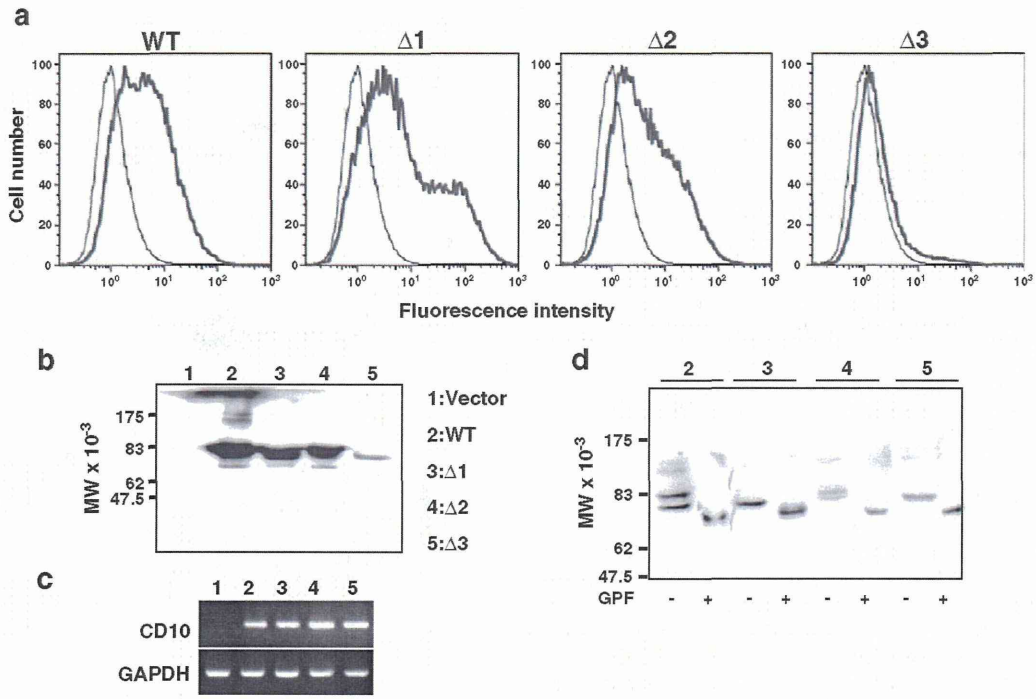

Fig. 6. Expression and glycosylation of CD10 in HEK293 cells transfected with wild type and mutated c-DNA of NALM-6 CD10. a, Surface expression of CD10 by the transfected HEK293 cells. HEK293 cells were harvested at day 2 after transfection and their expression of CD10 was analyzed by flow cytometry. Thin line, negative control; bold line, IF6. b, Protein expression of CD10 by transfected HEK 293 cells. 2×10^5 transfectants were solubilized and subjected to Western analysis with anti-CD10. c, RT-PCR analysis of CD10 and GAPDH mRNA expression. d, *N*-glycosylation of WT- and mutated CD10. Each lysate was mock-incubated (GPF−) or incubated with glycopeptidase F (GPF+) under denaturing conditions, and subjected to Western analysis with anti-CD10. Lanes 2, 3, 4 and lane 5 contain 3×10^5 and 6×10^5 cells, respectively. 1, Vector; 2, WT: 3, Δ1; 4, Δ2; 5, Δ3.

N-glycosylated. It is suggested that *N*-glycans are necessary for full expression of NEP activities because of the complete loss of NEP activities upon glycopeptidase F treatment. Interestingly, site-directed mutagenesis of *N*-glycosylation sites of CD10 reveals that an *N*-glycan at Asn_{628} is indispensable not only for NEP activities but also for surface expression. The proteins of Δ3-CD10 lacking an *N*-glycan at Asn_{628} seem to be eliminated via a certain protein quality control pathway. Since either lactacystin or chloroquine did not cause the accumulation of Δ3-CD10, the low expression of Δ3-CD10 cannot be explained by the degradation either in proteasome or lysosome. However, Δ3-CD10 transfectants increased the expression upon brefeldin A treatment. The addition of brefeldin A to cells is known to result in the tubulation of the endosomal system, the transe-Golgi network, and lysosomes [28]. It should be cleared how the proteins of Δ3-CD10, which is deficient in NEP activity due to lack of an *N*-glycan at Asn_{628}, are degraded.

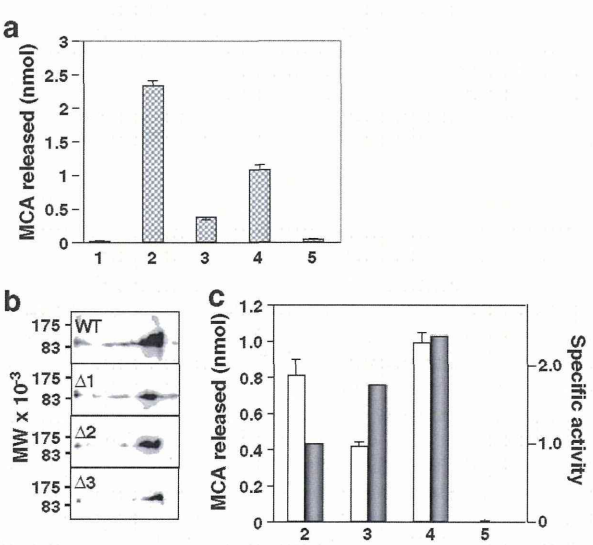

Fig. 7. NEP activities of HEK293 cells transfected with WT or mutated CD10. a, NEP activities of 5×10^4 transfectants. b, 2D Western analysis of CD10 immunoprecipitated from transfectants with anti-CD10. c, NEP activities of CD10 purified from transfectants. CD10 immunoprecipitated from 1.3×10^6 WT-, Δ1-, and Δ2-CD10 transfectants, and 1.7×10^6 Δ3-CD10 transfectants on PA/agarose beads was used as an enzyme source for NEP assay. After that, the beads were subjected to Western analysis with anti-CD10 for determination of the amount of CD10. The open column represents nmol MCA released by 5×10^4 cells into 50 μL of the assay buffer for 30 min and the solid column represents the specific activity. The value for WT-CD10 was set to 1.0. 1, Vector; 2, WT: 3, Δ1; 4, Δ2; 5, Δ3.

Previous studies have shown that changes in *N*-glycans of rabbit NEP affect its stability and enzymatic activity [29]. It has been demonstrated that glycosylation at any sites was not required for expression of membrane-bound NEP, whereas the presence of *N*-glycans at either N_{145} or N_{628} was sufficient to recover close-to-normal enzymatic activities. In our study, *N*-glycans at N_{628} of human membrane-bound CD10 were revealed to be necessary not only for expression, but also for enzymatic activities, whereas *N*-glycans at N_{145} were nonessential for both expression and enzymatic activities.

The experimental removal of sialic acid by *Vibrio cholerae* neuraminidase in cultured myotubes was shown to result in reduced expression and enzymatic activity of NEP in vitro [30]. We demonstrated that complete removal of sialic acid by *Anthrobacter ureafaciens* neuraminidase did not affect the enzymatic activity of human CD10. Therefore, sialylation should not be necessary at least for enzymatic activity.

Although the transcription level of Δ3-CD10 was almost the same as that of WT or other mutants, its surface expression was extremely low compared with that of the others. We have to keep in mind that quantification of the mRNA of CD10 does not reflect the protein expression or catalyzing activity of NEP.

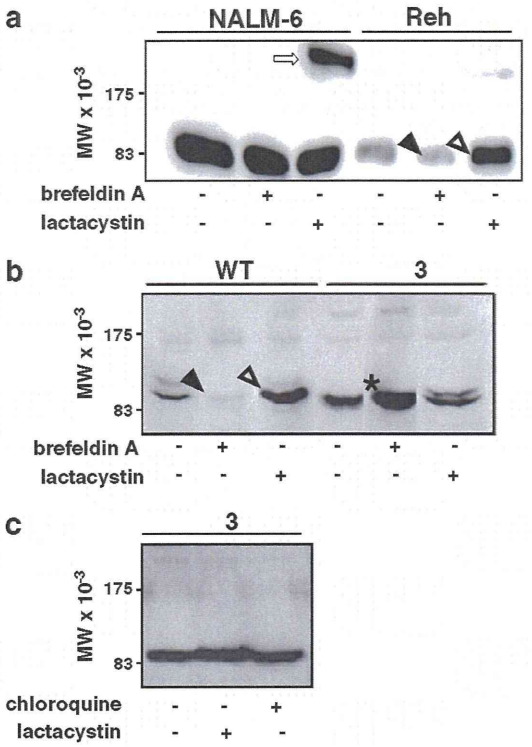

Fig. 8. Effect of brefeldin A, lactacystin and chloroquine on the expression of CD10. a, Cells were cultured in the presence or absence of brefeldin A or lactacystin, and harvested for Western analysis. Each lane contains 14 μg of protein of NALM-6 and Reh. b, 3×10^5 WT- and 6×10^5 Δ3-CD10 transfectants were loaded. c, 8×10^5 Δ3-CD10 transfectants were cultured in the presence or absence of lactacystin or brefeldin A, and harvested for Western analysis.

Abbreviations

NEP	neutral endopeptidase
ALL	acute lymphoblastic leukemia
CALLA	common acute lymphoblastic leukemia antigen
WT	wild type
mAb	monoclonal antibody
2D	two dimensional
PBS	phosphate-buffered saline
CBB	Coomassie brilliant blue
MCA	4-methyl-coumaryl-7-amides
pI	isoelectric point

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ORIGINAL ARTICLE

Effects of insulin-like growth factor-1 on B-cell precursor acute lymphoblastic leukemia

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Abstract Insulin-like growth factor-1 (IGF-1) is known to be a major growth factor with effects on various cell types, including hematopoietic cells, as well as neoplasms, and is regulated by IGF-binding proteins (IGFBPs). In this study, we investigated the effects of IGF-1 on B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells. When the expression of IGF-1R in clinical samples of BCP-ALL was examined, five of thirty-two cases showed IGF-1R expression, whereas IGF-1R was expressed in most BCP-ALL cell lines. We observed that IGF-1 enhanced the proliferation of BCP-ALL cell lines that can be partially inhibited by IGFBP-1, -3, and -4, but not other IGFBPs. IGF-1 also partially inhibited dexamethasone-induced apoptosis, but not apoptosis mediated by VP-16 and irradiation. Interestingly, the proliferative effect of IGF-1 was partially blocked by inhibitors of MAPK and AKT, whereas the inhibition of dexamethasone-induced apoptosis was completely blocked by both inhibitors. Our data indicate that IGF-1 is involved in cell proliferation and apoptosis regulation in BCP-ALL cells. Since some BCP-ALL cases express IGF-1R, it appears to be a plausible target for prognostic evaluation and may represent a new therapeutic strategy.

Keywords IGF-1 · IGFBP · B-cell precursor ALL · Cell growth · Apoptosis

Introduction

Insulin-like growth factor-1 (IGF-1) is known to be a major growth factor affecting various types of cell [1]. IGF-1 can bind to the homodimer of IGF-1 receptor (IGF-1R) and the heterodimer of IGF-1R and insulin receptor with different affinities. Upon binding with these receptors, IGF-1 induces intracellular signaling, including Akt and MAPK pathways, leading to the enhancement of cell cycle progression, cell proliferation, and cell differentiation [2, 3]. In regard to neoplastic cells, many studies have provided evidence for roles of IGF-1 in several tumor developments. For example, an elevated plasma concentration of IGF-1 has been shown to be linked to a higher risk of several solid tumors, including bladder [4], breast [5–7], colorectal [5, 8], lung [5, 9], pancreatic [10], and prostate cancer [5, 11], and malignant melanoma [12]. In hematological malignancies, an autocrine effect of IGF-I signaling on the growth and survival of acute myeloid leukemia (AML) cells has been reported [13]. In the case of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells, the expression of IGF system components in primary leukemic blasts has been shown [14], whereas its biological significance still remains unclear.

The bioactivity of IGF-1 is known to be modulated by a family of proteins termed IGF binding proteins (IGFBPs). IGFBPs have a high binding affinity for IGF-1, while their effects are variable and some of them limit IGF-1 access to

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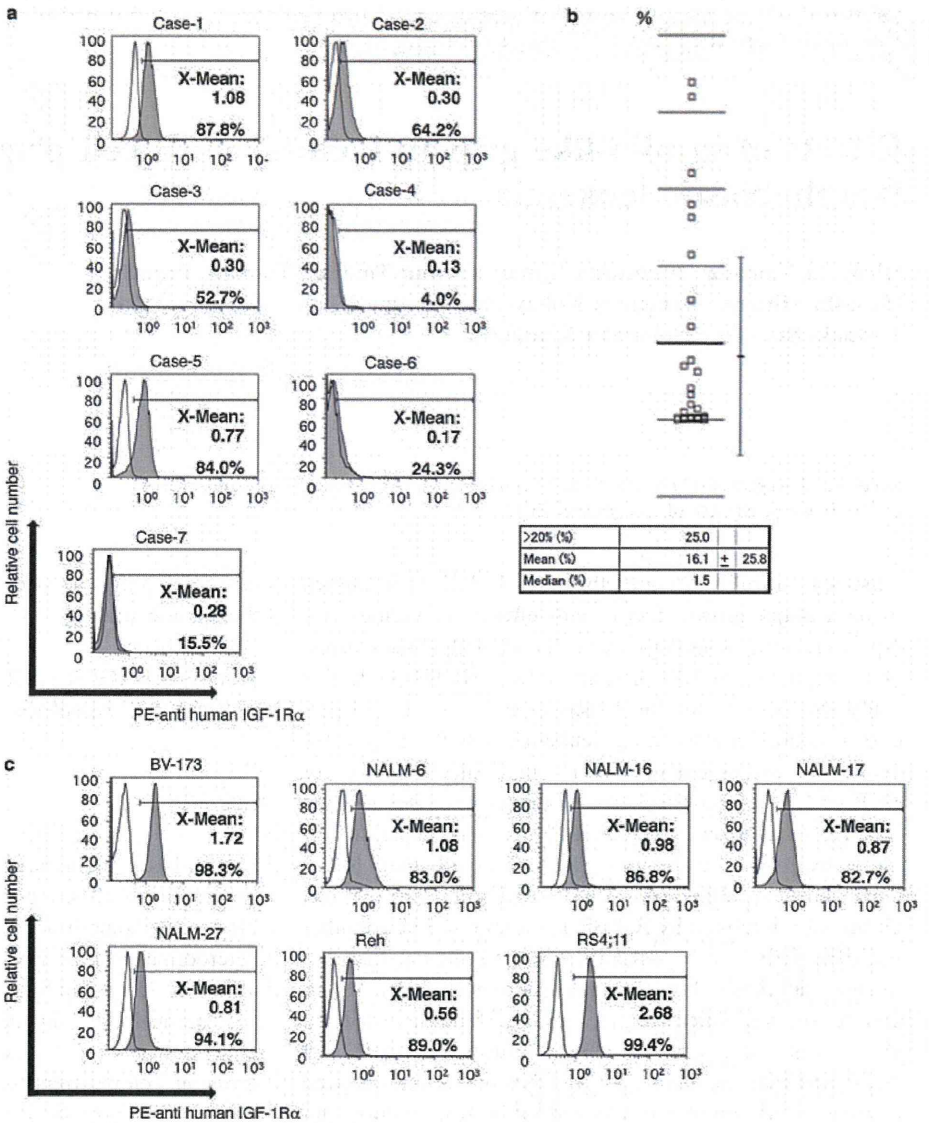

Fig. 1 Expression of insulin-like growth factor-1 receptor (IGF-1R) in B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells. **a** Leukemic cells obtained from seven patients with BCP-ALL were stained with phycoerythrin (PE)-labeled anti-human IGF-1Rα antibody and analyzed by flow cytometry. **b** IGF-1R positivity (percentage) of BCP-ALL [$n = 32$, including the cases presented in **a**] was plotted on a scattergram. Percentage of IGF-1R -positive cases (more than 20 % expression in blasts) is indicated below. **c** Seven BCP-ALL cell lines were stained and analyzed, as shown in **a**. The histograms obtained (*dark areas*) are shown superimposed on those of the negative control (cells stained with isotype-matched control mouse Ig, light areas, cut-off: 2 %). Positivity (%) and mean fluorescence (X-mean) values are presented. *X*-axis, fluorescence intensity; *Y*-axis, relative cell number

the IGF-1 receptor (IGF-1R) [15], but the others serve as a carrier protein for IGF-1 [16] and help to lengthen the half-life of circulating IGF-1 [17]. We previously reported the regulatory effects of IGF-1 and IGFBPs on early B-cell development by employing an in vitro culture system of human hematopoietic stem cells cocultured with bone marrow stromal cells, and observed that IGF-1 is essential for pro-B-cell development from $CD34^+$ cells and IGFBP-3 inhibited but IGFBP-6 was required for pro-B-cell development [18]. Considering the fact that BCP-ALL cells are tumor counterparts of B-cell precursors, IGF-1 and IGFBPs should play an important role in the biology of ALL cells.

In the present report, we investigated the functions of IGF-1 and IGFBPs in the proliferation and survival of BCP-ALL cells. We show that IGF-1 promotes the proliferation of BCP-ALL cells and inhibits apoptosis under specific circumstances. The effects of IGFBPs on ALL cells were also shown.

Methods

Reagents

The antibodies (Abs) used for flow cytometry were phycoerythrin (PE)-conjugated mouse monoclonal anti-human IGF-1 Rα from Becton Dickinson Biosciences (BD, San Diego, CA, USA), Alexa Fluor® 488-conjugated rabbit

polyclonal anti-Phospho-p44/41 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204), and Alexa Fluor® 488-conjugated rabbit monoclonal anti-Phospho-Akt (Ser473) from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibodies (Abs) used for immunoblot analysis were mouse monoclonal anti-ERK antibody and anti-Akt antibody from BD, anti-IGF-1R antibody, anti-phospho-IGF-1R antibody, anti-phospho-p44/42-MAPK antibody, and anti-Phospho-Akt antibody from Cell Signaling Technology, Inc. As a negative control, isotype-matched control Igs were also used.

Recombinant human IGF-1, IGFBP-2, IGFBP-7, and rabbit anti-human IGF-1 antibody for neutralization were obtained from PeproTech EC Ltd. (London, UK). Recombinant human IGFBP-1 was obtained from R&D SYSTEMS (Minneapolis, MN, USA), IGFBP-3, -4, and -5 were obtained from Sigma-Aldrich, Co. (St. Louis, MO, USA) and IGFBP-6 was obtained from G-T Research Products (Minneapolis, MN, USA). The IGF-1R kinase inhibitor I-OMe-AG538 and the MAPK extracellular signal-regulated kinase (MEK) 1/2 inhibitor U0126 were obtained from Calbiochem-Novabiochem Co. (San Diego, CA, USA). The Phosphoinositide 3-kinase (PI3K) inhibitor LY294002 was obtained from Cell Signaling Technology, Inc. Dexamethasone (DEX) and etoposide (VP-16) were obtained from Sigma-Aldrich.

Clinical specimens

Clinical specimens from pediatric patients, consisting of thirty-two patients with BCP-ALL, were used upon obtaining informed consent. All of the experiments included in this study adhered to the tenets of the Declaration of Helsinki and were performed with the approval of the local ethics committee.

Cells and cultures

The human BCP-ALL cell lines BV-173, NALM-16, NALM-17, and NALM-27 (Dr. Y. Matsuo, Hayashibara Biochemical Laboratories, Inc. Research Center Cell Biology Institute, Okayama, Japan), NALM-6 (Tohoku University Cell Bank, Sendai, Japan), Reh (American Type Culture Collection), and RS4;11 (Japanese Cancer Research Resource Bank, JCRB, Tokyo, Japan) were used. Cells were maintained in RPMI-1640 supplemented with

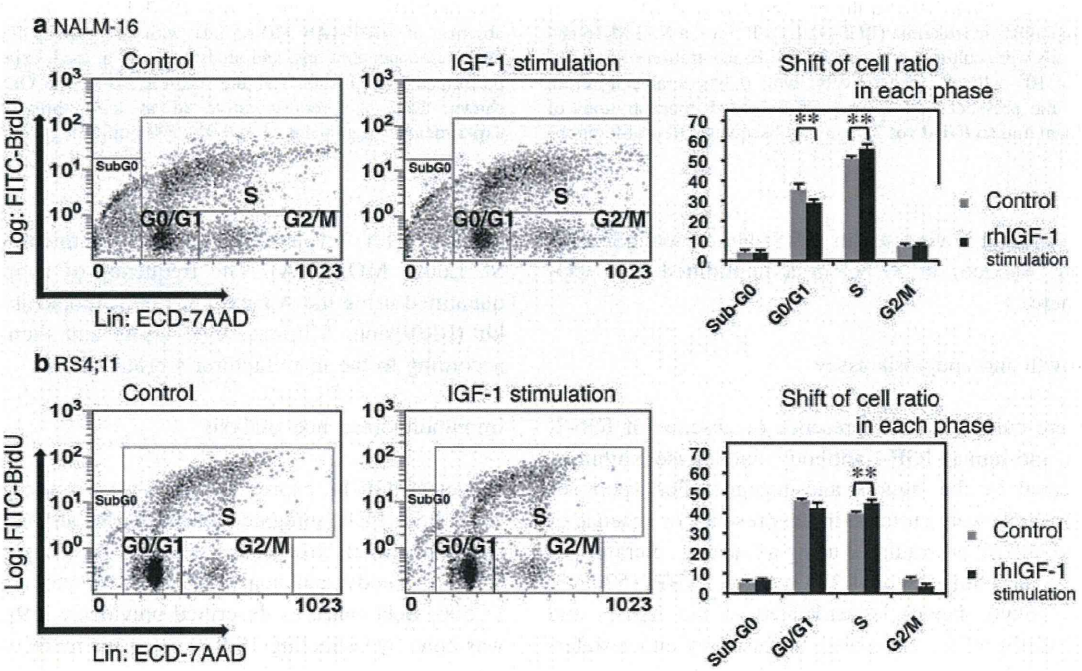

Fig. 2 Effect of insulin-like growth factor-1 (IGF-1) stimulation on cell cycle progression. NALM-16 and RS4;11 cells at a starting cell concentration of 5×10^5 cells/mL with the serum-free condition were incubated with or without 100 ng/mL of IGF-1 for 24 h followed by 4-h labeling with bromodeoxyuridine (BrdU). After staining with fluorescein isothiocyanate (FITC)-labeled (*X*-axis) anti-BrdU antibody and 7-amino-actinomycinD (7AAD, *Y*-axis), cells were analyzed with flow cytometry. Experiments were performed in triplicate, and the mean ± SD of the percentage of each fraction are shown. Data are representative of at least three independent experiments. **significant ($p < 0.05$)

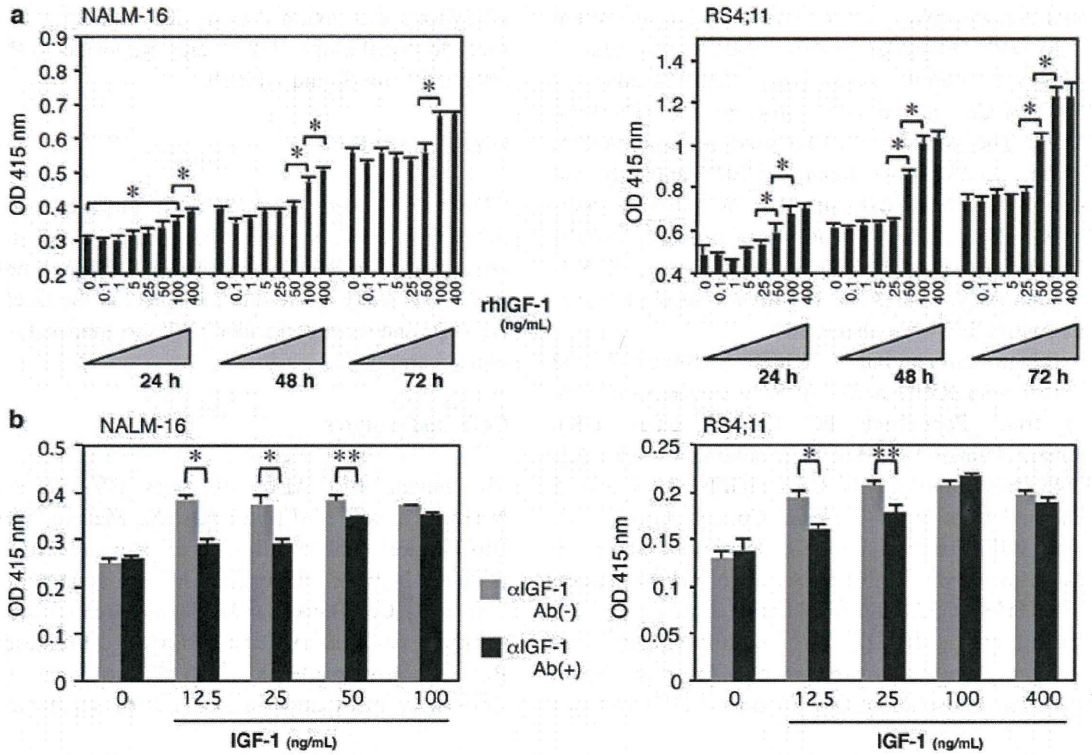

Fig. 3 Effect of insulin-like growth factor-1 (IGF-1) and anti-human IGF-1 antibody (αIGF-1Ab) on the proliferation of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cell lines. **a** NALM-16 and RS4;11 cells were cultured at a starting cell concentration of 5×10^5 and 2.5×10^5 cells/mL, respectively, with 0.5 % fetal calf serum (FCS) in the presence and absence of indicated concentrations of recombinant human IGF-1 for 72 h, and subsequent cell proliferations were assessed by WST assays. **b** The cells at the same starting cell concentration were cultured with 0.5 % FCS in the presence and absence of αIGF-1Ab (10 μg/ml) with or without IGF-1 at the indicated concentrations, and analyzed as in **a**. Each experiment was performed in triplicate, and the mean ± SD of the OD values are shown. Data are representative of at least three independent experiments. *significant ($p < 0.01$); **significant ($p < 0.05$)

10 % (v/v) fetal bovine serum (FBS; NescoHealthScience Pty, Ltd. Mexico) at 37 °C in a humidified 5 % CO_2 atmosphere.

Cell growth and apoptosis assay

Cells were cultured in the presence or absence of IGF-1, IGFBPs, anti-human IGF-1 antibody, and kinase inhibitors, as indicated in the figures and legends. For apoptosis induction, cells were cultured in the presence or absence of DEX and VP-16, or irradiated using a linear accelerator (Al 0.5 + Cu 0.3 mm filter, 1.27 Gy/min; MBR-1520R-3; Hitachi, Tokyo, Japan), as indicated in the figures and legends. Cell proliferations were assessed by either water-soluble tetrazolium salt (WST) assays (Cell Counting Kit-8, DojinDo, Kumamoto, Japan) or quantitative measurement of the adenosine 5′-triphosphate (ATP) levels using the luciferin–luciferase assay solution (TOYO B-Net CO., LTD., Tokyo, Japan) according to the manufacturer's protocol. In parallel, viable cell counts were made after staining with Trypan blue (Sigma Chemical Company, St. Louis, MO, USA). The frequency of apoptosis was quantified using the Annexin V-FITC Apoptosis Detection kit (BioVision, Milpitas, CA, USA) and then analyzed according to the manufacturer's protocol.

Immunofluorescence analysis

To detect IGF-1R expression, cells were stained with phycoerythrin (PE)-conjugated anti-IGF-1Rα antibody in combination with electron-coupled dye (ECD)-conjugated anti-CD45 antibody and analyzed by flow cytometry (FCM, FC500, Beckman), as described previously [19]. Analysis was done by collecting 10,000 gated list mode events, and selecting an appropriate blast gate for the combination of CD45 and side scatter. The IGF-1R was considered positively expressed when at least 20 % of the gated cells expressed IGF-1R. The cell cycle was assessed with the BrdU Flow Kit (Becton Dickinson Biosciences) and analyzed by flow cytometry according to the manufacturer's

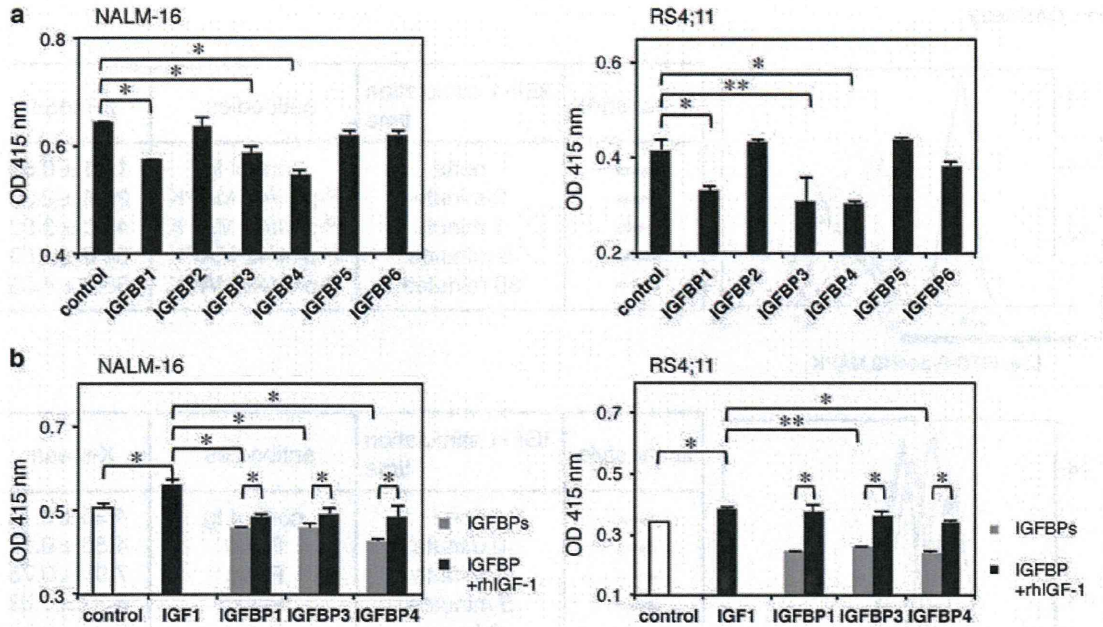

Fig. 4 Effect of insulin-like growth factor binding proteins (IGFBPs) on the proliferation of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cell lines. **a** Each IGFBP (at a concentration of 200 ng/mL) as indicated was added alone to the culture of NALM-16 (starting at cell concentration of 5×10^5 cells/mL with 0.5 % fetal calf serum) and RS4;11 [starting at cell concentration of 2.5×10^5 cells/mL with 10 % fetal calf serum (FCS)] and resulting cell proliferations after a 72-h cultivation were assessed by WST assay. The data are indicated as OD values. **b** IGFBP-1, -3, and -4 were added to the culture under the same experimental conditions as in **a** in the presence and absence of IGF-1 (100 ng/mL) and analyzed similarly to **a**. Each experiment was performed in triplicate, and the mean ± SD of the data are shown. Data are representative of at least three independent experiments. $*p < 0.01$; $**p < 0.05$

protocol. To detect phosphorylations of p44/42 MAPK and Akt, cells stimulated with IGF-1, as indicated in the figures and legends, were fixed with formaldehyde (final concentration: 2 %, room air temperature for 10 min), permeabilized with methanol (final concentration 90 %, −20 °C, 30 min), blocked with 0.5 % bovine serum albumin (room temperature for 10 min), and then incubation with phospho-specific Ab against each kinase (room temperature for 60 min), followed by FCM analysis.

Immunoblotting

Immunoblotting was performed as described previously [20]. Briefly, a 50-μg sample of each cell lysate was electrophoretically separated on an SDS-poly acrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with an appropriate combination of primary and secondary Abs, washed, and examined with an enhanced chemiluminescence reagent system.

Statistical analysis

Statistical analysis was performed by means of a non-parametric Mann–Whitney test, and correlations were determined using nonparametric statistics. A p-value <0.05 was considered significant.

Results

Expression of IGF-1R in BCP-ALL cells

First, we examined whether the clinical materials of BCP-ALL cells expressed IGF-1R. As shown in Fig. 1a and b, five out of thirty-two cases apparently expressed IGF-1Rα (>50 %) and three cases weakly expressed IGF-1R. The frequency of positive IGF-1R expression (>20 %) of our cases was 25.0 %. On the other hand, when BCP-ALL cell lines were similarly examined, all of the cell lines expressed IGF-1Rα (Fig. 1c).

Effect of IGF-1 on cell proliferation of BCP-ALL cell lines

Using NALM-16 and RS4;11 cells, we next investigated the effect of IGF-1 on the cell cycle and proliferation of BCP-ALL cell lines. When cells treated with or without IGF-1 were examined by BrdU assay, IGF-1 stimulation increased the ratio of cells in the S phase and the cells in the G0/G1

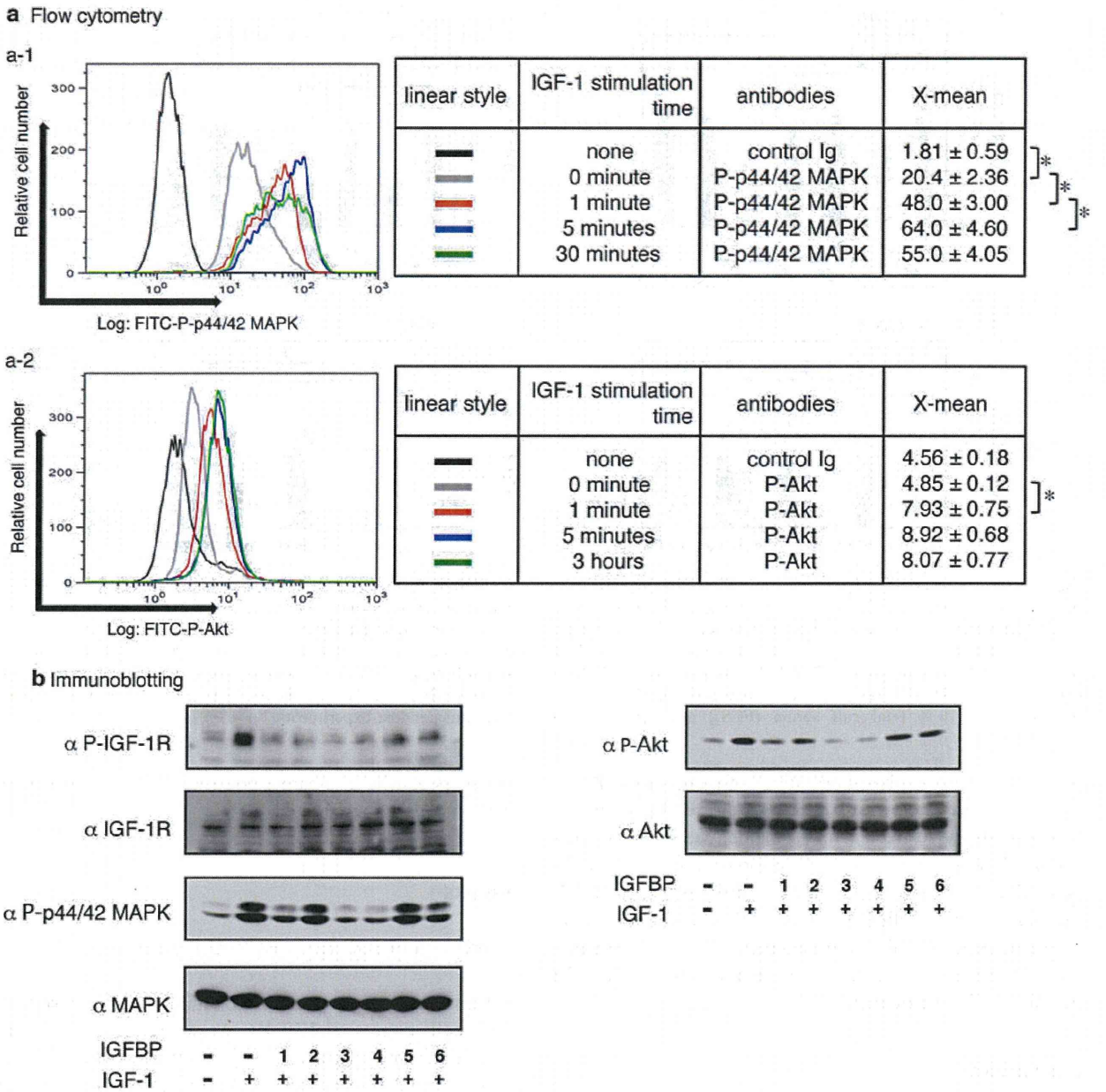

Fig. 5 Effects of insulin-like growth factor binding proteins (IGFBPs) on insulin-like growth factor-1 (IGF-1)-mediated signaling. **a** NALM-16 cells were stimulated with 100 ng/mL of IGF-1 under serum-free conditions for the indicated periods, and phosphorylations of p44/42 mitogen-activated protein kinase (MAPK) (*a-1*) and Akt (*a-2*) were detected by anti-phospho-specific antibodies using flow cytometry. Each experiment was performed in triplicate, and average mean fluorescence (X-mean) values are presented. Control Ig, staining with isotype-matched control rabbit Ig. Data are representative of at least three independent experiments. *X*-axis, fluorescence intensity; *Y*-axis, relative cell number. **b** NALM-16 cells were stimulated with 100 ng/mL of IGF-1 under serum-free conditions for 1 min in the presence and absence of 200 ng/mL each of IGFBP, as indicated. Cell extracts were prepared, and immunoblotting with indicated antibodies was performed. Data are representative of three independent experiments. *significant ($p < 0.01$)

phase decreased (Fig. 2). Then, we examined the proliferative effect of IGF-1 on BCP-ALL cell lines. As shown in Fig. 3a, the addition of IGF-1 leads to a dose-dependent increase in cell proliferation in each cell line and the concentration of 100 ng/ml had already achieved a maximum effect. Furthermore, when cell proliferation was inhibited by anti-IGF-1Ab, IGF-1 also had a recovery effect on cell proliferation in a dose-dependent manner (Fig. 3b).

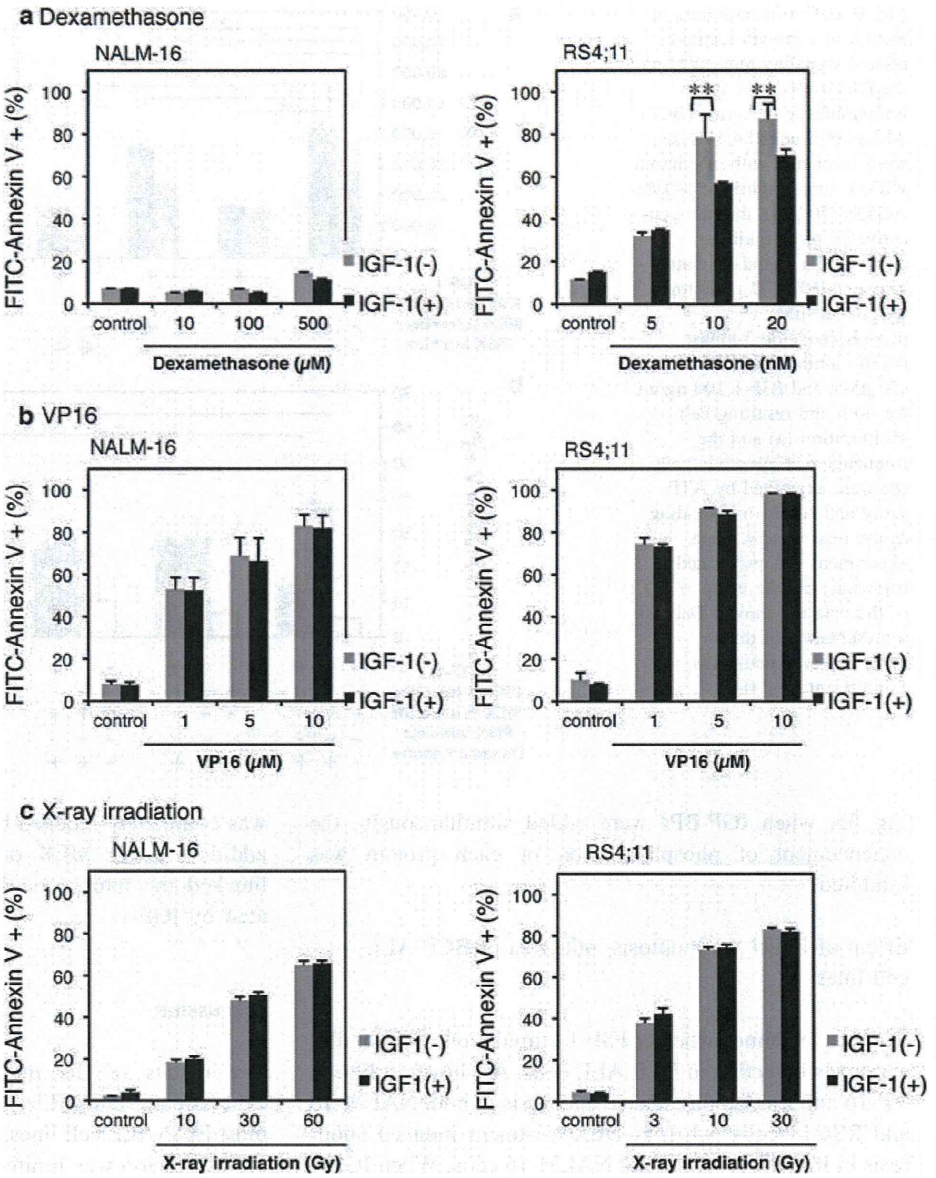

Fig. 6 Effect of insulin-like growth factor-1 (IGF-1) on apoptosis in B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cell lines. NALM-16 and RS4;11 cells were cultured with or without the indicated doses of dexamethasone (**a**), VP16 (**b**), and irradiation (**c**), and the resulting apoptotic cells were examined by Annexin V-binding using flow cytometry. Each experiment was performed in triplicate, and the mean ± SD of the percentage of Annexin V-binding cells are presented. Data are representative of at least three independent experiments. **significant ($p < 0.05$)

Effect of IGFBPs on the proliferation of BCP-ALL cell lines

We then examined the effect of IGFBPs on IGF-1-mediated cell proliferation. As shown in Fig. 4a, when each IGFBP alone was added to the culture, IGFBP-1, -3, and -4 inhibited the cell proliferation. Inhibitions of the cell proliferation effect were reduced by the simultaneous addition of IGF-1, indicating that IGFBP-1, -3, and -4 specifically inhibited IGF-1-mediated cell proliferation (Fig. 4b).

IGF-1-mediated intracellular signaling in BCP-ALL cells

Subsequently, we investigated the IGF-1-mediated intracellular signaling in BCP-ALL cells. When the phosphorylation state of p44/42 MAPK and Akt induced by IGF-1-stimulation was examined by flow cytometry, a time-dependent increase in phosphorylation of these proteins was observed (Fig. 5a). The IGF-1-mediated enhancement of phosphorylation on IGF-1R, MAPK, and Akt was also confirmed by immunoblotting (Fig. 5b). As shown in