IKAROS isoform 6 enhances BCR-ABL1-mediated proliferation of human CD34⁺ hematopoietic cells on stromal cells

KEI SUZUKI^{1,2}, RYOICHI ONO¹, KOHSHI OHISHI^{2,3}, MASAHIRO MASUYA², ITARU KATAOKA⁴, BING LIU², YOSHIKI NAKAMORI², KAZUKO INO², FUMIHIKO MONMA², HIROFUMI HAMADA⁵, TOSHIO KITAMURA⁶, NAOYUKI KATAYAMA² and TETSUYA NOSAKA¹

Departments of ¹Microbiology and Molecular Genetics, ²Hematology and Oncology, Mie University Graduate School of Medicine; ³Division of Blood Transfusion, Mie University Hospital, 2-174 Edobashi, Tsu, Mie 514-8507; ⁴Department of Hematology and Oncology, Okayama University Graduate School of Medicine, 2-5-1 Shikatacho, Okayama 700-8558; ⁵Department of Molecular Medicine, Sapporo Medical University, Sapporo 060-8556; ⁶Division of Cellular Therapy, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

Received July 5, 2011; Accepted August 16, 2011

DOI: 10.3892/ijo.2011.1192

Abstract. The BCR-ABL1 induces chronic myelogenous leukemia (CML) and Ph⁺ acute lymphoblastic leukemia (ALL). Recent studies revealed high ratios of loss of the IKZF1 gene which encodes IKAROS in BCR-ABL1+ ALL and lymphoblastic crisis (LBC) of CML. However, little is known about the cooperativity between the aberrant IKAROS and BCR-ABL1 in primary human hematopoietic cells. We investigated the effects of expression of BCR-ABL1 and/or IK6, a natural dominant negative isoform of IKAROS, on proliferation and differentiation of human CD34⁺ cord blood cells with or without human bone marrow-derived stromal cells which support early B cell differentiation. Cell proliferation was remarkably enhanced by co-expression of BCR-ABL1 and IK6, with reduced expression of glycophorin A and increased expression of CD41, especially on stromal cells, compared with expression of BCR-ABL1 alone that resulted in expansion of erythroid progenitors. Interestingly, p190^{BCR-ABL1} showed higher dependency on stromal cells to stimulate cell growth with IK6, than p210^{BCR-ABL1}. Furthermore, the cooperation was found to depend on direct cell adhesive interaction of hematopoietic progenitors with stromal cells. These findings suggest that IK6 and BCR-ABL1 synergistically contribute to leukemogenesis in human bone marrow stromal microenvironment, and may provide a clue to elucidate the mechanisms of leukemogenesis of Ph⁺ ALL and CML-LBC.

Introduction

The BCR-ABL1 fusion gene, which originates from a reciprocal translocation between chromosome 9q34 and 22q11, is closely involved in chronic myelogenous leukemia (CML) and a subgroup of acute lymphoblastic leukemia (ALL). p190^{BCR-ABL1} is found almost exclusively in BCR-ABL1⁺ ALL, whereas p210^{BCR-ABL1} is found in nearly all cases of CML and approximately one-third of the patients with BCR-ABL1+ ALL. Several studies using mouse models suggest that forced expression of BCR-ABL1 alone in hematopoietic stem cells can induce CML-like myeloproliferative disorder. However, cooperating oncogenic gene alterations are required for the generation of blastic leukemia (1,2). On the other hand, forced expression of BCR-ABL1 alone in human hematopoietic cells does not develop leukemia (3). Since BCR-ABL1+ ALL is a more aggressive disease than CML, factors other than BCR-ABL1 are considered to be involved in its development and progression with decreased responses to the BCR-ABL1 tyrosine kinase inhibitors (TKIs) (4). Yet the reasons for aggressive nature of BCR-ABL1+ ALL and molecular basis of transformation of CML into lymphoblastic crisis (CML-LBC) remain to be clarified.

In nearly a decade, several groups reported the expression of aberrant IKAROS isoforms in CML-LBC and BCR-ABL1+ ALL (5-7). IKAROS is a zinc-finger nuclear protein that is essential for normal lymphoid development (8-10). Mice homozygous for the Ikaros null allele lack B, NK, and prenatal T cells (11), while T cell leukemia and lymphoma develop in mice heterozygous for the dominant-negative (DN) allele with 100% penetrance (12). By alternative splicing, multiple IKAROS isoforms are generated, several of which, including IK6, lack the aminoterminal zinc fingers required for DNA binding (10,13). IK6 is normally expressed at very low levels, retaining the dimerization domain, but lacking the four amino-terminal DNA-binding zinc fingers. Therefore, IK6 shows a DN effect by titrating out the DNA-binding activities of other IKAROS isoforms (14). The reduction of IKAROS activity may be an important step in

Correspondence to: Dr Tetsuya Nosaka, Department of Microbiology and Molecular Genetics, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan E-mail: nosaka@doc.medic.mie-u.ac.jp

Key words: BCR-ABL, IKAROS, CD34⁺ cells, stromal cells, acute leukemia

leukemogenesis (15), and normal IKAROS may function as a tumor suppressor in human hematopoietic cells. In addition, a recent study (16) revealed that *IKZF1* gene on 7p12, encoding IKAROS, is genomically deleted in most cases of CML-LBC and *BCR-ABL1*⁺ ALL samples. *IKZF1* deletion typically results in the expression of the DN IKAROS variant such as IK6 (4,17). The knock-in mice expressing the DN Ikaros developed leukemia earlier than the control mice on mating with the *BCR-ABL* transgenic mice, with fewer additional chromosomal abnormalities (18). These findings suggest that IK6 serves as second hit in BCR-ABL1-mediated leukemogenesis or vice versa. In addition, forced expression of IK6 in human B cells was reported to confer drug resistance (19). Moreover, recent clinical studies revealed that the deletion of *IKZF1* has negative prognostic impact in human ALL (20,21).

Although limited studies using human CD34⁺ cells demonstrated that BCR-ABL1 expression results in erythroid differentiation and expansion of erythroid progenitors (3,22,23), the consequence of co-expression of IK6 and BCR-ABL1 in primary human hematopoietic cells has not hitherto been addressed.

To investigate whether IK6 collaborates with BCR-ABL1 in human hematopoietic cells, we examined the combinatory effect of BCR-ABL1 and IK6 on proliferation and differentiation of human CD34⁺ hematopoietic progenitor cells in stroma-free culture or in co-culture with human stromal cells, which support not only myeloid but also early B cell differentiation, using retroviral vectors. Here we demonstrate a synergistic effect of IK6 and BCR-ABL1 in the growth of CD34⁺ progenitor cells, which is enhanced on human stromal cells.

Materials and methods

Cells and the cell line. Cord blood (CB) samples were collected from normal deliveries after obtaining written informed consent in accordance with guidelines from the human subject ethics committee at Mie University (No. 995). Mononuclear cells were separated by Ficoll-Hypaque centrifugation (Sigma Chemical Co., St. Louis, MO, USA). The CD34⁺ cells were enriched by two passages using immunoselection with the MACS CD34 Isolation Kit (Miltenyi Biotec, Auburn, CA, USA) as described previously (24). The percentage of CD34⁺ cells was >95% by flow cytometry analysis after this procedure.

The telomerized human stromal cell line (25) was cultured in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA) in the presence of antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). The cells were incubated at 37°C in a humidified atmosphere flushed with 5% CO₂.

Construction of the plasmids. The complementary DNAs (cDNAs) for p190^{BCR-ABL1} (B1A2) and p210^{BCR-ABL1} (B3A2) (26) cloned in a pMXneo vector (27) (kindly provided by Dr Tsuyoshi Tanabe, Shimane University, Izumo, Japan) were inserted into an *Eco*RI site of a pMYs-IRES (internal ribosomal entry site)-EGFP (enhanced green fluorescent protein) retroviral vector (28) to generate pMYs-p190^{BCR-ABL1}-IRES-EGFP and pMYs-p210^{BCR-ABL1}-IRES-EGFP, respectively. The *IK6* cDNA was obtained from Ph⁺ human cell line BV173 (29) by reverse transcription (RT)-polymerase chain reaction (PCR)

as described previously (19). The *IK6* cDNA was introduced between *Bam*HI and *Not*I sites of pMYs-IRES-Kusabira Orange (KO) vector which is a derivative of pMXs-IRES-KO (30), to generate pMYs-IK6-IRES-KO. The pMYs-IRES-EGFP (GFP mock) and pMYs-IRES-KO (KO mock) vectors were used as control vectors.

Retrovirus production and transduction of CB CD34⁺ cells. Transfection of the retroviral vector and pCMV-VSV-G (kindly provided by Dr Hiroyuki Miyoshi, RIKEN, Tsukuba, Japan) into PLAT-gp cells (28) was carried out using FuGENE 6 (Roche Diagnostic Corp., Indianapolis, IN, USA) according to the manufacturer's recommendations. The retroviruses (pseudo-type) were harvested twice 48 and 72 h after transfection, passed through a 0.45- μ m filter, concentrated by centrifugation at 6,000 g for 16 h, and resuspended in α -minimum essential medium (α -MEM), then stored at -80°C until use for transduction.

For retroviral transduction, CB CD34⁺ cells were first stimulated for 48 h in α -MEM with 20% FBS and human thrombopoietin (TPO; 20 ng/ml), stem cell factor (SCF; 50 ng/ml), and FLT3 ligand (FLT3-L; 50 ng/ml), which were purchased from PeproTech (Rocky Hill, NJ, USA), in 6-well plates. During the last 4 h of pre-stimulation, the virus stock was preloaded onto Retronectin (Takara, Otsu, Japan)-coated 24-well plate according to the manufacturer's recommendation as described (30). The pre-stimulated cells resuspended in the fresh medium were transferred to the virus-loaded 24-well plate at a concentration of 1x10⁵ cells/ml. The cells were cultured for another 48 h, and the transduction efficiency (positive rates of GFP and KO) was analyzed by flow cytometry.

Proliferation and differentiation assays. For stroma-free culture, 0.5×10^5 cells resuspended in 0.5 ml of the same fresh medium as that used in pre-stimulation were plated in duplicate in 24-well plates after 48 h of transduction and passaged thereafter before they reached confluence.

For stromal culture, the same post-transduced cells resuspended in the same medium as that for the stroma-free culture were plated onto the 25 cm² flask pre-sheeted with the semiconfluent human stromal cells, which support early B cell differentiation (Nakamori *et al*, unpublished data). The cells were fed twice a week by half medium change.

The cell numbers were counted by trypan blue dye exclusion at days 14 and 21 after isolation of CD34⁺ cells. To analyze immunophenotype of cultured cells, the cells were collected and their phenotype was assessed with flow cytometry at days 14 and 21 after isolation of CD34⁺ cells. Cytospin preparations were stained with Wright-Giemsa stain and viewed with a BX50F4 microscope (Olympus Optical, Tokyo, Japan) at magnification x400.

Assays using transwell plates. To test dependency on contacting to stromal cells in cell growth and survival, transwell plates (5 μ m pores; Corning, NY, USA) were used. The 0.5x10⁵ post-transduced cells were resuspended in the same medium described above, and were added onto the plate pre-sheeted with semi-confluent human stromal cells (lower compartment), or into transwell inserts (upper compartment) to avoid direct contact to the stromal cells. Thus the upper compartment separated with transwell membrane reflects the stroma-free culture



Figure 1. Retroviral transduction of the *BCR-ABL1* and *IK6*. (A) Schematic representation of the retroviral constructs used. LTR, long terminal repeat; IG, IRES-*EGFP*; IKO, IRES-*Kusabira Orange*. (B) Experimental design of this study. Human cord blood CD34⁺ cells were transduced with the retroviral vector encoding the p190^{*BCR-ABL1*} or p210^{*BCR-ABL1*}, along with the *EGFP* marker gene, or with a control vector encoding the *EGFP* only; and the retroviral vector encoding the *IK6* isoform, along with the *KO* marker gene, or with a control vector encoding the *KO* only. (C) Western blot analyses of proteins extracted from PLAT-gp cells transfected with the constructs shown in (A) were performed with an anti-BCR antibody (a) or with an anti-IKAROS antibody (b) (see Materials and methods). The expression of α -tubulin served as a loading control. (D) RT-PCR analyses of p190^{*BCR-ABL1*}, p210^{*BCR-ABL1*}, and *IK6* in the transduced human cord blood cells at day 21 after CD34⁺ separation. β_2 -microglobulin (MG) was used as an internal control. None, non-transduced cells.

condition with probable soluble factors from the stromal cells and the lower compartment is in stromal condition.

Flow cytometry. Immunofluorescent staining was performed as described previously (24). Monoclonal antibodies directed against CD10, conjugated with fluorescein isothiocyanate (CD10-FITC) (BD Bioscience, San Jose, CA, USA), CD14, conjugated with allophycyanin (CD14-APC) (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA), CD15-APC (BD Pharmingen, San Diego, CA, USA), CD19-APC (BD Pharmingen), CD41-APC (Becton-Dickinson Immunocytometry Systems), and glycophorin A (GPA)-APC (BD Pharmingen) were used. Mouse immunoglobulin (Ig) M-APC (BD Pharmingen), IgG_1 -APC (Becton-Dickinson Immunocytometry Systems), IgG_{2b} -APC (BD Pharmingen), and IgG_1 -FITC (BD Bioscience) served as isotype-matched controls. Cells were analyzed with a FACSCalibur (Becton-Dickinson Immunocytometry Systems). Dead cells were excluded by gaiting out the propidium iodide-positive cells.

RT-PCR. Total RNA was extracted from the cells and reverse transcribed to cDNA with random hexamers as described previously (31). PCR amplification was run for 28 cycles. The condition and reagents for RT-PCR were previously described (31) using the following primers: p190^{BCR-ABL1}-S, 5'-CTA TACCCCGGACTGCAGCT-3', and p190^{BCR-ABL1}-AS, 5'-CCA

GCGAGAAGGTTTTCCTTGGA-3' for the B1A2 p190 transcript, p210^{BCR-ABL1}-S, 5'-GAGTCACTGCTGCTGC TTATGTCT-3', and p210^{BCR-ABL1}-AS, 5'-CCAGCGAGAAGG TTTTCCTTGGA-3' for the B3A2 p210 transcript, IK6-S, 5'-CCCCTGTAAGCGATACTCCAGA-3', and IK6-AS, 5'-CTTGGAGAGCAGCAGCAGCAGGTT-3' for the *IK6* transcript.

Immunoprecipitation and Western blot analyses. Immunoprecipitation (IP) and Western blotting were performed as described previously (32). In brief, PLAT-gp cells transfected with each construct were harvested with the lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Noridet P-40, 1 mM EDTA, 0.2 mM Na₃VO₄, 2 mM phenylmethylsulfonyl fluoride]. Cell lysates for BCR-ABL1 detection were cleared of debris by centrifugation at 12,000 g for 30 min, and the supernatants were incubated with an anti-BCR monoclonal antibody G6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 2 h. Immune complexes were precipitated with protein G-Sepharose (Amersham Bioscience, Uppsala, Sweden), washed with the lysis buffer, and proteins were eluted with an SDS (sodium dodecyl sulfate) sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue] for SDS-PAGE. Cell lysates for IK6 detection were directly mixed with an equal volume of 2X SDS sample buffer. The eluted proteins were electrophoresed and transferred to nitrocellulose. Membranes were probed with antibodies (the anti-BCR antibody G6 for BCR-ABL1 detection and an anti-IKAROS antibody E20 (Santa Cruz Biotechnology) for IK6 detection), and visualized with the enhanced chemiluminescence detection system (Amersham-Pharmacia, Orsay, France) as described previously (31).

Statistical analyses. Data were analyzed for significance between groups using a Mann-Whitney U test. All differences were considered significant at p<0.05.

Results

Transduction and expression of BCR-ABL1 and IK6 in human CD34⁺ cells. To investigate how co-expression of BCR-ABL1 and IK6 affects proliferation and differentiation of human CD34+ hematopoietic progenitors, BCR-ABL1 and/or IK6 were retrovirally introduced into the CD34+ cells using bicistronic retroviral vectors encoding GFP (BCR-ABL1) or KO (IK6). The experimental schemes are summarized in Fig. 1A and B. Two days after transduction, p190^{BCR-ABL1}, p210^{BCR-ABL1}, IK6, GFP mock, and KO mock were expressed in 5.62±3.24, 3.22±1.76, 7.56±3.83, 14.67±2.53, and 12.30±3.36% of the cells, respectively. These transduction efficiencies did not significantly vary in five independent experiments. Successful expression of BCR-ABL1 or IK6 transgene in PLAT-gp packaging cells was confirmed by Western blot analysis (Fig. 1C). Furthermore, RT-PCR analysis of total RNA from transduced CD34+ cells verified the transgene expression (Fig. 1D).

IK6 enhances proliferative effects by BCR-ABL1 in human $CD34^+$ cells. During 3-week stroma-free culture with cytokines, cells transduced with either p210^{*BCR-ABL1*} (p210⁺) or p190^{*BCR-ABL1*} (p190⁺) gave rise to higher number of cells than the mock-



Figure 2. Effects of BCR-ABL1 and IK6 in stroma-free cultures. (A) Comparison of total cell numbers among the human cord blood CD34⁺ cells transduced with each retroviral construct on days 14 and 21 after CD34⁺ separation. (B) Percentages of GFP and KO double positive cells transduced with each retroviral construct in seven independent experiments. Av, average.

transduced cells as in a previous report (23). The cells expressing IK6 and GFP mock (BCR-ABL1⁻/IK6⁺) had no proliferative advantage compared with the double mock cells (BCR-ABL1⁻/IK6⁻). However, the cells co-expressing BCR-ABL1 and IK6 (BCR-ABL1⁺/IK6⁺) expanded more, compared with the cells expressing BCR-ABL1 and KO mock (BCR-ABL1⁺/IK6⁻) in seven independent experiments with high reproducibility. One representative example is shown in Fig. 2A. The percentages of BCR-ABL1⁺/IK6⁺ cells were considerably increased from day 14 to 21, albeit not in BCR-ABL1⁺/IK6⁻ control cells (n=7; Fig. 2B).

We next analyzed the immunophenotype of each cell fraction at day 21 after transduction in stroma-free culture (Fig. 3A). As previously reported (23), the majority of p210⁺ or p190⁺ cell fraction exhibited a CD34⁻CD14⁻CD15⁻GPA⁺ erythroid phenotype, regardless of the co-expression of IK6. Interestingly, the percentages of the cells positive for a megakaryocytic lineage marker CD41 were higher in the BCR-ABL1⁺/IK6⁺ fraction than in the BCR-ABL1⁺/IK6⁻ fraction. Consistent with this, Wright-Giemsa staining revealed that the majority of the cells transduced with p190^{BCR-ABL1} or p210^{BCR-ABL1} without *IK6* were basophilic erythroblasts, but some populations of the cells transduced with both *BCR-ABL1* and *IK6* showed morphological characteristics of



Figure 3. Immunophenotype and morphology of the cells in stroma-free cultures at day 21 after CD34⁺ separation. (A) The cells were stained with the APCconjugated antibodies against human CD34, CD14, CD15, CD41, and GPA, respectively. The cells gated for GFP expression are shown. Numbers in each quadrant represent the percentages. A representative result out of five independent experiments is shown. (B) Wright-Giemsa staining images of a representative result out of five independent experiments are shown. Bars represent 50 μ m.

megakaryocytic cells with multilobulated nuclei (Fig. 3B). No significant difference was observed in expression of CD34, CD14, and CD15 between the BCR-ABL1⁺/IK6⁺ cells and the BCR-ABL1⁺/IK6⁻ cells (n=5). Transduction of either of the

p190^{BCR-ABL1} or *IK6* did not induce CD41 expression, while that of p210^{BCR-ABL1} alone induced CD41 expression moderately. The cells without *BCR-ABL1* with or without *IK6* were classified as of various stages of myeloid differentiation.



Figure 4. Immunophenotype of the cells in stromal cultures at day 21 after $CD34^+$ separation. (A) Control cord blood $CD34^+$ cells generated a distinct population of $CD19^+$ $CD10^-$ pro-B lymphocytes following 3 weeks of culture. (B) Transduced cord blood $CD34^+$ cells were stained with the APC-conjugated antibodies against human CD19, CD41, and GPA, respectively. The cells gated for GFP expression are shown. Numbers in each quadrant represent the percentages. A representative result out of five independent experiments is shown.

Co-expression of BCR-ABL1 and IK6 in human CD34⁺ *cells leads to erythroid cell expansion even by co-cultures with human stromal cells which support early B cell differentiation.* We next examined the effects of IK6 and BCR-ABL1 on the proliferation and differentiation of hematopoietic progentiors by co-culture with telomerized human stromal cells that support myeloid and early B cell development. We observed that non-transduced CD34⁺ cells gave rise to ~15-20% of CD19⁺ B lymphoid precursors, following 3 weeks of culture (Fig. 4A). However, the CD34⁺ cells expressing p210 or p190 failed to generate CD19⁺ B-lineage cells (Fig. 4B), regardless of presence or absence of IK6. Rather, these cells differentiated mainly into GPA⁺ erythroid cells. The percentages of GPA⁻ cells in BCR-ABL1⁺/IK6⁺ cells were increased significantly in KO⁺ (i.e., IK6⁺) fraction, compared

with those of BCR-ABL1⁺/IK6⁻ cells. Other immunophenotypes regarding to the expression of CD34, CD14, CD15 (data not shown), and CD41 were almost similar to those in stroma-free culture (Figs. 3A and 4B). Thus, co-expression of BCR-ABL1 and IK6 in human CD34⁺ cells mainly leads to erythroid cell expansion even in the circumstances that support early B cell differentiation.

Notably, we observed that the synergistic growth-promoting effects between *IK6* and p190^{*BCR-ABL1*} became more remarkable in stromal culture, compared with the findings obtained with stroma-free culture (Fig. 5).

Synergistic effect of BCR-ABL1 and IK6 on cell proliferation is enhanced by co-cultures with stromal cells. To compare the



Figure 5. Synergistic effects of BCR-ABL1 and IK6 in stromal cultures. (A) Comparison of total cell numbers among the human cord blood CD34⁺ cells transduced with each retroviral construct on days 14 and 21 after CD34⁺ separation. (B) Percentages of GFP and KO double positive cells transduced with each retroviral construct in seven independent experiments. Av, average.



Figure 6. Percentages of the cells expressing both BCR-ABL1 and IK6 at day 21 after CD34⁺ separation in comparison with those expressing BCR-ABL1 alone. (A) Comparison of the effects of $p190^{BCRABL1}$ with and without *IK6* in stroma-free culture. (B) $p210^{BCRABL1}$ with and without *IK6* in stroma-free culture. (C) $p190^{BCR-ABL1}$ with and without *IK6* in stromal culture. (D) $p210^{BCR-ABL1}$ with and without *IK6* in stromal culture. (D) $p210^{BCR-ABL1}$ with and without *IK6* in stromal culture. (D) $p210^{BCR-ABL1}$ with and without *IK6* in stromal culture. (D) $p210^{BCR-ABL1}$ with and without *IK6* in stromal culture. (D) $p210^{BCR-ABL1}$ with and without *IK6* in stromal culture. (D) $p210^{BCR-ABL1}$ with and *IK6* in stromal culture was excluded because the transduction itself was unsuccessful. Open circles represent the averages in each panel.

co-operativity of BCR-ABL1 and IK6 between stroma-free and stromal cultures, GFP/KO-double positive rates were analyzed from the aspect of growth advantage. The percentages of the BCR-ABL1+/IK6+ populations were significantly higher than those of the BCR-ABL1⁺/IK6⁻ populations in stromal culture (Fig. 6C and D), while statistically not in stroma-free culture in seven independent experiments (Fig. 6A and B). Nevertheless, the p210⁺/IK6⁺ population in stroma-free culture also showed tendency of higher growth than the p210^{+/} IK6⁻ population (Fig. 6B), albeit not so much in the p190⁺/ IK6⁺ population (Fig. 6A). Therefore, the outgrowth driven by BCR-ABL1 and IK6 in stromal culture relative to stroma-free culture is prominent in p190, but not in p210 (p=0.010 and 0.365, respectively; Fig. 7). These findings indicate that the co-operation between IK6 and BCR-ABL1, especially p190, to stimulate cell proliferation is enhanced on stromal cells.

Growth enhancement by co-expression of BCR-ABL1 and IK6 on stromal cells requires cell adhesive interaction. During the cell culture experiments, we noticed that a higher

proportion of the cells transduced with BCR-ABL1 adhered to the stromal cells compared with the control cells (data not shown). Therefore, we investigated whether direct cell to cell interaction between transduced hematopoietic progenitors and stromal cells is required for the synergistic effect of BCR-ABL1 and IK6 on cell proliferation by using transwell plates. To this end, the percentages of the BCR-ABL+/IK6+ cell population and of the BCR-ABL1+/IK6- cell population in both upper and lower compartments were analyzed at day 21 after CD34⁺ separation. The percentages of the BCR-ABL1⁺/IK6⁺ cell populations were increased significantly in lower compartment, albeit not in upper compartment (Fig. 8). However, the percentages of BCR-ABL1+/IK6- cell populations were not significantly different in upper and lower compartments in three independent experiments. These results suggest that the enhanced cell proliferation induced by BCR-ABL1 and IK6 in stromal culture is mediated by direct cell adhesive interaction rather than the soluble factors produced by the stromal cells.



Figure 7. Percentages of the cells expressing both BCR-ABL1 and IK6 at day 21 after CD34⁺ separation in comparison between stroma-free and stromal cultures. (A) Comparison (stroma-free vs stromal) of the cells expressing both $p190^{BCR-ABL1}$ and IK6. (B) Comparison of the cells expressing both $p210^{BCR-ABL1}$ and IK6. One out of seven independent experiments with $p210^{BCR-ABL1}$ and IK6 on stromal culture was excluded because the transduction itself was unsuccessful. Open circles represent the averages in each panel.

Discussion

In this study we demonstrated the synergistic effect of BCR-ABL1 and IK6 on cell proliferation and differentiation in human CD34⁺ cells, and that this effect was enhanced on stromal cells, especially between p190 and IK6.

Forced expression of BCR-ABL1 and that of IK6 in human CD34⁺ cells were reported to induce erythroid cell expansion and to impair B cell differentiation, respectively (10,22,23).

Despite the use of human, not murine stromal cells which can support early B cell development, expression of BCR-ABL1 and IK6 in human CD34⁺ cells resulted in GPA⁺ erythroid cell expansion and inhibition of B cell differentiation. Notably, major populations of the cells transduced with both BCR-ABL1 and IK6 expressed GPA and CD41. These findings led us to speculate that coexpression of BCR-ABL1 and IK6 induced the growth of more immature progenitors with both erythroid and megakaryocytic differentiation potentials. Although precise mechanism remains to be elucidated, impaired IKAROS function may perturb the differentiation of hematopoietic progenitors that have proliferative and/or survival advantage by BCR-ABL1-mediated signaling. Interestingly, IKAROS was recently reported to behave as tumor suppressor to induce cell cycle arrest in BCR-ABL1⁺ cells through cooperation with downstream molecules of the pre-B cell receptor signaling pathway, and this effect is reversed by coexpression of IK6 (33).

The synergistic effect was greatly enhanced on stromal cells in our experiments. The assays using transwell plates suggested that the co-operative effect depends on cell adhesive interaction of hematopoietic progenitors and stromal cells. The bone marrow stromal microenvironment may provide anti-apoptotic signals to the leukemic cells. Indeed in clinical observations, TKIs-resistant cells from relapsed patients are still sensitive to the proapoptotic effects of TKIs when administrated *ex vivo* (34). Further study may uncover the role of stromal microenviroment in leukemogenesis of Ph⁺ ALL with impaired IKAROS function.

To date, strong clinical evidence exists supporting the idea that BCR-ABL1 and impaired IKAROS function play pivotal roles in *BCR-ABL1*-related aggressive lymphoid leukemogenesis. However, these 'two hits' are unlikely to be sufficient to fully transform human CD34⁺ cells *in vitro*, because CD34⁺ cells were not immortalized after transduction in our experimental systems (data not shown). It is plausible that additional key players are required to recapitulate BCR-ABL1-related human lymphoid leukemia. Yet preferential co-operation of IK6 and



Figure 8. Enhanced cell proliferation induced by BCR-ABL1 and IK6 requires cell adhesive interaction in the assays using transwell plates. Percentages of the cells expressing BCR-ABL1 with or without IK6 at day 21 after CD34⁺ separation are shown. The upper compartment reflects the stroma-free culture condition, whereas the lower compartment pre-sheeted with semi-confluent human stromal cells is in stromal culture condition. Numbers in each quadrant represent the percentages. One representative result out of three independent experiments is shown.

Although it is challenging to study the effect of coexpression of BCR-ABL1 and IK6 in human CD34⁺ cells transplanted into immunodeficient mice (3,23), it could unveil the long-term effects of BCR-ABL1 and IK6 in human cells under *in vivo* conditions. However, we have been unable to achieve promising results at present. Further trial on different target cells, and/or introduction of additional molecules is necessary to solve this important issue.

In conclusion, we found that the co-operativity between BCR-ABL1 and IK6 was enhanced in primary human CD34⁺ cells through direct interaction with human stromal cells, especially between p190 and IK6. These findings would provide a clue to elucidate the mechanism of lymphoid leukemogenesis by BCR-ABL1, and also the mechanism underlying the difference of the disease phenotype between p190^{BCR-ABL1} and p210^{BCR-ABL1}. Further studies are required to see whether IK6 controls BCR-ABL1 expression and activity during disease progression, and how IK6 influences the survival and self renewal of the BCR-ABL1⁺ leukemia-initiating cells in the bone marrow microenvironment.

Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology in Japan (20659151 to T.N.), the Novartis Foundation (Japan) for the Promotion of Science (to T.N.), the Japan Leukaemia Research Fund (to T.N.), and Grants-in-Aid for scientific research from Mie University Graduate School of Medicine (to K.S.). The authors declare no conflict of interest.

References

- Daley GQ, van Etten RA and Baltimore D: Blast crisis in a murine model of chronic myelogenous leukemia. Proc Natl Acad Sci USA 88: 11335-11338, 1991.
- Williams RT, Roussel MF and Sherr CJ: Arf gene loss enhances oncogenicity and limits imatinib response in mouse models of Bcr-Abl-induced acute lymphoblastic leukemia. Proc Natl Acad Sci USA 103: 6688-6693, 2006.
- Chalandon Y, Jiang X, Christ O, Loutet S, Thanopoulou E, Eaves A and Eaves C: BCR-ABL-transduced human cord blood cells produce abnormal populations in immunodeficient mice. Leukemia 19: 442-448, 2005.
- 4. Iacobucci I, Lonetti A, Messa F, Cilloni D, Arruga F, Ottaviani E, Paolini S, Papayannidis C, Piccaluga PP, Giannoulia P, Soverini S, Amabile M, Poerio A, Saglio G, Pane F, Berton G, Baruzzi A, Vitale A, Chiaretti S, Perini G, Foà R, Baccarani M and Martinelli G: Expression of spliced oncogenic Ikaros isoforms in Philadelphia-positive acute lymphoblastic leukemia patients treated with tyrosine kinase inhibitors: implications for a new mechanism of resistance. Blood 112: 3847-3855, 2008.
- 5. Nakayama H, Ishimaru F, Avitahl N, Sezaki N, Fujii N, Nakase K, Ninomiya Y, Harashima A, Minowada J, Tsuchiyama J, Imajoh K, Tsubota T, Fukuda S, Sezaki T, Kojima K, Hara M, Takimoto H, Yorimitsu S, Takahashi I, Miyata A, Taniguchi S, Tokunaga Y, Gondo H, Niho Y, Nakao S, Kyo T, Dohy H, Kamada N and Harada M: Decreases in Ikaros activity correlate with blast crisis in patients with chronic myelogenous leukemia. Cancer Res 59: 3931-3934, 1999.
- 6. Nakase K, Ishimaru F, Avitahl N, Dansako H, Matsuo K, Fujii K, Sezaki N, Nakayama H, Yano T, Fukuda S, Imajoh K, Takeuchi M, Miyata A, Hara M, Yasukawa M, Takahashi I, Taguchi H, Matsue K, Nakao S, Niho Y, Takenaka K, Shinagawa K, Ikeda K, Niiya K and Harada M: Dominant negative isoform of the Ikaros gene in patients with adult B-cell acute lymphoblastic leukemia. Cancer Res 60: 4062-4065, 2000.

- Nishii K, Katayama N, Miwa H, Shikami M, Usui E, Masuya M, Araki H, Lorenzo F, Ogawa T, Kyo T, Nasu K, Shiku H and Kita K: Non-DNA-binding Ikaros isoform gene expressed in adult B-precursor acute lymphoblastic leukemia. Leukemia 16: 1285-1292, 2002.
- Georgopoulos K, Moore DD and Derfler B: Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. Science 258: 808-812, 1992.
- 9. Georgopoulos K, Bigby M, Wang JH, Molnar A, Wu P, Winandy S and Sharpe A: The Ikaros gene is required for the development of all lymphoid lineages. Cell 79: 143-156, 1994.
- Tonnelle C, Bardin F, Maroc C, Imbert A-M, Campa F, Dalloul A, Schmitt C and Chabannon C: Forced expression of the Ikaros 6 isoform in human placental blood CD34⁺ cells impairs their ability to differentiate toward the B-lymphoid lineage. Blood 98: 2673-2680, 2001.
- Wang JH, Nichogiannopoulou A, Wu L, Sun L, Sharpe AH, Bigby M and Georgopoulos K: Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. Immunity 5: 537-549, 1996.
- 12. Winandy S, Wu P and Georgopoulos K: A dominant mutation in the Ikaros gene leads to rapid development of leukemia and lymphoma. Cell 83: 289-299, 1995.
- 13. Molnár A, Wu P, Largespada DA, Vortkamp A, Scherer S, Copeland NG, Jenkins NA, Bruns G and Georgopoulos K: The Ikaros gene encodes a family of lymphocyte-restricted zinc finger DNA binding proteins, highly conserved in human and mouse. J Immunol 156: 585-592, 1996.
- Sun L, Liu A and Georgopoulos K: Zinc finger-mediated protein interactions modulate Ikaros acitivity, a molecular control of lymphocyte development. EMBO J 15: 5358-5369, 1996.
- Ruiž A, Williams O and Brady HJ: The Ikaros splice isoform, Ikaros 6, immortalizes murine haematopoietic progenitor cells. Int J Cancer 123: 1240-1245, 2008.
- 16. Mullighan CG, Miller CB, Radtke I, Phillips LA, Dalton J, Ma J, White D, Hughes TP, Le Beau MM, Pui CH, Relling MV, Shurtleff SA and Downing JR: BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. Nature 453: 110-114, 2008.
- 17. Iacobucci I, Storlazzi CT, Cilloni D, Lonetti A, Ottaviani E, Soverini S, Astolfi A, Chiaretti S, Vitale A, Messa F, Impera L, Baldazzi C, D'Addabbo P, Papayannidis C, Lonoce A, Colarossi S, Vignetti M, Piccaluga PP, Paolini S, Russo D, Pane F, Saglio G, Baccarani M, Foà R and Martinelli G: Identification and molecular characterization of recurrent genomic deletions on 7p12 in the IKZF1 gene in a large cohort of BCR-ABL1-positive acute lymphoblastic leukemia patients: on behalf of Gruppo Italiano Malattie Ematologiche dell'Adulto Acute Leukemia Working Party (GIMEMA AL WP). Blood 114: 2159-2167, 2009.
- Virely C, sMoulin S, Cobaleda C, Lasgi C, Alberdi A, Soulier J, Sigaux F, Chan S, Kastner P and Ghysdael J: Haploinsufficiency of the IKZF1 (IKAROS) tumor suppressor gene cooperates with BCR-ABL in a transgenic model of acute lymphoblastic leukemia. Leukemia 24: 1200-1204, 2010.
- Sezaki N, Ishimaru F, Takata M, Tabayashi T, Nakase K, Kozuka T, Fujii K, Nakayama H, Teshima T, Harada M and Tanimoto M: Over-expression of the dominant-negative isoform of Ikaros confers resistance to dexamethasone-induced and anti-IgM-induced apoptosis. Br J Haematol 121: 165-169, 2003.
- 20. Mullighan CG, Su X, Zhang J, Radtke I, Phillips LA, Miller CB, Ma J, Liu W, Cheng C, Schulman BA, Harvey RC, Chen IM, Clifford RJ, Carroll WL, Reaman G, Bowman WP, Devidas M, Gerhard DS, Yang W, Relling MV, Shurtleff SA, Campana D, Borowitz MJ, Pui CH, Smith M, Hunger SP, Willman CL and Downing JR, Children's Oncology Group: Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med 360: 470-480, 2009.
- 21. Martinelli G, Iacobucci I, Storlazzi CT, Vignetti M, Paoloni F, Cilloni D, Soverini S, Vitale A, Chiaretti S, Cimino G, Papayannidis C, Paolini S, Elia L, Fazi P, Meloni G, Amadori S, Saglio G, Pane F, Baccarani M and Foà R: IKZF1 (Ikaros) deletions in BCR-ABL1-positive acute lymphoblastic leukemia are associated with short disease-free survival and high rate of cumulative incidence of relapse: a GIMEMA AL WP report. J Clin Oncol 27: 5202-5207, 2009.
- 22. Chalandon Y, Jiang X, Hazlewood G, Loutet S, Conneally E, Eaves A and Eaves C: Modulation of p210^{BCR-ABL} activity in transduced primary human hematopoietic cells controls lineage programming. Blood 99: 3197-3204, 2002.

- 23. Järås M, Johnels P, Agerstam H, Lassen C, Rissler M, Edén P, Cammenga J, Olofsson T, Bjerrum OW, Richter J, Fan X and Fioretos T: Expression of P190 and P210 *BCR/ABL1* in normal human CD34⁺ cells induces similar gene expression profiles and results in a STAT5-dependent expansion of the erythroid lineage. Exp Hematol 37: 367-375, 2009.
- 24. Yamamura K, Ohishi K, Katayama N, Yu Z, Kato K, Masuya M, Fujieda A, Sugimoto Y, Miyata E, Shibasaki T, Heike Y, Takaue Y and Shiku H: Pleiotropic role of histone deacetylases in the regulation of human adult erythropoiesis. Br J Haematol 135: 242-253, 2006.
- 25. Kawano Y, Kobune M, Yamaguchi M, Nakamura K, Ito Y, Sasaki K, Takahashi S, Nakamura T, Chiba H, Sato T, Matsunaga T, Azuma H, Ikebuchi K, Ikeda H, Kato J, Niitsu Y and Hamada H: Ex vivo expansion of human umbilical cord hematopoietic progenitor cells using a coculture system with human telomerase catalytic subunit (hTERT)-transfected human stromal cells. Blood 101: 532-540, 2003.
- 26. Nosaka T and Kitamuta T: Pim-1 expression is sufficient to induce cytokine independence in murine hematopoietic cells, but is dispensable for BCR-ABL-mediated transformation. Exp Hematol 30: 697-702, 2002.
- 27. Onishi M, Nosaka T, Misawa K, Mui ALF, Gorman D, McMahon M, Miyajima A and Kitamura T: Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. Mol Cell Biol 18: 3871-3879, 1998.
- Kitamura T, Koshino Y, Shibata F, Oki T, Nakajima H, Nosaka T and Kumagai H: Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. Exp Hematol 31: 1007-1014, 2003.

- Pegoraro L, Matera L, Ritz J, Levis A, Palumbo A and Biagini G: Establishment of a Ph1-positive human cell line (BV173). J Natl Cancer Inst 70: 447-453, 1983.
- 30. Ono R, Kumagai H, Nakajima H, Hishiya A, Taki T, Horikawa K, Takatsu K, Satoh T, Hayashi Y, Kitamura T and Nosaka T: Mixed-lineage-leukemia (MLL) fusion protein collaborates with Ras to induce acute leukemia through aberrant Hox expression and Raf activation. Leukemia 23: 2197-2209, 2009.
- Ono R, Nakajima H, Ozaki K, Kumagai H. Kawashima T, Taki T, Kitamura T, Hayashi Y and Nosaka T: Dimerization of MLL fusion proteins and FLT3 activation synergize to induce multiple-lineage leukemogenesis. J Clin Invest 115: 919-929, 2005.
- 32. Nosaka T, Kawashima T, Misawa K, Ikuta K, Mui AL-F and Kitamura T: STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. EMBO J 18: 4754-4765, 1999.
- Trageser D, Iacobucci I, Nahar R, Duy C, von Levetzow G, Klemm L, Park E, Schuh W, Gruber T, Herzog S, Kim Y-M, Hofmann W-K, Li A, Storlazzi CT, Jäck H-M, Groffen J, Martinelli G, Heisterkamp N, Jumaa H and Müschen M: Pre-B cell receptor-mediated cell cycle arrest in Philadelphia chromosome-positive acute lymphoblastic leukemia requires IKAROS function. J Exp Med 206: 1739-1753, 2009.
 Perrotti D, Jamieson C, Goldman J and Skorski T: Chronic
- Perrotti D, Jamieson C, Goldman J and Skorski T: Chronic myeloid leukemia: mechanisms of blastic transformation. J Clin Invest 120: 2254-2264, 2010.