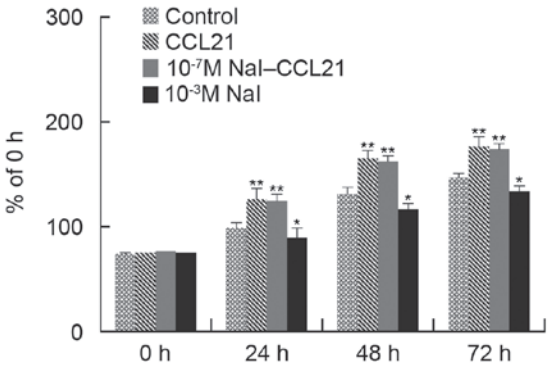

Figure 3. Effect of CCL21/C-C chemokine receptor type 7 interaction on PTC3 cell proliferation after treatment with 10^{-3} or 10^{-7} M NaI. PTC3 cells were treated with CCL21 (100 ng/ml) for 24, 48 or 72 h following 1-h treatment with 10^{-3} or 10^{-7} M NaI. Cell viability was estimated using the Cell Counting Kit-8 assay. Error bars represent the mean ± standard deviation of three independent experiments. $^{*}P<0.05$ or $^{**}P<0.01$ vs. control cells. CCL21, chemokine (C-C motif) ligand 21; PTC, papillary thyroid carcinoma.

10^{-5} M NaI significantly abrogated the effects of CCL21/CCR7 interaction on cell proliferation (Figs. 3 and 4).

CCL21/CCR7 interaction augments the proportion of cells in G_2/M. Cell cycle analysis was performed using flow cytometry to verify whether the effect of CCL21/CCR7 interaction on PTC1 and PTC3 cell proliferation is associated with an alteration in cell cycle distribution. CCL21/CCR7 interaction significantly enhanced the proportion of cells in G_2/M, however had no significant effect on the proportion of cells in the G_0/G_1 or S phases as compared with control cells. NaI (10^{-5} M) significantly abolished this effect of CCL21, however did not have a significant effect on cell cycle distribution (Tables I and II).

Table I. Effect of CCL21 and NaI on cell cycle distribution in PTC1 cells.

Group	G_0/G_1 phase (%)	S phase (%)	G_2/M phase(%)
Control	73.35±9.43	21.35±11.28	2.13±2.76
CCL21	65.72±1.23	19.60±2.91	11.25±1.54[a]
10^{-5} M NaI-CCL21	64.08±9.08	22.90±8.28	6.50±3.49
10^{-5} M NaI	71.73±4.32	17.09±3.33	7.19±3.46

Data presented are the mean ± standard deviation of three independent experiments. [a]P<0.01 vs. control cells. CCL21, chemokine (C-C motif) ligand 21; PTC, papillary thyroid carcinoma.

Table II. Effect of CCL21 and NaI on cell cycle distribution in PTC3 cells.

Group	G_0/G_1 phase (%)	S phase (%)	G_2/M phase(%)
Control	59.50±3.70	33.33±4.41	7.22±2.63
CCL21	61.38±6.35	28.08±4.16	13.54±2.95[a]
10^{-5} M NaI-CCL21	68.25±4.44	20.40±3.11	6.02±2.77
10^{-5} M NaI	62.57±2.11	27.00±9.66	4.43±1.10

Data presented are the mean ± standard deviation of three independent experiments. [a]P<0.01 vs. control cells. CCL21, chemokine (C-C motif) ligand 21; PTC, papillary thyroid carcinoma.

NaI (10^{-5} M) significantly abrogates the upregulatory effects of CCL21/CCR7 interaction on cyclin A, cyclin B1, CDK1, and P-ERK expression (Fig. 5). To determine the possible mechanism by which CCL21/CCR7 interaction influences G_2/M distribution in PTC cells, cyclin A, cyclin B1 and CDK1 expression were assessed using western blotting. Compared with the control cells, cells with CCL21/CCR7 interaction exhibited significantly upregulated protein levels of cyclin A, cyclin B1 and CDK1 (Fig. 5A). It was identified that 10^{-5} M NaI significantly abrogated the effects of CCL21; however, it had no significant effect on cyclin A, cyclin B1 or CDK1 expression. To verify whether the CCL21/CCR7 interaction may additionally enhance P-ERK expression in PTC cells, ERK and P-ERK expression levels were measured using western blotting. CCL21/CCR7 interaction upregulated P-ERK expression significantly, however, had no significant effect on ERK expression. Following treatment with 10^{-5} M NaI, the significant upregulation of P-ERK expression by CCL21/CCR7 interaction was abrogated (Fig. 5B).

Induced by CCL21/CCR7 interaction, P-ERK interacts with cyclin A, cyclin B1 and CDK1. Co-immunoprecipitation was used to determine whether there is interaction between P-ERK and cyclin A, cyclin B1 or CDK1. PTC3 cells were incubated for 24 h with or without CCL21, then were immunoprecipitated with antibodies against P-ERK or IgG, followed by western blotting for cyclin A, cyclin B1 and CDK1. There was a pronounced, specific interaction between P-ERK and cyclin A, cyclin B1 and CDK1, particularly subsequent to 24-h treatment with CCL21. Reciprocal immunoprecipitation was assessed with antibodies against cyclin A, cyclin B1, CDK1 or IgG using western blotting for P-ERK. The resulst were consistent with those above mentioned, with the interaction

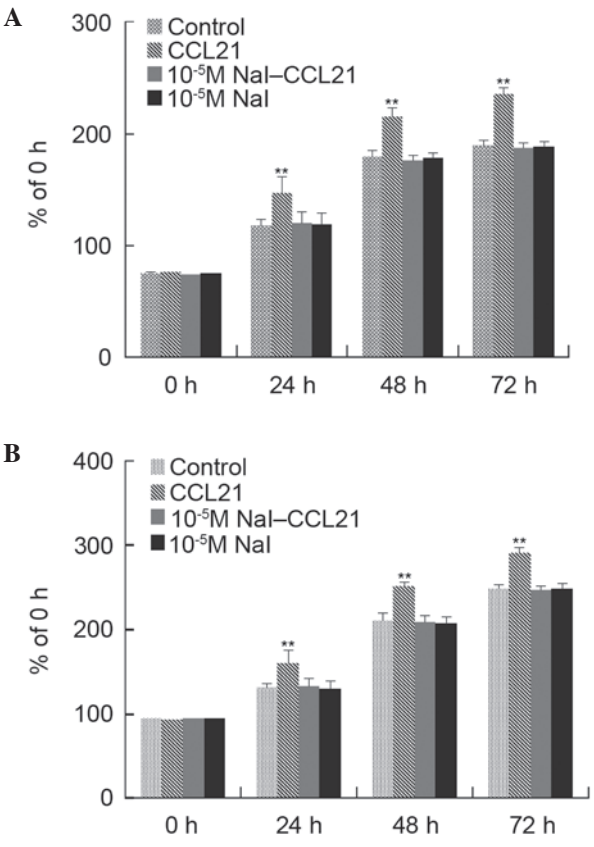

Figure 4. Effect of CCL21/C-C chemokine receptor type 7 interaction on PTC1 and PTC3 cell proliferation after treatment with 10^{-5} M NaI. (A) PTC1 and (B) PTC3 cells were treated with CCL21 (100 ng/ml) for 24, 48 or 72 h following 1-h treatment with 10^{-5} M NaI. Cell viability was estimated using the Cell Counting Kit-8 assay. Error bars represent the mean ± standard deviation of three independent experiments. **P<0.01 vs. control cells. CCL21, chemokine (C-C motif) ligand 21; PTC, papillary thyroid carcinoma.

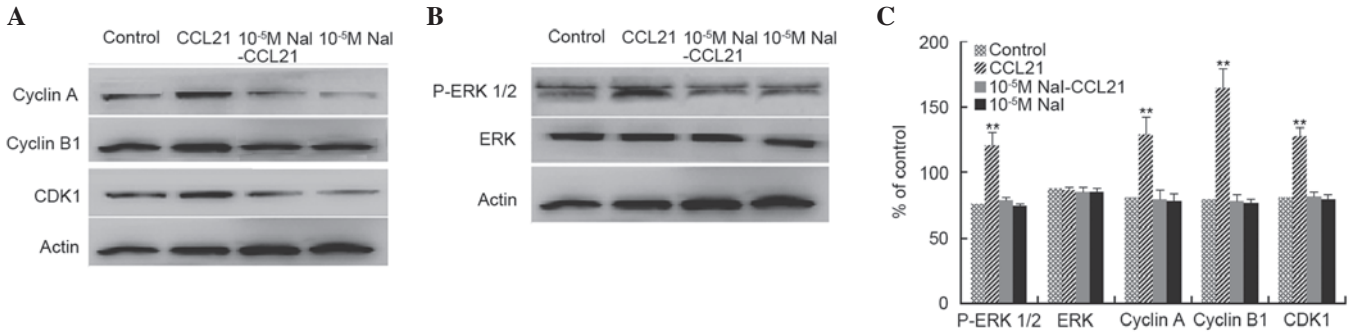

Figure 5. Effect of CCL21/C-C chemokine receptor type 7 interaction on P-ERK, ERK, cyclin A, cyclin B1 and CDK1 expression subsequent to inhibition of ERK activation. PTC3 cells were treated with CCL21 (100 ng/ml) for 24 h following 1-h treatment with 10^{-5} M NaI. The expression levels of (A) cyclin A, cyclin B1 and CDK1, and (B) P-ERK and ERK, were estimated using western blotting, and (C) quantified. Error bars represent the mean ± standard deviation of three independent experiments. **P<0.01 vs. control cells. CCL21, chemokine (C-C motif) ligand 21; P-, phosphorylated; ERK, extracellular signal-related kinase; CDK1, cyclin-dependent kinase 1; PTC, papillary thyroid carcinoma.

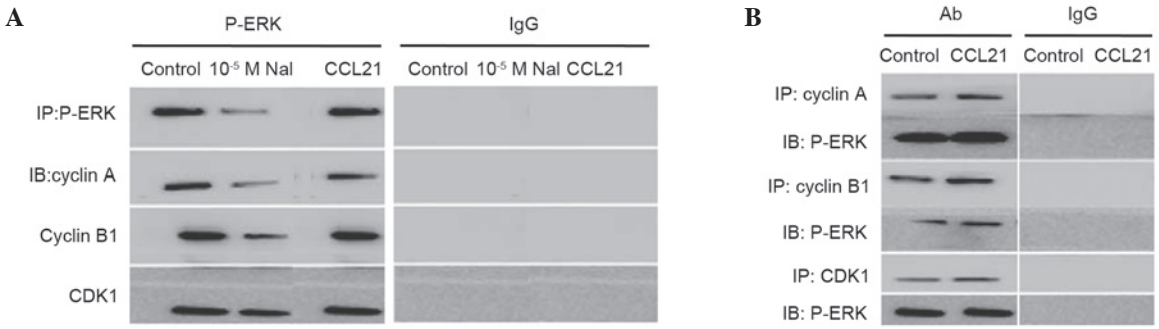

Figure 6. Interaction between P-ERK and cyclin A, cyclin B1 or CDK1 with or without CCL21 or 10^{-5} M NaI. (A) Western blot analysis for cyclin A, cyclin B1 and CDK1 in PTC3 cells incubated with or without CCL21 (100 ng/ml) or 10^{-5} M NaI for 24 h. (B) Western blot analysis for P-ERK was used to analyze reciprocal immunoprecipitation with antibodies against cyclin A, cyclin B1, CDK1 or IgG. P-, phosphorylated; ERK, extracellular signal-related kinase; CDK1, cyclin-dependent kinase 1; CCL21, chemokine (C-C motif) ligand 21; PTC, papillary thyroid carcinoma; IgG, immunoglobulin G; IP, immunoprecipitation; IB, immunoblotting; Ab, antibody.

between P-ERK and cyclin A, cyclin B1 or CDK1 observed to be clear, particularly in the presence of CCL21. PTC3 cells incubated for 1 h with or without 10^{-5} M NaI were immunoprecipitated with antibodies against P-ERK or IgG, followed by western blotting for cyclin A, cyclin B1 and CDK1. There was a reduced interaction between P-ERK and cyclin A, cyclin B1 and CDK1 in response to the 10^{-5} M NaI exposure (Fig. 6).

Discussion

Iodine serves an important role in regulating differentiation and proliferation in the normal thyroid follicular cell, whereas during thyroid oncogenic activation, excess iodine serves an anti-oncogenic role (5). Previous studies have reported that CCR7 activation mediates survival in certain cancer cell lines by promoting cell migration and proliferation or by inhibiting apoptosis (17,18). At present, the association between iodine concentrations and CCL21/CCR7 interaction and the cell cycle in RET/PTC remains to be fully elucidated. In the current study, CCL21/CCR7 interaction significantly enhanced human RET/PTC cell proliferation in a time-dependent manner, and involved cyclin A, cyclin B1 and CDK1 upregulation, possibly via the ERK pathway. In addition, 10^{-5} M NaI regulates G_2/M progression induced by CCL21/CCR7 interaction in primary cultures of PTC1 and PTC3 cells.

CCR7 activation has been previously reported to increase P-ERK levels (18,19). ERK belongs to the MAPK family; activated by mitogenic stimuli, the ERK cascade is critical for cell proliferation and survival (20) and is required for normal progression to mitosis (18). The cell cycle is regulated by cyclins and CDKs: cyclin A is essential for progression through the S phase (21,22). Cyclin A and cyclin B1 associate with CDK1 to promote entry into mitosis (18,21,22). In the current study, both protein and mRNA levels of cyclin A, cyclin B1 and CDK1 in PTC1 and PTC3 cells were observed to be significantly upregulated when cells had been treated with CCL21 for 24 h, indicating that CCL21/CCR7 interaction accelerates G_2/M progression to promote cell proliferation. This observation demonstrates that CCL21/CCR7 interaction drives cell cycle progression involving the G_2/M phase in PTC1 and PTC3 cells.

As CCL21/CCR7 interaction increases P-ERK expression, it was determined whether there was an interaction between P-ERK and cyclin A, cyclin B1 or CDK1. Co-immunoprecipitation and reciprocal immunoprecipitation strongly suggested interaction between P-ERK and cyclin A, cyclin B1 and CDK1, particularly in the presence of CCL21; inhibiting ERK with 10^{-5} M NaI weakened this interaction. And 10^{-5} M NaI abolished the effect of CCL21/CCR7 interaction on PTC1 and PTC3 cell proliferation and G_2/M progression and downregulated P-ERK, cyclin A, cyclin B1 and CDK1 expression. These results demonstrate the

effect of CCL21/CCR7 interaction on cell proliferation and that cyclin A, cyclin B1 and CDK1 upregulation may occur via the ERK pathway in association with 10^{-5} M NaI in PTC cells.

The present study suggests that activating CCR7 with CCL21 significantly promotes PTC1 and PTC3 cell proliferation in a time-dependent manner and involves cyclin A, cyclin B1 and CDK1, potentially via the ERK pathway. In addition, it was identified that iodine regulates G_2/M progression induced by CCL21/CCR7 interaction in primary cultures of PTC cells with RET/PTC expression. This information may aid in clarifying the mechanisms of cancer cell survival and identify potential targets for PTC treatment.

Acknowledgements

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