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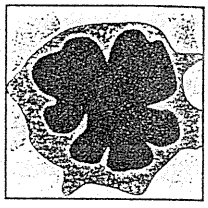

Bcl-x is a regulatory factor of apoptosis and differentiation in megakaryocytic lineage cells

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Abstract

Differentiation- and lineage-related differences in the expression of two anti-apoptotic molecules, bcl-x and bcl-2, were examined using various human hematopoietic cell lines. Bcl-x was strongly expressed in cell lines with erythroid and megakaryocytic properties (K562, HEL, CMK, and Mo7E), and was moderately expressed in immature myeloid cell lines (KG-1 and KCL-22). Bcl-2 expression was relatively weak in these cells. On the other hand, bcl-x was not expressed in more mature myeloid cell lines (HL-60 and PL-21), but bcl-2 was strongly expressed in these cells and in monocytoid cell lines (U937, THP-1, and JOSK-I). We investigated the biological significance of high levels of bcl-x expression in erythroid and megakaryocytic lineage cells. When K562 cells were specifically differentiated into megakaryocytic lineage by phorbol ester, the amounts of bcl-x increased by 10-fold. In contrast, bcl-x was gradually downregulated during erythroid differentiation induced by cytosine arabinoside. Apoptosis was observed following erythroid differentiation of K562 cells, but it was not associated with megakaryocytic differentiation in consistent with the increase in bcl-x. Moreover, phorbol ester–induced megakaryocytic differentiation was facilitated by the overexpression of bcl-x in K562 cells. Finally, in situ hybridization revealed that *bcl*-x mRNA expression was strongest in megakaryocytes among normal bone marrow cells. These results suggest that bcl-x is a regulatory factor in the apoptosis and differentiation of megakaryocytes.

Key words: Bcl-x—Apoptosis—Megakaryocytes—Differentiation

Introduction

Megakaryocytopoiesis is a complex phenomenon that includes the proliferation of committed progenitor cells, nuclear polyploidization, size growth, and generation of specific cytoplasmic proteins during terminal differentiation [1]. Following the completion of platelet release in bone marrow sinusoids, senescent megakaryocytes undergo death by apoptosis [2]. In contrast to other hematopoietic lineages, the mechanisms regulating megakaryocytic differentiation and death have not been fully elucidated because of the unavailability of samples and adequate assay methods.

The K562 cell line, which was established from a patient with chronic myeloid leukemia in blastic crisis, has properties similar to multipotent progenitor cells and has therefore been used as a model system for hematopoietic cell differentiation [3]. The K562 cell line can be differentiated into erythroid lineage by treatment with hemin [4], sodium butyrate [3], or cytosine arabinoside (Ara-C) [5]. In contrast, phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [6] or phorbol 12,13-dibutyrate (PDBu) [7] induce megakaryocytic differentiation. These properties of K562 are especially useful for separately studying the mechanisms of erythroid and megakaryocytic differentiation.

Apoptosis is a genetically controlled process leading cells to self-elimination [8]. Among several genes responsible for induction and suppression of apoptosis, the *bcl*-2/ced-9 genes are a growing family that negatively regulate cell death [9–11]. Boise et al. [12] have reported that bcl-x, which is structurally related to bcl-2, functions as a bcl-2–independent regulator of apoptotic cell death. Although bcl-2 and bcl-x act similarly as repressors of apoptosis in various cell types [13–15], there may be some functional differences between them. For example, bcl-x but not bcl-2 may prevent FK506-induced apoptosis of B lymphocytes [16]. No hematopoietic cell abnormalities were observed in bcl-2–deficient mice [17], whereas immature hematopoietic cells underwent massive apoptosis in bcl-x–deficient mice [18]. These differences can be partially explained by their different expression patterns, i. e., only bcl-x is expressed in the most primitive hematopoietic precursors [19]. However, little is known about the differential roles of bcl-2 and bcl-x in each hematopoietic cell lineage.

Recent investigations have revealed that apoptosis also plays a major role in homeostatic control of hematopoietic cells [20–22], and the mechanisms regulating apoptotic program are becoming clear. For example, we and others have demonstrated that downregulation of bcl-2 is implicated in apoptosis associated with myeloid cell differentiation [23–25]. The Fas antigen system was described in apoptosis of granulocytes and immature progenitor cells [26]. Despite these advances in understanding the mechanisms of apoptosis in other hematopoietic cells, those at work in megakaryocytes are largely unknown. Interestingly, it has been reported that megakaryocytes can survive longer than other lineage cells and hardly undergo apoptosis until the end of their normal

life span in bone marrow [27]. This finding prompts the speculation that megakaryocytes have a distinct mechanism to prevent apoptosis.

In the present study, we examined the role of bcl-x in the regulation of apoptosis and differentiation of megakaryocytes using the K562 cell line as a model system.

Materials and methods

Reagents and antibodies

Rabbit polyclonal antibodies against human bcl-2 and bcl-x proteins were obtained from Pharmingen (San Diego, CA) and Transduction Laboratories (Lexington, KY), respectively. Monoclonal antibody (mAb) reactive with glycoprotein IIb/IIIa (CD41) was a gift from Dr. Peter J. Newman (Blood Center of Southeastern Wisconsin). All chemical reagents, including PDBu, Ara-C and 3,3'-dimethoxybenzidine (o-dianisidine), were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

cDNA clones and expression vector

A 0.8 kb EcoRI fragment of full-length *bcl*-x_L cDNA was kindly provided by Dr. Craig B. Thompson (University of Chicago, IL) [12]. A 0.96-kb XhoI fragment of full-length *bcl*-2α cDNA was a gift from Dr. Yoshihide Tsujimoto (Osaka University, Osaka, Japan) [28]. Bcl-x expression vector was prepared by ligation of *bcl*-x_L cDNA into the EcoRI site of pcDNA3 mammalian expression vector (Invitrogen, San Diego, CA).

Cell lines and culture

Human erythroid leukemic cell line (HEL), megakaryocytic (CMK and Mo7E), myeloid (KG-1, KCL-22, HL-60, and PL-21), and monocytic leukemia cell lines (U937, THP-1, and JOSK-1) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). K562 was routinely maintained in GIT medium (Wako Pure Chemicals, Osaka, Japan). For differentiation induction, the cells were seeded at an initial concentration of 2×10^5 cells/mL in serum-free medium (10% GIT in HamF12/DMEM medium) and grown in the presence of either 50 nM PDBu or 1 μM Ara-C. Wright-Giemsa staining of the cytospin specimens was used for morphologic assessment of apoptotic cells. The appearance of an apoptotic body was defined as a morphologic marker of apoptosis in individual cells.

Dianisidine staining

Erythroid differentiation was scored by dianisidine staining as described previously [29]. O-dianisidine in 3.9% acetic acid was mixed with 1/10 volume of 30% hydrogen peroxide and added directly to 150 μL of cell suspension. After 30 minutes, the percentage of dianisidine staining–positive cells was determined microscopically by counting more than 200 cells on cytospin slides.

Flow cytometry

To assess megakaryocytic differentiation, cell surface expression of glycoprotein IIb/IIIa (CD41) was detected by indirect immunofluorescence using flow cytometry. The cells were incubated with primary antibodies for 30 minutes, washed, and developed with fluorescein-isothiocyanate–conjugated goat anti-mouse immunoglobulin (Ig)G + IgM (TAGO Products, Burlingame, CA). Human AB serum was included throughout the procedure to avoid nonspecific binding of antibodies to Fc receptors on the cells. The stained cells were analyzed on a FACScan system with Lysis II software (Becton-Dickinson, Mountain View, CA).

Analysis of DNA fragmentation in agarose gels

DNA was extracted with phenol/chloroform following proteinase K and RNase digestion of the cell lysates. Ten micrograms of each sample was analyzed by horizontal electrophoresis. DNA was visualized by ethidium bromide (EtBr) staining.

DNA fragmentation assay

DNA fragmentation was measured by quantitation of cytosolic oligonucleosome–bound DNA by using a Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Germany) as described elsewhere [22,24].

DNA transfection and isolation of stable transformants

Transfection of bcl-x expression vector into K562 cells was performed by electroporation with a Bio-Rad Gene Pulser basically as previously described [23]. In brief, a pulse was delivered to a 0.7-mL suspension containing 1.5×10^7 cells and 50 μg of the linearized plasmid. The cells were appropriately diluted and were seeded after 48 hours at 5×10^4 cells/mL in growth medium containing 350 μg/mL geneticin (G418 sulfate, Life Technologies, Grand Island, NY). After 3 to 4 weeks, surviving cells were subcloned in agar. Stable transformants were maintained with 200 μg/mL of the drug.

RNA and protein blotting

Expression of bcl-2 and bcl-x was examined by Northern and Western blotting according to the standard methods. An enhanced chemiluminescence system (Amersham Life Science, Arlington Heights, IL) was used to visualize the signals.

In situ hybridization

In situ hybridization was performed with digoxygenin-labeled probes as described previously [30]. Sense and antisense riboprobes were generated by T7 and SP6 RNA polymerases (Boehringer Mannheim) after linearizing pcDNA3 plasmid containing *bcl*-x_L cDNA with Xho I and Hind III, respectively. Paraformaldehyde-fixed bone marrow specimens from healthy volunteers were rehydrated in phosphate-buffered saline (PBS), immersed in 0.2 N HCl, and rinsed in PBS again. These specimens were treated with proteinase K (1 μg/mL) for 20 minutes at 37°C and washed in glycine (2 mg/mL) in PBS. They were then refixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride/0.1 M triethanolamine-HCl (pH 8) for 10 minutes. Hybridization was performed at 50°C overnight with the application of 10 pM digoxigenin-labeled riboprobes in 50 μL of hybridization buffer (50% deionized formamide, 10 mM Tris-HCl [pH 7.6], 0.6 M NaCl, 1 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, 0.25% sodium dodecyl sulfate (SDS), and 0.2 mg/mL tRNA). Posthybridization wash was carried out at 50°C in 2× standard saline citrate (SSC)/50% formamide for 30 minutes, in TNE solution (10 mM Tris-HCl [pH 7.6], 500 mM NaCl and 1 mM EDTA) containing RNase A (10 μg/mL) for 30 minutes, in 2× SSC for 20 minutes, and in 0.2× SSC for 40 minutes. Finally, anti-digoxigenin–alkaline phosphatase conjugate was applied for 2 hours. The signals were detected with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphatase (BCIP).

Results

Expression of bcl-x and bcl-2 in various human hematopoietic cell lines

Differentiation- or lineage-related difference in the expression of *bcl*-x and *bcl*-2 mRNA was examined by Northern blot analysis using various hematopoietic cell lines. As shown in Figure 1A, *bcl*-x mRNA was strongly expressed in erythroid and megakaryocytic cell lines such as K562, HEL, CMK, and Mo7E, but *bcl*-2 mRNA expression was relatively weak in these cells. Immature myeloid cell lines such as KG-1 and KCL-22 expressed *bcl*-x mRNA moderately and *bcl*-2 mRNA weakly. In contrast, HL-60 and PL-21, which are more differentiated myeloid cell lines, expressed *bcl*-2 but not *bcl*-x mRNA. Monocytoid cell lines such as U937, THP-1, and JOSK-I expressed both *bcl*-x and *bcl*-2 mRNAs.

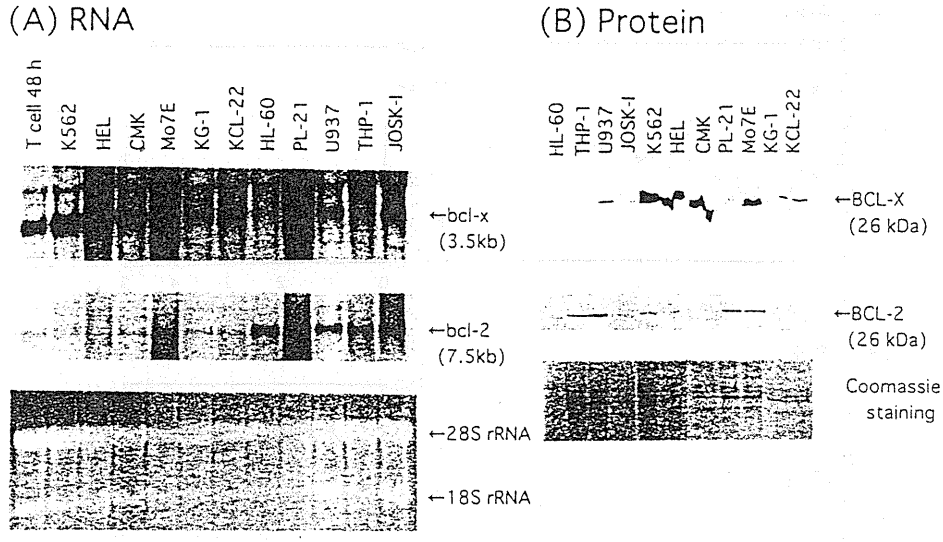

Fig. 1. Expression of bcl-x and bcl-2 in various hematopoietic cell lines. Total cellular RNA and protein samples were isolated from various human hematopoietic cell lines. **A.** Expression of *bcl-x* and *bcl-2* mRNA transcripts was examined by Northern blot analysis. EtBr staining of the gels is shown as a loading control (lowermost panel). **B.** Expression of bcl-x and bcl-2 proteins was examined by Western blot analysis. Coomassie blue staining of the membrane filters is shown as a loading control (lowermost panel).

We next investigated expression of bcl-x and bcl-2 proteins by Western blotting using specific antibodies. As shown in Figure 1B, the expression pattern of bcl-x and bcl-2 proteins was almost identical to that of mRNA transcripts, and confirmed the high level of bcl-x expression in K562, HEL, CMK, and Mo7E. These results suggest that bcl-x has a specific function in erythroid and/or megakaryocytic lineage cells.

Changes in bcl-x expression during erythroid and megakaryocytic differentiation of K562 cells

To investigate the functional significance of the high level of bcl-x expression in erythroid and megakaryocytic lineage cells, we examined the change in bcl-x expression during K562 cell differentiation. It has been reported that K562 cells can be differentiated into either erythroid or megakaryocytic lineage cells by using specific inducers [3–7]. To confirm this, we treated K562 cells with either Ara-C or PDBu and serially checked Hb synthesis and IIb/IIIa expression. When K562 cells were cultured with 1 μM Ara-C, the percentage of dianisidine-positive cells gradually increased from 23.7 ± 8.2% at day 0 to 67.3 ± 4.2% at day 5. On the other hand, the proportion of surface IIb/IIIa-positive cells decreased from 40.4 ± 5.0% at day 0 to 22.0 ± 6.0% at day 5 (Fig. 2A). When the cells were cultured with 50 nM PDBu, the percentage of dianisidine-positive cells decreased to 10.7 ± 3.3% at day 5. The proportion of IIb/IIIa-positive cells rapidly increased to 79.6 ± 6.0% at day 1 and maintained the same level of positivity until day 5 (Fig. 2B).

Using this system, we investigated the change in bcl-x expression during erythroid or megakaryocytic differentiation. Total cellular RNA was isolated from K562 cells after treatment with Ara-C and PDBu and subjected to Northern blot analysis. Untreated K562 cells expressed a high level of *bcl-x* mRNA transcript. When the cells were differentiated into erythroid lineage by Ara-C, *bcl-x* mRNA was gradually downregulated, reaching 20% of the initial amount by densitometric comparison after 72 hours (Fig. 3A). The amount of bcl-x protein, as detected by Western blotting, also decreased in parallel with the reduction in *bcl-x* mRNA expression (Fig. 3C). In contrast, when the cells were differentiated into megakaryocytic lineage by PDBu, the amount of *bcl-x* mRNA increased after 12 hours of culture following a transient decrease at approximately 6 hours, and became approximately 10-fold of the initial amount after 72 hours (Fig. 3B). This increase resulted in a concomitant increase in bcl-x protein (Fig. 3D). Because bcl-x is known to have two alternatively spliced forms, namely bcl-x_L and bcl-x_S, with opposite functions, we tried to determine whether bcl-x_L or bcl-x_S was expressed in K562 cells by RT-PCR. Only *bcl-*x_L mRNA was found to be present in K562 cells before and after differentiation (data not shown). Moreover, *bcl-2* mRNA was barely detectable even after differentiation (data not shown).

Induction of apoptosis during erythroid differentiation of K562 cells but not during megakaryocytic differentiation

Because K562 cells strongly expressed bcl-x_L, which has an anti-apoptotic potential, and because its expression was drastically modulated during differentiation, we next analyzed the functional consequences of the changes in bcl-x expression. When K562 cells were treated with Ara-C for 5 days, apoptotic cells, featuring compaction of the cytoplasm and chromatin condensation, were readily detected among proerythroblast-like differentiated cells (Fig. 4). As shown in Figure 5A, the percentage of apoptotic cells significantly increased from 1.3 ± 0.5% at day 0 to 48.7 ± 2.9% at day 5. DNA fragmentation was also increased 22.4-fold at day 5 according to a quantitative Cell Death Detection ELISA (Fig.

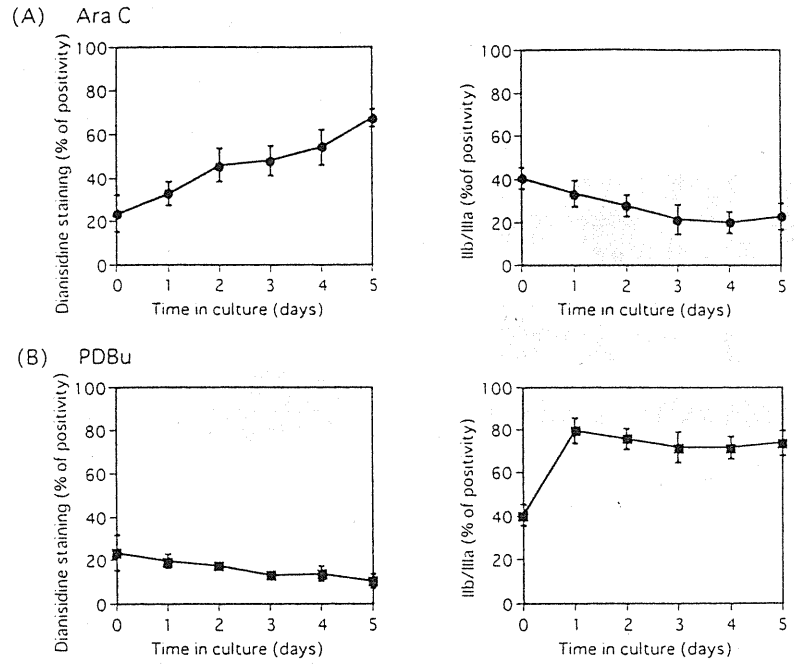

Fig. 2. Induction of Hb synthesis and IIb/IIIa expression during erythroid and megakaryocytic differentiation of K562 cells. K562 cells were treated with either 1 μM Ara-C **(A)** or 50 nM PDBu **(B)** over a 5-day period. Erythroid differentiation was microscopically scored by dianisidine staining (upper panel). Megakaryocytic differentiation was assessed by the expression of surface IIb/IIIa glycoprotein (lower panel).

5B), and oligonucleosomal fragments could be detected after day 3 on gel electrophoresis (Fig. 5C). When K562 cells were treated with PDBu, they showed the morphologic features of megakaryocytic cells, such as polyploidy and blebbing (Fig. 4). However, almost no apoptotic cells were observed. The absence of apoptosis associated with

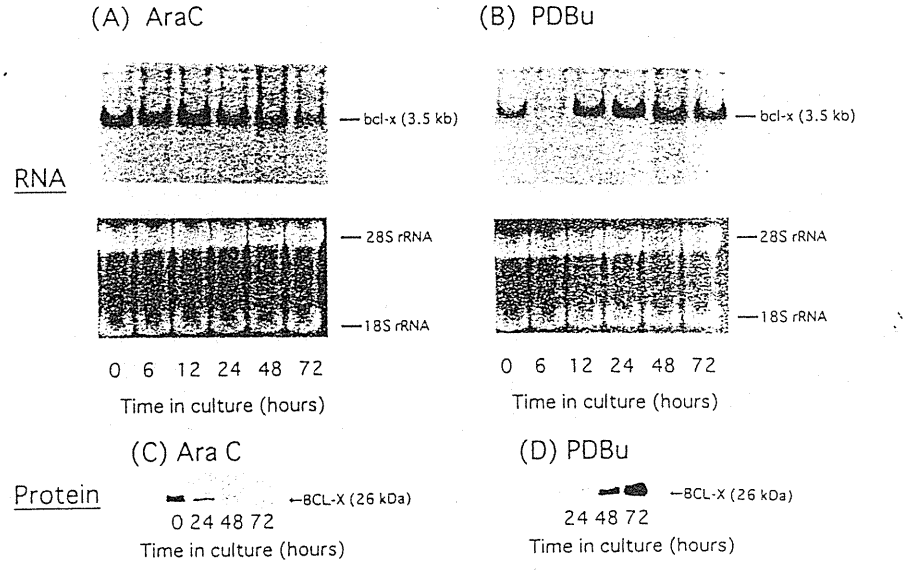

Fig. 3. Changes of bcl-x expression during erythroid and megakaryocytic differentiation of K562 cells. K562 cells were treated with either 1 μM Ara-C **(A, C)** or 50 nM PDBu **(B, D)**, and RNA samples and whole cell lysates were prepared at the given time points. Bcl-x expression was examined by Northern **(A, B)** and Western **(C, D)** blot analyses. The length of autoradiographic exposure was 3 times longer in **(A)** and **(C)** than in **(B)** and **(D)** to render the changes in signal intensity more clearly visible.

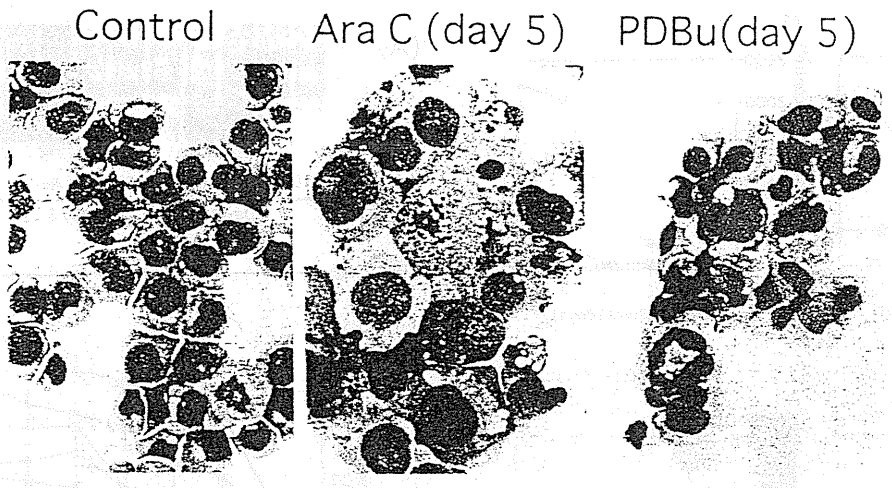

Fig. 4. Morphologic examination of apoptotic and differentiated cells during K562 cell differentiation. K562 cells were cultured in the absence (Control) or presence of either 1 μM Ara-C or 50 nM PDBu for 5 days, and morphologic examination was performed on Wright-Giemsa staining specimens. Magnification, ×100.

megakaryocytic differentiation was confirmed by morphologic assessment (Fig. 5A; 10.3 ± 1.9% at day 5), quantitative DNA fragmentation assay (Fig. 5B; 2.4-fold at day 5), and DNA electrophoresis (Fig. 5C). These results indicate that increased bcl-x_L actually acts as a suppressor of apoptosis during megakaryocytic differentiation, whereas downregulation of bcl-x_L during erythroid differentiation leads the cells to apoptotic death.

Effects of bcl-x overexpression on megakaryocytic differentiation of K562 cells

To further examine the role of bcl-x in megakaryocytic lineage cells, we have established K562 sublines that stably overexpress bcl-x under the control of cytomegalovirus promoter. Three sublines, designated as A6, A9, and A11, were used in this study. The parental pcDNA3-neo vector was also transfected into K562 cells as a control (hereinafter referred to as D5). Northern blot analysis revealed that the same amounts of endogenous *bcl*-x mRNA were expressed in all lines, including parental K562 cells before and after PDBu treatment. A 0.7-kb exogenous transcript was overexpressed in A6, A9, and A11 sublines and was upregulated after 5 days of treatment with PDBu in the A11 subclone (Fig. 6A). This increase in exogenous *bcl*-x transcript has been ascribed to the presence of phorbol ester–responsive elements in the cytomegalovirus promoter [31,32]. As is clearly shown in Figure 6B and C, overexpression of bcl-x in K562 cells resulted in enhanced differentiation toward megakaryocytic lineage, i.e., both the percentage of polyploid cells and the mean fluorescence intensity of surface IIb/IIIa molecules increased significantly during the course of PDBu treatment in A6, A9, and A11 compared with parental K562- and mock-transfected D5 cells. The extent of expression of differentiation-related phenotypes correlated well with the amount of overexpressed bcl-x in individual cell lines, i.e., it was strongest in the A6 subline. These results suggest that bcl-x is involved in megakaryocytic differentiation, probably through the suppression of apoptosis.

Bcl-x expression in normal bone marrow cells detected by in situ hybridization

Finally, *bcl*-x mRNA expression in normal bone marrow cells was examined by in situ hybridization. As shown in Figure 7B, antisense riboprobe could detect *bcl*-x signals in some components of bone marrow cells but sense probe did not produce any signal, indicating the specificity and validity of the method. Among *bcl*-x–positive bone marrow cells, megakaryocytes were found to have the strongest message (Fig. 7A). This observation is consistent with results obtained in K562 cells and strongly suggests that bcl-x plays a role in the regulation of apoptosis during normal megakaryocytic differentiation.

Discussion

In this study, we investigated differentiation- and lineage-related differences in the expression and function of two anti-apoptotic molecules, bcl-2 and bcl-x_L, in the hematopoietic cell system. First, we screened several hematopoietic cell lines for the expression of bcl-x and bcl-2 by Northern and Western blot analyses and found the cell lineage–specific pattern of their expression. Bcl-x was strongly expressed in cell lines with erythroid and megakaryocytic properties, and was moderately expressed in immature myeloid cell lines. Interestingly, bcl-2 was only weakly expressed in these cells. On the other hand, bcl-x was not expressed in more mature myeloid cell lines, whereas bcl-2 was strongly expressed in these cells. Monocytoid cell lines expressed both messages. Recently, Park et al. [19] reported that the most primitive human hematopoietic precursors expressed bcl-x but not bcl-2, and suggested that bcl-x might be essential for the long-term survival of the stem cell population. As primitive progenitors undergo proliferation and differentiation, there is a concurrent induction of bcl-2. Our present finding is fully consistent with their results.

It is of note that K562, which is one of the most primitive cell lines with properties of a pluripotent stem cell, predominantly expresses bcl-x. Krajewski et al. [33] have extensively investigated the expression of bcl-x and bcl-2 proteins in various tissues and have concluded that these two anti-apoptotic

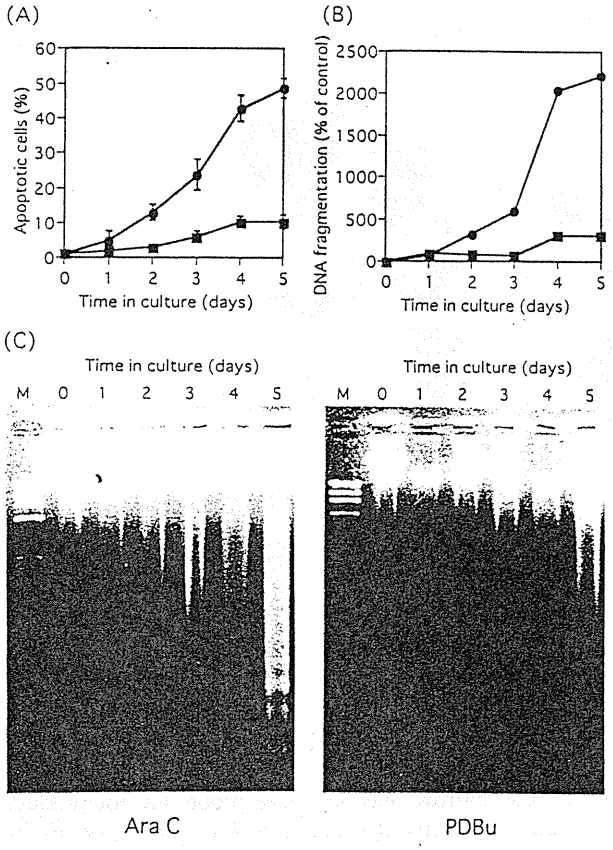

Fig. 5. Apoptosis was induced during erythroid differentiation of K562 cells but not during megakaryocytic differentiation. K562 cells were treated with either 1 μM Ara-C (●) or 50 nM PDBu (■) over a 5-day period. Morphologic examination **(A)**, quantitative DNA fragmentation assay **(B)**, and DNA electrophoresis **(C)** were performed at the given time points. Data are shown as means of three independent experiments. Standard deviations are shown by horizontal bars.

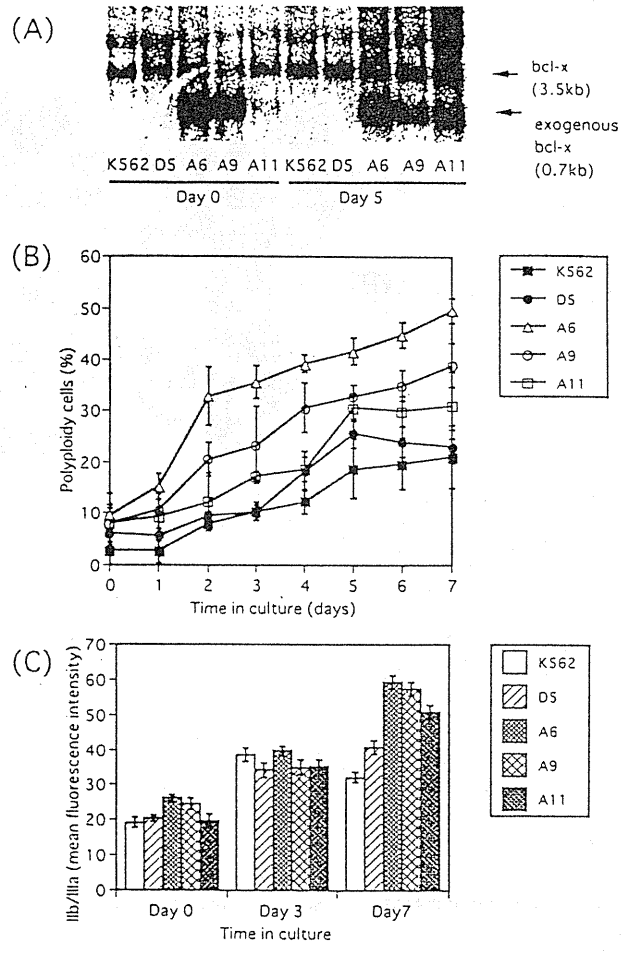

Fig. 6. Effect of overexpression of bcl-x on polyploidy and IIb/IIIa expression during megakaryocytic differentiation. Parent, mock-transfected (DS), and bcl-x–overexpressing (A6, A9, A11) K562 cells were treated with 50 nM PDBu over a 7-day period. **A.** Expression of endogenous and exogenous *bcl-x* mRNA was examined by Northern blot analysis before and after 5 days of PDBu treatment. **B.** The percentage of polyploid cells was determined microscopically on Wright-Giemsa–stained cytospin slides. **C.** Mean fluorescence intensity of surface IIb/IIIa was obtained by a FACScan/Lysis II analysis. Data are shown as means of three independent experiments. Standard deviations are shown by horizontal bars.

molecules regulate cell life and death at different stages of cell differentiation through tissue-specific control of their expression. However, because their study was conducted with immunohistochemical staining at the tissue level, the precise role of bcl-x and bcl-2 at different stages of hematopoietic cell differentiation or in specific individual lineage is still unclear. Therefore, we examined changes in bcl-x expression during hematopoietic cell differentiation using K562 cells. We found that the level of *bcl-x* mRNA was downregulated during erythroid differentiation, but that it was upregulated during megakaryocytic differentiation. The survival of differentiated cells of each lineage was affected in a manner dependent on bcl-x expression. Approximately half of the bcl-x–losing erythroid cells underwent apoptosis, whereas little if any apoptosis was observed in megakaryocytic cells whose bcl-x expression increased after differentiation. We also showed that normal bone marrow megakaryocytes strongly express *bcl-x* mRNA by in situ hybridization. Although the population of megakaryocytic cells is very small in bone marrow, they can continuously produce a large number of platelets. This may be attributable to the fact that megakaryocytes in normal bone marrow have a long life span and hardly undergo apoptosis [27]. The resistance of these cells to apoptotic cell death is a result at least in part of their high levels of bcl-x expression. Myelodysplastic syndrome (MDS) is characterized by ineffective hematopoiesis, with progenitor cells showing a reduced life span in culture [34]. Megakaryocytes in patients with MDS exhibit dysplastic changes such as micromegakaryocytes. Hatfill et al. [35] reported that the number of apoptotic megakaryocytes was elevated in MDS marrow, suggesting that the normal resistance of megakaryocytes to apoptosis is lost in MDS. To ascertain the mechanisms of this defect in

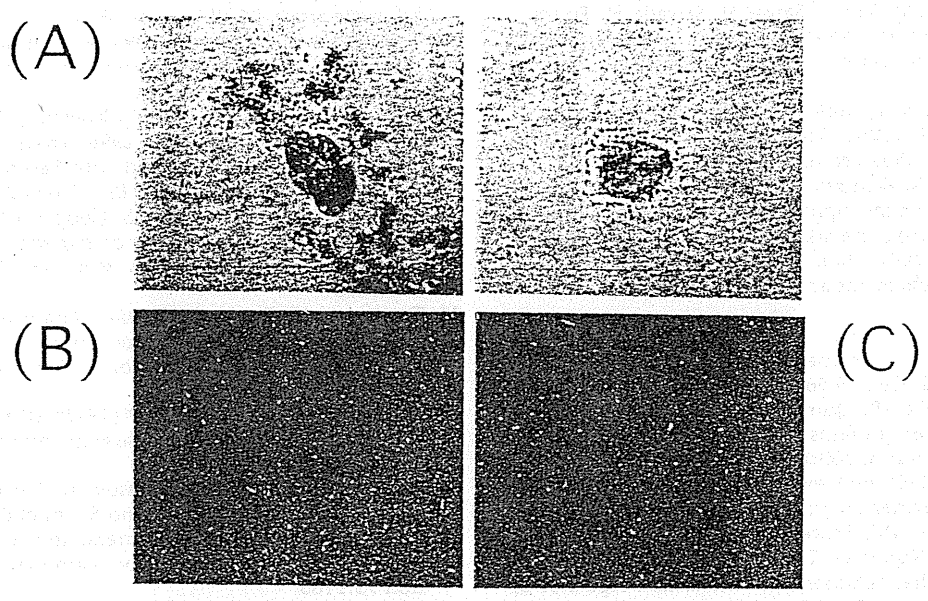

Fig. 7. Bcl-x expression in normal bone marrow cells by in situ hybridization. *Bcl-x* mRNA was visualized by in situ hybridization with a digoxigenin-labeled *bcl-x* riboprobe. Megakaryocytes in normal bone marrow are shown in **(A)**. Antisense **(A, B)** and sense **(C)** *bcl-x* probes were used for in situ hybridization. Original magnification, 200× **(A)**; 20× **(B, C)**.

MDS megakaryocytes, we investigated *bcl-x* mRNA expression in bone morrow cells from patients with MDS by Northern blotting and in situ hybridization. As anticipated, the level of *bcl-x* mRNA was significantly lower than that of normal bone marrow cells and other types of leukemic cells (Y. Terui and Y. Furukawa, unpublished observation). Taken together, these results suggest that bcl-x may play an important role in regulating the survival and function of megakaryocytic cells.

The mechanism of upregulation of *bcl-x* mRNA during megakaryocytic differentiation is at present unknown. It has been reported that treatment of M1 myeloid leukemic cells with IL-6 or dexamethazone upregulates *bcl-x* mRNA [36], and that stimulation of immature B cell line WEHI-231 by anti-CD40 antibody induces bcl-x expression [37]. The level of bcl-x did not increase in WEHI-231 cells treated with phorbol esters and ionomycin. Therefore, upregulation of bcl-x during megakaryocytic differentiation of K562 cells is not a nonspecific action of PDBu. Nonetheless, it is interesting to identify the factors regulating transactivation of the *bcl-x* gene during hematopoietic cell differentiation.

Finally, we found that overexpression of bcl-x in K562 cells facilitates megakaryocytic differentiation, as judged by the increased expression of surface IIb/IIIa molecules and polyploidization in the presence of PDBu. Induction of multilineage differentiation by suppression of apoptosis has been similarly described in bcl-2–transfected murine FDCP-Mix cells [38]. Furthermore, we observed that another megakaryocytic cell line, UT-7, is also resistant to apoptosis and becomes hyperploid after induction of differentiation by phorbol ester [39]. These observations are of particular interest in considering the causal relationship between hematopoietic cell differentiation and apoptosis, although recent evidence indicates that these two processes are regulated separately [23,25]. In a previous study, we showed that overexpression of bcl-2 did not affect expression of differentiation-related phenotypes in nonmegakaryocytic HL-60 cells [23]. Thus it seems likely that the effect of bcl-x on the differentiation process is specific to megakaryocytic cells; alternately, bcl-x may have the ability to directly affect differentiation independently of the modulation of apoptosis. It is possible that bcl-x facilitates differentiation by inducing cell cycle arrest, as recently described for bcl-2 by Mazel et al. [40]. The abnormalities of neuronal and hematopoietic systems in bcl-x–deficient mice resemble those in the retinoblastoma gene (RB)-deficient mice [41]. Moreover, bcl-x–deficient mice have a lymphoid cell disorder similar to c-abl–deficient mice [42]. It is known that RB and c-abl proteins are functionally associated in the regulation of the cell cycle [43]. Therefore, it is also possible that bcl-x induces megakaryocytic differentiation through some aspects of cell cycle control.

In summary, our study suggests that bcl-x, which is strongly expressed in megakaryocytic cells, may be an important regulator of apoptosis and differentiation in megakaryocytes.

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References

1. Hoffman R (1989) Regulation of megakaryocytopoiesis. Blood 74:1196
2. Radley JM, Holder CJ (1983) Fate of senescent megakaryocytes in the bone marrow. Br J Haematol 53:277
3. Lozzio CB, Lozzio BB (1975) Human chronic myelogenous cell line with positive Philadelphia chromosome. Blood 45:321
4. Anderson LC, Jokinen M, Gahmberg CG (1979) Induction of erythroid differentiation in the human leukemia cell-line K562. Nature 278:364

5. Ogawa K, Tashima M, Toi T, Sawai H, Sawada H, Fujita J, Maruyama Y, Okuma M (1994) Inhibition of erythroid differentiation by stem cell factor in K562 cells expressing the c-kit gene. Exp Hematol 22:45
6. Tetteroo PAT, Massaro F, Mulder A, Schreuder-van Gelder R, von dem Borne AEG (1984) Megakaryoblastic differentiation of proerythroblastic K562 cell-line cells. Leuk Res 8:197
7. Zutter MM, Fong AM, Kringman HR, Santoro SA (1992) Differential regulation of the alpha 2 beta 1 and alpha iib beta3 integrin genes during megakaryocytic differentiation of pluripotential K562 cells. J Biol Chem 267:20233
8. Steller H (1995) Mechanisms and genes of cellular suicide. Science 267:1445
9. Hengartner MO, Horvitz HR (1994) C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian protooncogene bcl-2. Cell 76:665
10. Boise LH, Gottschalk AR, Quintans J, Thompson CB (1995) Bcl-2 and Bcl2-related proteins in apoptosis regulation. Curr Topic Microbiol Immunol 200:107
11. Farrow SN, Brown R (1996) New members of the bcl-2 family and their protein partners. Curr Opin Gene Develop 6:45
12. Boise LH, Gonzalez GM, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB (1993) Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597
13. Dole MG, Jasty R, Cooper MJ, Thompson CB, Nunez G, Castle VP (1995) Bcl-xL is expressed in neuroblastoma cells and modulates chemotherapy-induced apoptosis. Cancer Res 55:2576
14. Ma A, Pena JC, Chang B, Margisian E, Davidson L, Alt FW, Thompson CB (1995) Bcl-x regulates the survival of double-positive thymocytes. Proc. Natl. Acad. Sci. U.S.A. 92: 4763
15. Boise LH, Minn AJ, June CH, Lindsten T, Thompson CB (1995) Growth factors can enhance lymphocyte survival without committing the cell to undergo cell division. Proc Natl Acad Sci USA 92:5491
16. Gottschalk AR, Boise LH, Thompson CB, Quintans J (1994) Identification of immunosuppressant-induced apoptosis in a murine B-cell line and its prevention by bcl-x but not bcl-2. Proc Natl Acad Sci USA 91:7350
17. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ (1993) Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75:229
18. Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Negishi I, Senju S, Zhang Q, Fujii S (1995) Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science 267:1506
19. Park JR, Bernstein ID, Hockenbery DM (1995) Primitive human hematopoietic precursors express Bcl-x but not Bcl-2. Blood 86:868
20. Koury MJ, Bondurant MC (1990) Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 248:378
21. Furukawa Y, Kikuchi J, Terui Y, Kitagawa S, Ohta M, Miura Y, Saito M (1995) Preferential production of interleukin-1α over interleukin-1 receptor antagonist contributes to proliferation and suppression of apoptosis in leukemic cells. Jpn J Cancer Res 86:208
22. Terui Y, Furukawa Y, Sakai T, Sugahara H, Kanakura Y, Kitagawa S, Miura Y (1996) Up-regulation of VLA-5 expression during monocytic differentiation and its role in negative control of the survival of peripheral blood monocytes. J Immunol 156:1981
23. Terui Y, Furukawa Y, Sakoe K, Ohta M, Saito M (1995) Expression of differentiation-related phenotypes and apoptosis are independently regulated during myeloid cell differentiation. J Biochem 117:77
24. Terui Y, Furukawa Y, Kikuchi J, Saito M (1995) Apoptosis during HL-60 cell differentiation is closely related to a G0/G1 cell cycle arrest. J Cell Physiol 164:74
25. Naumovski L, Cleary ML (1994) Bcl2 inhibits apoptosis associated with terminal differentiation of HL-60 myeloid leukemia cells. Blood 83:2261
26. Furukawa, Y, Iwase S, Terui Y, Kikuchi J, Sakai T, Nakamura M, Kitagawa S, Kitagawa M (1996) Transcriptional activation of the cdc2 gene is associated with Fas-induced apoptosis of human hematopoietic cells. J Biol Chem 271:28469
27. Zucker-Franklin D, Termin CS, Cooper MC (1989) Structural changes in megakaryocytes of patients infected with the human immune deficiency virus (HIV-1). Am J Pathol 134:1295
28. Tsujimoto Y, Croce CM (1986) Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci USA 83:5214
29. Orkin SH, Harasi FI, Leder P (1975) Differentiation in erythroleukemic cells and their somatic hybrids. Proc Natl Acad Sci USA 72:98
30. Takahashi M, Kitagawa S, Masuyama J, Ikeda U, Kasahara T, Takahashi Y, Furukawa Y, Kano S, Shimada K (1996) Human monocyte-endothelial cell interaction induces synthesis of granulocyte-macrophage colony-stimulating factor. Circulation 93:1185
31. Chan YJ, Tseng WP, Hayward GS (1996) Two distinct upstream regulatory domains containing multicopy cellular transcription factor binding sites provide basal repression and inducible enhancer characteristics to the immediate-early IES (US3) promoter from human cytomegalovirus. J Virol 70:5312
32. Chan YJ, Chiou CJ, Huang Q, Hayward GS (1996) Synergic interactions between overlapping binding sites for the serum response factor and ELK-1 proteins mediate both basal enhancement and phorbol ester responsiveness of primate cytomegalovirus major immediate-early promoters in monocytes and T-lymphocyte cell types. J Virol 70:8590
33. Krajewski S, Krajewska M, Shabaik A, Wang HG, Irie S, Fong L, Reed JC (1994) Immunohistochemical analysis of in vivo patterns of Bcl-x expression. Cancer Res 54:5501
34. Greenberg PL, Mara B (1979) The preleukemic correlation of in-vitro parameters of granulopoiesis with clinical features. Am J Med 66:951
35. Hatfill SJ, Fester ED, Steyler JG (1992) Apoptotic megakaryocyte dysplasia in the myelodysplastic syndrome. Hematol Pathol 6:87
36. Lotem J, Sachs L (1995) Regulation of bcl-2, bcl-x_L and bax in the control of apoptosis by hematopoietic cytokines and dexamethasone. Cell Growth Differ 6:647
37. Choi MS, Boise LH, Gottshalk AR, Quintans J, Thompson CB, Klaus GG (1995) The role of bcl-x_L in CD40-mediated rescue from anti-mu-induced apoptosis in WEHI-231 B lymphoma cells. Eur J Immunol 25:1352
38. Fairbairn LJ, Cowling GJ, Reipert BM, Dexter TM (1993) Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. Cell 74:823
39. Kikuchi J, Furukawa Y, Iwase S, Terui Y, Nakamura M, Kitagawa S, Kitagawa M, Komatsu N, Miura Y (1997) Polyploidization and functional maturation are two distinct processes during megakaryocytic differentiation: Involvement of cyclin-dependent kinase inhibitor p21 in polyploidization. Blood 89:3980
40. Mazel S, Burtrum D, Petrie HT (1996) Regulation of cell division cycle progression by bcl-2 expression: A potential mechanism for inhibition of programmed cell death. J Exp Med 183:2219
41. Lee EY-HP, Chang C-Y, Hu N, Wang Y-CJ, Lai C-C, Herrup K, Lee W-H, Bradley A (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis.

Nature 359:288

42. Tybulewicz VLJ, Crawford CE, Jackson PK, Bronson RT, Mulligan RC (1991) Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. Cell 65:1153

43. Welch PJ, Wang JYJ (1993) A c-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-abl tyrosine kinase in the cell cycle. Cell 75:779

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CD95 predicts responsiveness to tretinoin in acute promyelocytic leukemia

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Abstract. We describe a predictive marker (CD95) for the responsiveness to tretinoin (RA) in acute promyelocytic leukemia (APL). Functional CD95 expression during RA treatment have been observed only in those patients who responded to RA. Expression of CD95 (Fas antigen), which plays a major role in apoptosis, was determined by fluorescence activated cell sorter (FACS) analysis. APL cases in which no enhancement of CD95 expression was observed showed no response to RA and did not obtain complete remission. We propose that CD95 can predict the clinical response to RA probably due to differentiation.

Introduction

Treatment of acute promyelocytic leukemia (APL) has become a model of differentiation therapy, and tretinoin (RA) showed good complete remission (CR) rate for APL (1). Extensive reviews concerning RA treatment of APL have been published (2,3), and the recent application of genetic analysis to examine was also reported (4). Continuation or discontinuation of RA is very important because side effects including retinoic acid syndrome are serious. The presence of PML/RARα or translocation of chromosome (15;17) are correlated with the response to RA treatment (5). A subgroup with PML/RARα break/fusion sites in PML exon 6 showed decreased *in vitro* responsiveness to RA (6). On the other hand, isoforms of PML/RARα fused transcripts affect neither clinical features of APL nor prognosis after treatment with RA (7).

The cDNA encoding CD95 (Fas antigen) is involved in apoptotic cell death (8). We observed CD95 expression by monoclonal antibody staining and analysis of gene expression in hematological disorders (9). CD95 expression enhanced in the bone marrow cells from patients of myelodysplastic syndrome. It has been reported that increased expression of CD95 in blasts from patients with AML is associated with a better initial response to chemotherapy (10). However, CD95 expression in newly diagnosed or relapsed APL has not been further evaluated, and its clinical significance remains to be elucidated. In this report we demonstrate that increased expression of CD95 in blasts from patients with APL during differentiation therapy is associated with a better response to RA therapy.

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Key words: acute promyelocytic leukemia, tretinoin, CD95

Materials and methods

Patients. Bone marrow (BM) samples were collected from eight patients (6 with newly diagnosed and 2 relapsed APL). Mononuclear cells were separated from the above samples and used immediately for fluorescence activated cell sorter (FACS) analysis. In our institution all APL cases (newly diagnosed and relapsed cases) are treated with RA.

FACS analysis and cell sorting. We purchased antibodies against CD33 from Beckton Dickinson, and CD95 analysis was performed using FITC-conjugated or PE-conjugated anti-CD95 (11) from MBL. Cells ($1x10^6$ cells/sample) were incubated with appropriate monoclonal antibodies for 30 min on ice, followed by washing twice. Analysis was performed using the LYSIS II program (Beckton Dickinson, San Jose, CA, USA). Gating was performed for $CD33^+$ to determine the percentages of $CD95^+$ cells in the leukemic cells. For detection of apoptosis or cell death, we used propidium iodide staining as reported previously (12). To determine whether the percentage of normal progenitor cells increased or APL cells differentiated into $CD33^+CD95^+$ cells, we separated the cells into $CD33^+CD95^-$ and $CD33^+CD95^+$ fractions (13).

RT-PCR. We analyzed PML/RARα mRNA transcripts in each cell fraction. Oligonucleotide probes corresponding to each gene were synthesized by PCR mate DNA synthesizer (Takara,

Table I. Profiles of 8 patients with APL and expression of PML/RARα, CD19, HLA-DR, CD11b and CD33 on BM cells.

No.	Case	Age/sex	Disease status	PML/RARα	CD19	HLA-DR	CD11b	CD33	CD95
1	K.S.	52F	Untreated	bcr1	33.2	9.2	11.3	99.4	3.3
2	H.O.	44F	Untreated	ND	37.5	2.6	47.7	93.9	1.7
3	D.K.	63M	1st relapse	bcr1	ND	ND	ND	ND	2.0
4	A.I.	46M	2nd relapse	bcr1	ND	ND	ND	ND	2.2
5	Y.E.	42M	Untreated	bcr1	56.3	77.8	61.7	97.5	77.2
6	S.A.	47M	Untreated	bcr1	33.3	4.1	34.9	99.3	3.6
7	S.S.	36F	Untreated	ND	ND	ND	ND	ND	2.5
8	N.K.	48M	Untreated	ND	ND	3.1	ND	95.3	1.6

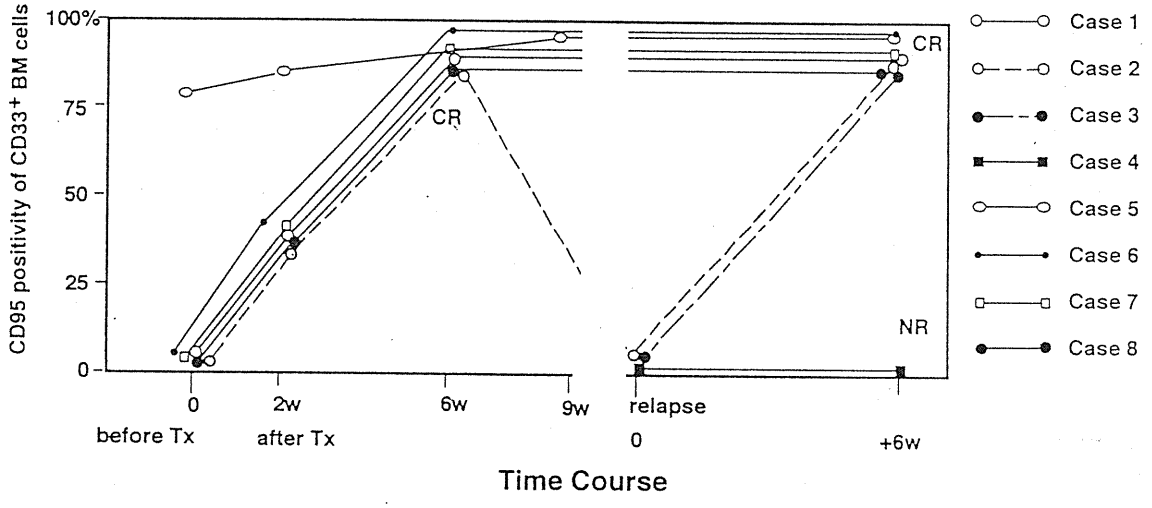

Figure 1. The percentages of CD95 immunoreactivity on marrow APL cells prior to treatment, 2 weeks and 6 weeks after treatment, and relapse and 6 weeks after treatment.

Kyoto, Japan) as previously reported (14). After two-step nested PCR, Southern hybridization was performed to determine the levels of PML/RARα transcripts in both $CD33^+CD95^+$ and $CD33^+CD95^-$ cells.

Immunoelectron microscopic analysis of sorted cells. Sorted $CD33^+CD95^+$ and $CD33^+CD95^-$ cells were stained using colloidal gold (5 nm)-conjugated anti-CD95 antibody.

Assay for cytolytic activities. Fresh marrow cells were cultured at 37°C for 12 h in the presence of 5, 50, 500 ng/ml anti-Fas Ab (IgM). After incubation, viable and dead cells were quantified by flow-cytometric analysis using propidium iodide as reported previously (12).

Effect of ATRA and Fas Ab. RA-responsive NB-4 cell line (5) and RA-resistant UF-1 cell line (15) were used. We analyzed CD95 and CD11b expression after 5-days co-culture with or without 10^{-7} M all-trans retinoic acid (ATRA). We also examined whether after 5 days co-culture with 10^{-7} M ATRA, 12 h co-culture with 500 ng/ml Fas Ab (IgM) or control Ab (IgM) would make cytolytic effects on NB-4 cells or UF-1 cells.

Results

We analyzed $CD95^+$ cells in newly diagnosed and relapsed APL patients by FACS analysis. In all APL patients except case 5, $CD95^+$ cells were present in very small numbers (less than 5%) before RA therapy. In case 5, HLA-DR and CD95 were positive. Five cases showed the same break site of PML/RARα (bcr1) (Table I), and four cases obtained complete remission (CR) at 6 weeks. Percentages of $CD95^+$ cells in gated $CD33^+$ cells were shown at the time of diagnosis, partial remission (PR) at two weeks after the beginning of the RA treatment, CR, and relapse. The percentage of $CD95^+$ cells increased during RA treatment and reached a significantly

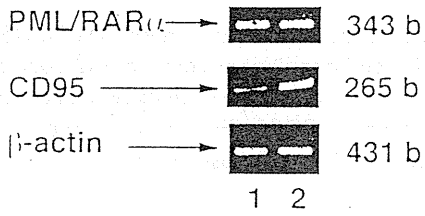

Figure 2. CD95 mRNA expression in sorted CD33+CD95- (lane 1) or CD33+CD95+ (lane 2) cells detected by RT-PCR. PML-RARα mRNA expression in CD33+CD95- or CD33+CD95+ cells detected by use of RT-PCR. Differentiated CD33+CD95+ cells also contained PML-RARα mRNA.

Table II. Effect of Fas Ab on freshly obtained leukemic marrow cells from APL patients *ex vivo*.

		Dead cells	
		Case 4	Case 5
Fas Ab (ng/ml)	5	0.1±0.2	18.2±2.3
	50	0.2±0.2	33.4±3.3
	500	0.2±0.3	58.8±3.6

higher level (more than 70%) at the time of CR, and decreased to the level of pre-treatment level (less than 5%) at relapse (Fig. 1). All untreated 6 cases but case 5, reached CR at 6 weeks after administration of RA. As for case 5, CD95 were 80% at 9 week, proved CR by bone marrow aspirates. Unresponsive case 4 showed no changes in the rate of positivity for CD95 even after administration of RA.

Two of 8 cases were followed up during their clinical course by FACS analysis and RT-PCR for PML/RARα. Sorted CD33+CD95+ cells contained PML/RARα transcript similar to sorted CD33+CD95- cells (Fig. 2), indicating the former cell population contained cells which had derived from promyelocytic leukemia cells. PML/RARα mRNA expression decreased after consolidation therapy (data not shown). Immunoelectron microscopic examination showed the presence of microvilli-like structures only on the surface of CD33+CD95+ cells (Fig. 3). Neither normal CD34+CD33+ nor CD34+CD33- cells showed such microvilli-like structures on their surfaces.

Table II showed the effect of Fas antibody (Ab) on freshly CD95-negative leukemic marrow cells (case 4) and positive

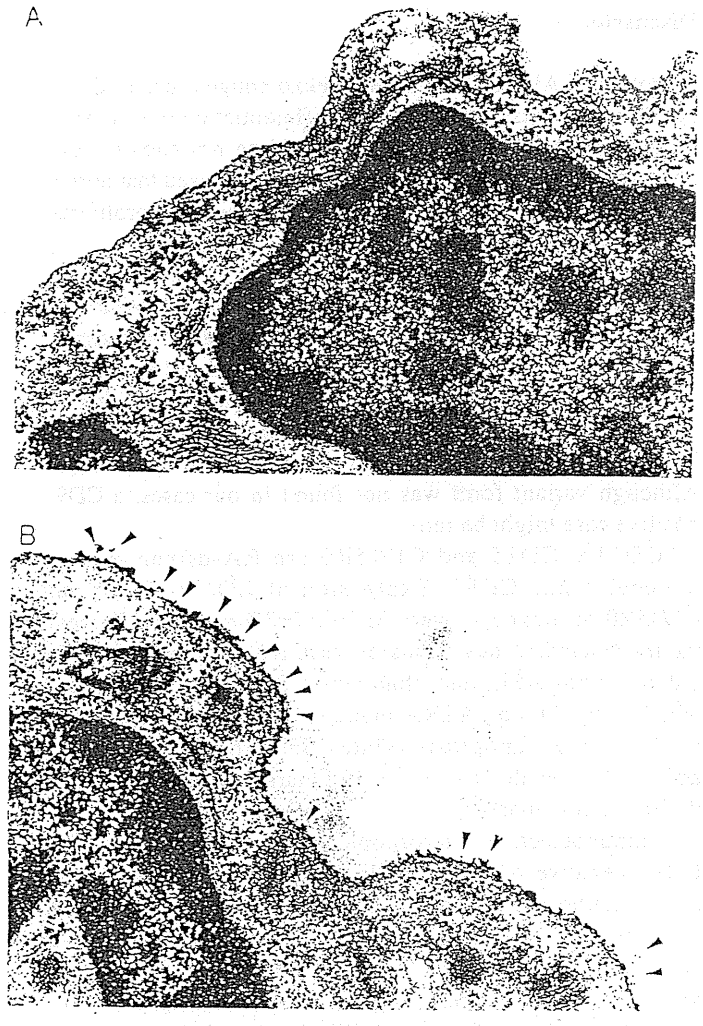

Figure 3. Immunoelectron microscopic observation of CD33+ leukemic cells. A, CD33+CD95- cell; B, CD33+CD95+ cell. Microvilli-like structure (arrows) on CD33+CD95+ cells were stained by anti-CD95 antibody.

cells (case 5). Case 4 showed that Fas Ab had no effect on CD95 negative cells. Case 5 showed that the cells were dose-dependently killed by Fas Ab.

To examine other myeloid differentiation markers, we used CD11b (16). RA-responsive NB-4 cells became CD11b positive, and RA-resistant UF-1 cells remained CD11b negative after ATRA co-culture. NB-4 and UF-1 cells did not express CD95, and were not killed by Fas Ab after co-culture (Table III). NB-4 cells proliferated with ATRA, therefore, viability of NB-4 cells decreased due to overgrowth.

Table III. CD95/CD11b expression and effect of ATRA/Fas Ab co-culture on NB-4 and UF-1 cells.[a]

Cell line	ATRA-			ATRA+			
	CD95	CD11b	Viability	CD95	CD11b	Viability	
						Control Ab	Fas Ab
NB-4	1.5±0.6	7.7±3.8	97.2±2.3	2.2±0.8%	78.8±5.4[b]	80.8±2.4	81.7±2.4
UF-1	3.3±1.2	5.0±2.3	98.4±1.3	3.5±1.4%	7.2±3.3	90.5±2.5	94.5±3.2

[a]ATRA-, 5-days culture without ATRA; ATRA+, 5 days culture with ATRA. [b]$p<0.001$ versus ATRA- control (Student t-test).

Discussion

Pathological APL cells express myeloid antigen such as CD33, but do not express CD95 prior to differentiation in contrast to normal hematopoietic progenitors (data not shown). Our observations of normal bone marrow cells showed that normal $CD33^+$ cells express CD95 and have no ability to proliferate *in vitro* in semi-solid culture (17).

The fact that 7 of 8 our cases and two APL cell lines did not express CD95 demonstrated that almost all fresh APL cells express little CD95. Paietta *et al* demonstrated that almost all APL cells showed HLA-DR negativity (18). Case 4 expressed both HLA-DR and CD95 at diagnosis. Significantly fewer APL blast cells were positive for HLA-DR (18) and HLA-DR positive cases tended to be of rare variant form (6). Although variant form was not found in our cases, a CD95 positive case might be rare.

CD11b, CD15 and CD45R0 are RA-driven surface molecules like CD95. Expression of CD11b, CD15 and CD45R0 at diagnosis were 8±5%, 7±7% and 11±8% mean values ± standard deviations of fresh leukemic cells from 30 patients with APL, and changed to 85±15%, 77±19% and 80±20% by 7th day ATRA inductions (18). We demonstrate that CD95, an apoptosis-related antigen which is not an adhesion molecule, can be a useful marker as well as myeloid differentiation marker.

Immunoelectron microscopic examination revealed that CD95 negative APL blast cells changed to positive by RA action. Although normal promyelocytes were rarely seen at two weeks' RA treatment, blastic promyelocytes were rare at six week's RA treatment. From two to six weeks after RA therapy, APL blastic cells might differentiate or die.

In this study, we found that CD95 expression is associated with a response to RA. In our APL cases, expression of members of the *bcl*-2 family such as *bcl*-2, *bcl*-x and *bax* showed no differences between $CD33^+CD95^+$ and $CD33^+CD95^-$ cells (data not shown). We have already demonstrated that apoptosis-related genes such as CD95 might also be important in normal hematopoietic stem cells (17). To observe whether APL cells are killed by Fas Ag-Fas ligand system, we examined APL cell lines. *Ex vivo* NB-4 and UF-1 cells did not express CD95 by adding RA, whereas fresh APL cells expressed CD95 by adding RA *in vitro* (data not shown). *In vivo* APL cells expressed CD95 by RA treatment. Cell lines may have some apoptosis inhibitory mechanism. RA at pharmacological (10^{-6} and 10^{-7} M) concentrations showed significant effects on the proliferation and differentiation of human $CD34^+$ hematopoietic progenitors, and especially stimulation of CFU-GM with a shift from mixed GM to pure G colonies (19). RA down-regulates both p55 and p75 tumor necrosis factor receptors, and this mechanism may involve Fas antigen expression in APL (20). In our case, the expression of p55 and p75 tumor necrosis factor receptors has not been demonstrated by FACS analysis using antibodies against both receptors (data not shown). Active apoptotic cell death *in vivo* was not confirmed in our cases. RA may act not only on differentiation of APL cells but also on Fas Ag-Fas L system and life span of the cells.

In conclusion, the increases in number of $CD33^+CD95^+$ cells in the APL cell population seems to be a good marker to predict the response to RA in APL. In differentiated promyelocytic leukemic cells, CD95 (Fas antigen) expression, may play an important role in apoptosis or tumor cell eradication.

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References

1. Huang ME, Ye YI, Chen SR, Chai JR, Lu JX, Zhoa L, Gu LJ and Wang ZY: Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood 72: 567-572, 1988.
2. Warrell RP Jr, de Thé H, Wang ZY and Degos L: Acute promyelocytic leukemia. N Eng J Med 29: 77-189, 1993.
3. Degos L, Domberet H, Chomienne C, Daniel MT, Miclea JM, Chastang C, Castaigne S and Fenaux P: All-trans-retinoic acid as a differentiating agent in the acute promyelocytic leukemia. Blood 85: 2643-2653, 1995.
4. Grignani F, Fagioli M, Alcalay M, Longo L, Pandolfi PP, Donti E, Biondi A, LoCoco F, Grignani F and Pelicci PG: Acute promyelocytic leukemia: from genetics to treatment. Blood 83: 10-25, 1994.
5. Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F and Berger R: NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood 77: 1080-1086, 1991.
6. Gallagher RE, Li YP, Rao S, Paietta E, Andersen J, Etkind P, Bennett JM, Tallman MS and Wiernik PH: Characterization of acute promyelocytic leukemia cases with PML-RAR alpha break/fusion sites in PML exon 6: identification of a subgroup with decreased *in vitro* responsiveness to all-trans retinoic acid. Blood 86: 1540-1547, 1995.
7. Fukutani H, Naoe T, Ohno R, Yoshida H, Miyawaki S, Shimazaki C, Miyake T, Nakayama Y, Kobayashi H and Goto S: Isoform of PML-retinoic acid receptor alpha fused transcripts affect neither clinical features of acute promyelocytic leukemia nor prognosis after treatment with all-trans retinoic acid. Leukemia 9: 1478-1482, 1995.
8. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y and Nagata S: The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66: 233-243, 1991.
9. Hatake K, Tomizuka H, Ikeda M, Tsunoda J, Hoshino Y, Ohtsuki T, Kasahara S, Yonehara S and Miura Y: The presence of apoptosis in refractory anemia of myelodysplasia. In: Molecular Biology of Haematopoiesis. Vol 3. Intercept, Andover, MD, pp127-133, 1994.
10. Min YH, Lee S, Lee JW, Chong SY, Hahn JS and Ko YW: Expression of Fas antigen in acute myeloid leukaemia is associated with therapeutic response to chemotherapy. Br J Haematol 93: 928-930, 1996.
11. Yonehara S and Yonehara M: A cell-killing monoclonal antibody (Anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med 69: 1747-1756, 1989.
12. Nicoletti I, Migliorati G, Paglicci MC, Grignani F and Riccardi C: A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139: 271-279, 1991.
13. Gunji Y, Nakamura M, Hagiwara T, Hayakawa K, Matsushita H, Osawa H, Nagayoshi K, Nakauchi H, Yanagisawa M, Miura Y and Suda T: Expression and function of adhesion molecules on hematopoietic stem cells: CD34+LFA- 1- cells are more primitive than CD34+LFA-1+ cells. Blood 80: 429-436, 1992.
14. Ikeda K, Sasaki K, Tasaka T, Nagai M, Kawanishi K, Takahara J and Irino S: Reverse transcription-polymerase chain reaction for PML-RARα fusion transcripts in acute promyelocytic leukemia and its application to minimal residual leukemia detection. Leukemia 7: 544-548, 1993.

15. Kizaki M, Matsushita H, Takayama N, Muto A, Ueno H, Awaya N, Kawai Y, Asou H, Kamada N and Ikeda Y: Establishment and characterization of a novel acute promyelocytic leukemia cell line (UF-1) with retinoic acid-resistant features. Blood 88: 1824-1833, 1996.
16. Noto RD, Schiavone EM, Ferrara F, Manzo C, Lo Pardo C and Del Vecchio L: Expression and ATRA-driven modulation of adhesion molecules in acute promyelocytic leukemia. Leukemia 8: 1900-1905, 1994.
17. Tomizuka H, Hatake K, Ikeda M, Nagayoshi Y, Gunji Y, Hoshino Y, Ohtsuki T, Miyazato A, Okada S, Kasahara T, Ohta S, Yonehara S and Miura Y: Apoptosis-related gene expression in human bone marrow CD34-positive cells. In: Myelodysplastic Syndromes; Advances in Research and Treatment. Excerpta Medica, Elsevier Science, pp269-279, 1995.
18. Paietta E, Anderson J, Gallagher R, Bennett J, Yunis J, Cassileth P, Rowe J and Wiernik PH: The immunophenotype of acute promyelocytic leukemia (APL): an ECOG study. Leukemia 8: 1108-1112, 1994.
19. Zauli G, Visani G, Vitale M, Gibellini D, Bertolaso L and Capitani S: All-trans retinoic acid shows multiple effects on the survival, proliferation and differentiation of human fetal CD34+ haemopoietic progenitor cells. Br J Haematol 90: 274-282, 1995.
20. Toptal K, Chaturvedi MM, LaPushin R and Aggarwal BB: Retinoids downregulate both p60 and p80 forms of tumor necrosis factor receptors in human histiocytic lymphoma U-937 cells. Blood 85: 3547-3555, 1995.