Immunosuppressant FTY720 Induces Apoptosis by Direct Induction of Permeability Transition and Release of Cytochrome c from Mitochondria

Yukitoshi Nagahara, Masahiko Ikekita and Takahisa Shinomiya

*J Immunol* 2000; 165:3250-3259; doi: 10.4049/jimmunol.165.6.3250

http://www.jimmunol.org/content/165/6/3250

---

**Why The JI?**

- **Rapid Reviews! 30 days**\(^*\) from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

\(^*\)average

---

**References**

This article cites 61 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/165/6/3250.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2000 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Immunosuppressant FTY720 Induces Apoptosis by Direct Induction of Permeability Transition and Release of Cytochrome c from Mitochondria

Yukitoshi Nagahara,*† Masahiko Ikekita, † and Takahisa Shinomiya1*

FTY720 has immunosuppressive activity in experimental organ transplantation and shows a prompt and protracted decrease of blood T lymphocytes upon oral administration. The blood lymphocyte decrease in vivo was mainly a result of FTY720-induced apoptosis. However, this apoptotic mechanism is not well understood. We examined the mechanism of FTY720-induced apoptosis in lymphoma. Western blotting and fluorescent caspase-specific substrate revealed that caspase-3 is involved in FTY720-induced apoptosis, whereas caspase-1 is not. Apoptotic cell death was inhibited by the pan-caspase inhibitor, Z-VAD-FMK, suggesting that caspase activation is essential for FTY720-induced apoptosis. FTY720 reduced mitochondrial transmembrane potential and released cytochrome c from the mitochondria of intact cells as well as in a cell-free system even in the presence of Z-VAD-FMK. As these mitochondrial reactions occurred before caspase activation, we concluded that FTY720 directly influences mitochondrial functions. The inhibition of mitochondrial permeability transition by Bcl-2 overexpression or by chemical inhibitors prevented all apoptotic events occurring in intact cells and in a cell-free system. Moreover, using a cell-free system, FTY720 did not directly affect isolated nuclei or cytosol. These results indicate that FTY720 directly affects mitochondria and triggers permeability transition to induce further apoptotic events. The Journal of Immunology, 2000, 165: 3250–3259.

Caspases play important roles in the process of apoptosis (1–3). Caspase-3, -6, and -7 are so-called “effector” caspases, which cleave intracellular substrates leading to further cell death (4, 5). These are activated by “initiator” caspases. The initiator, caspase-8, is activated by the Fas (CD95/APO-1) system. Upon Fas ligand/receptor interaction, caspase-8 is cleaved and processed to effectors, which allow apoptosis to proceed (6). Another initiator is caspase-9. Mitochondria activated by apoptotic signals undergo a permeability transition (PT),2 including the reduction of mitochondrial transmembrane potential (ΔΨm), and release apoptotic inducing factor (AIF) and/or cytochrome c from the intermembrane space into the cytosol (7–14). Recent studies have revealed that AIF release is ΔΨm dependent, and that cytochrome c release is ΔΨm independent (8–10, 15, 16). Released AIF directly affects nuclei and triggers chromatin condensation as well as large-scale (~50 kb) DNA fragmentation without the help of caspases and endonucleases (9). The other releasing substrate, cytochrome c, binds to Apaf-1, and this complex in turn activates caspase-9. Activated caspase-9 processes effector caspases and induces cell death (13, 14, 17, 18). The anti-apoptotic Bcl-2 family proteins, Bcl-2 and Bcl-xL, inhibit this phenomenon by stabilizing the membrane and preventing PT (19–24). However, the basis for these actions remains to be clarified. To add to the confusion, activated caspases can also induce PT, so that caspases can act either upstream or downstream of mitochondria. Activated caspases and mitochondria can engage in a circular self-amplification loop (25).

Some types of chemotherapeutic agents are known to trigger apoptosis (7, 19, 26, 27). Thus, to apply such agents in therapeutic strategies requires an understanding of the site and mechanism of apoptosis. The novel immunosuppressant, FTY720, 2-amino-2-(2-(4- octylphenyl) ethyl)-1,3-propanediol hydrochloride, was screened from synthesized analogs of ISP-1, which is an immunosuppressive metabolite of Isaria sinclairii (28–31). The oral administration of FTY720 prolongs allograft survival in experimental organ transplantation without producing any noticeable side effects (31–35). The action mechanism of orally administered FTY720 to suppress graft rejection is regarded as a significant decrease in the number of blood lymphocytes, especially T cells (36). Chiba et al. have demonstrated that this decrease in blood lymphocytes is the result of FTY720-accelerated lymphocyte homing to lymph nodes and Peyer’s patches (37). However, administration of FTY720 to aly/aly mice, which lack lymph nodes and Peyer’s patches, suppresses immune functions in the same manner as in normal mice (38). This result indicates that another mechanism must also be involved in the immunosuppressive effect of FTY720. Based on these observations, we demonstrated that the decrease in blood lymphocytes caused by oral administration of FTY720, with doses commonly used in organ transplantation, is mainly a result of FTY720-induced apoptosis (39). It is important to estimate the mechanism of FTY720-induced apoptosis due to the potential use of this drug in clinical organ transplantation. In vitro studies have revealed a number of apoptotic events during FTY720-induced apoptosis. FTY720-induced apoptosis is blocked by Bcl-2 overexpression (40), but is not associated with the Fas pathway (41). However, little is known about the site of action of this drug.

*Division of Research Promotion, National Children’s Medical Research Center, Tokyo, Japan; and †Department of Applied Biological Science, Faculty of Science and Technology, University of Tokyo, Noda, Chiba, Japan

Received for publication December 2, 1999. Accepted for publication July 5, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Address correspondence and reprint requests to Dr. Takahisa Shinomiya, Division of Research Promotion, National Children’s Medical Research Center, 3-35-31 Tasshido, Setagaya-ku, Tokyo 154-8509, Japan. E-mail address: shinomy@rch.go.jp

2 Abbreviations used in this paper: PT, permeability transition; ΔΨm, mitochondrial transmembrane potential; AIF, apoptotic inducing factor; CsA, cyclosporin A; BA, bongkrekic acid; CCCP, carbonylcyanide-m-chlorophenylhydrazone; DIOC (3), 3,3’-diohexyloxacarbocyanide iodide; Bet A, betulinic acid; ERK, extracellular signal-regulated kinase.

Copyright © 2000 by The American Association of Immunologists

0042-1767/00/$02.00
This study examines the mechanism of FTY720-induced apoptosis from the viewpoint of mitochondria dysfunction. We initially examined caspase involvement. A previous study showed that Bcl-2 overexpression abolished the effect of FTY720 (40), and thus we examined whether ΔΨm was reduced and cytochrome c was released from the mitochondria of FTY720-induced cells. We investigated whether FTY720 directly affects mitochondria functions in the intracellular signal transduction pathways of apoptosis.

Materials and Methods

Drugs

FTY720 was synthesized and supplied in powder form by Taito (Tokyo, Japan) in cooperation with Yoshitomi Pharmaceutical Industries (Osaka, Japan). FTY720 was dissolved in saline (1 mM). Atractylloside (Sigma, St. Louis, MO) was dissolved in saline (25 mM) and used at a concentration of 5 mM. The pan-caspase inhibitor Z-VAD-FMK (Bachem, Bubendorf, Switzerland) was dissolved in DMSO (10 mM) and used at a concentration of 40 μM. Cyclosporin A (CsA; Sigma) was dissolved in DMSO (10 mM) and used at a concentration of 10 μM. Bongkrekic acid (BA) was provided by Dr. J. A. Duine (University of Delft, Delft, The Netherlands). Oligomycin (Sigma) was dissolved in ethanol (10 mM) and used at a concentration of 10 μM. Carbonylecyanide-m-chlorophenylhydrazone (CCCP; Sigma) was dissolved in ethanol (50 mM) and used at a concentration of 10 μM.

Cells

The human myelogenous leukemia cell line HL-60 was provided by Dr. T. Miyashita, National Children’s Medical Research Center (Tokyo, Japan). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 75 mg/L kanamycin (Sigma) and maintained at 37°C in a humidified chamber under an atmosphere of 95% air and 5% CO2. All cells were washed, suspended at a density of 2 × 106 cells/ml in fresh culture medium, and incubated with drugs.

Determination of apoptosis-associated parameters in intact cells

The ΔΨm value was determined using 3,3′-dihexyloxacarbocyanide iodide (DiOC(3); Molecular Probes, Eugene, OR) as described (10, 12, 16). DNA fragmentation (1 × 106 cells/lane) was determined by agarose gel electrophoresis (41).

Caspase activity assay

The caspase activity assay described by Zapata et al. was improved (42). Cells (1 × 107) were lysed by RIPA buffer (25 mM Tris, pH 7.4, 150 mM KC1, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), and cell extracts were obtained by centrifugation at 10,000 × g for 5 min at 4°C. The protein concentration was determined using the bichinonic acid protein assay (Pierce, Rockford, IL). After an incubation with or without drugs for 30 min at 37°C, the mitochondrial suspensions were further incubated for 15 min with 50 nM DiOC(3). Mitochondrial ΔΨm was examined by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA). To immunodetect cytochrome c, isolated mitochondria were washed and resuspended in CFS buffer at a concentration of 100 μg mitochondrial protein in 100 μl CFS buffer. After incubating with or without drugs for the indicated periods at 37°C, reaction mixtures were centrifuged (10,000 × g, 30 min, 4°C). The mitochondria pellet and supernatant were separated and immediately used or stored at −80°C until the immunodetection of cytochrome c. To allow for observation of the Csa–c effect, EDTA was excluded from the CFS buffer.

Determination of mitochondrial membrane potential and cytochrome c release

To determine ΔΨm, isolated mitochondria were washed and resuspended in CFS buffer at a concentration of ~25 μg mitochondrial protein in 100 μl CFS buffer. Protein concentrations were determined by the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA). After an incubation with or without drugs for 30 min at 37°C, the mitochondrial suspensions were further incubated for 15 min with 50 nM DiOC(3). Mitochondrial ΔΨm was examined by flow cytometry (FACSscan; Becton Dickinson, Mountain View, CA). To immunodetect cytochrome c, isolated mitochondria were washed and resuspended in CFS buffer at a concentration of ~100 μg mitochondrial protein in 100 μl CFS buffer. After incubating with or without drugs for the indicated periods at 37°C, reaction mixtures were centrifuged (10,000 × g, 30 min, 4°C). The mitochondria pellet and supernatant were separated and immediately used or stored at −80°C until the immunodetection of cytochrome c. To allow for observation of the Csa–c effect, EDTA was excluded from the CFS buffer.

Determination of mitochondrial NAD(P)H level

Isolated mitochondria were washed and resuspended in CFS buffer at a concentration of ~25 μg mitochondrial protein in 100 μl CFS buffer. The mitochondrial pyridine nucleotide (NAD(P)H) level was determined by measuring autofluorescence of NAD(P)H at excitation and emission wavelengths of 360 and 450 nm, respectively, using a spectrofluorometer as described (43).

Cell-free system of apoptosis

Nuclei from HeLa or HL-60 cells were isolated as described (44, 45). To detect a direct effect upon the nuclei, isolated HL-60 nuclei (1011/μl) were resuspended in CFS buffer and incubated with or without FTY720 for 4 h at 37°C. The nuclei were then stained with 10 μg/ml propidium iodide (Sigma) and analyzed by flow cytometry. To detect a direct effect upon the cytosol, isolated HeLa nuclei (1011/μl) were resuspended in the isolated HL-60 cytosol fraction (100 μg protein in 100 μl CFS buffer) pretreated with 8 μM FTY720 for 4 h at 37°C. In the control experiment, HeLa nuclei (1011/μl) were resuspended in the isolated 8 μM, 4 h FTY720-induced apoptotic HL-60 cytosol fraction (100 μg protein in 100 μl CFS buffer) and nonapoptotic (normal) HL-60 cytosol fraction (100 μg protein in 100 μl CFS buffer). After incubating for 2 h at 37°C, nuclei were stained with 10 μg/ml propidium iodide (Sigma), and nuclear degradation was measured by flow cytometry.

Results

FTY720-induced caspase activation

Apoptotic cell death is provoked in vitro by FTY720 especially in PBLs and lymphomas at concentrations above ~4 μM within 4 h (32). Fig. 1A shows the ladder formation of nuclear DNA and caspase activation, confirming apoptosis. Caspase-3 was evaluated by Western blotting the cytosol obtained from FTY720-treated HL-60 cells (Fig. 1B). The level of 32-kDa unprocessed procaspase-3 was time-dependently decreased slightly, whereas a 17-kDa active caspase-3 large fragment appeared and was increased within 3 h in the presence of 8 μM FTY720. The 11-kDa active caspase-3 fragment was also cleaved from the pro-form, but this was not detected by the Abs used here (42, 46). Thus, FTY720 was dissolved in ethanol (50 mM) and used at a concentration of 4°C, and the remaining supernatant was used as the cytosol fraction. Both samples were used immediately or stored at −80°C until immunodetection of caspase-3 and cytochrome c.

Western blots

Mitochondria and cytosol fractions prepared as described above were mixed in the same volume of SDS sample buffer (4% SDS, 125 mM Tris, pH 6.8, 10% glycerol, 0.02 mg/ml bromophenol blue, 10% 2-ME) and heated at 65°C for 10 min. Proteins were separated by 4–20% gradient SDS-PAGE and electrically transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking the membrane using 3% skim milk, caspase-3, cytochrome c, and Bcl-2 were immunodetected using mouse anti-cytochrome c mAb (1:300, Pharmingen, San Diego, CA), rabbit anti-caspase-3 polyclonal Ab (1:100, Pharmingen), or rabbit anti-Bcl-2 polyclonal Ab (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA). Thereafter, HRP-conjugated anti-mouse IgG or anti-rabbit IgG was applied as second Abs, and positive bands were detected by enhanced chemiluminescence (Amersham Life Science, Buckinghamshire, U.K.).
activated caspase-3. The results using the cell-permeable fluorogenic caspase substrate PhiPhlux-G1D2 were the same (data not shown).

Caspase-1 activity was also determined by the caspase specificity of the proteolytic activity detected by the Ac-YVAD-MCA hydrolysis assay. No Ac-YVAD-MCA hydrolytic activity was detected even after exposure to 8 μM FTY720 for 5 h, when caspase-3 was already activated and a DNA ladder was evident. By contrast, the level of Ac-DEVD-MCA hydrolytic activity, which is processed by caspase-3, was greatly increased within 3 h (Fig. 1, C and D). These results suggested that caspase-3 is involved in FTY720-induced apoptosis, whereas caspase-1 is not.

**FTY720-induced mitochondrial perturbation is independent of caspase activation**

Our previous study showed that Bcl-2 overexpression inhibits the apoptotic cell death induced by FTY720 (40). Because Bcl-2 is localized in the mitochondrial membrane, we considered that mitochondria are somewhat involved in FTY720-induced apoptosis. To verify this hypothesis, we examined the release of cytochrome c from mitochondria, which is the major event of mitochondriamediated apoptosis. Release of cytochrome c from mitochondria into the cytosol was detected by Western blotting. FTY720 induced a time-dependent release of cytochrome c from mitochondria into HL-60 cytosol (Fig. 2A). Cytochrome c was released within 1 h after exposure to FTY720, which was slightly faster than caspase-3 activation, assuming that cytochrome c release from mitochondria precedes caspase activation. Cytochrome c in mitochondria disappeared within a 3-h exposure of FTY720. To determine whether mitochondrial cytochrome c release or caspase activation is the prior action, we used the pan-caspase inhibitor, Z-VAD-FMK. After a 1-h preincubation with 40 μM Z-VAD-FMK, FTY720-induced nuclear fragmentation was completely blocked (Fig. 2B). However, Z-VAD-FMK did not inhibit the release of mitochondrial cytochrome c (Fig. 2C). These results indicate that FTY720-induced mitochondrial cytochrome c release precedes caspase activation, which demonstrates that it is caspase independent.

The reduction in the ΔΨm value, which is another major event in mitochondria perturbation, was observed after FTY720-induced apoptosis.
HL-60 cells were stained with DiOC₆(3). The level of ∆Ψₘ was dose dependently reduced within 45-min exposure to FTY720, and this reduction was not inhibited by Z-VAD-FMK (Fig. 3). Hence, FTY720 triggered mitochondrial transmembrane depolarization without activating caspases. Therefore, FTY720 has specific effects upon mitochondria that induce further caspase activation and nuclear fragmentation.

FTY720 directly affects mitochondria and induces ∆Ψₘ and cytochrome c release
We considered whether FTY720 directly affects mitochondria. Mitochondria isolated from HL-60 cells were incubated with drugs, then stained with DiOC₆(3) to assess ∆Ψₘ (Fig. 4). After incubation with the direct mitochondrial activator, atractyloside (5 mM), ∆Ψₘ was reduced. However, ∆Ψₘ reduction was not affected by the potent protein kinase C inhibitor, staurosporine (1 μM; data not shown). In a similar manner as atractyloside, ∆Ψₘ was reduced by 10 μM FTY720. Thus, 10 μM FTY720 potently influenced mitochondria, because far higher concentrations of the other direct mitochondrial activators, atractyloside, tert-butyl hydroperoxide, and betulinic acid (Bet A), were required to activate mitochondria (19, 26). However, coincubation with 40 μM Z-VAD-FMK failed to recover the FTY720-induced ∆Ψₘ reduction. Z-VAD-FMK alone did not induce a reduction in ∆Ψₘ at all, indicating that Z-VAD-FMK has no potential to affect ∆Ψₘ. These findings indicate that FTY720 directly affects mitochondria in a caspase-independent fashion.

Mitochondrial cytochrome c was examined in isolated mitochondria. Isolated HL-60 mitochondria were incubated with FTY720, then cytochrome c in isolated mitochondria and mitochondrial supernatant was detected by Western blotting (Fig. 5). Exposure to 10 μM FTY720 induced a time-dependent release of cytochrome c from the mitochondria into the mitochondrial supernatant. Cytochrome c was released within 15-min exposure to FTY720. However, coincubation with 40 μM Z-VAD-FMK did not inhibit cytochrome c release (data not shown).

PT inhibitors block FTY720-induced apoptosis
FTY720-induced apoptosis was further examined using the anti-apoptotic endogenous molecule, Bcl-2, which inhibits PT and cytochrome c release. Jurkat cells overexpressing Bcl-2, Jurkat (bcl-2), completely blocked FTY720-induced apoptotic phenomena, such as nuclear DNA fragmentation (Fig. 6A), activation of caspase-3 (Fig. 6B), and cytochrome c release into the cytosol of intact cells (Fig. 6C). In addition, ΔΨₘ reduction was nearly inhibited (when compared with the control experiment by Jurkat (neo)) (Fig. 6D), and cytochrome c release was completely inhibited (Fig. 6E) when isolated Jurkat (bcl-2) mitochondria were

**FIGURE 3.** Caspase-independent ∆Ψₘ reduction by incubating HL-60 cells with FTY720. HL-60 cells (1 × 10⁵) were incubated with or without 40 μM Z-VAD-FMK for 1 h, then with 8 μM FTY720 for 4 h. DiOC₆(3) (50 nM) was added 45 min before ending culture, and ∆Ψₘ was analyzed by flow cytometry. Values in parentheses indicate median fluorescence intensity. Data are representative of three independent experiments.

**FIGURE 4.** FTY720 reduced ∆Ψₘ directly in isolated HL-60 mitochondria. Mitochondria from HL-60 cells (25 μg mitochondrial protein in 100 μl CFS buffer) were prepared as described in Materials and Methods. Mitochondria were incubated with no additives, 10 μM FTY720, 40 μM Z-VAD-FMK, FTY720 and Z-VAD-FMK together, and 5 mM atractyloside (positive control) for 30 min at 37°C. Mixtures were then incubated with DiOC₆(3) (50 nM) for 15 min then ∆Ψₘ was analyzed by flow cytometry. Values in parentheses indicate median fluorescence intensity. Data are representative of three independent experiments.

**FIGURE 5.** FTY720 induced cytochrome c release from HL-60 mitochondria. Mitochondria of HL-60 cells (100 μg mitochondrial protein in 100 μl CFS buffer) were prepared as described in Materials and Methods and incubated with no additives, 10 μM FTY720, 40 μM Z-VAD-FMK, and FTY720 and Z-VAD-FMK together at 37°C for the indicated periods. Mitochondria and mitochondrial supernatant were prepared, and cytochrome c was detected by Western blotting. PC indicates incubation with 5 mM atractyloside for 60 min (positive control). Data are representative of three independent experiments.
treated with FTY720. However, cells that do not overexpress Bcl-2, Jurkat (neo) showed the same effects as HL-60 (Fig. 6, A–E). These findings indicate that FTY720-induced apoptosis is dependent upon PT and cytochrome c release. The time course for the amount of Bcl-2 in the mitochondria was unchanged by FTY720 in Jurkat (bcl-2) cells and in Jurkat (neo) cells, indicating

**FIGURE 6.** FTY720-induced apoptosis blocked by overexpression of bcl-2. A, Both Jurkat (neo) and Jurkat (bcl-2) cells (1 × 10⁶) were incubated with 8 μM FTY720 for 0, 2, 4, and 6 h. At the indicated times, the cells were lysed and DNA fragmentation was analyzed by agarose gel electrophoresis. PC indicates incubation with 1 μM staurosporine for 4 h (positive control). Data are representative of three independent experiments. B, Both Jurkat (neo) and Jurkat (bcl-2) cells (1 × 10⁷) were incubated with 8 μM FTY720 for the indicated periods. Cytosolic fractions were prepared, and pro-caspase-3 and caspase-3 were detected by Western blotting. Open arrow indicates pro-caspase-3 (32 kDa); filled arrow indicates caspase-3 (17 kDa); PC indicates incubation with 1 μM staurosporine for 4 h (positive control). Data are representative of three independent experiments. C, Both Jurkat (neo) and Jurkat (bcl-2) cells (1 × 10⁷) were incubated with 8 μM FTY720 for the indicated periods. Mitochondria and cytosolic fractions were prepared at various times, and cytochrome c in cytosolic fraction was detected by Western blotting. PC indicates treatment with 1 μM staurosporine for 4 h (positive control). Data are representative of three independent experiments. D, Mitochondria from both Jurkat (neo) and Jurkat (bcl-2) cells (25 μg mitochondrial protein in 100 μl CFS buffer) was prepared as described in Materials and Methods. Mitochondria were incubated with no additives, 10 μM FTY720, 40 μM Z-VAD-FMK, FTY720 and Z-VAD-FMK together, and 5 mM atractyloside (positive control) for 30 min at 37°C. Thereafter, DiOC₆(3) (50 nM) was added and incubated for a further 15 min, and ΔΨₘ was analyzed by flow cytometry. Values in parentheses indicate median fluorescence intensity. Data are representative of three independent experiments. E, Mitochondria from both Jurkat (neo) and Jurkat (bcl-2) cells (100 μg mitochondrial protein in 100 μl CFS buffer) prepared as described in Materials and Methods were incubated with no additives, 10 μM FTY720, 40 μM Z-VAD-FMK, and FTY720 and Z-VAD-FMK together at 37°C for indicated times. Mitochondria and mitochondrial supernatant were prepared, and cytochrome c was detected by Western blotting. In addition, Bcl-2 levels in mitochondria were measured. PC indicates incubation with 5 mM atractyloside for 60 min (positive control). Data are representative of three independent experiments.
that FTY720-induced PT and cytochrome c release in isolated mitochondria was not the result of diminishing Bcl-2 levels (Fig. 6E).

Apoptotic mitochondrial PT appears to be mediated by opening of the PT pore complex (10, 19, 46). This complex consists of several proteins, including hexokinase, cyclophilin D, adenine nucleotide translocator, and voltage-dependent anion channel (11). Some chemicals inhibit PT by targeting this pore complex as well as Bcl-2. CsA and BA directly target cyclophilin D and adenine nucleotide translocator, respectively (10, 19, 46, 47). Fig. 7, A and B, shows that both CsA and BA inhibited FTY720-induced ΔΨ\textsubscript{m} reduction and cytochrome c release, suggesting that the PT pore complex is involved in FTY720-induced PT.

Moreover, the pro-apoptotic Bcl-2 family protein Bax, which is also a direct PT inducer (21–24), requires F\textsubscript{1}F\textsubscript{0}-ATPase when yeast cells undergo apoptosis (48). The F\textsubscript{1}F\textsubscript{0}-ATPase inhibitor oligomycin completely inhibits Bax-induced ΔΨ\textsubscript{m} reduction and cytochrome c release, and, as a result, cells do not die (21). Similarly, 10 μM oligomycin completely inhibited FTY720-induced ΔΨ\textsubscript{m} reduction and cytochrome c release (Fig. 7, A and B). These findings indicate that F\textsubscript{1}F\textsubscript{0}-ATPase is involved in PT induced by FTY720.

In addition, ΔΨ\textsubscript{m} is modulated by several different physiological events, such as Ca\textsuperscript{2+} overload, intramitochondrial proton influx, and intramitochondrial NAD(P)H reduction. In a previous study, Ca\textsuperscript{2+} (about 200 nM) was shown to be released from the intracellular calcium pool of intact HL-60 in the presence of FTY720 (49). As shown in Fig. 8, A and B, a slight ΔΨ\textsubscript{m} reduction was observed but no cytochrome c was released in response to 500 nM Ca\textsuperscript{2+} treatment of mitochondria. These results suggest that the increase of intracellular Ca\textsuperscript{2+} due to FTY720 treatment may be an independent effect of FTY720-induced PT. Protonophores are known to directly facilitate the transport of protons into the matrix space and reduce ΔΨ\textsubscript{m}. After incubation with a protonophore, CCCP, ΔΨ\textsubscript{m} was significantly reduced (data not shown). However, no cytochrome c release was observed after CCCP treatment (Fig. 8C). These results indicate that unlike FTY720, CCCP reduces ΔΨ\textsubscript{m} but does not release cytochrome c, and thus FTY720 does not act like a protonophore. Fig. 8D shows intramitochondrial levels of NAD(P)H, suggesting that NAD(P)H reduction is not involved in FTY720-induced PT.

**FTY720 has no effect on the nuclei and cytosols**

The possibility of other cellular effects of FTY720 were also examined. A cell-free apoptosis system was established to determine direct effects upon nuclei by FTY720. After incubating intact HL-60 cells for 4 h with 8 μM FTY720, nuclear DNA fragmented due to apoptosis; 57.3% of the DNA was hypoploid in nuclei isolated from apoptotic HL-60 induced by 8 μM FTY720 (Fig. 9A; Ref. 2). In contrast, less hypoploidy (24.0%) was observed in HL-60 nuclei exposed to 8 μM FTY720 after isolation (Fig. 9A; Ref. 1). DNA hypoploidy between the isolated nuclei and nuclei in the intact cells was not significantly changed, indicating minimal DNA fragmentation during isolation (data not shown). The effects of incubating isolated nuclei with various concentrations of FTY720 are shown in Fig. 9B. Although the FTY720 concentration increased to 20 μM, at which necrosis of intact cells is induced (data not shown), little DNA fragmentation was observed compared with control nuclei. These findings indicated that FTY720 did not directly affect the nuclei.

We examined the FTY720 effect upon cytosol. HeLa nuclei were isolated and coincubated with isolated nonapoptotic HL-60 cytosol. Nuclei fragmented after coincubation with cytosol isolated from FTY720-treated HL-60 (apoptotic), as shown in Fig. 9C (2). This suggested that fragmentation was caused by apoptotic factors such as activated caspases, including those in cytosol. However, coincubating cytosol isolated from normal cells with FTY720 did not affect nuclei (Fig. 9C; Ref. 1). We concluded from these experimental results that FTY720 had no potency to activate cytosolic apoptotic factors directly, such as caspases and endonucleases.
FIGURE 8. Ca$^{2+}$ overload, proton influx, and NAD(P)H decrease were not associated with FTY720-induced apoptosis. A, Mitochondria from HL-60 cells (25 µg mitochondrial protein in 100 µl CFS buffer excluding EDTA) prepared as described in Materials and Methods were incubated with no additives, 10 µM CaCl$_2$, or 5 mM atractyloside (positive control) for 30 min at 37°C. Thereafter, DiOC$_{6}(3)$ (50 nM) was added for a further 15 min and $\Delta \Psi_m$ was analyzed by flow cytometry. Values in parentheses indicate median fluorescence intensity. Data are representative of three independent experiments. B, Mitochondria from HL-60 cells (100 µg mitochondrial protein in 100 µl CFS buffer excluding EDTA) prepared as described in Materials and Methods were incubated with no additives, 10 µM CaCl$_2$, or 5 mM atractyloside (positive control) at 37°C for 1 h. Mitochondria supernatants were prepared, and cytochrome $c$ was detected by Western blotting. Data are representative of two independent experiments. C, Mitochondria from HL-60 cells (100 µg mitochondrial protein in 100 µl CFS buffer) prepared as described in Materials and Methods were incubated with no additives, 10 µM CCCP, or 5 mM atractyloside (positive control) at 37°C for 1 h. Mitochondria supernatants were prepared, and cytochrome $c$ was detected by Western blotting. Data are representative of two independent experiments. D, Mitochondria from HL-60 cells (25 µg mitochondrial protein in 100 µl CFS buffer) prepared as described in Materials and Methods were incubated with no additives, 10 µM CCCP (positive control), or 10 µM FTY720 at 37°C for 45 min. NAD(P)H level was measured as described in Materials and Methods. Bars denote SD (n = 3).

Discussion

The novel immunosuppressant, FTY720, specifically induces apoptosis in PBLs in vitro and in vivo (32, 39). This effect is thought to be the main reason for its action, and thus this drug has a novel suppression mechanism compared with that of classical immunosuppressants such as CsA (50), FK506 (51), and azathioprine (52). Immunosuppressants are used in combination to lessen the side effects of each immunosuppressant and to strengthen the immunosuppressive effect. In light of this, it is important to develop a new immunosuppressive drug with a novel immunosuppressive mechanism. Thus, FTY720 is expected to be a unique immunosuppressant and promising drug. However, little is known about the mechanism of inducing apoptosis; therefore, we examined precisely how FTY720-induced apoptosis is executed.

In this study, caspase-3 was activated in HL-60 and Jurkat exposed to FTY720 in vitro (Fig. 1, B and D). This activation preceded FTY720-induced nuclear DNA fragmentation, indicating that caspase-3 is involved in FTY720-induced apoptosis. Recent studies by Wang et al. have revealed that caspase-3 is activated by FTY720 in the prostate cancer cell line, DU145, indicating that caspase-3 activation in FTY720-induced apoptosis is not cell specific (53). In addition, the pan-caspase inhibitor, Z-VAD-FMK, completely blocked subsequent FTY720-induced nuclear DNA fragmentation in this study. Thus, caspase-3 activation is essential to FTY720-induced apoptosis. However, in agreement with the findings of Wang et al., caspase-1 was not activated in HL-60 and Jurkat lymphoma cells as shown in Fig. 1C (53).

Though upstream caspase-3 events occur through several pathways according to this study and others, caspase-1 and Fas-mediated pathways were presumed not to be involved in FTY720-induced apoptosis (41). The overexpression of Bcl-2 inhibits apoptotic cell death induced by FTY720 (40). Because Bcl-2 is localized in the mitochondrial membrane, we speculated that a mitochondrial-mediated pathway is involved in the FTY720-induced apoptosis pathway. In this study, cytochrome $c$ was released from the mitochondrial intermembrane into the cytosol, and $\Delta \Psi_m$ was reduced in FTY720-treated HL-60 cells (Figs. 2 and 3). These phenomena occurred at the same time or before caspase-3 activation, and were not inhibited by preincubating the cells with Z-VAD-FMK. This evidence suggested that releasing cytochrome $c$ from the mitochondria and reducing $\Delta \Psi_m$ are upstream events before caspase activation. However, the amount of cytochrome $c$ released was less than that in the absence of Z-VAD-FMK, indicating that caspases adversely affected mitochondria to release more cytochrome $c$.

In contrast, studies using a cell-free system revealed that FTY720 directly triggers the reduction of $\Delta \Psi_m$ and the release of cytochrome $c$ (Figs. 4 and 5). Inhibition of PT by Bcl-2 overexpression prevented all events associated with FTY720-induced apoptosis that also occur in intact cells, such as nuclear DNA fragmentation, activation of caspase-3, releasing of cytochrome $c$ from mitochondria, and reduction of $\Delta \Psi_m$ (Fig. 6). The other PT inhibitors, CsA and BA, also prevented FTY720-induced reduction of $\Delta \Psi_m$ and the release of cytochrome $c$ in isolated mitochondria, indicating that FTY720 affects PT pore complexes to open and induces PT and cytochrome $c$ release (Fig. 7). However, PT pore complex studies revealed that the PT pore allows only molecules
FTY720 did not directly affect nuclei and cytosol. A. Isolated nuclei were incubated with 8 μM FTY720 for 4 h at 37°C (1). Nuclei isolated from HL-60 cells incubated with 8 μM FTY720 for 4 h (2) were used as positive controls. Nuclear apoptosis is described in Materials and Methods. Values in parentheses indicate cell hypoploidy percentage. Data are representative of three independent experiments. B. Isolated nuclei were incubated with various concentrations of FTY720, and percent hypoploidy was estimated. Experimental protocols are the same as those described in A. Bars denote SD (n = 3). C, HeLa nuclei were resuspended in HL-60 cytosol fraction that had been incubated with FTY720 for 4 h at 37°C (1). As a control experiment, HeLa nuclei were resuspended in FTY720-induced apoptotic (2) and nonapoptotic (normal) HL-60 cytosol fractions (3). After incubating for 2 h at 37°C, nuclear apoptosis was determined as described in Materials and Methods. Values in parentheses indicate hypoploidy percentage. Data are representative of three independent experiments.

FIGURE 9. FTY720 did not directly affect nuclei and cytosol. A. Isolated nuclei were incubated with 8 μM FTY720 for 4 h at 37°C (1). Nuclei isolated from HL-60 cells incubated with 8 μM FTY720 for 4 h (2) were used as positive controls. Nuclear apoptosis is described in Materials and Methods. Values in parentheses indicate cell hypoploidy percentage. Data are representative of three independent experiments. B. Isolated nuclei were incubated with various concentrations of FTY720, and percent hypoploidy was estimated. Experimental protocols are the same as those described in A. Bars denote SD (n = 3). C, HeLa nuclei were resuspended in HL-60 cytosol fraction that had been incubated with FTY720 for 4 h at 37°C (1). As a control experiment, HeLa nuclei were resuspended in FTY720-induced apoptotic (2) and nonapoptotic (normal) HL-60 cytosol fractions (3). After incubating for 2 h at 37°C, nuclear apoptosis was determined as described in Materials and Methods. Values in parentheses indicate hypoploidy percentage. Data are representative of three independent experiments.

of <1500 Da to pass through, and that 15-kDa cytochrome c cannot be released from the PT pore complex (11, 47, 54). However, cytochrome c release may be the result of indirect induction by FTY720-induced PT, similar to Bax-induced PT (21, 23). PT may activate other molecules that locate in the mitochondrial transmembrane to release cytochrome c. The exact mechanism of this PT-induced cytochrome c release remains to be explained.

The possibility exists that opening of the PT pore complex is a result of direct interaction of FTY720 as well as an indirect effect whereby FTY720 sensitizes physiological PT- inducers or itself acts as a protonophore. In this study, we demonstrated that these indirect possibilities were presumably not involved in FTY720-induced PT (Fig. 8). FTY720 induced intracellular Ca2+ release (2), but the level was not sufficient for inducing PT. This increase may play a role in other signal transduction processes. Mitochondrial NAD(P)H level was unchanged even after FTY720 treatment. If NAD(P)H is oxidized or, alternatively, if the citric acid cycle is inhibited, mitochondrial NAD(P)H decreases, and if the mitochondrial respiratory chain is inhibited, mitochondrial NAD(P)H increases. The effect of FTY720 on mitochondria did not indicate either case, and thus FTY720 presumably did not inhibit respiration directly. Moreover, we indicated that a protonophore, CCCP, failed to release cytochrome c. Similar results were reported by Shimizu et al., whereby protonophores did not induce PT or cytochrome c release from mitochondria (55, 56). However, inhibition of F$_0$F$_1$-ATPase by oligomycin inhibited FTY720-induced ΔΨm reduction and cytochrome c release (Fig. 7). An investigation of the involvement of F$_0$F$_1$-ATPase in Bax-induced cell death showed that this inhibition was caused by an alteration of the ADP/ATP ratio to promote PT pore closure (21, 57). This may also be the cause of FTY720-induced PT, although release of cytochrome c was not completely inhibited. Moreover, one recent study revealed that F$_1$-ATPase bound to the PT pore complex, and this may have resulted in a close relationship between the PT pore complex and F$_0$F$_1$-ATPase (47). These findings suggest a direct effect of FTY720 on the PT pore complex, although there is no evidence that FTY720 interacts with PT pore complex proteins. Further analysis is necessary to elucidate the exact mechanisms underlying these phenomena.

Moreover, to our surprise, after exposure to FTY720 in a cell-free system, Bcl-2 was not degraded during PT pore opening and allowed ΔΨm to reduce and cytochrome c to be released (Fig. 6E). These findings can be explained by recent studies that Bcl-2 is degraded by caspase-3 and/or by ubiquitin-dependent proteasome, which are both cytosolic factors (58, 59). As a cell-free system has no cytosolic factors, our evidence suggested that no degradation occurred in Bcl-2 and that FTY720 itself had no potential to degrade Bcl-2. The original amounts of mitochondrial Bcl-2 differed greatly between Jurkat (neo) and Jurkat (bcl-2) mitochondria, suggesting that induction of PT and cytochrome c release is dependent on a regular amount of Bcl-2.

However, other apoptotic pathways of FTY720 cannot be excluded. Therefore, we determined here that FTY720 had no direct effect upon nuclei or cytosol in a cell-free system. Therefore, FTY720 itself has no apparent ability to degrade DNA, caspases, or endonucleases (Fig. 8). Also, FTY720-induced apoptosis is suppressed by the activation of extracellular signal-regulated kinase (ERK) in intact Jurkat cells (60), suggesting that the ERK pathway is involved in FTY720 apoptosis. Recent studies revealed that the ERK pathway confers protection against apoptosis at the level of caspase activation, downstream of the release of cytochrome c in mitochondria (61). Also, the ERK pathway phosphorylates mitochondrial Bcl-2 and inhibits Bcl-2 degradation (59). These findings suggest that the ERK pathway inhibits the mitochondria-mediated apoptosis pathway (the assumed FTY720-induced apoptotic pathway), with no involvement of c-Jun N-terminal kinase- or mitogen-activated protein kinase-induced apoptosis.
FTY720 specifically induces apoptosis in lymphocytes and lymphoma (32). If FTY720 directly activates mitochondria, then why does this drug not induce apoptosis in all types of cells? We considered the following two reasons. The first is that lymphocytes have different types of mitochondria from other cells. However, this is questionable because mitochondria isolated from FTY720 apoptosis-resistant HeLa cells were as susceptible to those from HL-60 cells (data not shown). Another direct mitochondrial activator, Bet A, also cell specifically induces apoptosis, but when Bet A is added to isolated mitochondria in a cell-free system, Bet A induces ΔΨm reduction without any specificity (26). Thus, there is no drug specificity for the mitochondrial type. Therefore, we considered that FTY720 has the potential to pass through various cellular membranes at different concentrations. The second possibility is that the apoptotic potential differs among cell types. Consistent with this possibility, human breast carcinoma MCF7 cells lacking caspase-3 were highly resistant to FTY720 (data not shown). Further studies are needed to resolve these issues.

We present the FTY720-induced apoptosis pathway as follows: FTY720 induces mitochondrial PT and cytochrome c release via an influence on the PT pore complex and F1F0-ATPase. Thereafter, released cytochrome c binds to Apaf-1 and activates further caspasas, including caspase-3. Activated caspases then induce apoptotic cell death.

Because FTY720 directly triggers PT in mitochondria at extremely low doses and induces further apoptotic effects in lymphocytes, this drug has a potent immunosuppressive effect. In addition, FTY720 may be effective in tumor therapy because FTY720 can also significantly induce apoptosis in lymphomas. Moreover, as this drug induces apoptosis Fas and TNF-α independently because FTY720 can bypass those upstream signal ligands/receptors and induce apoptosis, this drug could remove lymphomas that have mutated cellular membranes and cannot induce Fas-and/or TNF-α-mediated apoptosis. Thus, FTY720 may yield important information about mitochondria-mediated apoptosis pathways as well as assist organ transplantation and improve tumor therapy due to its unique and potent effects.

Acknowledgments

We thank H. Terada and Y. Shinohara of Tokushima University and J. A. Duine of University of Delft for providing BA, and K. Saito of our department for excellent technical support.

References


