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Reduced expression of Bax in ceramide-resistant HL-60 subline

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Abstract

Ceramide, the backbone of sphingolipids, has been reported to be involved in various cellular responses including apoptosis. We recently established and characterized a C2-ceramide-resistant HL-60 subline designated HL-CR. HL-CR cells were resistant to not only ceramide but also anti-cancer drugs including daunorubicin, etoposide, and cytosine arabinoside. To elucidate the mechanisms by which HL-CR cells became resistant to various apoptosis-inducing stimuli, the levels of Bcl-2 family proteins, which play crucial roles in drug-induced apoptosis, were compared between HL-CR and parental HL-60 cells. Among Bcl-2 family members, Bax, a pro-apoptotic Bcl-2 family protein, was highly expressed in HL-60 but was hardly detected in HL-CR cells. Transient transfection of bax-expressing plasmid, but not the vector alone, induced apoptosis in HL-CR cells. These results suggest that reduced expression of Bax might play a role in resistance to various apoptosis-inducing stimuli in HL-CR cells.

Keywords: Ceramide; Bax; Bcl-2; Apoptosis; HL-60; HL-CR

Ceramide, the backbone of sphingolipids, has been reported to function as an intracellular signal mediator involved in various cellular responses including growth inhibition, apoptosis, and differentiation [1–3]. Since increase of ceramide has been observed in apoptosis induced by various stimuli such as TNF, cross-linking of Fas, anti-cancer drugs, withdrawal of growth factors, heat, and irradiation, it has been proposed that ceramide might be the key mediator of apoptosis [4].

The mechanism of apoptosis has been extensively investigated in the last decade, and various components of apoptosis have been elucidated [5,6]. Among them, caspases (especially caspase-3) seem to be the main executioners of apoptosis [7–9]. Mitochondria are involved in intrinsic apoptotic pathway induced by various stresses including anti-cancer drugs, heat, and irradiation, and Bcl-2 family members play key roles in this pathway [10,11]. Anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-xL inhibit apoptosis, whereas pro-apoptotic Bcl-2 family members such as Bax and Bak induce apoptosis.

To investigate the role for ceramide in apoptosis, we recently established ceramide-resistant HL-60 subline designated HL-CR [12]. In this report we examined the expression levels of Bcl-2 family proteins in HL-CR cells, and found that the expression of pro-apoptotic Bax was greatly reduced in HL-CR compared with parental HL-60 cells. Furthermore, overexpression of Bax in HL-CR cells induced apoptosis. These results suggest that the reduced expression of Bax might play a role in resistance to various apoptotic stimuli in HL-CR cells.

Materials and methods

Cell lines. Human promyelocytic leukemia cell line HL-60 cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. Ceramide-resistant HL-60 subline HL-CR was established as described [12]. HL-CR cells had been maintained in medium described above with 40 μM C2-ceramide for more than 1 year. In this study, HL-CR cells were cultured in medium without C2-ceramide.

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Western blot analysis. Cells were washed once in phosphate-buffered saline (PBS) and lysed in buffer containing 25 mM Tris/HCl (pH 7.4), 1% Triton X-100, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Cell lysate was centrifuged at 10,000*g* for 10 min at 4 °C and the supernatant was used for Western blot analysis. The samples (50 μg) were denatured by boiling in Laemmli's sample buffer for 2 min, subjected to 12% SDS–polyacrylamide gel electrophoresis, and electroblotted to Immobilon-P membranes (Millipore, MA, USA). Nonspecific protein binding was blocked by incubation of the membranes for 1 h in PBS containing 0.1% Tween 20 (PBS-T) plus 5% skim milk (Nacalai Tesque, Kyoto, Japan). The membranes were incubated for 1 h with either rabbit anti-Bcl-x, anti-Bcl-2, anti-Mcl-1, anti-Bax, anti-Bid, anti-Bik, goat anti-Bak or mouse anti-Bad antibody (Santa Cruz Biotechnology, CA, USA). After washing in PBS-T for 10 and 5 min, the membranes were incubated for 45 min with either goat anti-rabbit, bovine anti-goat or goat anti-mouse IgG horseradish peroxidase conjugated (Santa Cruz Biotechnology, CA, USA). After washing three times for 5 min in PBS-T, the membranes were incubated with Supersignal West Pico chemiluminescent substrate (Pierce, IL, USA) according to the manufacturer's protocol. The membranes were re-hybridized with goat anti-Actin IgG horseradish peroxidase conjugated (Santa Cruz Biotechnology, CA, USA) to confirm equal protein loading.

Construction of bax-expressing plasmid. Total RNA was purified from HL-60 cells by using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized by using SuperScript first-strand synthesis system (Invitrogen, CA, USA). bax cDNA was amplified by polymerase chain reaction (PCR) using PfuTurbo DNA polymerase (Stratagene, CA, USA), 5′-CCGGAAATTCAGCGGCGGTGATGGAC-3′ as the 5′-primer and 5′-CCGGAATTCCTCAGCCCATCTTCTTCCAGAT-3′ as the 3′-primer. The PCR product was digested by *Eco*RI and then ligated into pIRESneo2 vector (Clontech, CA, USA). The construct containing bax cDNA was designated pIRESbax.

Plasmid transfection. pIRESneo2 or pIRESbax together with pEGFP-N1 (Clontech, CA, USA) was transfected into HL-CR cells by using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. Briefly, 0.5 ml of cells at 5×10^5/ml was seeded in 24-well plate. Two μg of either pIRESneo2 or pIRESbax plus 1 μg pEGFP-N1 was incubated with 4 μl of Lipofectamine 2000 in 100 μl OPTI-MEM I reduced-serum medium (Invitrogen, CA, USA) for 20 min. The plasmid mixture was then added to the cells and incubated for 24 h.

Analysis of apoptosis. Only cells expressing green fluorescence under fluorescent microscope were examined for apoptosis. Apoptosis was assessed under microscope by typical morphological changes such as membrane blebbing and formation of apoptotic bodies.

Results

Differential expression of anti-apoptotic Bcl-2 family proteins between ceramide-resistant HL-CR and parental HL-60 cells

Since Bcl-2 family members play crucial roles in intrinsic apoptosis induced by various stresses including anti-cancer drugs [10,11], we investigated the expression of Bcl-2 family proteins in HL-CR cells, which were resistant not only to ceramide but also various anti-cancer drugs such as etoposide, daunorubicin, and cytosine arabinoside [12]. Among anti-apoptotic Bcl-2 family members, the level of Bcl-xL was increased in HL-CR cells compared with parental HL-60 cells (Fig. 1A), consistent with the fact that overexpression of Bcl-xL conferred resistance to apoptotic stimuli in various types of cells [13,14]. However, the level of Bcl-2, another anti-apoptotic Bcl-2 family member, was greatly reduced in HL-CR cells compared with parental HL-60 cells (Fig. 1B). The level of Mcl-1, another anti-apoptotic Bcl-2 family protein, was increased in HL-CR compared with HL-60 cells (Fig. 1C). These conflicting results among anti-apoptotic Bcl-2 family members prompted us to investigate pro-apoptotic Bcl-2 family members in HL-CR cells.

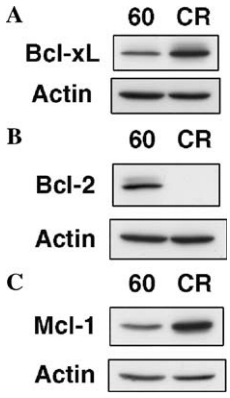

Fig. 1. Differential expression of anti-apoptotic Bcl-2 family proteins between parental HL-60 (60) and ceramide-resistant HL-CR (CR) cells. (A) Bcl-xL; (B) Bcl-2; and (C) Mcl-1. The membranes were re-hybridized with anti-Actin antibody to confirm equal protein loading. The results are representative of at least three different experiments.

Differential expression of pro-apoptotic Bcl-2 family proteins between ceramide-resistant HL-CR and parental HL-60 cells

Among pro-apoptotic Bcl-2 family members, Bax could hardly be detected in HL-CR cells, whereas it was highly expressed in parental HL-60 cells (Fig. 2A). Since Bax plays a crucial role in intrinsic apoptosis and overexpression of Bax-induced apoptosis [10,11,15], greatly reduced expression of Bax seemed consistent with resistance to various apoptotic stimuli in HL-CR cells. The level of Bak, another pro-apoptotic Bcl-2 family member, was slightly decreased in HL-CR cells compared with parental HL-60 cells (Fig. 2B). The level of Bid, a pro-apoptotic BH3-only protein, was slightly increased in HL-CR cells compared with HL-60 cells (Fig. 2C). Since Bid plays a crucial role in extrinsic but not in intrinsic apoptosis [5], increase of Bid seemed compatible with drug-resistance in HL-CR cells. Other pro-apoptotic Bcl-2 family members such as Bad and Bik were not detected in either HL-CR or parental HL-60 cells (data not shown). These results suggest that greatly reduced expression of Bax might play a role in resistance to various apoptosis-inducing stimuli in HL-CR cells.

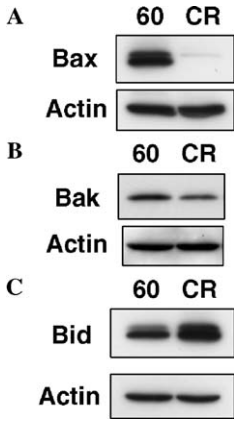

Fig. 2. Differential expression of pro-apoptotic Bcl-2 family proteins between parental HL-60 (60) and ceramide-resistant HL-CR (CR) cells. (A) Bax; (B) Bak; and (C) Bid. The membranes were rehybridized with anti-Actin antibody to confirm equal protein loading. The results are representative of at least three different experiments.

Induction of apoptosis by overexpression of Bax in HL-CR cells

To investigate whether reduced expression of Bax might be involved in resistance to apoptotic stimuli in HL-CR cells, HL-CR cells were transiently transfected with bax-expressing plasmid (designated pIRESbax). Since the efficiency of transient transfection assessed by transfection of green fluorescent protein (GFP)-expressing plasmid was up to 20%, either bax-expressing plasmid or control vector was transiently transfected together with GFP-expressing plasmid, and only GFP-expressing cells were analyzed for apoptosis. Transfection of control vector did not induce more apoptosis in HL-CR cells, whereas transfection of bax-expressing plasmid induced apoptosis in approximately 15% of GFP-expressing cells (Fig. 3). These results showed that overexpression of Bax induced apoptosis in at least a part of HL-CR cells, suggesting that reduced expression of Bax might play a role in resistance to apoptotic stimuli in HL-CR cells.

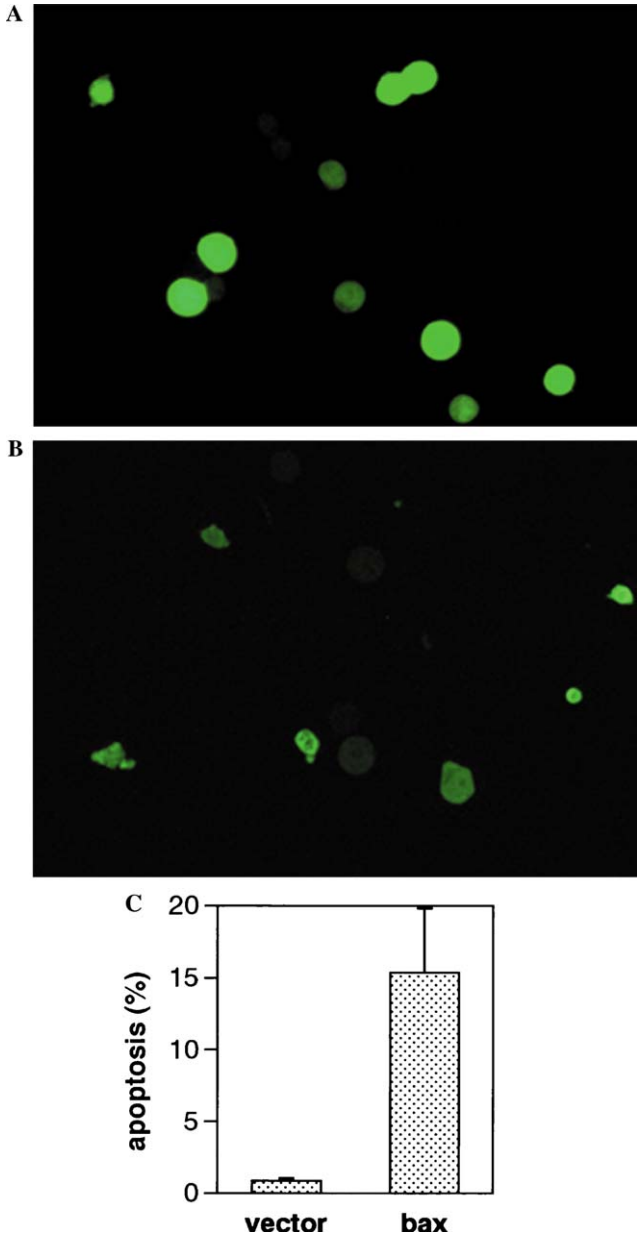

Fig. 3. Induction of apoptosis by overexpression of Bax in HL-CR cells. HL-CR cells were transfected with either pIRESneo2 (A) or pIRESbax (B) together with pEGFP-N1. After 24 h cells were observed under fluorescent microscope and only GFP-expressing cells were analyzed for apoptosis. (C) Apoptosis was assessed by characteristic morphological changes such as membrane blebbing and formation of apoptotic bodies. The data show means of three different experiments and the bars indicate one SD.

Discussion

Although ceramide has been proposed as an important intracellular signal mediator involved in various cellular responses especially in apoptosis, the mechanisms by which ceramide induce apoptosis remain to be elucidated. Several recent reports suggested that ceramide might target mitochondria, key component of intrinsic apoptotic pathway [16–18].

We recently established ceramide-resistant HL-60 subline (HL-CR), which was resistant not only to C2-ceramide but also to various anti-cancer drugs including daunorubicin, etoposide, and cytosine arabinoside [12]. To elucidate the mechanisms for drug-resistance in HL-CR cells, we focused on Bcl-2 family proteins, which play crucial roles in intrinsic apoptotic pathway induced by various stresses including anti-cancer drugs. As shown in Figs. 1 and 2, the levels of some of the Bcl-2 family members varied between HL-CR and parental HL-60 cells. Among anti-apoptotic Bcl-2 family mem-

bers, Bcl-xL was increased whereas Bcl-2 and Mcl-1 were decreased in HL-CR compared with in HL-60 cells. These conflicting results among anti-apoptotic Bcl-2 family members prompted us to investigate pro-apoptotic Bcl-2 family proteins. Among pro-apoptotic members, the expression of Bax could hardly be detected in HL-CR whereas Bax was highly expressed in parental HL-60 cells. Furthermore, overexpression of Bax induced apoptosis in at least a part of HL-CR cells. These results suggest that reduced expression of Bax might play a role in resistance to apoptotic stimuli in HL-CR cells.

In the previous paper, we reported that reduced expression of PKC δ might be involved in drug-resistance in HL-CR cells [12]. Since resistance to C2-ceramide was only slightly recovered by overexpression of PKC δ in HL-CR cells, it seemed that mechanisms other than reduced expression of PKC δ might exist for drug-resistance in HL-CR cells. In the case of PKC δ, overexpression of PKC δ itself did not induce apoptosis in HL-CR cells, therefore, the cells stably overexpressing PKC δ were generated and used for further experiments [12]. On the other hand, the cells stably overexpressing Bax could not be obtained in this study, probably because Bax-overexpressing cells died by apoptosis. Since transfection efficiency in HL-CR cells was at most 20% by the method we used in this paper, GFP-expressing vector was co-transfected with bax-expressing vector and only GFP-expressing cells were analyzed for apoptosis. Under these experimental conditions, transfection of bax-expressing plasmid induced apoptosis in approximately 15% of GFP-expressing cells (Fig. 3).

In conclusion, reduced expression of Bax might be involved in the mechanisms for drug-resistance in HL-CR cells. Further studies will be needed to elucidate the relationship between ceramide and Bax in apoptosis.

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