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CCR7 is involved in BCR-ABL/STAP-2-mediated cell growth in hematopoietic Ba/F3 cells

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ABSTRACT

Chronic myeloid leukemia is a clonal disease characterized by the presence of the Philadelphia chromosome and its oncogenic product, BCR-ABL, which activates multiple pathways involved in cell survival, growth promotion, and disease progression. We previously reported that in murine hematopoietic Ba/F3 cells, signal transducing adaptor protein-2 (STAP-2) binds to BCR-ABL and up-regulates BCR-ABL phosphorylation, leading to enhanced activation of its downstream signaling molecules. The binding of STAP-2 to BCR-ABL also influenced the expression levels of chemokine receptors, such as CXCR4 and CCR7. For the induction of CCR7 expression, signals mediated by the MAPK/ERK pathway were critical in Ba/F3 cells expressing BCR-ABL and STAP-2. In addition, STAP-2 cooperated with BCR-ABL to induce the production of CCR7 ligands, CCL19 and CCL21. Our results demonstrate a contribution of CCR7 to STAP-2-dependent enhancement of BCR-ABL-mediated cell growth in Ba/F3 cells.

1. Introduction

The BCR-ABL fusion oncogene is responsible for the pathogenesis of chronic myeloid leukemia (CML) [1], which is characterized by the premature release of leukemia cells from bone marrow as well as a substantial accumulation of those with a potential of differentiation in the blood, spleen, and bone marrow. Owing to its elevated tyrosine kinase activity, BCR-ABL activates a number of signaling pathways, including the Ras, PI3K/AKT, Janus kinase/signal transducer and activator of transcription, and NF-κB signaling pathways [1,2]. Moreover, in various human and mouse models, hematopoietic cells expressing BCR-ABL show a growth advantage, resistance to apoptosis, and altered adhesion and homing properties.

Signal transducing adaptor protein-2 (STAP-2) is a novel adaptor molecule, which was isolated as a c-FMS interacting protein [3]. The human homolog of STAP-2 is a known substrate of breast tumor kinase (BRK) [4]. Previous work in our laboratory found that STAP-2 can associate with and influence a variety of signaling or transcriptional molecules [3,5–9], including STAT3 and STAT5 [3,5], and FcεRI and Toll-like receptor-mediated signals [6,9]. Further, thymocytes and peripheral T cells from STAP-2-deficient mice show enhanced IL-2-dependent cell growth and integrin-mediated T-cell adhesion and impaired SDF-1α-induced T-cell migration [5,7,8]. Because STAP-2 is expressed in a variety of tissues and cells, such as lymphocytes, macrophages, and hepatocytes, STAP-2 is also likely to function in a variety type of cells. Importantly, we identified BCR-ABL as a novel binding partner of STAP-2, and STAP-2 augmented BCR-ABL activity and activation of downstream signaling molecules, including ERK, STAT5, BCL-xL, and BCL2 [10]. We also found that STAP-2 over-expression in murine hematopoietic Ba/F3 cells confers a growth advantage *in vitro* and is able to induce leukemia *in vivo*. In addition, STAP-2 controls the migration and homing of BCR-ABL-expressing cells by influencing chemokine receptor expression levels.

In the present study, we found that chemokine receptor CCR7 influences the enhancement of BCR-ABL-dependent cell growth via STAP-2 and that CCR7 mRNA levels are mediated through the MAPK/ERK pathway in murine hematopoietic Ba/F3 cells.

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2. Materials and methods

2.1. Reagents and antibodies

BCR-ABL tyrosine kinase inhibitor STI571, imatinib mesylate, was kindly gifted by Novartis Pharmaceuticals (Basel, Switzerland). A6730 and U0126 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Expression vectors for human CXCR4 (hCXCR4) was generated by PCR methods and sequenced (primer sequences are available upon request). Anti-ABL, anti-Stat5, anti-BCL-xL, and anti-phospho ERK (pERK) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Myc, anti-pStat5, and anti-Actin mAb were purchased from Sigma–Aldrich. Anti-ERK, anti-Akt, and anti-pAkt antibodies was purchased from Cell signaling Technologies (Beverly, MA).

2.2. Cell culture, establishment of cell lines and siRNA transfection

An interleukin (IL)-3-dependent murine pro-B cell line, Ba/F3 was maintained in RPMI1640 medium supplemented with 10% FCS with 10% of WEHI-3B conditioned medium as a source of IL-3. Stable Ba/F3 transformants expressing pcDNA3 (Ba/F3-pcDNA), pcDNA3-STAP-2 (Ba/F3-STAP-2) or pcDNA3-p210 BCR-ABL (Ba/F3-p210) were established as described previously [3,10]. Stable Ba/F3 transformants expressing human CXCR4 (Ba/F3-p210/STAP-2/hCXCR4#1 and #2) were established by transfection with pcDNA3-hCXCR4. Stable CCR7 knockdown Ba/F3-p210/STAP-2 cell lines (Ba/F3-p210/STAP-2/shCCR7#1 and #2) were established by transfection of with pGPU6/GFP vector (Shanghai GenePharma, Shanghai, China) bearing short hairpin RNA (shRNA) targeting CCR7 (5′-GCATCTTTGGCATCTATAAGTT-3′). Similarly, control shRNA (non-sliencing; 5′-TTCTCCGAACGTGTCACGT-3′)-transfected Ba/F3-p210/STAP-2 cell lines (Ba/F3-p210/STAP-2/shControl) were also established. Ba/F3-p210/STAP-2 cells transfected with control or Stat5a/b siRNAs using a Nucleofector (Amaxa Biosystems, Cologne, Germany). Cells were transfected with 200 pmol siRNA in Nucleofector solution V. The siRNAs targeting mouse Stat5a and Stat5b used in this study were as follows: Stat5a, 5′-AGGUCUUUGCCAAGUAUUATT-3′; Stat5b, 5′-GCGUGAUGGAAGUAUUGAATT-3′. Control siRNA was obtained from Qiagen (non-silencing; cat. no. 1022076).

2.3. RT-PCR and quantitative real-time PCR

Cells were harvested, total RNAs from the transfected cells were prepared by using Isogen (Nippon Gene, Tokyo, Japan) and used in RT-PCR. RT-PCR was performed using RT-PCR high -Plus- Kit (TOYOBO, Tokyo, Japan). Primers used for RT-PCR were: *CXCR4*: 5′-GGTGGTCTATGTTGGCGTCT-3′ (sense), 5′-TGGAGTGTGACAGCTTG GAG-3′ (antisense); *Ccl19*: 5′-AGACTGCTGCCTGTCTGTGA-3′ (sense), 5′-GCCTTTGTTCTTGGCAGAAG-3′ (antisense); *Ccl21*: 5′-GTCCGAGG CTATAGGAAGCA-3′ (sense), 5′-GCCCTTTCCTTTCTTTCCAG-3′ (antisense); *G3pdh*: 5′-GAAATCCCATCACCATCTTCCAGG-3′ (sense), 5′-CAGTAGAGGCAGGGATGATGTTC-3′ (antisense). Quantitative real-time PCR analyses of *Ccr7* as well as the control *G3pdh* or *Actin* mRNA transcripts were carried out using the assay-on-demandTM gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems, Tokyo, Japan).

2.4. Western blot analysis

Western blot analyses were performed as described previously [3]. Briefly, cells were harvested and lysed in a lysis buffer (50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA).

2.5. Cell proliferation assay

The numbers of viable Ba/F3 cells after the indicated treatments were measured using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Cell Counting Kit-8; Wako Pure Chemicals) [10]. Briefly, 10 μl of WST-8 solution was added to the cells in each well and incubated for 2 h. The absorbances were measured at a test wavelength of 450 nm and a reference wavelength of 620 nm using a microplate reader (Bio-Rad, Hercules, CA).

2.6. FACS analysis

The following monoclonal antibodies were used: PE-anti-mouse CCR7 and APC-anti-human CXCR4 antibodies (eBioscience, San Diego, CA). All analyses were conducted on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.7. Animal tumorigenesis

Ba/F3-p210/STAP-2/shControl and Ba/F3-p210/STAP-2/shCCR7#1 cells (1×10^7) were injected s.c. into BALB/c nude mice aged 4 weeks. After 4 weeks, the animals were sacrificed and the weighs of the tumor, lymph node, and spleen were measured. Mice were housed and bred in the Pharmaceutical Sciences Animal Center of Hokkaido University. All animals were maintained under specific pathogen-free conditions and in compliance with national and institutional guidelines. All protocols were approved by the Hokkaido University animal ethics committee.

2.8. Statistical methods

The significance of differences between group means was determined by Student's t-test.

3. Results

3.1. STAP-2 cooperates with BCR-ABL to modify the expression of chemokine receptors in BCR-ABL-expressing Ba/F3 cells

We previously reported chemokine receptor expression profiles in Ba/F3 cells and showed that the mRNA levels of CCR7 were higher and those of CXCR4 were lower in Ba/F3 cells expressing both BCR-ABL and STAP-2 compared with those expressing BCR-ABL alone. To further investigate these altered levels of CXCR4 and CCR7 expression, we here used four lines of Ba/F3 cells transfected with a control vector (Ba/F3-pcDNA), STAP-2 (Ba/F3-STAP-2), p210 BCR-ABL (Ba/F3-p210), or co-STAP-2/BCR-ABL (Ba/F3-p210/STAP-2) (Fig. 1A). We re-confirmed the expression levels of CCR7 and CXCR4 by quantitative real-time PCR analysis. As shown in Fig. 1B, the mRNA levels of CCR7 were up-regulated in Ba/F3-p210/STAP-2 cells compared with Ba/F3-p210 cells, whereas mRNA levels of CXCR4 were down-regulated in both Ba/F3-p210 and Ba/F3-p210/STAP-2 cells. The enhanced cell surface expression of CCR7 in Ba/F3-p210/STAP-2 cells was confirmed by flow cytometry analysis using an anti-CCR7 mAb (Fig. 1C). Therefore, STAP-2 positively regulates CCR7 expression but down-regulates CXCR4 expression in BCR-ABL-expressing Ba/F3 cells.