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An immunosuppressive agent, FTY720, increases intracellular concentration of calcium ion and induces apoptosis in HL-60

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SUMMARY

We previously reported that FTY720 is an efficient inducer of apoptosis in lymphocytes and cultured cell lines. In the present study, HL-60 human promyerocytoma cells also induced apoptosis through *in vitro* treatment with the drug, demonstrating extensive DNA fragmentation 6 hr after incubation. The major target of FTY720 was the common signalling pathway of apoptosis, since a rapid (<1 min) increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was found in the cells treated with the drug. Calcium chelation in the culture medium with EGTA did not affect the $[Ca^{2+}]_i$ mobilization. A phospholipase C inhibitor, U73122, inhibited the increase in $[Ca^{2+}]_i$ as well as the fragmentation of the nuclear DNA, whereas U73343, a non-effective analogue of U73122, had little effect. These results suggest that FTY720-induced apoptosis is mediated through an activation of phospholipase C and the subsequent release of Ca^{2+} from intracellular calcium pools. In addition, the treatment of HL-60 with pertussis toxin (PTX) did not inhibit Ca^{2+} mobilization or apoptosis, suggesting that the activation of phospholipase C is independent of PTX-sensitive G-proteins.

INTRODUCTION

The immunosuppressant FTY720,[1,2] 2-amino-2-[2-(4-octylphenyl) ethyl]-1,3-propanediol hydrochloride, was screened from compounds chemically synthesized on the model of ISP-1[3] (myriocin,[4] thermozymocidin[5]), which was purified from culture filtrates of *Isaria sinclairii* (an ascomycete; a parasite on the larva of the cicada *Meimuna opalifera Walker*). FTY720 has a sphingosine-like chemical structure that is completely different than conventional immunosuppressants. The drug has no toxic effect on rats and prolongs skin allograft survival[2] with an oral dose ranging from 0·1–30 mg/kg, although its original product, ISP-1, induced severe digestive disorders at a dose of 1 mg/kg, resulting in the death of the animals.[1] In our previous report,[6] the oral administration of a single dose of FTY720 at 10 mg/kg into normal rats induced a marked reduction of peripheral lymphocytes. Rat spleen cells incubated with FTY720 demonstrated features characteristic of apoptosis: the chromatin condensation and the formation of apoptotic bodies observed by electron microscopy, and genomic DNA fragmentation by agarose gel electrophoresis. When liver-grafted rats and kidney-grafted dogs were administered FTY720 orally, the survival of the recipients was remarkably prolonged. Daily administration of FTY720 in combination with cyclosporine resulted in a synergistic prolongation of graft survival in canine kidney recipients.

Thymocytes from MRL-*lpr/lpr* mice with a mutant *fas* gene were sensitive to FTY720, indicating that the drug-induced apoptosis was not related to Fas-antigens.[7] In addition, Jurkat lymphoma cells with an overexpressed *bcl*-2 gene were resistant to the drug.[8] These results suggest that FTY720 induces *bcl*-2-associated apoptotic cell death.

Thus, it is important to investigate the intracellular action mechanisms of a drug that induces apoptosis. Sphingolipid and ceramide have recently been studied in relation to apoptosis using HL-60 cells.[9–12] These sphingolipid-related substances increased the intracellular calcium ions[13,14] and induced apoptosis.[11,12] Based on these results, we examined whether increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) would trigger apoptosis in HL-60 cells treated with FTY720. The mobilization of $[Ca^{2+}]_i$ was tested in the presence of effector drugs for the signalling pathway, such as a phospholipase C inhibitor (U73122)[15] and its non-inhibitory analogue (U73343),[15] thapsigardin,[16,17] and pertussis toxin.[18–20] The results strongly suggest that FTY720 induces apoptosis in HL-60 cells through phospholipase C activation and $[Ca^{2+}]_i$ mobilization.

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MATERIALS AND METHODS

Drugs and cells

The FTY720, donated in powder form and synthesized by Taito Co. Ltd (Tokyo, Japan) in cooperation with Yoshitomi Pharmaceutical Industries (Osaka, Japan), was dissolved in

saline at 1 mM. The U73122, U73343, psychosine, thapsigargin, and pertussis toxin (PTX or IAP) were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). The HL-60[21] cells were obtained from Dr Ikekita (Faculty of Science and Technology, Science University of Tokyo, Japan). The human lymphoid T-cell lines, Jurkat(bcl-2) and Jurkat(neo), stably transfected previously with human *bcl*-2 expression plasmid and its vector, were provided by Dr T. Miyashita (Oncogene & Tumour Suppressor Gene Program, La Jolla Cancer Research Foundation, La Jolla, CA; see ref. 22).

Cell cultures

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and maintained in a humidified chamber with 95% air and 5% CO_2. The cells were pretreated with PTX by adding the toxin (50 ng/ml) to the culture medium 4–12 hr before the FTY720 treatment.[13]

Measurement of $[Ca^{2+}]_i$

The cells were sedimented, resuspended in HEPES–BSS (20 mM HEPES, 127 mM NaCl, 3·1 mM KCl, 0·8 mM KH_2PO_4, 0·1 mM Na_2HPO_4, 1·4 mM $NaHCO_3$, 0·42 mM $CaCl_2$ and 0·27 mM $MgCl_2$, pH 7·4) containing 0·1% bovine serum albumin (BSA), and incubated for 20 min with 2 μM Fura-2/AM (Dojindo Co. Ltd, Kumamoto, Japan). The labelled cells were washed and suspended in the same buffer. The $[Ca^{2+}]_i$ was estimated from the fluorescence intensity of the Fura-2 loaded cells[23,24] using a CAF-110 fluorescence cytometer (JASCO, Tokyo, Japan). The data were incorporated and calculated using a Maclab analogue-digital transducer and software (AD Instruments Pty. Ltd, Sydney, Australia).

Assessment of apoptosis by DNA fragmentation

Apoptosis was determined by genomic DNA fragmentation[25,26] assessed by agarose gel electrophoresis. The treated cells were rinsed once with 10 mM Tris-HCl, pH 8·7, containing 3 mM $MgCl_2$ and 2 mM 2-mercaptoethanol, then dissolved in 50 mM Tris-HCl buffer, pH 7·8, containing 10 mM EDTA, 0·5% sodium lauryl sarcosinate and 0·5 mg/ml RNase A. After incubation at 50° for 15 min, proteinase K was added at a concentration of 1 mg/ml, and the lysates were incubated further for 30 min at 50°. The samples were mixed with an equal volume of loading buffer containing 1 × TBE, (89 mM Tris, 2·5 mM EDTA, 89 mM boric acid, pH 8·4) 20% glycerol and 0·01% BPB and electrophoresed on 1·8% agarose gels in 1 × TBE containing 0·5 mg/ml ethidium bromide.

MTT assay

The cells were incubated for 4 hr in a 0·1-ml medium in 96-well flat-bottomed plates (Falcon Oxnard, CA) at $1–2 \times 10^4$ cells per well with various concentrations of the drugs. Ten microlitres of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co., St Louis, MO) stock solution[27] was added to each well, and the plates were further incubated at 37° for 1–2 hr. The plates were centrifuged at 400 ***g*** for 5 min and the supernatants were discarded. The coloured formazan in the living cells was developed by adding 0·1 ml of dimethyl sulfoxide and the absorbance was measured on a microplate reader using 550 nm and 650 nm double filters. The number of viable cells was determined from the standard curve of the values of the MTT assay measured using different numbers of control cells.

RESULTS

Induction of apoptosis in HL-60-treated with FTY720

Genomic DNAs extracted from FTY720-treated HL-60 cells were analysed by agarose gel electrophoresis. As shown in Fig. 1(a), a DNA ladder formation was clearly observed when the HL-60 cells were incubated for 6 hr with FTY720 at concentrations between 4 and 8 μM. However, the DNA treated with 20 μM of FTY720 was diffusely mobilized on the gel, mainly because of necrotic cell death. In addition, when the HL-60 cells were incubated with 6 μM of FTY720, DNA fragmentation began to form as early as 2 hr after incubation (Fig. 1b). The cell viability, assessed by MTT assay, is shown in Fig. 2. The time-related cell growth was markedly inhibited

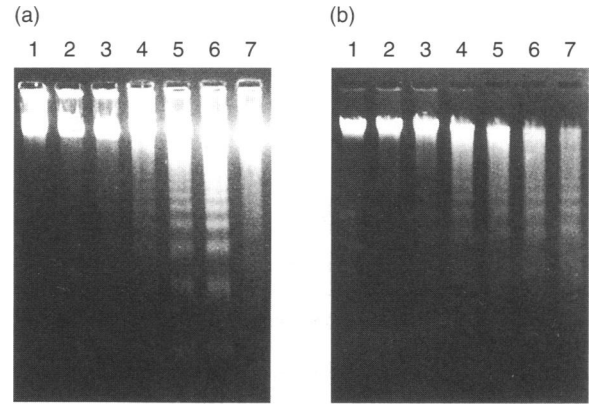

Figure 1. DNA fragmentation of FTY720-treated HL-60 cells on agarose gel electrophoresis. (a) Lane 1 is DNA prepared from HL-60 cells incubated without FTY720; lanes 2, 3, 4, 5, 6 and 7 are DNAs from the cells incubated for 6 hr with FTY720 at doses of 1, 2, 4, 6, 8 and 20 μM, respectively. (b) Lane 1 is DNA prepared from untreated HL-60 cells; lanes 2, 3, 4, 5, 6 and 7 are DNAs from the cells incubated for 1, 2, 4, 6, 8 and 12 hr, respectively, with 6 μM FTY720.

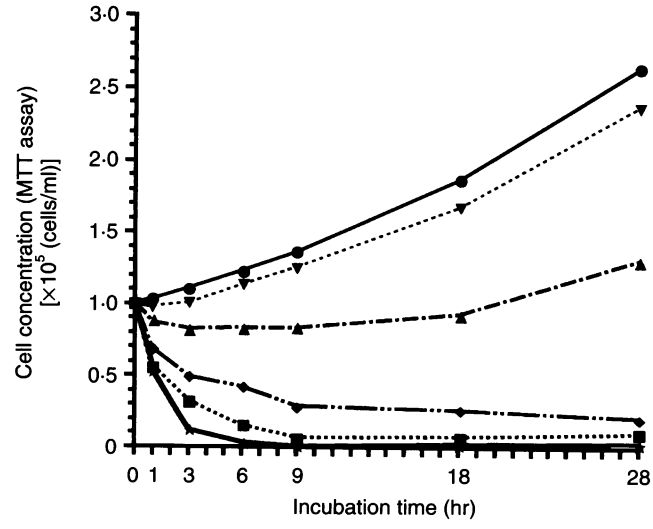

Figure 2. Effect of FTY720 treatment on the HL-60 cell viability. HL-60 cells were incubated for 1 to 28 hr with various concentrations of FTY720. MTT solution was added 1 hr before the end of culture, and the viable cells were measured by MTT assay. Concentrations of FTY720 are as follows: ●, none; ▼, 2 μM; ▲, 4 μM; ◆, 6 μM; ■, 8 μM; ☆, 10 μM.

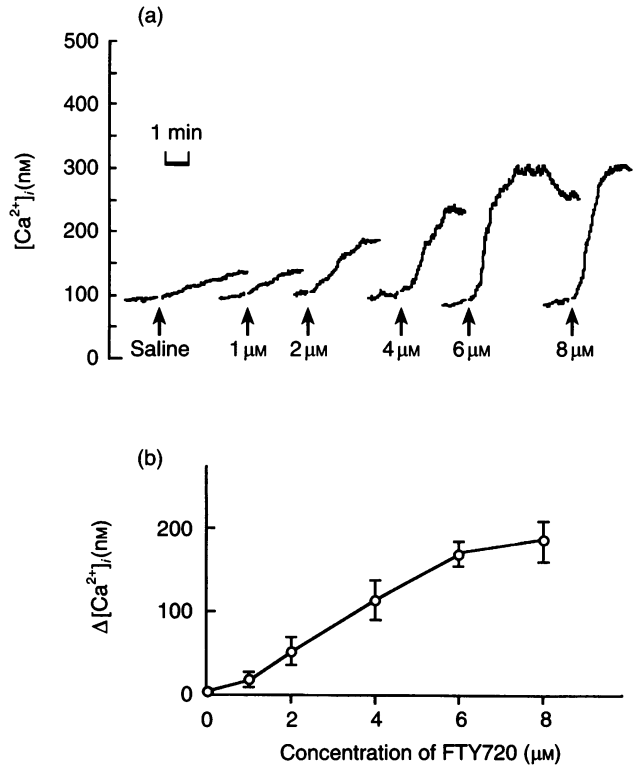

Figure 3. Effect of FTY720 treatment on $[Ca^{2+}]_i$ in HL-60 cells. (a) representative spectra of Fura-2-labelled HL-60 cells show a dose-dependent increase of $[Ca^{2+}]_i$ by the addition of FTY720 on CAF-110 fluorescence cytometer. (b) Means ± SD of $\Delta[Ca^{2+}]i$ (peak value − basal value) from four separate experiments.

at a dose of 4 μM of FTY720; doses greater than 6 μM resulted in a dose-dependent decrease of cell viability. Thus, there is a correlation between the development of DNA ladder formation on agarose gel and the decreased viability of the cells measured by MTT assay.

FTY720-induced increase of $[Ca^{2+}]_i$

The HL-60 cells were labelled with Fura-2/AM to study $[Ca^{2+}]_i$ mobilization. The concentration of intracellular calcium ions was immediately increased by the addition of FTY720 and reached a maximum within 1–2 min (Fig. 3a). The net peak height ($\Delta[Ca^{2+}]_i$), increased with an increased dose of the drug (Fig. 3b). The $[Ca^{2+}]_i$ remained at a high level for several minutes after the addition of FTY720. The long incubation time resulted in the release of inner Fura-2 outside of the cells, where the Fura-2 reacts with the Ca^{2+} in the assay medium, causing a deceptive increase of $[Ca^{2+}]_i$.

FTY720 also induced a dose-dependent increase of $[Ca^{2+}]_i$ in both the Jurkat(bcl-2) and Jurkat(neo) cell lines; there was no difference in the $[Ca^{2+}]_i$ levels between the two types of Jurkat cells incubated with the drug (Fig. 4).

Mobilization of Ca^{2+} from the internal pool of HL-60

As shown in Fig. 5(a), the addition of excess (3 mM) O,O'-Bis(2-aminoethyl)ethylene-glycol-N,N,N',N'-tetra acetic acid (EGTA, Dojindo Co. Ltd, Kumamoto, Japan) to the HL-60

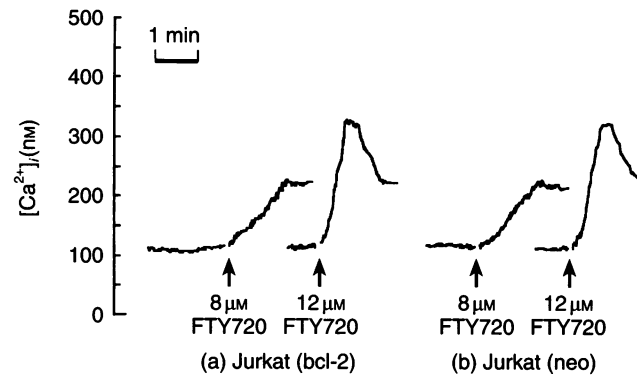

Figure 4. Effect of FTY720 treatment on $[Ca^{2+}]_i$ in Jurkat(bcl-2) and Jurkat(neo) cells. Both Jurkat(bcl-2) (a) and Jurkat(neo) (b) increased $[Ca^{2+}]_i$ to a similar extent by the addition of FTY720 at doses of 8 and 12 μM. Representative spectra in the figure show the intensity of Fura-2 loaded cells with CAF-110 fluorescence cytometer.

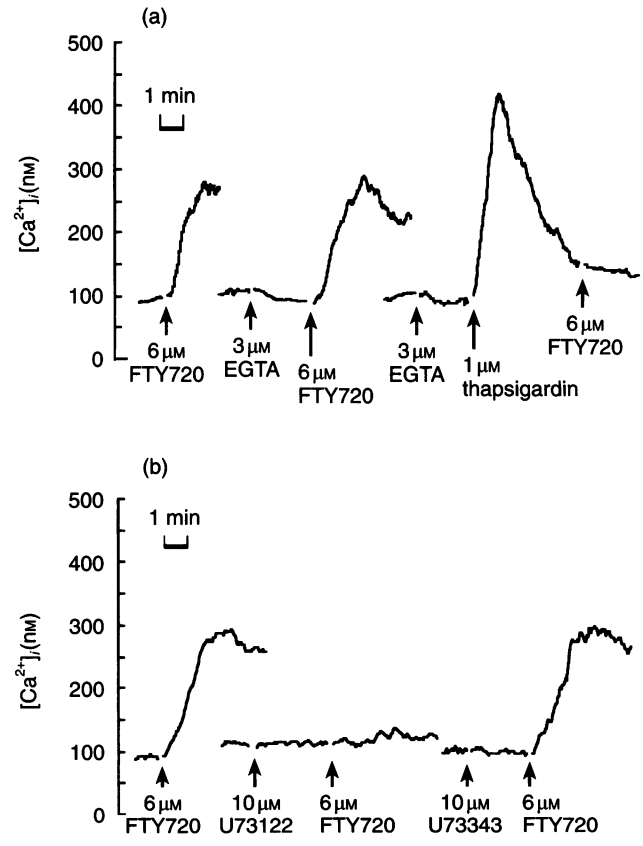

Figure 5. Effect of EGTA, thapsigargin and U73122 on FTY720-induced increase of $[Ca^{2+}]_i$. (a) Shows spectra of $[Ca^{2+}]_i$ change in HL-60 cells treated with FTY720 in the presence of either EGTA or EGTA plus thapsigargin. (b) shows spectra of $[Ca^{2+}]_i$ change in HL-60 cells pretreated with U73122 or U73343 by the addition of FTY720. All spectra are representative in three or four separate experiments.

culture medium did not affect the FTY720-induced increase of $[Ca^{2+}]_i$. After the simultaneous addition of 3 mM EGTA and 1 μM thapsigargin, a transient increase of $[Ca^{2+}]_i$ was observed, although further increase of $[Ca^{2+}]_i$ did not occur

with the additional challenge with FTY720 (Fig. 5a). Thapsigargin inhibits the Ca^{2+} shift into its intracellular pool by inhibiting Ca^{2+}-ATPase, resulting in an increase in $[Ca^{2+}]_i$.[16,17] This suggests that the $[Ca^{2+}]_i$ increased by FTY720 is derived predominantly from intracellular calcium pools.

The mechanism to increase $[Ca^{2+}]_i$ by FTY720 treatment was further studied in HL-60 cells preincubated with U73122, a potent inhibitor of phospholipase C. The intracellular calcium mobilization was completely eliminated by pretreatment of 10 μM U73122 2 min before the addition of FTY720, while pretreatment of U73343 (a control substance for U73122) had no effect on the FTY720-induced $[Ca^{2+}]_i$ increase (Fig. 5b). A similar inhibitory effect was obtained when the HL-60 cells were treated with U73122 after the addition of excess EGTA in the medium (data not shown). These results indicate that the increase in $[Ca^{2+}]_i$ was based mainly on provisions from the intracellular calcium stores by the activation of phospholipase C in the FTY720-treated cells. Moreover, the $[Ca^{2+}]_i$ increase by ionomycin, a calcium ionophore, or thapsigargin was not affected by treating the cells with U73122 (data not shown), suggesting that a $[Ca^{2+}]_i$ increase by FTY720 is mediated by completely different action mechanisms than those in ionomycin.

Inhibition of cell death by pretreatment with U73122

To further confirm the role of increased $[Ca^{2+}]_i$ on induction of apoptosis in FTY720-treated HL-60 cells, the cells were preincubated with 0–10 μM of U73122 for 10 min at 37°. After the cells were washed, incubation was continued for 4 hr in the presence of 6 μM of FTY720. As shown in Fig. 6, the treatments with U73122 alone induced a little DNA fragmentation in HL-60 cells by agarose gel electrophoresis, whereas pretreatment of U73122 prevented the FTY720-induced DNA degradation in a dose-dependent manner.

The MTT assay showed the viability percentages of the HL-60 cells after incubation for 4 hr in the absence of FTY720

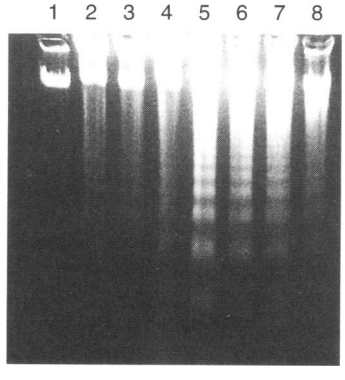

Figure 6. Inhibition of FTY720-induced DNA fragmentation by the pretreatment with U73122. HL-60 cells were preincubated with U73122, washed, and subsequently incubated for 4 hr in the presence or absence of 6 μM FTY720. Thereafter, DNAs were extracted and analysed on agarose gel electrophoresis. Lanes 1, 2, 3 and 4 are control electrophoresis of DNAs prepared from HL-60 cells incubated without FTY720 after the treatment with 0, 2·5, 5 and 10 μM, respectively, of U73122; lanes 5, 6, 7 and 8 are DNAs from the cells incubated with FTY720 after the pretreatment with U73122 at the same concentration as control experiments.

Table 1. Effect of pretreatment with U73122 on FTY720 induced cell death of HL-60. Results are given as percentage of value obtained from the cells treated without any drug. All results are mean ± SD in four experiments

Pretreatment (10 min)	MTT assay (1 hr)	
U73122 (μM)	None	6 μM FTY720
0	100·0 ± 1·8	68·8 ± 1·2
2·5	99·2 ± 0·9	73·3 ± 2·4
5	98·7 ± 2·5	81·1 ± 3·5
10	99·9 ± 3·8	81·8 ± 4·4

to be 98·7 ± 2·9, 97·6 ± 3·2 and 94·7 ± 3·6 (% mean ± SD) after pretreatment with 2·5, 5 and 10 μM U73122 for 10 min. Pretreatment of the cells with U73122 resulted in a reduction of FTY720-induced cell death after incubation for 1 hr by the MTT assay (Table 1). These results suggest that the activation of phospholipase C is important for apoptosis mediated by FTY720.

The activation pathway of FTY720

The HL-60 cells were pretreated with PTX[18–20] to study whether FTY720 activates the phospholipase C and $[Ca^{2+}]_i$ pathway through receptors coupling to PTX-sensitive G-proteins. As shown in Fig. 7(a), overnight incubation of HL-60 with 50 ng/ml of PTX had no effect on the FTY720-treated increase of $[Ca^{2+}]_i$. To the contrary, PTX pretreatment inhibited the $[Ca^{2+}]_i$ increase in the cells incubated with psychosine, which is known to activate phospholipase C via the PTX-sensitive G-proteins.[13] The MTT assay also showed no effect of PTX-pretreatment on the viability of FTY720-treated HL-60 cells (Fig. 7b). These results indicate that a different activation pathway than that via PTX-sensitive G-proteins may be involved in FTY720-induced apoptosis.

DISCUSSION

The present study demonstrated that FTY720 may activate the phospholipase C-Ca^{2+} pathway to induce apoptosis in HL-60 cells. This action mechanism is quite unique and not observed in other current immunosuppressive drugs, including cyclosporin A,[28,29] FK506[30,31] and rapamycin.[32,33] Likewise, glucocorticoids[34,35] caused a Ca^{2+} influx, increased the $[Ca^{2+}]_i$, then induced cell death in immature lymphocytes, but the Ca^{2+} influx occurred much later than 30 min after *in vitro* treatment.[35] The increase in $[Ca^{2+}]_i$ may activate Ca-dependent degradative enzymes, including Ca-dependent endonucleases and Ca-activated proteinases, accompanied by mitochondrial damage.[36,37] Thus, a continuous increase of $[Ca^{2+}]_i$ through stimulation by FTY720 may trigger apoptotic cell death.

The pharmacological effect of FTY720 was first found in the *in vitro* inhibition of MLR and later in the *in vivo* disappearance of peripheral lymphocytes from lymphocyte deletion.[6] The lymphocytes with apoptotic morphology were ingested in the phagocytic cells in the thymus, spleen, and lymph nodes in *lpr/lpr*-mice treated with FTY720. These mice exhibited recovery from symptoms such as an unusual pro-

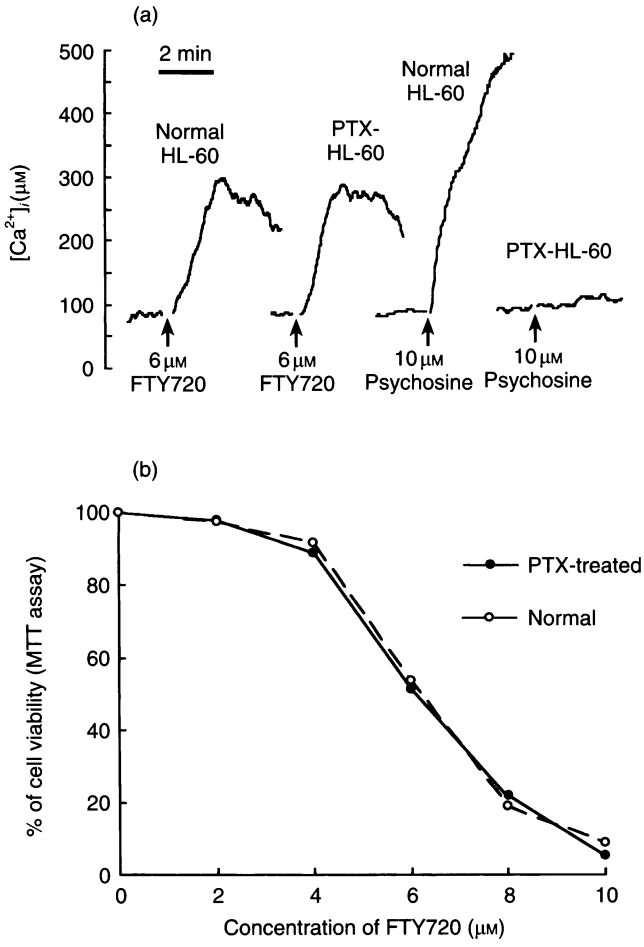

Figure 7. Effect of PTX on FTY720-induced increase of $[Ca^{2+}]_i$ and cell death in HL-60. (a) Both PTX-treated and non-treated HL-60 cells show an increase of $[Ca^{2+}]_i$ to a similar extent by the addition of FTY720. As a control experiment, PTX-treated HL-60 cells did not increase $[Ca^{2+}]_i$ by the addition of psychosine, a $[Ca^{2+}]_i$-mobilizing agent via activation of PTX-sensitive G-protein. (b) HL-60 cells pretreated for overnight with 50 ng/ml PTX show no difference of cell viability from the non-pretreated cells, when cultured for 6 hr with 0–10 μM FTY720.

portion of $CD3^+B220^+$ cells in all lymphoid organs, and their lives were prolonged.[7]

The DNA fragmentation and cell death were related to a sustained increase in the cytosolic Ca^{2+} concentration, as shown in Figs 2 and 3. A slight difference was observed in the concentrations of FTY720 between the onset of cell death by MTT assay (4 μM FTY720) and calcium mobilization (1 μM FTY720). This may be due to the serum used in the culture medium; a low concentration of FCS enhances the killing effect of FTY720. In addition, HL-60 grows slowly in a medium with a low concentration of FCS. The effective dose of FTY720 may also depend on the medium components used in the cell culture.

We previously reported that Jurkat(bcl-2) cells were resistant to FTY720 and that Jurkat(neo) cells were susceptible to the drug.[8] However, no difference in $[Ca^{2+}]_i$ increase was seen between the two types of cells in the present experiment, as shown in Fig. 4. These results may confirm that the expression of the oncoprotein Bcl-2 plays a role in protection from apoptosis induced by the stimuli of high $[Ca^{2+}]_i$ in cells exposed to the Ca^{2+} ionophore and ionomycin, which was also reported in other studies.[38,39]

The increased $[Ca^{2+}]_i$ was not inhibited in the FTY720-treated HL-60 cells, even in the presence of a chelating reagent for calcium ions, suggesting that the Ca^{2+} was mobilized from the intracellular calcium pool. This evidence was further confirmed by treatment of the cells with thapsigargin. The pathway for induction of apoptosis could be triggered by the activation of phospholipase C, which would then increase $[Ca^{2+}]_i$.

Conversely, it is also possible that the Ca^{2+} activated the phospholipase C (inositol triphosphate-induced entry of the outer calcium into the cytoplasm, an IP_3-sensitive calcium channel) or directly activated the intracellular Ca^{2+} pools to increase the $[Ca^{2+}]_i$ (calcium-induced calcium release, CICR), as described by Berridge.[40,41] Okajima *et al.*[13] described a deficiency of Ca^{2+}-induced phospholipase C activation in HL-60 cells, since ionomycin and thapsigargin did not activate phospholipase C in these cells. The present study showed that HL-60 cells treated with FTY720 had increased $[Ca^{2+}]_i$, even in a culture medium containing excess EGTA. This observation suggests that neither the IP_3-sensitive calcium influx nor CICR were mediated for increasing the $[Ca^{2+}]_i$ in FTY720-treated HL-60 cells.

Phospholipase C is activated by various types of GTP-binding proteins (G-protein), tyrosine kinase, and other factors.[41] Evidence that PTX-treated cells increase the $[Ca^{2+}]_i$ through treatment with FTY720 (Fig. 7) suggests that the PTX-sensitive G-proteins did not cooperate with the drug. We are now searching for clear evidence of other possible activation pathways.

FTY720 has a similar chemical structure to sphingosine, a naturally existing substance. Sphingosine and sphingolipids were recently reported to increase Ca^{2+} in HL-60 cells. We also know that sphingosine inhibits protein kinase C[42] and induces apoptosis.[43,44] However, FTY720 could not inhibit protein kinase C in cell lysates, even at high concentrations (20–30 μM) (our preliminary data), hence it is unlikely that FTY720-induced apoptosis was triggered by the direct inhibition of protein kinase C.

ISP-1, an original compound of FTY720, inhibited the proliferation of the IL-2-dependent mouse cytotoxic T-cell line CTLL-2 at nanomole concentrations, and inhibited serine palmitoyltransferase activity at picomole concentrations,[45] suggesting that serine palmitoyltransferase may be the primary target of ISP–1. Thus, the sphingolipids-related pathways for the action site of FTY720 are not yet clarified.

In summary, FTY720 acts via intracellular Ca^{2+} mobilization, which may trigger an apoptotic reaction in HL-60. The drug has demonstrated potent immunosuppressive activity in various experimental animals. Therefore, FTY720 may yield important knowledge regarding the signal pathway of lymphocyte apoptosis and lymphocyte developments as well as the fundamental process of the immunological response in transplantation immunity.

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