Cephalostatin 1 Inactivates Bcl-2 by Hyperphosphorylation Independent of M-Phase Arrest and DNA Damage

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ABSTRACT

Cephalostatin 1 is a marine product that induces a novel cytochrome c-independent apoptotic pathway in Jurkat leukemia T cells (Cancer Res 63:8869–8876, 2003). Here, we show that overexpression of the antiapoptotic protein Bcl-2 protects cells only partially against cephalostatin 1-induced apoptosis. The mechanism of Bcl-2 inactivation by cephalostatin 1 is based on hyperphosphorylation of Bcl-2 on Thr69 and Ser87 because Jurkat cells overexpressing a Bcl-2 protein with mutations on both phosphorylation sites were completely protected against cephalostatin 1. In search of the kinase responsible for Bcl-2 phosphorylation, c-Jun NH2-terminal kinase (JNK) was found to be activated by cephalostatin 1. Reduction of Bcl-2 phosphorylation by the specific JNK inhibitor (anthra(1,3-cd)pyrazol-6(2H)-one) SP600125 suggested a crucial role for JNK in this process. JNK activation was not a consequence of DNA damage, a known stimulus of JNK, because cephalostatin 1 did not induce DNA lesions as shown by the comet assay. Arrest in M-phase is also demonstrated to be associated with JNK activation. However, cephalostatin 1 does not evoke an arrest in M-phase as shown by flow cytometry. Together, cephalostatin 1 is shown to induce JNK activation with subsequent Bcl-2 phosphorylation and inactivation. Reported triggers, such as the induction of an M-phase arrest or DNA damage are not involved in this process, suggesting a novel mechanism for cephalostatin 1-mediated Bcl-2 hyperphosphorylation.

The cephalostatins, isolated from the Indian ocean hemi-chordate Cephalodiscus gilchristi Ridewood for the first time in 1988 (Pettit et al., 1988), belong to the most cytotoxic marine natural products ever tested by the National Cancer Institute (Bethesda, MD). Cephalostatin 1 proved to be the most active of the 19 cephalostatins (~1 nM mean GI50 in the 2-day NCI-60 screen) (LaCour et al., 1999). Besides the in vitro tests, it was shown to inhibit murine leukemia and brain tumor xenografts in vivo (Pettit, 1994).

We showed recently that cephalostatin 1 induces a unique apoptotic signaling pathway that activates caspase-9 independently of an apoptosome because neither the release of cytochrome c from mitochondria nor an interaction of apoptotic protease-activating factor 1 with caspase-9 was detected. Remarkably, the protein Smac/DIABLO was selectively released from mitochondria in response to cephalostatin 1 (Dirsch et al., 2003).

We show here that overexpression of Bcl-2 in Jurkat cells confers only partial protection against cephalostatin 1-induced apoptosis, whereas overexpression of Bcl-xL was found to abolish cephalostatin 1-mediated cell death (Dirsch et al., 2003).

Bcl-2 and Bcl-xL are members of an evolutionarily conserved family of proteins that control apoptosis. Both proteins belong to the antiapoptotic subgroup of the family, whereas the Bcl-2 homology domain 3-only polypeptides and the Bax/Bak-like proteins are involved in the initiation of apoptosis. Bcl-2 is localized on the mitochondrial outer membrane but was also found at membranes of the ER and nucleus (Cory and Adams, 2002).

Because of its important role in regulation of apoptotic processes, the Bcl-2 family has been associated with cancer development and with resistance to anticancer treatment. Occurrence of Bcl-2 overexpression, first found in human follicular B-cell lymphomas and generated by the chromosomal translocation t(14;18) (Reed et al., 1988), has been discovered in most chronic lymphocytic lymphomas (Hanada...
et al., 1993), indolent lymphomas, and several other tumors (Reed, 1999). High expression of Bcl-2 alone is only weakly oncogenic (Strasser et al., 1993) but provides an extended life span of the cell for secondary mutations to develop (Kaufmann and Vaux, 2003). As a result, the two events in concert are able to provoke formation of neoplasms. Studies using Bcl-2-overexpressing leukemic cell lines showed that Bcl-2 conveys resistance to a large number of anticancer drugs, including DNA damaging agents (Pratesi et al., 2001). The clinical manifestation of raised Bcl-2 levels in acute myelogenous leukemia, acute lymphocytic leukemia, and also prostatic cancer is correlated with a poor prognosis (Campos et al., 1993). Hence, finding substances that abrogate the antiapoptotic function of Bcl-2 and thereby enhance sensitivity of the cell to drug treatment is of great therapeutic importance. This prompted us to investigate whether cephalostatin 1 is able to inactivate Bcl-2 and to characterize the underlying mechanism.

Materials and Methods

Materials. Cephalostatin 1 was isolated from C. gilchristi as described previously (Pettit et al., 1988). Etoposide and the JNK inhibitor SP600125 were purchased from Calbiochem (Bad Soden, Germany). Paclitaxel and propidium iodide were obtained from Sigma Chemie (Deisenhofen, Germany). All used anticancer drugs were dissolved in dimethyl sulfoxide.

Cells. Jurkat human T cells (clone J16) and Jurkat cells stably transfected with vector control, Bcl-2, or a mutant form of Bcl-2 (Yamamoto et al., 1999) (kindly provided by Dr. S. Korsmeyer, Harvard Medical School, Boston, MA, and Drs. P. H. Krammer and H. Walczak, German Cancer Research Center, Heidelberg, Germany) were maintained in RPMI 1640 medium containing 2 mM l-glutamine (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (PAA Laboratories, Co ¨lbe, Germany). Medium of transfected cells was supplemented with 1 mg/ml G418 (Genetecin; Invitrogen, Egggenstein, Germany) every fifth passage.

Quantification of Apoptosis and Cell Cycle Analysis. Quantification of apoptosis was carried out according to Nicoletti et al. (1991). In brief, cells were incubated for 24 h in a hypotonic buffer (0.1% sodium citrate, 0.1% Triton-X-100, and 50 μg/ml propidium iodide) and analyzed by flow cytometry on a FACSCalibur (BD Bio-

Western Blot Analysis. Western blotting was performed as described previously (Antlsperger et al., 2003). In brief, cells were lysed by adding lysis buffer (2 mM EDTA, 137 mM NaCl, 10% glycerol, 2 mM tetrasodium pyrophosphate, 20 mM Tris, 1% Triton-X-100, 20 mM sodium glycerophosphate hydrate, 10 mM NaF, 2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride, supplemented with the protease inhibitor complete; Roche Diagnostics, Mannheim, Germany). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (12%) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Eschborn, Germany). Equal protein loading was controlled by Coomassie Blue staining of gels. Membranes were blocked by 5% fat free milk powder in Tris-buffered saline with Tween and incubated with specific antibodies against Bcl-2 (Upstate Biotechnology, Lake Placid, NY), phospho-Bcl-2 (Cell Signaling Technology Inc., Franklin, Germany), and phospho-JNK (Cell Signaling Technology Inc.). Detection of the proteins of interest was accomplished with secondary antibodies conjugated to horseradish peroxidase and ECL Plus substrate solution (Amerham Biosciences Inc., Freiburg, Germany).

Comet Assay. A single cell gel electrophoresis assay was performed using the Trevigen Comet assay kit (Trevigen, Gaithersburg, MD) under alkaline conditions according to the manufacturer’s conditions. After treatment, cells were suspended in low point melting agar and placed onto a microscope slide. After solidification of agarose, slides were submerged in lysis solution (2.5 M sodium chloride, 100 mM EDTA, pH 10, 10 mM Tris base, 1% sodium lauryl sarcosinate, and 1% Triton-X-100) for 40 min and then transferred to alkaline solution for 40 min. Electrophoresis was performed at 300 mA for 30 min. Finally, slides were neutralized and DNA was stained with SYBR Green. Pictures were taken at an Axiosvert 25 microscope (Carl Zeiss, Munich, Germany).

Statistical Analysis. All experiments were performed three times. Results are expressed as mean ± S.E. Statistical analysis was performed by ANOVA followed by a Bonferroni multiple comparison test or by an unpaired two-tailed Student’s t test. p values <0.05 were considered significant.

Results

Overexpression of Bcl-2 Protects Jurkat Cells Only Partially against Cephalostatin 1-Induced Apoptosis. Both Bcl-2 and Bcl-xL inhibit apoptosis at the mitochondrial level. In a previous study, we showed that Bcl-xL overexpression confers full protection against cephalostatin 1-induced apoptosis in Jurkat cells (Dirsch et al., 2003). Interestingly, Bcl-2-overexpressing cells were only partially protected (Fig. 1B) against cephalostatin 1 (Fig. 1A, chemical structure).
Inactivation of Bcl-2 by Cephalostatin 1 Is Provoked by Bcl-2 Hyperphosphorylation. The incomplete protection in Bcl-2-overexpressing cells suggests that cephalostatin 1 is able to inactivate Bcl-2. Because hyperphosphorylation at Thr⁶⁹ and Ser⁸⁷ has been reported to be one mechanism of inactivation (Ruvolo et al., 2001), we clarified whether cephalostatin 1 uses this mechanism to disable Bcl-2. As depicted in Fig. 2A, Bcl-2 is indeed hyperphosphorylated 8 h after cephalostatin 1 treatment visible by an additional upper band at 28 kDa in the Western blot. Etoposide (E; 25 μg/ml, 16 h) used as control showed no effect. Figure 2B demonstrates that the overall level of Bcl-2 remained unchanged upon cephalostatin 1 treatment.

To prove that the observed Bcl-2 hyperphosphorylation is indeed the mechanism of Bcl-2 inactivation, we used Jurkat cells overexpressing a mutant form of Bcl-2. In this mutant Bcl-2 protein, all three phosphorylation sites (Thr⁶⁹, Ser⁷⁰, and Ser⁸⁷) are substituted by alanine to prevent phosphorylation and thus inactivation of Bcl-2 (Yamamoto et al., 1999). In comparison with cells carrying the vector alone (Jurkat/neo) and cells overexpressing the wild-type Bcl-2 protein (Jurkat/bcl-2), the mutant cell line (Jurkat/mbl-2) was completely protected against cephalostatin 1 (Fig. 2C). To prove that all three cell lines respond as reported previously (Yamamoto et al., 1999), they were exposed to 1 μM paclitaxel for 24 h. Figure 2D shows that the Jurkat/mbl-2 cells were more protected against paclitaxel-induced apoptosis than cells overexpressing the wild-type Bcl-2 protein, whereas Jurkat/neo cells succumbed freely to apoptosis. In
summary, hyperphosphorylation of Bcl-2 by cephalostatin 1 diminishes the protective function of Bcl-2.

**Jun-Terminal Kinase Is Involved in Cephalostatin 1-Triggered Bcl-2 Phosphorylation.** Next, we examined which kinase may be responsible for the cephalostatin 1-induced Bcl-2 hyperphosphorylation. As depicted in Fig. 3A, both JNK1 and JNK2 are phosphorylated already 2 h after cephalostatin 1 treatment, and phosphorylation increases up to 8 h after stimulation. To link JNK activation to Bcl-2 phosphorylation, we pretreated Jurkat cells with the specific JNK inhibitor SP600125 (Fig. 3B, chemical structure) before stimulation with cephalostatin 1. Figure 3C reveals that the inhibitor reduces Bcl-2 hyperphosphorylation after treatment with cephalostatin 1 or paclitaxel. Etoposide showed no effect.

It is noteworthy that paclitaxel seems to lead to a stronger hyperphosphorylation than cephalostatin 1. To clarify whether this difference in Bel-2 hyperphosphorylation has an impact on Bcl-2 inactivation, we compared the levels of apoptosis induced by these two drugs in Jurkat/neo, Jurkat/bcl-2, and Jurkat/mbcl-2 cells as shown in Fig. 3D. As expected, Bcl-2-overexpressing cells are more sensitive to paclitaxel than to cephalostatin 1, suggesting that the stronger hyperphosphorylation induced by paclitaxel leads indeed to a stronger inactivation of Bcl-2. However, Jurkat/mbcl-2 cells showed also a higher sensitivity toward paclitaxel compared with cephalostatin 1, suggesting that paclitaxel may have—next to the mechanism of Bcl-2 hyperphosphorylation—a further, unknown mechanism to inactivate Bcl-2.

**Bcl-2 Phosphorylation Is Not Mediated by G2/M-Phase-Dependent Events.** The majority of compounds reported to induce Bcl-2 hyperphosphorylation activate JNK and induce a cell cycle arrest in M phase as, for example, microtubule-damaging agents (Ruvolo et al., 2001). A causal link between M-phase arrest, JNK activation, and subsequent Bcl-2 hyperphosphorylation, however, has not been shown in all cases. To investigate whether cephalostatin 1-induced JNK activation and successive Bcl-2 phosphorylation depend on an M-phase arrest, we analyzed the cell cycle distribution of untreated Jurkat cells and cells incubated with 1 μM cephalostatin 1 or 1 μM paclitaxel for 8 h, a time point at which JNK activation was evident. As demonstrated by Fig. 4, A and B, paclitaxel induces a potent M-phase block, whereas cephalostatin 1 did not interfere with cell cycle progression. Interestingly, cells in G1 and S phase seem to be more susceptible to cephalostatin 1 because the overall percentage of cells in G1 and S phase decreased after cephalostatin 1 stimulation (Fig. 4B). The appearance of a sub-G1 peak (Fig. 4A, middle) reveals that cells formerly present in G1 and S underwent apoptosis. The percentage of cells in the G2/M phase was not altered after cephalostatin 1 treatment compared with control. These results indicate that both JNK activation and Bcl-2 phosphorylation induced by cephalostatin 1 occur independently of an M-phase blockade.

**Cephalostatin 1 Does Not Induce Bcl-2 Phosphorylation via DNA Damage.** JNK may be activated by cellular stress. Many genotoxic agents mediate JNK activation (Salleem et al., 1995), and some have been described to phosphorylate Bcl-2 (Pratesi et al., 2000). To elucidate whether cephalostatin 1 leads to DNA lesions, we performed a comet assay using cells treated with 1 μM cephalostatin 1 or 10 μM etoposide for 4 h. Both drugs induced comparable levels of apoptosis at these concentrations (data not shown). Figure 5 provides clear evidence that etoposide induces DNA damage visible as the typical comet tail, whereas cephalostatin 1 does not. This experiment suggests that DNA damage is not the
type of cell stress leading to cephalostatin 1-induced JNK activation.

**Discussion**

Bcl-2 overexpression has been shown to convey resistance to various chemotherapeutic agents (Domen and Weissman, 2003). Here, we show that cephalostatin 1 is able to inactivate Bcl-2 in Jurkat T cells via a mechanism that includes hyperphosphorylation. Several mechanisms are reported to inactivate Bcl-2: activated caspases have been shown to cleave Bcl-2, generating a 23-kDa proapoptotic product (Faddeel et al., 1999; Del Bello et al., 2001). Furthermore, down-regulation of Bcl-2 mRNA or Bcl-2 protein has been observed after treatment with several anticancer drugs (Sawada et al., 2000; Bandopadhayay et al., 2003). In cephalostatin 1-induced apoptosis, total Bcl-2 levels are not altered, suggesting that cleavage of the protein or alterations of mRNA levels do not apply here. Beyond that, Bcl-2 phosphorylation has been observed as mechanism for altering the activity of the protein. Under physiological conditions, Bcl-2 is phosphorylated on Ser70 during M phase (Ito et al., 1997). The kinases suggested to be responsible for this phosphorylation are PKC (May et al., 1994; Ito et al., 1997) and extracellular signal-regulated kinase (Deng et al., 2000). Phosphorylation of Ser70 seems to be important for the anti-apoptotic function of the protein (Ito et al., 1997). In contrast, hyperphosphorylation on Thr69 and Ser87 was proposed to inactivate Bcl-2 (Yamamoto et al., 1999) and abrogate its protective role by impairing its interaction with the proapoptotic protein Bax (Scatena et al., 1998).

Hyperphosphorylation of Bcl-2 was shown to be induced by drugs, such as antimitotic agents (Ruvolo et al., 2001) and some DNA damaging chemotherapeutics (Pratesi et al., 2000). However, cephalostatin 1-induced Bcl-2 hyperphosphorylation occurs independently of a mitotic arrest or DNA damage and is sufficient for the observed Bcl-2 inactivation. Compared with paclitaxel, the degree of hyperphosphorylation induced by cephalostatin 1 seemed to be lower, corresponding to the lower sensitivity of Bcl-2-overexpressing cells toward cephalostatin 1. Interestingly, Jurkat T cells carrying mutated phosphorylation sites (Jurkat/mbl-cbl-2) displayed a higher apoptosis rate after paclitaxel treatment compared with cephalostatin 1. This observation may be explained by an additional inactivating mechanism of paclitaxel, such as cleavage of Bcl-2 (Blagosklonny et al., 1999).

Several previous studies have focused on the signaling pathway leading to Bcl-2 hyperphosphorylation. Among the key enzymes responsible for Bcl-2 inactivation, various kinases have been described [Raf-1, Blagosklonny et al. (1997); protein kinase A, Srivastava et al. (1999); apoptosis signal-regulating kinase-1, Tang et al. (1994); and JNK, Yamamoto et al. (1999)], depending on cell type and stimulus. Activated JNK has been implied in the hyperphosphorylation of Bcl-2 in response to numerous antimitotic agents such as paclitaxel, Vinca alkaloids, or cryptophycins (Mollinedo and Gajate, 2003). In some settings, JNK activation but no Bcl-2 phosphorylation was evident (Figueroa-Masot et al., 2001), or no causal link between JNK activation and Bcl-2 phosphorylation could be proven (Wang et al., 1999). In fact, protein kinase A was favored as the kinase exclusively responsible for Bcl-2 phosphorylation (Srivastava et al., 1999).

In the present study, cephalostatin 1-triggered Bcl-2 hyperphosphorylation was strongly impaired by the specific JNK inhibitor SP600125, pointing again to JNK as the crucial kinase upstream of Bcl-2.

How does cephalostatin 1 induce JNK activation and subsequent Bcl-2 phosphorylation? JNK/stress-activated protein kinase activation is involved in the regulation of cell cycle progression at the transition from G_{1} to S phase (MacCorkle-Chosnek et al., 2001). JNK also fulfills functions in the M phase (Yamamoto et al., 1999). Beyond that, it is induced by diverse extracellular stimuli such as UV irradiation, proinflammatory cytokines, heat shock, and numerous cytotoxic agents (Mindern and Karin, 1997). Among these, microtubule-interfering agents inducing an arrest in G_{2}/M phase activate JNK as major proapoptotic player (Mollinedo and Gajate, 2003). JNK activation by microtubule-interfering agents was often linked to Bcl-2 hyperphosphorylation, suggesting that an arrest in M phase is a crucial factor in the signaling pathway leading to Bcl-2 inactivation and subsequent apoptosis (Yamamoto et al., 1999; Fan et al., 2000; Tseng et al., 2002). Cephalostatin 1, however, does not arrest cells in G_{2}/M phase, contradicting the view that an arrest in M-phase may be a prerequisite for JNK activation with a subsequent Bcl-2 hyperphosphorylation and inactivation.

JNK activation was also found in response to DNA damaging agents (Saleem et al., 1995; Hayakawa et al., 2003). Some of them, such as platinum compounds and doxorubicin, were shown to induce Bcl-2 phosphorylation (Pratesi et al., 2000). Up to now, the pathway leading to Bcl-2 phosphorylation in DNA damage-induced apoptosis has not been investigated, but involvement of JNK is conceivable. Cephalostatin 1, however, does not induce DNA damage, excluding this mechanism for JNK activation.

In conclusion, the activation of JNK by cephalostatin 1 which leads to hyperphosphorylation and inactivation of Bcl-2 is mediated by a mechanism yet to be identified. A promising possibility may be ER stress as activator of JNK (Urano et al., 2000). As to whether cephalostatin 1 induces ER stress and whether this ER stress accounts for JNK activation and Bcl-2 phosphorylation awaits to be shown. Regardless, cephalostatin 1 may have a potential for the treatment of drug-resistant cancers because of its ability to inactivate the antiapoptotic protein Bcl-2. Studies testing this hypothesis are in progress.

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References


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