Bcl-2 Inhibits Calcineurin-mediated Fas Ligand Expression in Antitumor Drug-treated Baby Hamster Kidney Cells

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It is well known that human leukemia cells, such as HL-60 and U937 are sensitive to antitumor drugs, but human normal lung fibroblasts, such as WI-38 cells are resistant to the drugs. However, the mechanisms of the different responses to apoptosis in these cell lines remain unclear. We report here that an increase of Fas and Fas ligand (FasL) expression was required for antitumor drug-induced apoptosis in WI-38 and baby hamster kidney (BHK) cells, but not in HL-60 cells. Then, we used BHK cells transfected with the bcl-2 gene to investigate the involvement of complex formation of Bcl-2 and calcineurin. Calcineurin was imported to the nucleus in response to the drug treatment. Overexpression of Bcl-2 and cyclosporin A treatment inhibited the nuclear import and FasL expression, and as a result, both inhibited apoptosis. Although a caspase inhibitor, z-Asp-CH2-DCB, suppressed the drug-induced apoptosis, it failed to inhibit the drug-induced expression of Fas and FasL. These findings suggest that initially the Fas/FasL system is activated by calcineurin-dependent transcription followed by activation of the downstream caspase cascade resulting in antitumor drug-induced apoptosis in BHK cells, but not in HL-60 cells. Furthermore, Bcl-2 inhibits the nuclear import of calcineurin and suppresses calcineurin-mediated FasL expression during antitumor drug-induced apoptosis.

Key words: Bcl-2 — Calcineurin — Fas — Pironetin

Induction of apoptosis specifically in cancer cells is a good approach for cancer chemotherapy. We have reported that human leukemia HL-60 and normal human lung fibroblast line WI-38 responded differently to antitumor drugs such as cytotrienin A.1, 2) In this report, we investigate whether the Fas/Fas ligand (FasL) system is involved in antitumor drug-induced apoptosis, because the differences in this system may determine the sensitivity of cells to antitumor drug-induced apoptosis.

Fas, a receptor for FasL, transduces the apoptotic signal into cells.3) The intracellular domain of Fas contains a death domain, which is required for the induction of apoptosis.4) The activated Fas recruits another death domain-containing protein FADD/MORT-1, which in turn recruits caspase-8.5, 6) Upon recruitment to activated Fas, caspase-8 undergoes autoproteolytic activation. Active caspase-8 then cleaves and activates downstream caspases.7) This cascade of sequential autoproteolytic events apparently transmits and amplifies the apoptotic signal.

It has been proposed that induction of Fas and FasL expression with subsequent autoclire and/or paracrine induction of apoptosis through binding to Fas accounts for chemotherapy-associated death in solid tumors, such as neuroblastoma, hepatoma and brain tumor cells.8−12) It is also suggested that Fas/FasL system may, at least in part, mediate drug-induced apoptosis. However, Fas signal-neutralizing antibodies had no effect on drug-induced apoptosis in leukemia cells.13, 14) Thus, it is likely that the role of the Fas/FasL system in the mediation of drug-induced apoptosis depends on the cell type.15)

Bcl-2 protects cells from apoptosis induced by various stimuli, including treatment with antitumor drugs,16) growth factor withdrawal17) and Fas engagement.18) Bcl-2-related proteins share homology in one to four regions designated the Bcl-2 homology domains BH1−4.19) Among them, the BH4 domain homology at the N-terminal region exists only in anti-apoptotic members except Bcl-xL.20) This domain is not required for heterodimer formation between family members, but is most probably involved in protein-protein interactions with regulatory proteins outside the Bcl-2 family, such as Raf-121) and calcineurin.22) Although many interpretations of Bcl-2 functions have been proposed, such as suppression of reactive oxygen species generation,17) inhibition of protease activation,23, 24) regulation of calcium flux,25) inhibition of cytochrome c release from mitochondria,26−28) formation of ion channels29) and suppression of the nuclear factor of activated T cells (NF-AT)-mediated signal transduction,30) the exact biochemical functions performed by Bcl-2 protein remain unclear.

We demonstrate here that the Fas/FasL system is involved in antitumor drug-induced apoptosis of WI-38 and baby hamster kidney (BHK) cells, but not in HL-60...
cells. It is suggested that the apoptosis machinery responding to antitumor drug treatment is ready in drug-sensitive leukemia cells, but not in drug-resistant fibroblasts. Furthermore, it is also demonstrated that Bcl-2 inhibits the drug-induced FasL expression, thereby inhibiting apoptosis in BHK cells.

MATERIALS AND METHODS

Materials Pironetin was isolated from Streptomyces sp. as described previously. Vinblastine sulfate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Paclitaxel, camptothecin, etoposide, adriamycin and cyclosporin A were obtained from Sigma (St. Louis, MO). An inhibitor for caspases, z-Asp-CH₂-DCB, was purchased from Peptide Institute (Osaka). The Fas antibody (ZB4) and the neutralizing FasL antibody (NOK-2) were obtained from Medical and Biological Laboratories (Nagoya) and Pharmingen (San Diego, CA), respectively.

Plasmids The cDNA encoding human Bcl-2 (provided by S. Korsmeyer) was inserted into pcDNA3 vector (Invitrogen, Carlsbad, CA) with the N-terminal Myc tag. Bcl-2 fragment was sequentially cloned into pUHD10-3-myc (provided by H. Bujard) to establish a tetracycline-inducible (Tet-Off expression system) BHK cell line as described before.

Cell culture BHK and WI-38 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), and BHK cells transfected with the vector of the Tet-Off expression system of Bcl-2 were cultured in DMEM containing 10% FBS, G418 (200 µg/ml), puromycin (2 µg/ml) and doxycycline (2 µg/ml) at 37°C in a 5% CO₂,95% air atmosphere.

Western blotting The cells were washed and lysed in lysis buffer (10 mM HEPES, pH 7.2, 142.5 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.2% NP-40, 0.1% aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF)) at 4°C with sonication. The lysates were centrifuged at 14 000 rpm for 15 min, and the amount of protein in each lysate was measured by staining with Coomassie Brilliant Blue G-250. The sample buffer (42 mM Tris-HCl, pH 6.8, 10% glycerol, 2.3% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 0.002% bromophenol blue) was then added to each lysate, which was subsequently boiled for 3 min and electrophoresed on an SDS-polyacrylamide gel (SDS-PAGE). Proteins were transferred to Immobilon membrane (Millipore, Bedford, MA) and immunoblotted with anti-Bcl-2 antibody (clone 124; DAKO, Glostrup, Denmark), anti-calcineurin subunit A antibody (clone 29; Transduction Laboratories, Lexington, KY), anti-poly(ADP-ribose) polymerase (PARP) antibody (sc-1561; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-FasL antibody (clone 33; Transduction Laboratories). Detection was performed with enhanced chemiluminescence reagent (Pierce, Rockford, IL).

Analysis of Fas expression on the cell surface Expression of Fas on the cell surface was assessed by flow cytometry (Epics Profile II; Coulter, Hialeah, FL). The cells were washed and trypsinized for 1 min. The cells were incubated at room temperature for 30 min with 5 µg/ml of anti-Fas antibody (ZB4). They were then washed with phosphate-buffered saline (PBS) (containing 2% FBS), stained at room temperature for 30 min with “Alexa” 488-conjugated anti-mouse IgG (H+L) antibody (Molecular Probes, Eugene, OR), further washed, and analyzed by flow cytometry.

Immunoprecipitation assay To detect Bcl-2-calcineurin complex, immunodepletion and immunoprecipitation assays were performed. For immunodepletion assay, the cells were lysed in buffer A (20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EGTA, 1 mM MgCl₂, 0.2% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM PMSF, 10% glycerol, 1 µg/ml leupeptin) and centrifuged at 14 000 rpm for 15 min at 4°C. Fifty-microgram aliquots of cell lysates were pre-cleared with protein A-Sepharose and incubated with or without anti-Bcl-2 antibody (sc-783; Santa Cruz Biotechnology) at 4°C for 2 h. The immune complexes in the cell lysate were removed by immunoprecipitation. Resultant cell lysates or whole cell lysates were subjected to SDS-PAGE, transferred to Immobilon membrane and immunoblotted with anti-Bcl-2 antibody (clone 124) or anti-calcineurin antibody. Immunoprecipitation assay was described before.

RESULTS

Activation of Fas/FasL system in antitumor drug-treated WI-38 and BHK cells We have reported that human leukemia HL-60 and normal human lung fibroblast WI-38 responded differently to antitumor drugs such as cytotoxin A. Antitumor drug-induced apoptosis was inhibited by cycloheximide treatment in WI-38 cells, but not in HL-60 cells (data not shown), suggesting that the mechanism of antitumor drug-induced apoptosis was transcription-dependent in WI-38 cells, but not in HL-60 cells. Therefore, we first examined the expression levels of apoptosis-related proteins during antitumor drug-induced apoptosis. Treatment with antitumor drugs such as tubulin binders (pironetin, vinblastine and paclitaxel) and DNA damaging agents (camptothecin, adriamycin and etoposide) increased FasL levels in WI-38 and BHK cells (Fig. 1 A). Fas expression on the WI-38 cell surface was detected before antitumor drug treatment, and was enhanced after antitumor drug treatment (Fig. 1B), as detected by a shift in the logarithmic fluorescence intensity to the right. Cell surface Fas levels in antitumor drug-treated BHK cells were also increased (Fig. 1B). Next, we examined the effect of Fas signal-neutralizing antibody in pironetin-induced cell death. After 24 h in culture, about...
60% of the cells were killed by 100 nM pironetin (Fig. 1C). Preincubation with FasL-neutralizing antibody (NOK2) significantly reduced apoptosis at least up to 24 h after the start of pironetin treatment in both cell lines (Fig. 1C). Similar results were observed in vinblastine-, paclitaxel-, camptothecin-, adriamycin- or etoposide-treated BHK cells (data not shown). These data suggest that antitumor drug-induced apoptosis is mediated by the FasL/Fas system in WI-38 cells, as in WI-38 cells. In HL-60 cells, Fas and FasL expression was not altered after drug treatment, and antitumor drug-induced apoptosis was not inhibited by FasL-neutralizing antibody (Fig. 2).

**Antitumor drug-induced FasL expression was mediated by calcineurin** Next, we examined the effect of cyclosporin A, an inhibitor of calcineurin, on antitumor drug-induced FasL expression in BHK cells, because FasL expression is known to be regulated by the calcineurin-NF-AT signaling pathway. The expression level of FasL in pironetin-treated BHK cells was dose-dependently increased, and the increase in FasL by pironetin was eliminated by the pretreatment with 500 nM cyclosporin A (Fig. 3A). Moreover, calcineurin was translocated to the nucleus when cells were treated with pironetin (Fig. 3B). Concomitant with the inhibition of FasL upregulation, cyclosporin A blocked pironetin-activated calcineurin nuclear import (Fig. 3B). Similar results were observed with vinblastine-, paclitaxel-, camptothecin-, adriamycin- and etoposide-treated BHK cells (data not shown). Therefore, it is suggested that antitumor drug-induced FasL expression was mediated by calcineurin activity.

**Inhibition of pironetin-induced apoptosis by Bcl-2** To examine the effect of Bcl-2 expression on the antitumor drug-induced FasL/Fas system and apoptosis, we established a Tet-Off expression system of Bcl-2 in BHK cells. The cells were cultured in the presence of doxycycline (Bcl-2 expression was suppressed; designated BHK-V) or 24 h after removal of doxycycline (Bcl-2 expression was induced; designated BHK-Bcl-2) (Fig. 4A). Pironetin led
Inhibition of FasL Upregulation by Bcl-2

Inhibition of antitumor drug-induced FasL expression and apoptosis by Bcl-2

It was reported that calcineurin bound to Bcl-2 at mitochondria was unable to promote the nuclear translocation of NF-AT and that expression of FasL was regulated by NF-AT. Therefore, we investigated the effect of Bcl-2 expression on the formation of Bcl-2-calcineurin complexes and antitumor drug-induced FasL expression. Cell extracts were prepared from BHK-V or BHK-Bcl-2 cells and Bcl-2-calcineurin complex was removed by immunoprecipitation with anti-Bcl-2 antibody. As shown in Fig. 5A, calcineurin was detected in the whole cell lysate before immunodepletion and the expression level of calcineurin in whole cell extracts was not affected by the Bcl-2 expression. After immunodepletion with anti-Bcl-2 antibody, Bcl-2 and calcineurin disappeared in the supernatant in BHK-Bcl-2 cells, but not in BHK-V cells. This observation suggests that Bcl-2 forms a complex with most calcineurin in the cells, but that the normal level of Bcl-2 is not sufficient to saturate calcineurin. Immunoprecipitation assay also demonstrated that Bcl-2 associated with calcineurin in the cells (Fig. 5B). In BHK-Bcl-2 cells, pironetin-induced FasL expression did not occur (Fig. 5C), whereas the drug-induced Fas expression was not affected by overexpression of Bcl-2 (Fig. 5D). Concomitant with the inhibition of FasL upregulation, Bcl-2 blocked pironetin-activated calcineurin nuclear import (Fig. 5E). These results indicate that overexpression of Bcl-2 augments the association of Bcl-2 and calcineurin and suppresses the NF-AT signaling, leading to inhibition of FasL upregulation by pironetin.

Activation of caspases downstream of the Fas/FasL system in antitumor drug-induced apoptosis

We next examined the role of caspases in antitumor drug-induced Fas/FasL system activation and apoptosis in BHK cells. As shown in Fig. 6A, z-Asp-CH₂-DCB (50 µM), pan-
caspase inhibitor protected the cells from pironetin-induced apoptosis, indicating that this apoptosis induction process requires activation of the caspase cascade. Activation of caspases in pironetin-induced apoptosis was confirmed by testing the cleavage of PARP, a known endogenous substrate for caspases. During the drug-induced apoptosis, loss of the 116-kDa PARP was accompanied by the appearance of the 28-kDa polypeptide, as demonstrated by western blot analysis using an antibody directed against the NH2 terminus of PARP (Fig. 6B). As expected, z-Asp-CH2-DCB or Bcl-2 overexpression inhibited the PARP cleavage in the antitumor drug-treated cells (Fig. 6B). Preincubation with the FasL-neutralizing antibody NOK2 markedly inhibited pironetin-induced PARP cleavage (Fig. 6B), suggesting that activation of the Fas/FasL system occurred upstream of caspase activation. To exclude the possibility that the Fas/FasL system also acts downstream of caspase activation, we next examined whether pironetin-induced FasL expression and cell surface Fas would be inhibited by z-Asp-CH2-DCB. As shown in Fig. 6C and D, z-Asp-CH2-DCB failed to influence the drug-induced FasL expression or the cell surface Fas level. Moreover, z-Asp-CH2-DCB did not affect pironetin-induced calcineurin nucleus translocation (data not shown). These results indicate that caspases do not act upstream of the Fas/FasL system in the pathway of drug-induced apoptosis.

Fig. 4. Inhibition of antitumor drug-induced FasL expression and apoptosis by Bcl-2. (A) Detection of Bcl-2 protein in BHK-V and BHK-Bcl-2 cells. (B) Inhibition of pironetin-induced cell death by Bcl-2. BHK-V (○) and BHK-Bcl-2 (●) cells were treated with pironetin at various concentrations for 24 h. Cell viability was assessed by trypan blue dye exclusion assay. Values are means±SD of quadruplicate determinations.

Fig. 5. Inhibition of pironetin-induced FasL expression by Bcl-2. (A) Effect of Bcl-2 expression on the formation of Bcl-2-calcineurin A complexes. Bcl-2-calcineurin complexes in BHK-V or BHK-Bcl-2 cell lysate were depleted by immunoprecipitation with anti-Bcl-2 antibody. Resultant supernatants and whole cell lysates were analyzed by immunoblotting using anti-Bcl-2 or anti-calcineurin antibody. (B) Bcl-2-calcineurin complexes in BHK-V or BHK-Bcl-2 cell lysate were detected by immunoprecipitation assay. (C) Inhibition of pironetin-induced FasL expression by Bcl-2. BHK-V or BHK-Bcl-2 cells were treated with 100 nM pironetin for 12 h. Aliquots of cell lysates were immunoblotted with anti-FasL or anti-calcineurin antibody. (D) Effect of pironetin-induced expression of cell surface Fas by Bcl-2. BHK-V or BHK-Bcl-2 cells were treated with 100 nM pironetin for 12 h. Aliquots of cell lysates were immunoblotted with anti-FasL antibody or anti-Fas antibody. (E) Suppression of pironetin-induced calcineurin nuclear import by Bcl-2. BHK-Bcl-2 cells were pre-treated with or without 500 nM cyclosporin A (CsA) for 1 h and then the cells were treated with 100 nM pironetin for 12 h. The cells were fixed, stained and observed under a fluorescence microscope.
DISCUSSION

In this report, we show that FasL-neutralizing antibody suppressed apoptosis in WI-38 and BHK cells through inhibiting the Fas signaling pathway (Fig. 1). These results suggest that activation of the Fas/FasL system is involved in apoptosis induced by pironetin in these cell lines. Moreover, similar results were obtained by using other drugs, irrespective of their mode of action, such as vinblastine, paclitaxel, camptothecin, adriamycin and etoposide. However, we can not exclude the possibility that the Fas/FasL system-independent pathway(s) is also involved in antitumor drug-induced apoptosis in these cell lines, because FasL-neutralizing antibody partially inhibited antitumor drug-induced apoptosis. Activation of the Fas/FasL system is not involved in apoptosis induced by antitumor drugs in human leukemia HL-60 cells (Fig. 2). In fact, it was reported that apoptosis induced by antitumor drugs in some leukemia cells did not require the Fas/FasL system.1, 2, 13, 14) Therefore, it appears that the Fas/FasL system is one of the mechanisms that determine drug sensitivity in antitumor drug-induced apoptosis.

Our results demonstrate that Fas/FasL system-mediated apoptosis induced by antitumor drugs occurred in wild-type p53-positive BHK and WI-38 cells, but not in HL-60 cells, which are p53−/−.35) Recently, it was demonstrated that induction of Fas gene transcription by p53 was mediated through a p53-responsive element located within the first intron of the gene and p53 activated the transcription of Fas gene in response to antitumor drugs.36) Therefore, it is suggested that the antitumor drugs we tested might activate p53, resulting in increased Fas levels in BHK and WI-38 cells. Indeed, it was reported that p53-dependent
apoptosis was reduced in lpr or gld mouse embryonic fibroblasts, which contain mutations in Fas and FasL, respectively. In addition, an antagonistic anti-FasL antibody partially reduced the apoptosis in human lung cancer H1299 cells transfected with wild-type p53 gene.

Calcineurin has been implicated in both transcription-dependent and -independent apoptosis, with the former attributed to calcineurin-mediated dephosphorylation of NF-AT and subsequent trans-activation of apoptosis genes. Recently, it was reported that calcineurin induced transcription-independent apoptosis by dephosphorylating Bad, thus allowing the Bad to dimerize with Bcl-xL or other Bcl-2 family proteins located in mitochondrial membranes. On the other hand, our results indicate that FasL expression was upregulated via a calcineurin-dependent pathway, suggesting that calcineurin is also a critical mediator of transcription-dependent apoptosis during antitumor drug-induced apoptosis. Indeed, apoptosis induced with antitumor drugs was inhibited by cycloheximide in BHK cells (data not shown). Moreover, this is the first report to show that calcineurin is translocated to the nucleus by treatment with antitumor drugs (Fig. 3B). These findings are consistent with other findings: i) a correlation between calcineurin phosphatase activity and induction of apoptosis was observed in T cell hybridomas; ii) several types of apoptosis were inhibited by treatment with cyclosporin A. Taken together, the accumulated data suggest that calcineurin activity is a critical factor for apoptosis, regulating FasL expression. Recently, it has been reported that cyclosporin A actually promotes tumor progression directly, independently of its effects on the host immune system. Our data indicate that cyclosporin A may contribute not only to immune system-dependent cancer progression, but also to resistance to activation of the Fas/FasL system-mediated apoptosis, like Bcl-2 protein.

Our findings also demonstrate that antitumor drug-induced FasL upregulation is inhibited by Bcl-2 overexpression (Fig. 5C). Moreover, mutant Bcl-2 (a serine residue at position 24, the only serine residue in the BH4 domain, to alanine; Bcl-2S24A) was not able to bind with calcineurin after pironetin treatment and was not able to inhibit pironetin-induced FasL expression and apoptosis (Simizu et al., unpublished observation). Therefore, it is suggested that inhibition of antitumor drug-induced apoptosis by Bcl-2 is due to the suppression of FasL expression. These findings are consistent with previous reports that Bcl-2 inhibits NF-AT signaling by targeting calcineurin to mitochondria, and that NF-AT regulates FasL expression. Very recently, Srivastava et al. reported that inhibition of apoptosis by Bcl-2 is due to the blocking of calcineurin-NF-AT-dependent FasL expression in human cancer cells. This strongly supports our independent conclusion. This finding may also help to solve the mechanism of Bcl-2 function.

The agonistic Fas antibody (CH-11) alone did not induce apoptosis and CH-11 reversed cyclosporin A-inhibited apoptosis induced by pironetin (data not shown), suggesting that p53-dependent Fas expression is the important factor in pironetin-induced apoptosis. Moreover, cyclosporin A inhibited pironetin-induced apoptosis (data not shown), indicating that calcineurin-dependent FasL expression is also a critical event. Thus, FasL induction by calcineurin and Fas expression by p53 are indispensible for antitumor drug-induced apoptosis. This signaling scheme (Fig. 7) should provide a basis for the development of new cancer chemotherapy.

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