Distinct Pathways of Apoptosis Triggered by FTY720, Etoposide, and Anti-Fas Antibody in Human T-Lymphoma Cell Line (Jurkat Cells)

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ABSTRACT

2-Amino-2-[2-(4-octylphenyl)ethyl] propane-1,3-diol hydrochloride (FTY720), a synthetic product derived from a metabolite of Isaria sinclairii, has been demonstrated to have a potent immunosuppressive activity that induces apoptotic cell death in T cells and several other cell lines. In this study, using the human T-lymphoma cell line, Jurkat cells, we investigated the apoptotic signal transduction mediated by FTY720, in particular comparing its role on the cleavage of caspases, with that mediated by etoposide or anti-Fas antibody. All of these agents cleaved caspases, inducing their active form in the affected cells. Pretreatment with a broad caspase inhibitor [benzyloxy-carbonyl-Val-Ala-Asp-(Ome) fluoromethyl ketone] markedly decreased the incidence of apoptotic cells induced by FTY720, etoposide, and anti-Fas antibody, through the abrogation of cleavage of Bid, poly(ADP-ribose) polymerase, and caspases 3, 8, and 9. The overexpression of Bcl-2 gene prevented FTY720- and etoposide-mediated apoptosis, but not Fas-mediated apoptosis. In addition, mitochondria were demonstrated to play a critical role in FTY720-triggered cell death, suggesting that this drug has a potent anticancer activity.

2-Amino-2-[2-(4-octylphenyl)ethyl] propane-1,3-diol hydrochloride (FTY720), a novel immunosuppressive agent, was synthesized by chemical modification of a natural product, ISP-1, from Isaria sinclairii (Adachi et al., 1995). The immunosuppressive mechanisms of its action are completely different from those of cyclosporin A or tacrolimus; FTY720 does not affect interleukin-2 production from mitogen-stimulated lymphocytes (Suzuki, 1999). The drug was reported to significantly accelerate lymphocyte homing to peripheral lymph nodes, mesenteric lymph nodes, and Peyer’s patches in a dose-dependent manner (Chiba et al., 1999). In addition, we have demonstrated that FTY720 induces cell death selectively in mature T lymphocytes, especially CD4-positive T cells in the peripheral blood, without any suppression of bone marrow (Enosawa et al., 1996). Lymphocyte death was ascribed to apoptosis that was not related to Fas-antigen (Suzuki et al., 1997). Using Jurkat cells, a human T-lymphoma cell line, we observed that the induction of apoptosis was mediated by the activation of caspase 3, but not caspase 1 (Matsuda et al., 1999; Wang et al., 1999). When Bcl-2 gene was overexpressed, Jurkat cells were resistant to the drug. In an androgen-independent prostate cancer cell line, we also demonstrated the involvement of caspase 3 activation by FTY720 treatment (Wang et al., 1999). Furthermore, Sonoda et al. (2001) reported that caspase 6 was activated in glioma cells treated with FTY720.

Apoptosis, an active process consisting of an evolutionarily conserved cascade, exhibits characteristic features, including cell shrinkage, condensation of chromatin, and formation of genomic DNA into specific oligonucleosomal fragments (DNA fragmentation) (Steller, 1995).

The mammalian caspase family is known to be critically involved in the apoptosis induced by many types of stimuli such as anti-Fas antibody and etoposide (Sun et al., 1999). Current evidence implies that apoptosis involves a sequentially activated caspase cascade (Muzio et al., 1996). At least 14 members of the caspase family have been identified (Van de Craen et al., 1998). All caspases are synthesized as proenzymes and activated by cleavage at specific aspartate resi-

ABBREVIATIONS: PARP, poly(ADP-ribose) polymerase; apaf-1, apoptotic protease-activating factor-1; Z-VAD-FMK, benzyloxy carbonyl-Val-Ala-Asp-(Ome) fluoromethyl ketone; Z-Asp-CH2-DOB, benzyloxy carbonyl-Asp-CH2-COC-2,6-dichlorobenzene; Ac, acetyl; YVAD, Try-Val-Ala-Asp; CHO, aldehyde; DEVD, Asp-Glu-Val-Asp; IETD, Ile-Glu-Thr-Asp; LEHD, Leu-Glu-His-Asp; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline.

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dies. They play a critical role in the induction phase of apoptosis and are responsible for the biochemical and morphological changes in the affected cells (Cohen, 1997). It was proposed that the “initiator” caspases with a long prodomain, such as caspases 8, 9, and 10, directly or indirectly activate the “effector” caspases, such as caspases 3, 6, and 7 (Cohen, 1997). The activated effector caspases then cleave intracellular substrates, such as poly(ADP-ribose) polymerase (PARP) and lamins, during the execution phase. Caspase 8 is the most apical in Fas-mediated apoptosis (Muzio et al., 1996). Triggering of the Fas receptor with its cognate ligand or agonistic antibody results in receptor trimerization and recruitment of Fas receptor-associated protein with death domains (FADD), which in turn binds to the death effector domains in the N-terminal region of caspase 8, resulting in its activation. Because caspase 8 can activate many caspases in vitro (Srinivasula et al., 1996), it is the prime candidate for initiator caspase in many forms of apoptosis. Pro-caspase 9 was also proposed as an initiator caspase in etoposide-mediated apoptosis (Sun et al., 1999). In the presence of dATP and cytochrome c, its long N-terminal domain interacts with Apaf-1, leading to the activation of caspase 9 (Zou et al., 1997). The activated caspase 9 then activates the effector caspases 3, 6, and 7 (Zou et al., 1997). Thus, there are at least two major mechanisms by which caspase cascades are activated, one involving caspase 8 and the other, caspase 9.

We compared caspase activation by FTY720, etoposide, and anti-Fas antibody in Jurkat cells, including overexpressed Bcl-2-gene, in the presence of several caspase inhibitors. We have demonstrated FTY720-induced apoptosis to be mediated by the activation of a mitochondrial caspase cascade similar to the etoposide-mediated one, but different from Fas-related apoptosis. The difference in caspase activation among the products indicates that FTY720 induction of caspase activation involves the mitochondria in a Bcl-2-dependent manner.

Materials and Methods

Reagents and Antibodies. FTY720, provided by Yoshitomi Pharmaceutical Industries (Osaka, Japan), was dissolved in sterilized water at 100 μM and stored at 4°C. Etoposide, purchased from Wako (Osaka, Japan), was dissolved in methanol at 50 mg/ml and stored at -20°C. Anti-Fas antibody was purchased from Immuno-Gene (Santa Cruz Biotechnology), a horseradish peroxidase-coupled anti-rabbit IgG or anti goat IgG (BD Pharmingen), a horseradish peroxidase-coupled anti-rabbit IgG or anti goat IgG (Santa Cruz Biotechnology), and mouse anti-caspase 8 (#9746) (1:8000; Cell Signaling Technology, Beverly, MA), rabbit anti-caspase 9 (PC336) (1:8000; Oncogene Research Products, Boston, MA), and mouse anti-Tubulin (CP06) (Oncogene Research Products) to detect the specific signals. After overnight incubation with agitation at 4°C, the nitrocellulose membrane was washed three times with PBS. A secondary antibody, a horse-radish peroxidase-coupled goat anti-mouse Ig antibody (BD PHARMINGEN), a horseradish peroxidase-coupled anti-rabbit IgG or anti goat IgG (Santa Cruz Biotechnology), was added at a dilution of 1:8000 and incubated for 2 h at 4°C. Thereafter, the membrane was washed three times (5 min) with PBS. The specific protein complexes were identified using an enhanced chemiluminescence substrate chemiluminescence reagent (Amersham Pharmacia Biotech, Ltd., Tokyo, Japan).

Determination of Protein. The protein concentration was determined with the DC protein assay kit (Bio-Rad) by using bovine serum albumin as a standard (Fujino et al., 2001).

Results

FTY720-Mediated Apoptosis in a Time-Dependent Manner. PARP, a DNA repairation enzyme, is one of the substrates of active caspase 3 and is an early marker of apoptosis (Patel et al., 1996). Cleavage of PARP was reported to cleave the 116-kDa precursor into a subunit of 85 kDa (Kaufmann et al., 1993). Treatment of Jurkat-Neo cells (without Bcl-2-gene transfection) with FTY720 caused a time-dependent increase of apoptosis. PARP cleavage became apparent 30 min after FTY720 treatment, 1 h after anti-Fas antibody treatment, and 4 h after etoposide treatment (Fig. 1, M–O). In addition, incubation of the cells with FTY720, etoposide, or anti-Fas antibody resulted in nuclear fragmentation. When DNA, from Jurkat-Neo cells incubated for 4 h with these compounds, was applied to agarose gel electrophoresis, a characteristic “ladder” pattern was generated, based on discontinuous nuclear fragments (Fig. 2). We also obtained the findings typical of apoptosis, when the treated cells were stained with Annexin V and Hoechist 33342 (data not shown).
Apoptosis Mediated by Cleavage of Caspase 3 after FTY720 Treatment. Proteolytic processing of pro-caspase 3 by FTY720 treatment was examined using specific antibodies against the active subunits. The caspase 3 in Jurkat-Neo cells was present primarily as the intact 32-kDa proform (Fig. 1, J–L). FTY720 treatment resulted in a decrease of the proform of caspase 3 and the appearance of three immunoreactive fragments of 20 (p20), 19 (p19), and 17 kDa (p17), followed by the initial cleavage at Asp-175, and then at Asp-9 and Asp-28 (Fernandes-Alnemri et al., 1996). As shown in Fig. 1J, activated caspase 3 was indicated when the p17 subunit was detected within 1 to 2 h after the addition of FTY720. Its level increased progressively in a time-dependent manner, suggesting that the increase of active forms was related to the increase of apoptotic cells. Therefore, the activation of caspase 3 may play an important role in apoptosis induced by FTY720. Similar results were obtained by treating the cells with etoposide and anti-Fas antibody (Fig. 1, K and L).

Cleavage of Apoptotic Signaling Proteins after FTY720 Treatment. Caspase 3 is processed by caspase 8 and 9 (Slee et al., 1999). The activation of caspase 8 and the subsequent proapoptotic cleavage of Bid are important events in the apoptotic signal transduction through death receptors (Luo et al., 1998). Several investigators have reported that anticancer drugs can also induce caspase 8 activation independently, via the death receptors (Slee et al., 2000). In this scenario, activation of caspase 8 occurs downstream of cytochrome c release and functions as an amplifying effector mechanism of the mitochondrial caspase cascade. In the untreated cells, caspase 8 is present primarily as two isoforms of 55 kDa (Fig. 1, A–C), possibly corresponding to caspase 8a and 8b (Scaffidi et al., 1997). Exposure of cells to the apoptotic agents initially resulted in cleavage of caspase 8 into two fragments of 43 and 41 kDa, corresponding to cleavage between the large and small subunits of caspases 8a and 8b. This was followed by the appearance of p18 subunit by the removal of death effector domains, 43- and 41-kDa fragments (Fig. 1, A–C) (Scaffidi et al., 1997). As shown in Fig. 1A, FTY720 activated caspase 3 in Jurkat-Neo cells, which was observed by the appearance of the p18 subunit within 30 to 60 min after the addition of FTY720. Etoposide and anti-Fas antibody also activated caspase 8; the appearance of the p18 subunit was first detected after 4 h of etoposide and after 30 min of anti-Fas antibody. A shorter exposure of the film showed that the proform of caspase 8 comprised two bands of 55 and 53 kDa (data not shown).

The cleavage of Bid is important for releasing mitochondrial cytochrome c in receptor- and chemical-mediated apoptosis (Sun et al., 1999). Bid was present as a protein of 26 kDa in the control cells and was initially cleaved to two major fragments of 15 and 14 kDa (Fig. 1, D–F). Like other apoptotic stimuli, FTY720 was able to induce the cleavage of Bid. As shown in Fig. 1D, the cleavage of Bid by FTY720 was indicated by the appearance of a p14 subunit within 2 to 4 h of the addition of the drug. Etoposide and anti-Fas antibody treatment also cleaved the Bid; the p14 subunit was first
Preventing FTY720-Mediated Apoptosis by Bcl-2. Cytoprotective Bcl-2 family members Bcl-2 and Bcl-xl have been reported to protect cells against diverse apoptotic stimuli (Yang et al., 1997). Therefore, we studied whether the apoptosis induced by FTY720 was controlled by these proteins. Jurkat cells stably transfected with Bcl-2 (Jurkat-Bcl-2) were found to be completely resistant to nuclear fragmentation and cleavage of PARP induced by FTY720 and etoposide, whereas these cells were not resistant to anti-Fas antibody (Figs. 4E and 5).

Inhibition of Caspase 8 and Bid Cleavages by Bcl-2 Overexpression. In accordance with the inhibition of apoptosis by Bcl-2 overexpression (Figs. 4F and 5), the cleavage of caspases 3, 8, and 9, and Bid in the Jurkat-Bcl-2 cells was completely prevented when treated with FTY720 and etoposide, but not with anti-Fas antibody (Fig. 4, A–E).

Discussion

FTY720 was originally developed for preventing allograft rejection (Adachi et al., 1995; Chiba et al., 1999; Suzuki, 1999). It prolongs graft survival in recipient rats with liver allografts and in canine kidney recipients, partly because of lymphocyte apoptosis, especially in CD4-positive cells (Enosawa et al., 1996). Furthermore, FTY720 is known to induce apoptosis in several cell lines and primary lymphocytes in vivo and in vitro (Shinomiya et al., 1997; Matsuda et al., 1998, 1999; Wang et al., 1999; Nagahara et al., 2000; Sonoda et al., 2001). Apoptosis, a programmed cell death, or the cellular suicide program, is a fundamental biological process that plays requisite roles in the development, differentiation, and maintenance of cells. Inappropriate or dysregulated apoptosis, or failure to undergo programmed cell death, has been implicated in a number of diseases and pathological conditions (Thompson, 1995).

Understanding of biochemical events in apoptosis was significantly advanced by the identification of a family of aspartate-specific cysteine proteases, named caspases, which are involved in the initiation and amplification of the cell death machinery (Kidd, 1998). Each caspase is synthesized as an inactive zymogen (30 to 50 kDa) and is converted by proteolytic cleavage to yield an active enzyme composed of 20- and 10-kDa subunits. In the caspase family, the initiator
Caspases (e.g., caspases 8 and 9) activate the downstream executioner caspases (e.g., caspases 3, 6, and 7) that are responsible for cleaving a limited set of proteins, resulting in the disassembly of the cell (Kidd, 1998). Accumulating evidence also suggests that mitochondria play an essential role in the apoptotic program, and release of cytochrome c from mitochondria is now emerging as an important step in the apoptotic pathway (Kroemer et al., 1997). Diverse apoptotic stimuli, including ultraviolet B, etoposide, staurosporine, ionizing radiation, cisplatin, Ara-c, doxorubicin, betulinic acid, photodynamic therapy, and cytokines, induce cytochrome c release, which can be prevented by overexpression of Bcl-2 and Bcl-xl in cells (Kroemer et al., 1997). Recently, Bid, a proapoptotic member of the Bcl-2 family, has been shown to be activated by caspase 8 and then translocated from the cytosol to mitochondria, where its truncated form (tBid) mediates the release of cytochrome c (Gross et al., 1999). Released cytochrome c, in turn, binds to Apaf-1, a mammalian homolog of the death-promoting protein CED-4 in Caenorhabditis elegans (Zou et al., 1997), resulting in recruitment and activation of caspase 9 (Li et al., 1997). The activated caspase 9 will directly cleave procaspases 3 and 7 (Srinivasula et al., 1998).

In the present study, we investigated the process of caspase activation by FTY720 compared with the anticancer drug etoposide, and with anti-Fas antibody. Etoposide, a semisynthetic epipodophyllotoxin, has become one of the most widely used anticancer drugs since its introduction in 1971 (Slevin, 1991). A variety of leukemias, including acute lymphocytic leukemia, are treated with this drug. Fas is a widely expressed cell-surface receptor molecule belonging to the tumor necrosis factor receptor family (Nagata, 1997), which transduces intracellular apoptotic signals by binding with an agonistic anti-Fas antibody or its natural ligand (Fas ligand) (Suda et al., 1993). Our studies demonstrated that 1) cleavage of caspase 8 and Bid occurred time dependently in the FTY720-treated Jurkat cells and similarly in the etoposide- and Fas-treated cells; 2) Ac-IETD-CHO and Ac-LEHD-CHO completely prevented the cleavage of caspase 8 and partially that of caspase 3, but did not prevent apoptosis; and 3) FTY720 induced apoptosis via a Bcl-2-dependent pathway and so did etoposide, whereas anti-Fas antibody induced apoptotic cell death in a Bcl-2-independent manner.

We demonstrated that treatment of Jurkat-Neo cells with FTY720 resulted in activation of executioner caspases, leading to cleavage of PARP and nuclear fragmentation (Figs. 1,
FTY720 triggered apoptosis via proteolytic processing of caspases that was blocked by the various inhibitors (Fig. 1). In addition, the overexpression of Bcl-2 conferred protection against FTY720-mediated apoptosis by blocking the cleavage of Bid, caspases, and PARP. As shown in Fig. 6, the apoptosis originated from mitochondria, whereas the molecular mechanisms by which FTY720 interacted with mitochondria are still unknown. The Fas-mediated pathway was observed to be clearly different from FTY720-mediated apoptosis. However, in the kinetics study, the cleavage patterns of caspases, Bid, and PARP were quite similar in the apoptosis induced by these two different products. When incubated with caspase inhibitors, anti-Fas antibody exhibited a different pattern of cleavage of caspases and Bid from FTY720. Cleavage of caspase 8 and Bid was prevented with Ac-DEVD-CHO and Ac-Asp-CH2-DCB in the cells treated with FTY720, but not anti-Fas antibody. In Fas-mediated apoptosis, Ac-DEVD-CHO strongly inhibited caspase 3, but not caspase 8. Thus, in Fas-mediated apoptosis, caspase 8 is an apical caspase that cleaves the downstream Bid. Therefore, inhibition of caspase 8 by Ac-DEVD-CHO may result from mitochondrial loop inhibition. Z-Asp-CH2-DCB did not inhibit caspase 8, whereas it did inhibit caspases 3 and 9, preventing apoptosis by anti-Fas antibody. In contrast, Z-Asp-CH2-DCB prevented caspase 8 in the FTY720-treated cells. FTY720-mediated cleavage of Bid, PARP, and caspases 3, 8, and 9 was abrogated completely in the Jurkat-Bcl-2 cells, whereas the cleavage of these proteins was not abrogated in Fas-mediated apoptosis. In addition, etoposide mediated the apoptosis with a cleavage pattern of caspases very similar to that of FTY720.

Ac-IETD-CHO and Ac-LEHD-CHO completely prevented the cleavage of caspase 8 and partially prevented that of caspases 3 and 9 (Ac-LEHD-CHO only), although these inhibitors did not prevent the cleavage of any other caspase in anti-Fas antibody-, etoposide-, and FTY720-mediated apoptosis. It was suggested that prevention of caspase 8 activity was inefficient for preventing apoptosis. Unexpectedly, 100 μM Ac-LEHD-CHO was unable to prevent the cleavage of caspase 9. Caspase 8 may be cleaved via a feedback mechanism in etoposide and FTY720 treatments. Prevention of caspase 8 is an apical event in the anti-Fas antibody-treated cells. Even when caspase 8 was prevented by Ac-IETD-CHO, apoptosis occurred in anti-Fas antibody-treated cells, which suggests that another caspase(s) (i.e., caspase 10) was involved with the Fas-mediated apoptosis.

In summary, our findings indicate that FTY720 activated caspases in a Bcl-2-dependent manner as demonstrated in Fig. 6. The apoptotic signal pathway mediated by FTY720 is similar to the etoposide-mediated pathway, but not to the Fas-related one. Furthermore, the inhibition of caspase 8 alone could not prevent apoptosis. FTY720 induced apoptosis more effectively than etoposide and anti-Fas antibody. Therefore, FTY720 may be a useful drug for treating cancer cells that have a defect in the upstream apoptotic enzymes.
Fig. 6. Scheme of the intracellular signal pathway in the FTY720, etopoïde, and Fas-mediated apoptosis. Fas stimulation activates caspase 8 directly and triggers the mitochondrial signal pathway via Bid cleavage. Caspase 3 is activated either directly by the activated caspase 8 or through the mitochondrial pathway. In turn, Bid cleavage is occurred by the activated caspase 8 or in a positive feedback via caspase 3. Even by the activated caspase 8 directly and triggers the mitochondrial signal pathway via Bid.

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References

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