

Increased Expression of Insulin-Like Growth Factor I is Associated with Ara-C Resistance in Leukemia

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ABE, S., FUNATO, T., TAKAHASHI, S., YOKOYAMA, H., YAMAMOTO, J., TOMIYA, Y., YAMADA-FUJIWARA, M., ISHIZAWA, K., KAMEOKA, J., KAKU, M., HARIGAE, H. and SASAKI, T. *Increased Expression of Insulin-Like Growth Factor I is Associated with Ara-C Resistance in Leukemia.* Tohoku J. Exp. Med., 2006, **209** (3), 217-228 — Resistance to cytosine arabinoside (Ara-C) is a major problem in the treatment of patients with acute myeloid leukemia (AML). In order to investigate the mechanisms involved in Ara-C resistance, the gene expression profile of Ara-C-resistant K562 human myeloid leukemia cells (K562/AC cells) was compared to that of Ara-C-sensitive K562 cells (K562 cells) by using a cDNA microarray platform. Correspondence analysis demonstrated that insulin-like growth factor I (IGF-I) gene was upregulated in K562/AC cells. The biological significance of IGF-I overexpression was further examined in vitro. When K562 cells were incubated with IGF-I ligand, they were protected from apoptosis induced by Ara-C. In contrast, a significant inhibition of growth and increase of apoptosis of K562/AC cells were induced by IGF-I receptor neutralizing antibody, or suramin, a nonspecific growth factor antagonist. Moreover, from the analysis of 27 AML patients, we have shown that IGF-I expression levels are higher in patients at refractory stage, after Ara-C combined chemotherapy, than those in patients at diagnosis. These results suggest that the inhibition of IGF-I and its downstream pathway is a valuable therapeutic approach to overcome Ara-C resistance in AML. — acute myeloid leukemia; Ara-C; insulin-like growth factor I; drug resistance; apoptosis

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The antimetabolite 1- β -D-arabinofuranosyl-cytosine (cytosine arabinoside [Ara-C], cytarabine) represents the prototype of the nucleoside analogue class of antineoplastic agents. Ara-C is one of the most commonly used agents in the treatment of acute myeloid leukemia (AML)

(Grant 1998). The cytotoxic effect of Ara-C is mediated by its metabolite Ara-CTP generated by deoxycytidine kinase (dCK) (Flasshove et al. 1994). Ara-C is catabolized to the non-toxic metabolite arabinoside uridine (Ara-U) by rapid deamination mediated by cytidine deaminase

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(CDD) (Laliberte and Momparler 1994). Ara-CTP inhibits DNA polymerase and competes with deoxycytidine triphosphate (dCTP) for incorporation into DNA. This incorporation causes chain termination, resulting in a block of DNA synthesis (Kufe et al. 1980; Wiley et al. 1982). Resistance to Ara-C is a major problem for the treatment of AML patients. Clinically, only approximately 30-40% patients treated with conventional chemotherapy have prolonged leukemia free survival (Schiller et al. 1998). Multiple drug resistance to Ara-C and anthracyclines is thought to reduce leukemia-free survival rate in patients with AML (Fujimaki et al. 2002). Various biochemical mechanisms of Ara-C resistance have been shown by experimental animal tumor models or in vitro systems, including reduced influx of Ara-C mediated by equilibrative nucleoside transporter 1 (hENT1) (White et al. 1987), enhanced degradation of Ara-C or excessive conversion to inactive Ara-U by high levels of CDD (Momparler and Laiberte 1990; Capizzi et al. 1991), deficiency of dCK (Bhalla et al. 1984; Stegmann et al. 1993) and high levels of intracellular dCTP (Liliemark and Plunkett 1986; Ohno et al. 1988).

Complementary DNA (cDNA) microarray is a useful tool to identify the genes that induce the phenotypic changes when cells are transformed. It has been used for studies of drug resistance and the results have shown that there are distinct subtypes of resistant cells as defined by their gene expression profiles (Shuerf et al. 2000; Staunton et al. 2001; Dan et al. 2002; Ichikawa et al. 2004; Selvanayagam et al. 2004). Previously, we established Ara-C resistant K562 cells (K562/AC cells) (Funato et al. 2000). Since characteristics of K562/AC cells, except for Ara-C resistance, should be same as Ara-C sensitive parental K562 cells (K562 cells), genes which are differentially expressed between K562/AC cells and parental K562 cells, may be involved in Ara-C resistance. In this study, to clarify the mechanisms of Ara-C resistance, we employed cDNA microarray technique and compared the expression profile between K562/AC cells and K562 cells.

MATERIALS AND METHODS

Cell line

The human myeloid leukemia cell line K562 was obtained from the Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University. The Ara-C resistant sub-line K562/AC was obtained by continuous exposure of K562 cells to increasing concentrations of Ara-C (from 4 μ M to 2,000 μ M) (Funato et al. 2000). Ara-C was kindly provided by Nihon-Shinyaku (Kyoto, Japan). The cells were grown in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco), 100 IU of penicillin and 100 mg/l streptomycin (Gibco) at 37°C in 5% CO₂.

Cytotoxicity assay for cell survival

Cell growth was determined by a dye reduction assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma, St.Louis, MO, USA). Briefly, a total of 5,000 cells/well was plated in 96-well plates and indicated different concentrations of Ara-C were added in the presence or absence of either IGF-I (BD, Bedford, MA, USA), suramin (Sigma), or IGF-I Receptor neutralizing antibody (α IGF-IR, Oncogene Research Products, San Diego, CA, USA), or LY294002 (Sigma) and further incubated for 72 hrs. Thereafter, 10 μ l of MTT solution was added to each well, and the plates were incubated for 4 hrs at 37°C. The absorbance of each well was measured at 540 nm using a microplate reader. The effect on growth inhibition could be assessed as follows: percent of growth inhibition = 1 - ([absorbance of drug treated cells/absorbance of untreated cells] \times 100).

Ribonucleic acid (RNA) preparation and microarray analysis

Total cellular RNA was isolated by Isogen-LS (Nippon Gene, Tokyo), following manufacturer's protocol. Human Cancer CHIP, Version 2 (Takara, Tokyo), was used in which 425 human cancer-related genes and 11 control house-keeping genes were spotted on glass slides. A fluorescent probe was synthesized by incorporating Cy3TM- or Cy5TM-dUTP (American Life Science Inc., Arlington Heights, IL, USA) using 1 μ g of the above mRNA as the template and 50 units of AMV reverse transcriptase (Takara). Cy3-labeled probe prepared from cells was mixed in the reaction buffer (6 \times SSC, 0.2% sodium dodecyl sulfate [SDS], 5 \times Denhardt's solution, 1.5 mg/ml human Control DNA, 0.8 mg/ml poly dA and 1 mg/ml yeast tRNA) and hybridized with

DNA CHIP at 65°C overnight. The hybridized CHIP was visualized and quantified using an Affimetrix 418 Array Scanner™ (Affimetrix, Woburn, MA, USA) and ImaGene™ software (BioDiscovery, Los Angeles, CA, USA).

Quantitative real time reverse-transcriptase polymerase chain reaction analysis

cDNA was synthesized using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) from extracted total RNA. For quantitative mRNA expression analysis, real-time RT-PCR was carried out with total RNA using a LightCycler instrument (Roche, Mannheim, Germany). In total, 2 µl of cDNA was mixed with 2 µl of DNA Master SYBR Green (Roche), 4 mmol/l MgCl₂ and 1 µmol/l of each 3′ and 5′ primer (Nihon Gene Research, Sendai, Japan) in a total volume of 20 µl solution. The sequences of the primers used were: IGF-I, forward 5′-TCTTGAAGGTGATGCACAC-3′, reverse 5′-AGCGAGCTGACTTGGCAGGC-3′, GAPDH, forward 5′-TGAAGGTCGGAGTCAACGGATTGGT-3′, reverse 5′-CATGTGGGCATGAGGTCCACCAC-3′. The cycling conditions are as follows: initial enzyme activation at 95°C for 10 min, followed by 45 cycles at 95°C for 10 s 60°C for 15 s and 72°C for 11 s. For quantification, the expression of IGF-I was normalized against the expression of GAPDH mRNA.

Flow cytometric analysis

Apoptosis was determined with an Annexin V-FITC Apoptosis Detection kit (Sigma) according to the manufacturer's instructions. Briefly, prepared cells were washed and resuspended in binding buffer at a concentration of 1×10^6 cells/ml. Five hundreds µl of this solution were added to a 5-ml culture tube containing 5 µl of annexin V-FITC and 10 µl of propidium iodide (PI), and incubated at room temperature for 10 min in the dark. These cells were analyzed by FACS Calibur (BD). The percentage of early apoptotic cells (annexin V⁺/PI⁻ cells) among the treated cells was compared with those in the untreated cells.

Quantification of caspase 3 activity

Caspase 3 activity was measured with an EnzChek Caspase 3 kit (Molecular Probes, Leiden, Netherlands), following manufacturer's protocol. This assay uses the fluorogenic substrate rhodamine 110, bis-(N-CBZ-L-aspartyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110).

Fluorescence was measured with a fluorometer with a 496 nm excitation filter and a 520 nm emission filter.

Western blot analysis

Collected cells (1×10^7) were washed twice and lysed on ice in 50 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 5 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM Na₃VO₄, 1 mM NaF, and phosphatase inhibitor cocktail (Roche) for 15 min. Protein extracts were cleared by centrifugation and protein concentration was determined using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Two hundreds µg of protein was boiled in 2 × SDS gel loading buffer (2% SDS, 50 mM Tris-HCl, 200 mM DTT, 0.2% bromophenol blue, 10% glycerol, 2% 2-Mercaptoethanol) for 5 min and separated in electrophoresis on 10% SDS-polyacrylamide gel, and then transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 2.5% bovine serum albumin in Tris-buffered saline (TBS) and incubated 2 hrs at room temperature with mouse polyclonal phospho-Akt1/PKBα (Ser473) (Upstate Biotechnology, Lake Placid, NY, USA) in TBS. After washing, membranes were incubated with secondary antibody for 1 hr and detection was performed using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA). The membranes were then stripped by incubation at 50°C for 30 min in 62.5 mM Tris-HCl, 2% SDS, 100 mM 2-Mercaptoethanol and washed, reprobbed by polyclonal rabbit Akt1/PKBα (Upstate).

Patients

After obtaining informed consent, we collected bone marrow aspirates from 27 adult AML patients. Our study with human subjects or materials was approved by the ethical committee of Tohoku University School of Medicine. The percentage of leukemia cells in samples was > 50% in all cases. The diagnosis was based on French-American-British (FAB) criteria. Patient characteristics are listed in Table 1. Patients received Ara-C (100 mg/m²/d) for 7 days and idarubicin (12 mg/m²/d) for 3 days as an induction therapy. Five patients with M3 received all trans retinoic acid along with Ara-C and idarubicin. The response criteria were defined as follows: complete response (CR) (cellular marrow with < 5% blast cells, neutrophil count > 1.5×10^9 /L, platelet count > 100×10^9 /L, and no evidence of leukemia in other sites); and refractory disease (cellular marrow with > 5% blast cells or evidence of leukemia in other sights, at least two courses of chemotherapy). Patients who

TABLE 1. Patient characteristics.

Age (year), median (range)	41(21-68)
Sex male/female	15/12
Leukocyte ($\times 10^9/l$), median (range)	41.8(0.8-281)
FAB subtype	
M1	4
M2	11
M3	5
M4	3
M5	4
Chromosomal finding	
Good prognosis	11
Intermediate prognosis	12
Poor prognosis	4

For chromosomal findings, Poor indicates t(9;22), 11q23 with MLL rearrangements, complex karyotype, -5, -7, 5q-; Good indicates inv(16), t(15;17), t(8;21); and Intermediate indicates normal and other karyotypes.

achieved CR had 3 cycles of consolidation therapy with high dose Ara-C (4 g/m²/d) for 5 days or Ara-C (200 mg/m²/d) for 5 days and anthracycline for 3 days. Mononuclear cells were isolated on ficoll density gradient and used for RNA preparation.

Statistical analysis

Statistical evaluation of the data was performed using Student's *t*-test. *P* values were 2-tailed and were considered to be statistically significant at *p* < 0.05.

RESULTS

Overexpression of IGF-I in K562/AC cells

To investigate the differences in mRNA expression between K562 and K562/AC cells, microarray analysis was carried out using Human Cancer CHIP, in which 425 human cancer-related genes and 11 control house-keeping genes were spotted on glass slides. As shown in Fig. 1a, The expression of six genes, 1. vimentin (VIM), 2. lactate dehydrogenase A (LDHA), 3. DNA-damage-inducible transcript III (DDIT-III), 4. early growth response I (EGR-I), 5. IGF-I and 6. amphiregulin (AR), was increased, whereas one gene, 7. Kirsten rat sarcoma viral oncogene

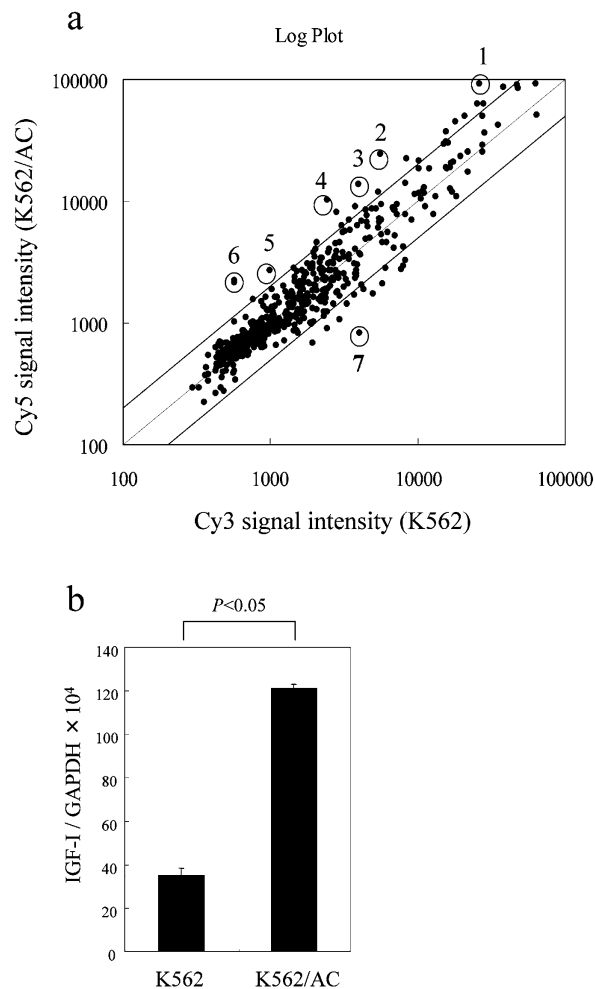


Fig. 1. IGF-I is highly expressed in K562/AC cells. (a) In Human Cancer CHIP, Version 2, 425 human cancer-related genes and 11 control house keeping genes were spotted on glass slides. The fluorescent probe was synthesized by incorporating Cy3TM-or Cy5TM-dUTP, and correspondence analysis was performed. 1; vimentin (VIM), 2; lactate dehydrogenase A (LDHA), 3; DNA-damage-inducible transcript III (DDIT-III), 4; early growth response I (EGR-I), 5; IGF-I, 6; amphiregulin (AR), 7; Kirsten rat sarcoma viral oncogene homolog (K-ras). (b) Quantitative real-time RT-PCR for IGF-I gene expression was performed in K562 and K562/AC cells. Data are shown as the relative ratio to the copies of the GAPDH gene. Data of three independent samples are shown.

homolog (K-ras), was decreased.

To confirm the differential gene expression profiles obtained by cDNA microarray analysis,

conventional RT-PCR for these seven genes was performed. Among these genes, insulin-like growth factor I (IGF-I) expression of K562/AC cells was significantly higher than that of K562 parental cells, while the expression of other genes appeared to be unchanged by conventional RT-PCR (data not shown). This result of IGF-I was confirmed by quantitative real time RT-PCR which revealed that the expression of IGF-I in K562/AC cells was almost three times higher than in K562 cells (Fig. 1b).

Protection by IGF-I from Ara-C induced apoptosis

Increased expression in K562/AC cells suggests that IGF-I may be involved in the development of Ara-C resistance. To examine this possibility, Ara-C sensitive K562 cells were incubated with IGF-I ligand and/or Ara-C, and cell growth was examined by MTT assay. In addition, to determine whether IGF-I ligand could inhibit apoptosis in K562 cells exposed to Ara-C, an in vitro apoptosis detection assay was carried out. As shown in Fig. 2a, the addition of 100 ng/ml or

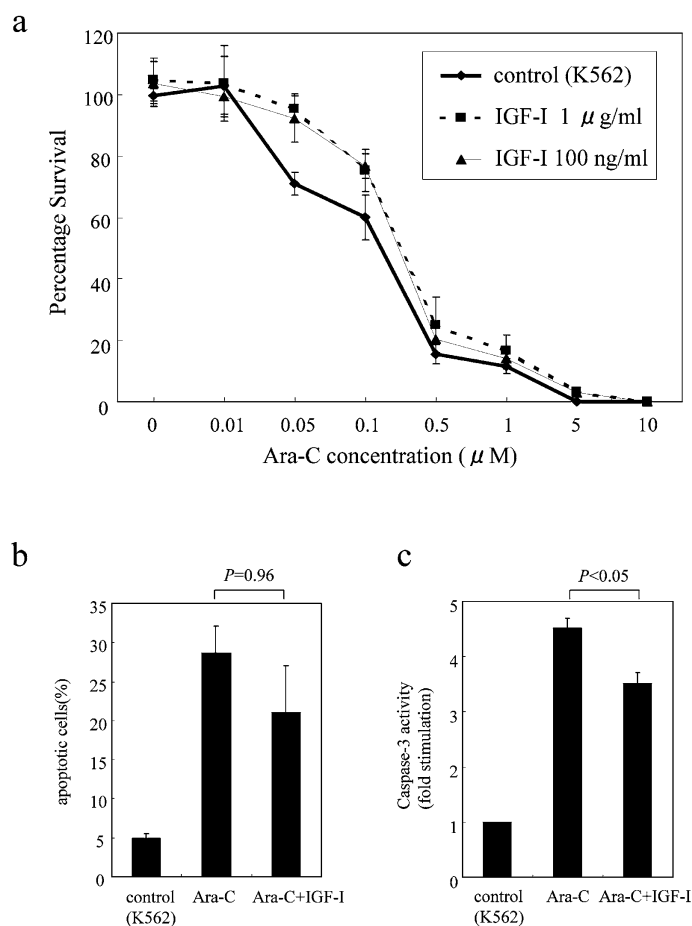


Fig. 2. Protection from Ara-C induced apoptosis in K562 cells, by IGF-I ligand.

(a) K562 cells were incubated for 72 hrs with various concentrations of Ara-C and two concentrations of IGF-I ligand. Cell growth was assessed by MTT assay as described in the *materials and methods*. Data are the means \pm s.d. of three separate experiments. (b) K562 cells were incubated with 100 nM of Ara-C with or without 100 μ g/ml of IGF-I ligand for 48 h. Cells were stained with annexin V-FITC and propidium iodide (PI). The percentage of early apoptotic cells (annexin V⁺/PI cells) of treated cells was compared with those of untreated cells. (c) Caspase 3 activity was determined using an EnzChek Caspase 3 kit (Molecular Probes) as described in the *materials and methods*. The presented data are mean values (\pm s.d.) from three independent samples.

1 $\mu\text{g/ml}$ IGF-I ligand protected K562 cells treated with Ara-C from cell death, and this effect was significant at 50 nM and 100 nM of Ara-C ($p < 0.05$), which is a clinical therapeutic concentration (Kufe et al. 1985; Muus et al. 1987). Furthermore, the addition of IGF-I ligand decreased the percentage of apoptotic cells (Fig. 2b), although it was not significant. Of note, the addition of IGF-I ligand significantly reduced caspase activity (Fig. 2c).

Sensitization of K562/AC cells by Suramin, a growth factor antagonist

Since IGF-I was thought to act as an auto-

crine anti-apoptotic factor of A562/AC cells, we examined whether a blockade of IGF-I signaling pathway results in the cancellation of Ara-C resistance. To this end, K562/AC cells were incubated with suramin, a non-specific growth factor antagonist, including IGF-I (Pollak and Richard 1990; Stein 1993). As a result, suramin significantly decreased a growth of K562/AC cells in wide range of concentrations of Ara-C, and the effect of suramin was most prominent at 300 $\mu\text{g/ml}$ ($p < 0.05$, Fig. 3a). Additionally, suramin increased a caspase activity and accordingly induced apoptosis in K562/AC cells at a significant level (Fig. 3b and c).

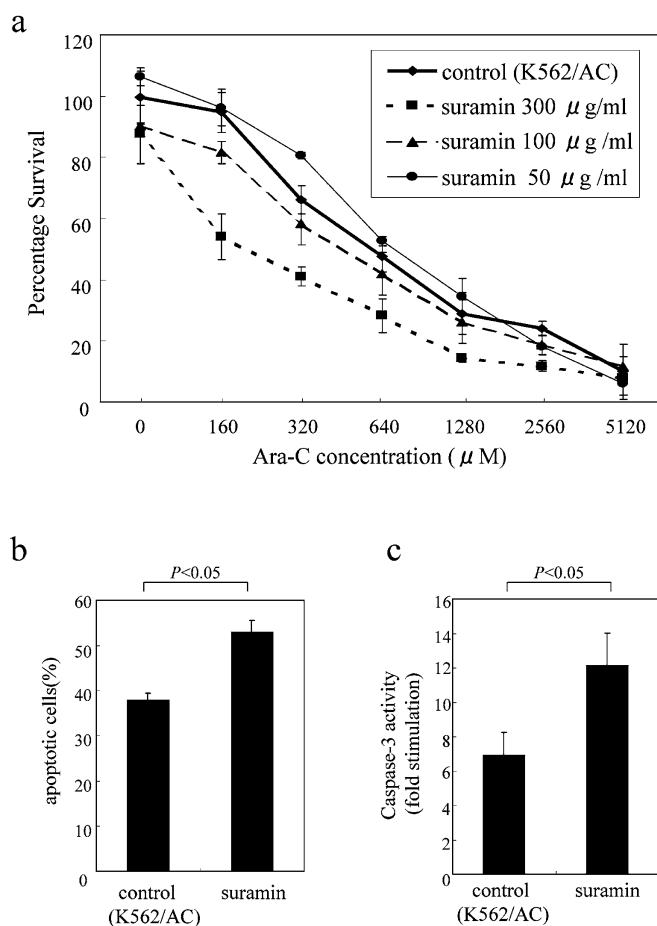


Fig. 3. Induction of apoptosis in K562/AC cells by Ara-C, in combination with suramin. (a) K562/AC cells were incubated for 72 hrs with various concentrations of Ara-C and three concentrations of suramin. Cell growth was assessed by MTT assay. (b) K562/AC cells were incubated with 1,000 μM of Ara-C with 300 $\mu\text{g/ml}$ of suramin or without suramin for 48 hrs. Apoptosis was determined by annexinV/PI staining. (c) Caspase activity was determined using an EnzChek Caspase 3 kit. Data were obtained from three independent samples.

Sensitization of K562/AC cells by IGF-I receptor neutralizing antibody α IGF-IR

Next, whether the specific inhibition of IGF-I signaling pathway influences the status of Ara-C resistance, K562/AC cells were incubated with the IGF-I receptor neutralizing antibody α IGF-IR, and the change of Ara-C sensitivity was examined. As shown in Fig. 4a, a growth of K562/AC cells was decreased by 1 μ g/ml of IGF-IR, and consistently, the number of apoptotic cells was increased (Fig. 4b and c). Taken together, IGF-I-IGF-IR system directly involved in the development of Ara-C resistance, and it may function in an autocrine or a paracrine fashion.

Involvement of PI3K-Akt pathway in Ara-C resistance

Because IGF-I is a powerful activator of the phosphoinositide 3-kinase (PI3K)-Akt pathway (Ge and Rudikoff 2000), which promotes cell survival and blocks apoptosis, we examined the Akt activation status by Western blot analysis. By using an antibody that specifically recognizes Akt1/PKB α (Akt1) phosphorylation at Ser473, we found that in K562/AC cells, Akt1 was constitutively activated compared with K562 cells (Fig. 5a). To verify this Akt1 activation depends on the PI3K pathway, LY294002, the specific inhibitor of PI3K, was added to K562/AC cells and the

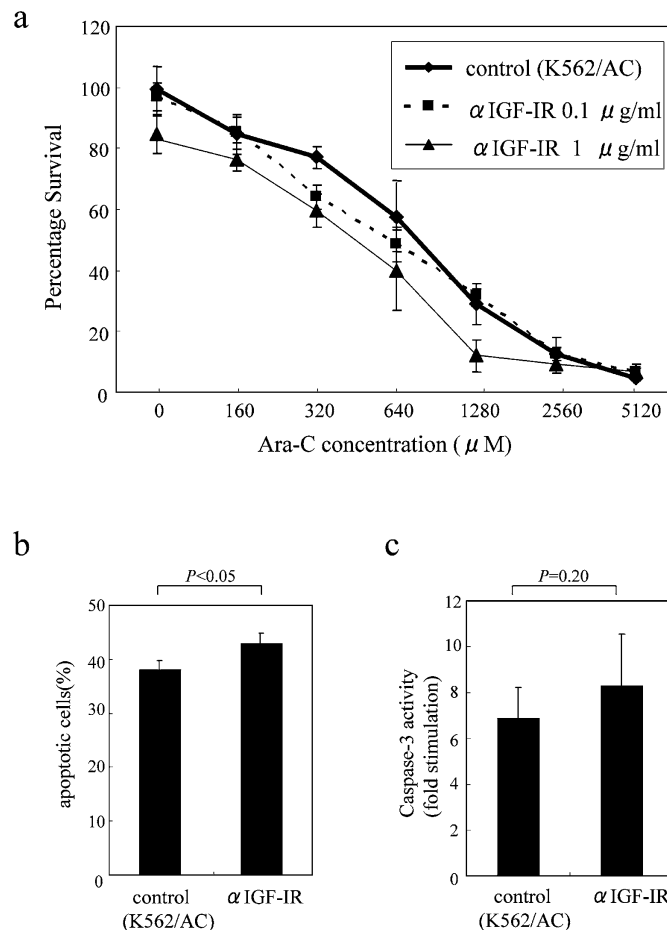


Fig. 4. Induction of apoptosis in K562/AC cells by Ara-C, in combination with α IGF-IR. (a) K562/AC cells were incubated for 72 hrs with various concentrations of Ara-C and two concentrations of α IGF-IR. Cell growth was assessed by MTT assay. (b) K562/AC cells were incubated with 1,000 μ M of Ara-C with 1 μ g/ml of α IGF-IR or without α IGF-IR for 48 hrs. Apoptosis was determined by annexinV/PI staining. (c) Caspase 3 activity was determined using an EnzChek Caspase 3 kit. Results were obtained as described in Fig. 3.

dysphosphorylation of Akt1 was confirmed (Fig. 5a). Next, in order to examine whether the blocking of PI3K biologically affects to the growth of K562/AC cells, cells were incubated with LY294002, and the changes of growth or apoptosis rate were examined. As a result, LY294002 significantly decreased the growth of K562/AC cells even at the low concentration of Ara-C (Fig. 5b). An increase of the caspase activity and the number of apoptotic cells were also found (Fig. 5

c and d). These results suggest that PI3K-Akt pathway activation is playing a role in Ara-C resistance in leukemia cells.

Increased IGF-I expression levels in refractory AML patients

Finally, the expression levels of IGF-I were examined in clinical AML samples by quantitative RT-PCR. Twenty seven AML patients were enrolled in our study. Bone marrow aspirates were

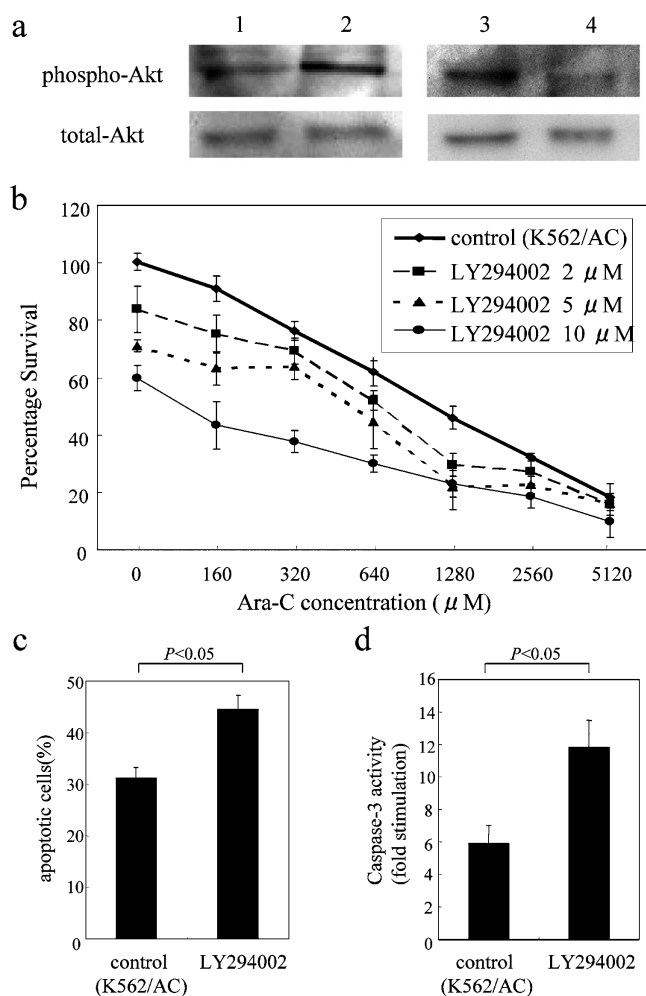


Fig. 5. Akt is activated in K562/AC cells. (a) Western blot analysis for Akt phosphorylation. Equal quantity of total Akt in each lane was confirmed as presented in lower panel. Lane 1, K562 cells; lane 2, K562/AC cells; lane 3, K562/AC cells; lane 4, K562/AC cells incubated for 4 hrs with 10 μ M LY294002. (b)-(d) Effect of Akt inhibition in K562/AC cells. (b) K562/AC cells were incubated for 72 hrs with various concentrations of Ara-C with LY294002, a specific PI3K inhibitor. Cell growth was assessed by MTT assay. (c) K562/AC cells were incubated with 1,000 μ M of Ara-C with 10 μ M of LY294002 or without LY294002 for 48 hrs. Apoptosis was determined by annexinV/PI staining. (d) Caspase 3 activity was determined using an EnzChek Caspase 3 kit. Data were obtained from two separate experiments, in triplicate.

obtained from patients as follows: 12 patients at diagnosis only, 7 patients who were refractory disease (after the Ara-C combined chemotherapy) at both diagnosis and refractory stage, 8 patients in refractory stage only. IGF-I expression levels of leukemia blasts in refractory patients were higher than those at diagnosis with statistical significance ($p < 0.05$, Fig. 6a). There was no specific relation between IGF-I levels and FAB subtype or chromosomal findings, however, the definite conclusion was not drawn because of the small number of patients. In 5 out of 7 patients who were at refractory stage, IGF-I expression levels in leukemia blasts were higher than those at diagnosis (Fig. 6b), while the level in the rest of 2 patients were unchanged. Although the number of patients was limited, these results may indicate that an increased expression level of IGF-I is involved in the refractory status of AML, after Ara-C combined chemotherapy.

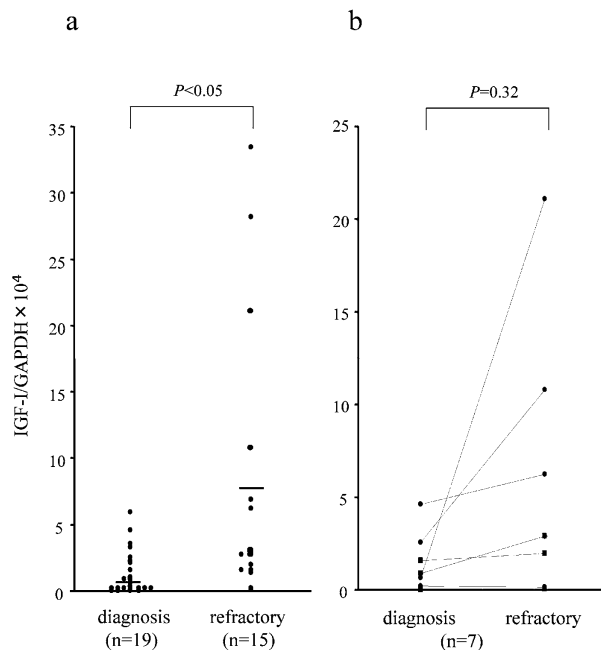


Fig. 6. IGF-I is highly expressed in refractory AML patients. (a) IGF-I expression levels of bone marrow blasts in 27 AML patients examined by quantitative RT-PCR. (b) IGF-I gene expression levels in bone marrow blasts of seven patients were compared between at diagnosis and refractory stage.

DISCUSSION

Many studies have been done to detect the genes related to Ara-C resistance by various methods (Galmarini et al. 2001). We also reported that the lack of dCK expression contributed to Ara-C resistance in vitro (Funato et al. 2000), but the clinical study showed the expression of dCK in leukemia blasts did not correlate with the patients prognosis (Galmarini et al. 2002). Through these efforts, the genes related to Ara-C resistance have been found, however, the roles of these genes are not fully understood.

In the present study, we have designed to investigate the expression pattern of genes between Ara-C-resistant and -sensitive cells in an attempt to identify the genes associated with Ara-C resistance. As a result, a significant increase of IGF-I expression in K562/AC cells was demonstrated, and additional in vitro studies suggest that the autocrine or paracrine loop of IGF-I is related to the acquisition of resistance to Ara-C.

Recently, the effect of the insulin-like growth factors (IGFs) in the progression of neoplasia has been studied (Grimberg and Cohen 2000; Khandwala et al. 2000; LeRoith and Roberts 2003). Several studies have shown that high levels of circulating IGF-I increase a risk for breast, prostate, colon, and lung cancer (Chan et al. 1998; Hankinson et al. 1998; Ma et al. 1999; Yu et al. 1999). IGF-I is a major mediator of the effects of growth hormones. It has, therefore, a strong influence on cell proliferation (Coppola et al. 1994; Sell et al. 1994) and is a potent inhibitor of apoptosis (Resnicoff et al. 1995; O'Connor et al. 1997). In fact, IGF-I has been shown to play an important role in maintaining growth and the survival of tumour cells in hematological malignancies (Georgii-Hemming 1996; Jelinek et al. 1997).

This study focused on IGF-I and examined the relationship between IGF-I and resistance to Ara-C. The action of IGF-I is predominantly mediated through IGF-IR, which can form heterodimers with the insulin receptor (LeRoith et al. 1995). When activated by specific ligands (IGF-I and IGF-II), IGF-IR induces cascades of intracel-

ular tyrosine phosphorylations including the Ras/Raf/Mitogen Activated Protein Kinase (MAPK) and the PI3K pathways that eventually culminate in the activation of transcription factors (Myers et al. 1994). PI3K provides an anti-apoptotic signal and is responsible for activating the downstream serine/threonine kinase Akt (Franke et al. 1995; Dudek et al. 1997). Akt has been shown to behave as an anti-apoptotic molecule functioning in phosphorylation of BAD, modulation of caspase-3, 9, upregulation of transcription factor FKHL1 (FOXO3a), or control of the synthesis of anti-apoptotic proteins such as c-IAPs, Bcl-2, and mcl-1 (Brazil and Hemmings 2001; Nicholson and Anderson 2002; Burgess et al. 2003). Recent findings revealed that Akt is constitutively activated and promotes cellular survival and resistance to chemotherapy in solid tumors (Ng et al. 2000; Brognard et al. 2001), normal myeloid cells (Grandage et al. 2005) and leukemic cells (Xu et al. 2003; Tabellini et al. 2004). We have shown that Akt is activated in K562/AC cells (Fig. 5a), but treatment of IGF-I inhibitors (suramin and IGF-IR) did not remarkably change the phosphorylation status of Akt (data not shown). These imply that IGF-I is involved (Neri et al. 2003), but not the sole factor for Akt activation responsible for Ara-C resistance in leukemia cells. Interestingly, Akt was dysphosphorylated after the treatment with PI3K inhibitor LY294002 resulting in an apoptosis of K562/AC cells (Fig. 5). These findings suggest that combination of a targeted therapy for IGF-I with that for PI3K-Akt pathway may be a promising approach to conquer Ara-C resistance.

We revealed that IGF-I expression levels of AML blasts of refractory patients who received Ara-C combined therapy were higher than those at diagnosis (Fig. 6). The results suggest that the IGF-I expression may influence on the outcome of treatment in AML. The previous study reported that Protein kinase C (PKC) is activated in leukemic cells to avoid apoptosis and cell death by induced Ara-C (Kharbanda et al. 1991; Emoto et al. 1996). Of note, PKC pathway positively regulates the IGF-I transcription (Nagaoka et al. 1990; Umayama et al. 2002). Based on these lines

evidence, PKC pathway might be involved in the increment of IGF-I in Ara-C resistant AML cells. Together with the results of in vitro study, inhibition of IGF-I and its downstream pathway appears to be a valuable therapeutic approach to overcome chemo-resistance to Ara-C.

Acknowledgments

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