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T cell selective apoptosis by a novel immunosuppressant, FTY720, is closely regulated with Bcl-2

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1 A novel immunosuppressant FTY720 caused a significant decrease in peripheral T lymphocytes, but not in B lymphocytes upon oral administration. This decrease was mainly a result of FTY720-induced apoptosis. In this study, we confirmed FTY720-induced T cell selective apoptosis using lymphoma cell lines *in vitro*.

2 Viability loss, DNA fragmentation, Annexin V binding, and caspases activation (caspase-3, -8, and -9) were observed in Jurkat cells (T lymphoma cells), but not significantly in BALL-1 cells (B lymphoma cells). These results indicated that FTY720 selectively induced apoptosis in T cell lymphoma to a greater extent than in B cell lymphoma, a finding that is similar to the result observed when FTY720 was treated with T lymphocytes and B lymphocytes *in vitro*.

3 FTY720 released cytochrome *c* from mitochondria in Jurkat intact cells as well as from isolated Jurkat mitochondria directly, but not from mitochondria in BALL-1 cells nor from isolated BALL-1 mitochondria.

4 BALL-1 cells and B cells had more abundant mitochondria-localized anti-apoptotic protein Bcl-2 than did Jurkat cells and T cells.

5 FTY720-induced apoptosis is inhibited by the overexpression of Bcl-2, suggesting that the cellular Bcl-2 level regulates the sensitivity to FTY720.

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Keywords: FTY720; immunosuppressant; apoptosis; mitochondria; lymphoma cells; Bcl-2

Abbreviations: FITC, fluorescein isothiocyanate; $\Delta\Psi_m$, mitochondrial membrane potential; mAb, monoclonal antibody; PBS, phosphate buffered saline; PE, phycoerythrin; PI, propidium iodide; PI3K, phosphatidylinositol 3′-kinase; PP2A, protein phosphatase 2A; PS, phosphatidylserine; PTPC, permeability transition pore complex

Introduction

Bcl-2 is localized mainly to the membranes of the mitochondria, and possesses anti-apoptotic activity (Krajewski *et al*., 1993). Bcl-2 prevents the opening of an apoptosis-related pore opening called mitochondrial permeability transition pore complex (PTPC), which is the basis for the mitochondria-mediated apoptosis pathway (Decaudin *et al*., 1997; Zamzami *et al*., 1998). This complex consists of several proteins, including hexokinase, cyclophilin D, and adenine nucleotide translocator, and a voltage-dependent anion channel (Zoratti & Szabo, 1995). PTPC opening by pro-apoptotic members of the Bcl-2 family of proteins, e.g., Bax, or through the modulation of PTPC by chemicals results in a rapid loss of mitochondrial membrane potential ($\Delta\Psi_m$) and organelle swelling (Fulda *et al*., 1998; Narita *et al*., 1998; Zamzami *et al*., 1998). PTPC opening also releases cytochrome *c*. Cytochrome *c*, with the involvement of apaf-1, activates the initiator caspase, caspase-9, and activated caspase-9 in turn activates the effector caspase, caspase-3. Activated caspase-3 initiates further apoptotic processes (Li *et al*., 1997; Liu *et al*., 1996; Zou *et al*., 1997). However, the release of cytochrome *c* can also occur in the absence of $\Delta\Psi_m$ loss, suggesting that PTPC may not be the only target of the Bcl-2 family proteins to release cytochrome *c* (Bossy-Wetzel *et al*., 1998; Eskes *et al*., 1998). In some types of apoptotic cell death, the anti-apoptotic effect of Bcl-2 is abrogated. Death receptors, e.g., Fas, aggregate and form a complex. This complex recruits through several adapter proteins and multiple procaspase-8, resulting in caspase-8 activation through induced proximity (Muzio *et al*., 1996; 1998). Activated caspase-8 in turn activates caspase-3, bypassing the mitochondria-mediated apoptotic pathway (Scaffidi *et al*., 1998).

FTY720, 2-amino-2- [2-(4-octylphenyl) ethyl]-1,3-propanediol hydrochloride, was screened from synthesized analogues of ISP-1, which is an immunosuppressive metabolite of *Isaria sinclairii* (Fujita *et al*., 1994; 1996). Unlike other conventional immunosuppressants that are currently used, the action mechanism of FTY720 to suppress graft rejection is thought to be a significant decrease in the number of peripheral blood lymphocytes, especially T cells (Enosawa *et al*., 1996). FTY720 does not interfere with cytokine production as do cyclosporine A and FK506 (Dumont *et al*., 1990; Nelson, 1984), nor does it inhibit DNA biosynthesis as do azathioprine, mizoribine and RS61443 (Behrend, 1996; Koyama & Tsuji, 1983; van Furth *et al*., 1975), suggesting that this drug has a unique immunosuppressive action. We have previously demonstrated that the

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blood lymphocyte decrease was mainly a result of FTY720-induced apoptosis (Nagahara *et al*., 2000a). FTY720 also induces apoptosis significantly and rapidly in lymphoma cells, demonstrating a potent ability for apoptosis induction (Suzuki *et al*., 1996b). The FTY720-induced cell death mechanism has been studied. FTY720-induced apoptosis is observed in splenocytes from MRL *lpr*/*lpr* mice, and is blocked by Bcl-2 overexpression, demonstrating that FTY720-induced apoptosis might be involved with the mitochondria-mediated pathway (Suzuki *et al*., 1996b; 1997). Recently, we clarified that FTY720 directly perturbed mitochondria function, which resulted in a decrease of mitochondrial permeability transition and a release of cytochrome *c* from the mitochondria to cytosol, followed by the activation of caspase-3 (Nagahara *et al*., 2000b).

Allograft rejection occurred by intragraft immune response, including the infiltration of mature T cells into the graft and the activation of these T cells by Th1-associated cytokines (Josien *et al*., 1995; Kupiec-Weglinski *et al*., 1993). Thus, modulating and investigating the apoptosis mechanisms of T cells would be greatly beneficial to the field of organ transplantation.

In this study, we examined the sensitivity to FTY720-induced apoptosis using T cells and B cells *in vitro*. We also described how these sensitivity differences occurred using T cell and B cell lymphomas.

Methods

Cells

The human lymphoid B cell line BALL-1 was obtained from Japanese Collection of Research Bioresources (Tokyo, Japan). The human lymphoid T cell line, Jurkat, stably transfected with a human bcl-2 expression plasmid (bcl-2) or neomycin resistance vector (neo) was provided by Dr T. Miyashita of the National Research Institute for Child Health and Development (Tokyo, Japan).

Animals

PVG rats were purchased from Crea Japan (Tokyo, Japan). All rats were 8–14 weeks of age. The rats were given *ad libitum* access to standard chow and water, and housed in our departmental animal room under specific pathogen-free conditions.

Drugs

2-amino-2-[2-(4-octylphenyl) ethyl]-1,3-propanediol hydrochloride (FTY720) was synthesized and supplied in powder form by Taito (Tokyo, Japan), in cooperation with Mitsubishi Pharma (Osaka, Japan). FTY720 was dissolved in saline (1 mM).

Medium and cell cultures

Cells were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum and 75 mg l^{-1}-Kanamycin, and maintained at 37°C in a humidified chamber under an atmosphere of 95% air and 5% CO_2.

Annexin V binding with selection of lymphocytes

Three to 5 ml of heparinized blood was diluted with an equal volume of phosphate buffered saline (PBS), overlaid on 3 ml of Ficoll-Conray solution ($d=1.090$, Lympho-Separ II; Immuno-Biological Laboratories, Gumma, Japan), and centrifuged at $300\times g$ for 30 min at room temperature. The lymphocytes in the interface were collected and washed three times with PBS. All cells were washed, suspended at a density of 2×10^5 cells ml^{-1} in fresh culture medium and incubated for 2 h with 8 μM FTY720. Lymphocytes (1×10^6) were suspended in Annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, 0.2% bovine serum albumin). Either biotinylated R73 monoclonal antibody (mAb) (anti-rat T cell receptor; Serotec, Oxford, U.K.) or biotinylated OX33 mAb (anti-rat B cell; Serotec) was added at a dilution of 1 : 100 and the solution was incubated for 20 min at room temperature. The cells were washed twice with Annexin V binding buffer and then suspended in the same buffer. Next, 2.5 μl of fluorescein isothiocyanate (FITC)-Annexin V and 1 : 25 dilution of streptavidin-phycoerythrin (PE)-conjugate (PharMingen, San Diego, CA, U.S.A.) were added and incubated for 20 min at room temperature. The stained cells were washed once with Annexin V binding buffer, and the pellets were then suspended in 0.5 ml of PBS containing 2 μg ml^{-1} propidium iodide (PI). Samples were analysed by 3-colour flow cytometry (FACScan; Becton Dickinson, Mountain View, CA, U.S.A.). Lymphocyte type was detected using a corresponding PE-mAb. PI positive cells were omitted from the analysis. PI negative and FITC-Annexin V positive cells were considered apoptotic.

MTT assay

Cells (2×10^4) were incubated in 96-well flat-bottomed plates for 3 h. Thereafter, 10 μl of 5 mg ml^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St. Louis, MO, U.S.A.) was added to each well and incubated further for 1 h. The plates were centrifuged at $400\times g$ for 5 min and the supernatants were discarded. Coloured formazan in the living cells was developed by adding 100 μl of dimethyl sulphoxide to each well, and the absorbance of each well was measured using a microplate reader with 570 nm. Cell viability was expressed as a percentage of control absorbance.

Nuclear morphology

Cells (4×10^5) were incubated in dish culture with or without FTY720. After incubation, 5 μM Hoechst 33342 and 2 μg ml^{-1} PI was added and the number of viable, apoptotic, and necrotic cells were counted by fluorescence microscopy.

DNA fragmentation

Apoptosis was determined by DNA fragmentation, which was assessed by agarose gel electrophoresis. Cells (2×10^6) were rinsed once with 10 mM Tris-HCl buffer, pH 8.7, containing 3 mM $MgCl_2$ and 2 mM 2-mercaptoethanol and dissolved in 50 mM Tris-HCl buffer, pH 7.8, containing 10 mM EDTA, 0.5% sodium lauryl sarcocinate, and

1 mg ml^{-1} proteinase K. After incubation at 50°C for 30 min, RNase A was added at a concentration of 0.5 mg ml^{-1} and further incubated at 50°C for 15 min. Lysates were mixed with an equal volume of loading buffer containing TBE buffer (89 mM Tris, pH 8.4, 2.5 mM EDTA, 89 mM boric acid), 20% glycerol, and 0.01% bromophenol blue. Samples were electrophoresed on 1.8% agarose gels in TBE containing 0.5 mg ml^{-1} ethidium bromide.

Annexin V binding

Cells (2×10^5) were washed with PBS, suspended in 0.1 ml of Annexin V binding buffer containing 2.5 μl of FITC-Annexin V, and incubated for 10 min at room temperature. Cells were washed with PBS, resuspended in 0.5 ml of PBS containing 2 μg ml^{-1} PI, and analysed by flow cytometry.

Preparation of mitochondria and cytosol fractions, or cell lysates

Cells (1×10^7) were washed with PBS, suspended in 5 volumes of CFS buffer (mM: mannitol 220, sucrose 68, NaCl 2, KH_2PO_4 2.5, EGTA 0.5, $MgCl_2$ 2, pyruvate 5, phenylmethylsulphonylfluoride 0.1, HEPES 10, pH 7.4) and swollen on ice for 20 min. Cells were disrupted by 10 aspirations through a 22 gauge needle and centrifuged at $750 \times g$ for 5 min at 4°C to remove the nuclei. The supernatant was centrifuged again ($10{,}000 \times g$, for 10 min, at 4°C) to recover mitochondria. The $10{,}000 \times g$ supernatant was ultracentrifuged at $100{,}000 \times g$ for 30 min at 4°C and the remaining supernatant was used as the cytosol fraction. Isolated mitochondria were used immediately for cell-free system assays. Cytosolic samples were used immediately or stored at −80°C until immunodetection of cytochrome *c*. For the preparation of cell lysates with immunodetecting cellular Bcl-2 or caspases, cells were lysed with lysis buffer (mM: Tris 50, pH 7.5, 1% Nonidet-P40 50, NaCl 150, NaF 50, EDTA 1, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, phenylmethylsulphonylfluoride 0.5, and sodium orthovanadate 0.1).

Western blots

Mitochondria and cytosol fractions or cell lysates (15 μg) 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^{-1} bromophenol blue, 10% 2-mercaptoethanol) and heated at 65°C for 10 min. Proteins were separated by 4–20% gradient polyacrylamide gel SDS-electrophoresis and electrically transferred to a PVDF membrane (Millipore, Bedford, MA, U.S.A.). After blocking the membrane using 3% skimmed milk, cytochrome *c*, Bcl-2, Bcl-x_L, caspase-3, caspase-8, and caspase-9 were immunodetected using mouse anti-cytochrome *c* mAb (1 : 3000; PharMingen), rabbit anti-Bcl-2 polyclonal Ab (1 : 2000; Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.), rabbit anti-Bcl-x_L polyclonal Ab (1 : 2000; Santa Cruz), rabbit anti-caspase-3 polyclonal Ab (1 : 500; Santa Cruz), rabbit anti-caspase-8 polyclonal Ab (1 : 500, Santa Cruz), or mouse anti-caspase-9 polyclonal Ab (1 : 1000; MBL, Nagoya, Japan). Thereafter, horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG were applied as second antibodies, and positive bands were detected by enhanced chemiluminescence (Amersham Life Science, Buckinghamshire, U.K.).

Detection of Bcl-2 by flow cytometry

Lymphocytes (2×10^6) were suspended in PBS. Either FITC-R73 mAb (PharMingen) or FITC-OX33 mAb (PharMingen) was added at a dilution of 1 : 50, and the solution was incubated for 20 min at room temperature. The cells were washed once with PBS, and permealized by incubating in permealizing buffer (0.5% saponin, 0.5% bovine serum albumin, 0.1% NaN_3 in PBS) for 10 min at room temperature. After centrifugation, a 1 : 50 dilution of rabbit anti-Bcl-2 polyclonal Ab was added and incubated for 30 min at room temperature. The stained cells were washed once with permealizing buffer and the pellets were then suspended in permealizing buffer including 1 : 100 dilution of PE-conjugated anti-rabbit IgG and incubated for 30 min at room temperature. After the cells were washed once, the samples were analysed by two-colour flow cytometry. Lymphocyte type was detected using a corresponding FITC-mAb.

Determination of isolated mitochondrial cytochrome c *release*

Isolated mitochondria were washed and resuspended in CFS buffer at a concentration of approximately 100 μg mitochondrial protein in 100 μl CFS buffer. After incubating with or without drugs for the indicated periods at 37°C, the reaction mixtures were centrifuged ($10{,}000 \times g$, for 10 min, at 4°C). The mitochondria pellet and supernatant were separated and immediately used or stored at −80°C until the immunodetection of cytochrome *c*.

Determination of isolated mitochondrial membrane potential

Isolated mitochondria were washed and resuspended in CFS buffer at a concentration of approximately 25 μg mitochondrial protein in 100 μl CFS buffer. After incubation with or without drugs for 30 min at 37°C, the mitochondrial suspensions were further incubated for 15 min with 50 nM $DiOC_6$ (3). Mitochondrial $\Delta\Psi_m$ was examined by flow cytometry.

Statistical analysis

All statistical analyses were performed using Student's *t*-test. Significance was established at the $P < 0.05$ level.

Results

Peripheral T cells were more sensitive to FTY720 than peripheral B cells

Oral administration of 10 mg kg^{-1} FTY720 to rats rapidly decreases the number of peripheral blood lymphocytes (by about half) within an hour, which is mainly a result of apoptosis. Most importantly, apoptosis is induced in peripheral T cells by the administration of FTY720, whereas

peripheral B cells are unaffected (Nagahara *et al*., 2000a). This FTY720-induced T cell selective apoptosis *in vivo* is assumed to be a main action mechanism of immunosuppression. Enosawa *et al*. (1996) revealed with PI staining that peripheral T cells were highly damaged with 3 h of 10 μM FTY720 treatment *in vitro*. However, peripheral B cells were relatively resistant to FTY720, though longer treatment (6 h) induced damage also to the B cells. First, we determined whether this T lymphocyte-selective damage was a result of FTY720-induced apoptosis *in vitro*. Rat peripheral blood lymphocytes were separated and incubated *in vitro* with 8 μM FTY720 for 2 h. Thereafter, lymphocytes were analysed by triple staining with FITC-labelled Annexin V, mAbs specific to T and B cells, and PI. After excluding the PI-positive cells as dead cells, we estimated the Annexin V binding ability of peripheral T or B cells. Annexin V specifically binds with phosphatidylserine (PS), which becomes exposed to the outer cell membrane from the inner cell membrane during apoptosis. PS exposure is believed to occur in the earlier phase of apoptotic cell death. Figure 1a shows the Annexin V binding ability to control cells and FTY720-treated cells. For estimating the effect of FTY720 to each cell types, Annexin V binding ratio of FTY720-treated groups was divided by the ratio of the control groups (Figure 1b). FTY720 markedly induced Annexin V binding to peripheral T cells. In contrast, peripheral B cells had about 50% less ability to bind with Annexin V than did T cells. This result suggested that after the lymphocytes fell into apoptosis, PS was exposed on the outer cell membrane, and thereafter the membrane collapsed (cells were stained with PI). Moreover, this result was coincident with the finding of Enosawa *et al*. (1996) that T cells were sensitive to FTY720. FTY720 induces apoptosis significantly in peripheral T cells *in vitro* just as it had in the *in vivo* experiment (Nagahara *et al*., 2000a).

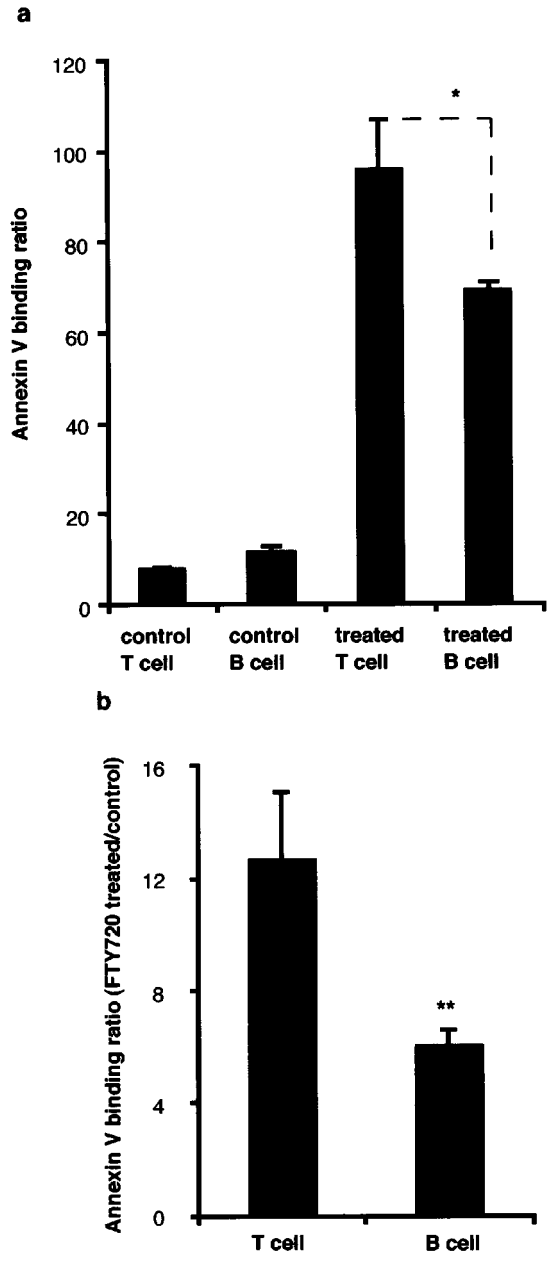

Figure 1 T cell selective apoptosis by FTY720 *in vitro*. Rat peripheral lymphocytes were corrected (1×10^6) and incubated with 8 μM FTY720 for 2 h. T cell and B cell apoptosis was detected with FITC-Annexin V, specific mAbs and PI as described in Methods. Annexin V positive and negative cells were counted and the Annexin V binding ratio was calculated for FTY720-treated T cells, FTY720-treated B cells, control T cells, and control B cells by dividing positive cells by negative cells (a). Finally, the Annexin V binding ratio of FTY720-treated groups was divided by the ratio of the control groups (b). Each bar denotes standard deviation ($n=3$). $^*P<0.05$, $^{**}P<0.01$ compared to T cells.

T lymphoma cells were sensitive to FTY720 than B lymphoma cells

For further analysis of peripheral T cell selective apoptosis by FTY720, we used T and B lymphoma cells. The cell viabilities of T lymphoma (Jurkat) and B lymphoma (BALL-1) under FTY720 treatment were estimated. As shown in Figure 2, the viability of Jurkat cells was significantly reduced by FTY720 in a dose-dependent

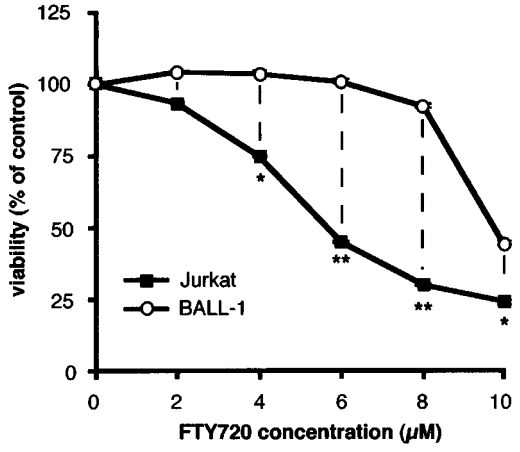

Figure 2 Viability of T, B cell lymphoma after treatment with FTY720. Jurkat cells and BALL-1 cells (2×10^4) were incubated for 4 h with and without FTY720 at concentrations ranging from 2 to 10 μM. At the indicated time, the cells were corrected and subjected to MTT assay. The results are presented as the percentage of absorbance in untreated cultures. Each bar denotes standard deviation ($n=4$). $^*P<0.05$, $^{**}P<0.01$ compared to the same dose of BALL-1 cells.

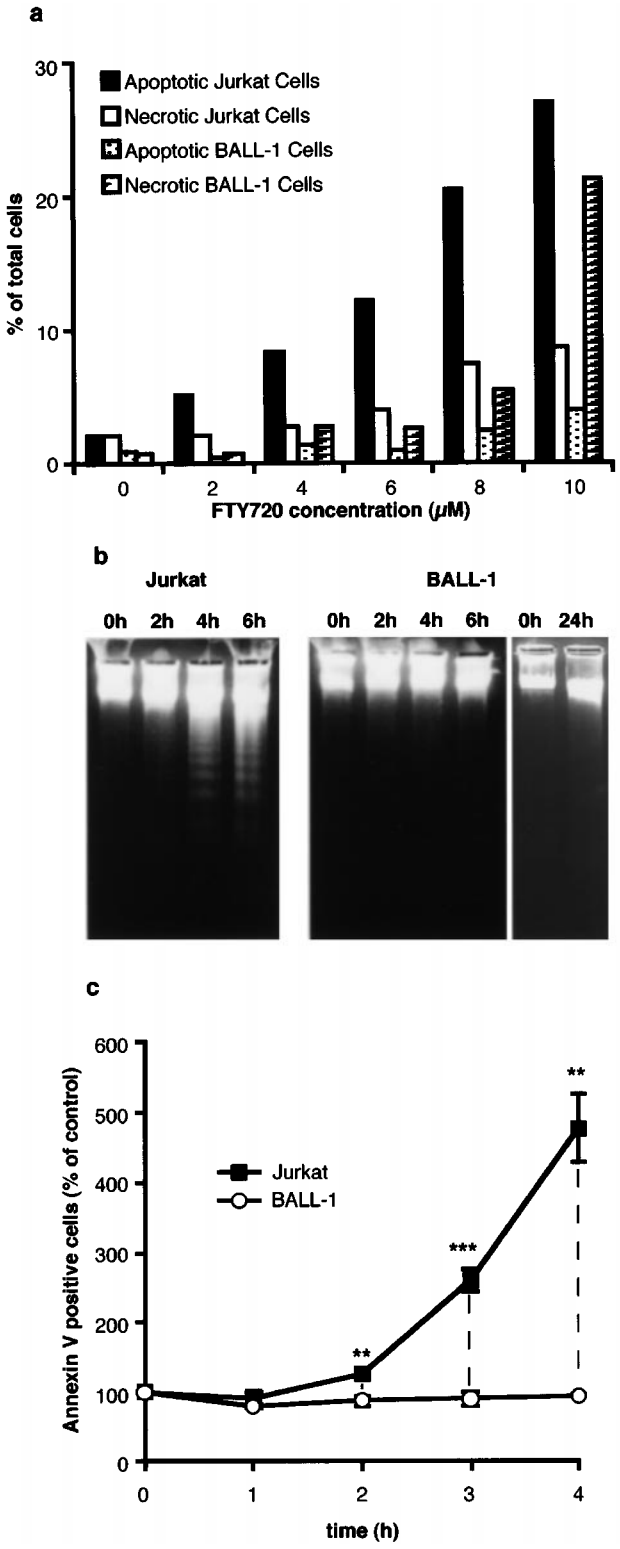

Figure 3 FTY720-induced T lymphoma selective apoptosis. (a) Jurkat (neo) cells and BALL-1 cells were incubated for 4 h with or without FTY720 at concentrations ranging from 2 to 10 μM. At the indicated times, the cells were corrected and stained with Hoechst 33342 and PI. Cells were counted using fluorescence microscopy and percentage of blue- and red-stained bubbled nuclei cells (apoptotic cells) and red-stained damaged cells (necrotic cells) in whole cells were estimated. Data are representative of three independent experiments. (b) Jurkat (neo) cells and BALL-1 cells were incubated with 8 μM FTY720 for 0, 2, 4, and 6 h, respectively and 24 h for BALL-1 cells. At the times indicated, the cells (1×10^6) were lysed and DNA was prepared. DNA fragmentation was analysed by agarose gel electrophoresis. (c) Jurkat (neo) cells and BALL-1 cells were incubated with 8 μM FTY720 for 0, 1, 2, and 3 h, respectively. At the times indicated, the cells (2×10^5) were stained with FITC-Annexin V. Binding of Annexin V to cells was indicated by FITC fluorescence with flow cytometry. The results are presented as the percentage of Annexin V positive cells (FTY720-treated per control). Each bar denotes standard deviation ($n=4$). $^{**}P<0.01$, $^{***}P<0.001$ compared to the same dose of BALL-1 cells.

manner. However, BALL-1 cells were not as susceptible to FTY720 as Jurkat cells. BALL-1 cells did not change their viability, even when 8 μM FTY720 was treated, though the viability of Jurkat cells was reduced to about 30%. In addition, when 10 μM FTY720 was treated, BALL-1 cells lost their viability to about 50%. Overall, T lymphoma is more sensitive to FTY720 than B lymphoma, just as T cells are more sensitive than B cells.

FTY720 selectively induced apoptosis in T lymphoma cells to a greater extent than in B lymphoma cells

We performed three experiments to detect whether apoptotic cell death occurred in these cell lines. Figure 3a shows the morphological change with FTY720. Staining with the cell permeable fluorogenic substrate Hoechst 33342 and cell impermeable fluorogenic substrate PI, the number of apoptotic cell (blue- or red-stained bubbled polynuclei cells) and necrotic cell (red-stained damaged cell) was counted. Jurkat cells gradually fell into apoptosis, though BALL-1 cells are resistant within 8 μM of FTY720 treatment. However, 10 μM FTY720 treatment to BALL-1 cells induced nectrotic cell death. In addition, 12 μM FTY720 treatment to Jurkat cells also induces necrosis (data not shown). Thus, we used 8 μM doses of FTY720 for following studies. Figure 3b shows DNA fragmentation, a hallmark of apoptosis, by agarose gel electrophoresis. The DNA of Jurkat cells was fragmented and made into a ladder form within 4 h of FTY720 treatment, though the DNA of BALL-1 cells did not fragment with 6 h of treatment. BALL-1 cells are resistant until 18 h of FTY720 treatment (Matsuoka *et al*., in preparation), and only a slight necrotic DNA degradation was observed with 24 h of treatment (Figure 3b). Moreover, the binding potential of FITC-Annexin V between FTY720-treated cells and control cells was estimated. Results similar to those shown in Figure 3a were obtained, namely that Jurkat cells exposed PS more quickly and to a greater extent than did BALL-1 cells. These results indicate that FTY720 selectively induces apoptosis in Jurkat cells to a greater extent than in BALL-1 cells (Figure 3b). Further experiments were performed to investigate the involvement of apoptosis-mediator, caspase family proteases using Western blots. FTY720 cleaved procaspase-9 (47 kD) and procaspase-3 (32 kD) to its active form (37/35 kD and 17 kD, respectively) in Jurkat cells within 4 h treatment (Figure 4). Again, no cleavage was observed in BALL-1 cells. BALL-1 cells have the same level of procaspases as Jurkat cells, suggesting that the procaspases' expression level was not attributable to the FTY720 sensitivity between those cell types. However, only slight activation was observed in caspase-8 (43/41 kD cleavage), in which FTY720 seemed to activate mitochon-

dria-involved caspases (Figure 4). No fully activated caspase-8 (18 kD) band was observed, even after 6 h treatment of FTY720 (data not shown). In conclusion, FTY720 induced caspases of the post-mitochondria-involved apoptosis pathway to Jurkat cells, but not to BALL-1 cells.

FTY720 induced cytochrome c *release from mitochondria to Jurkat cells but not to BALL-1 cells*

As previously reported, FTY720 induces cytochrome *c* release from mitochondria to cytosol. Using pan-caspase inhibitor Z-VAD-FMK, FTY720-induced DNA fragmentation and caspase activation is blocked, although cytochrome *c* release is not inhibited (Nagahara *et al*., 2000b). These results indicate that FTY720-induced cytochrome *c* release is independent of caspase activation, and this FTY720-induced mitochondrial perturbation was essential for FTY720-induced apoptosis. To determine cytochrome *c* release, both Jurkat and BALL-1 cytosol fraction were prepared and detected by Western blotting. FTY720 induced a cytochrome *c* release to cytosol within 4 h in Jurkat cells but not in BALL-1 cells (Figure 5), suggesting that BALL-1 mitochondria are resistant to FTY720 or that FTY720 intracellular concentration is different between those cells.

Bcl-2 was highly expressed in FTY720-insensitive BALL-1 cells

Because two hypotheses for FTY720-induced T lymphoma cells selective apoptosis arose, as described above, we considered the first hypothesis, that the difference in mitochondria regulated the susceptibility to FTY720, for the following reasons. A recent study described that FTY720 decreased the activity of Akt (also known as protein kinase B), which is the major mediator of survival signal that protect cells from undergoing apoptosis (Matsuoka *et al*., in preparation). Phosphorylation of Akt by tyrosine kinase receptor-phosphatidylinositol 3′-kinase (PI3K) pathway results in activation of this kinase (Toker, 2000). However, FTY720 did not inhibit PI3K pathway kinases or Akt itself, but activated protein phosphatase 2A (PP2A), suggesting that FTY720 activated PP2A and dephosphorylated Akt. In addition, FTY720 activated purified PP2A directly. FTY720 caused dephosphorylation of Akt in the same time-course in both Jurkat and BALL-1 cells, presuming that the intracellular concentrations of FTY720 in both cells were equivalent. Moreover, B lymphomas tend to express mitochondria-localized anti-apoptotic protein, Bcl-2, more than T lymphomas do (Akagi *et al*., 1994; Zutter *et al*., 1991).

Because our previous studies revealed that Bcl-2 overexpression prevented FTY720-induced apoptosis, including cytochrome *c* release and caspase activation (Nagahara *et al*., 2000b), we assumed that BALL-1 cells expressed Bcl-2 more highly than Jurkat cells. We detected the Bcl-2 protein expression level with Jurkat cells and BALL-1 cells. Western blotting of Bcl-2 clearly showed that BALL-1 cells expressed it more abundantly than did Jurkat cells (Figure 6a). The expression level of Bcl-2 in BALL-1 was the same as in the

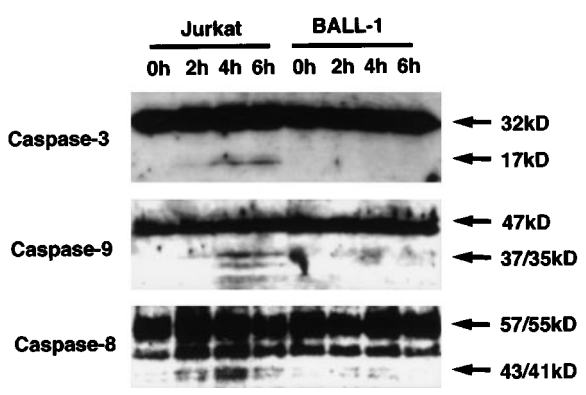

Figure 4 FTY720-induced caspases activation. Jurkat (neo) cells and BALL-1 cells (2×10^6) were incubated with 8 μM FTY720 for 0, 2, 4, and 6 h, respectively. At the times indicated, cells were lysed (15 μg) and caspase-3, caspase-9, and caspase-8 were detected by Western blotting.

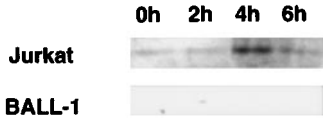

Figure 5 Cytochrome *c* released to cytosol with FTY720. Jurkat (neo) cells and BALL-1 cells (1×10^7) were incubated with 8 μM FTY720 for 0, 2, 4, and 6 h, respectively. At the times indicated, cytosolic fractions were prepared and cytosolic cytochrome *c* was detected by Western blotting.

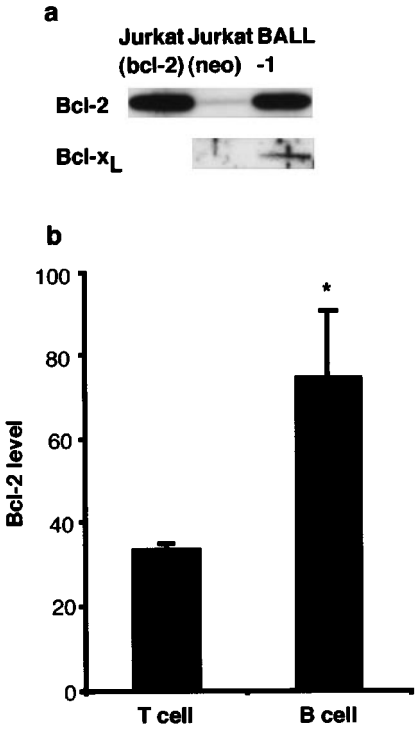

Figure 6 Differences of Bcl-2 expression among cell lines. (a) Jurkat (neo) cells, Jurkat (bcl-2) cells, and BALL-1 cells were lysed (15 μg) and Bcl-2 and Bcl-x_L was detected by Western blotting. (b) Rat peripheral lymphocytes were permealized and the T cell and B cell Bcl-2 level were detected by using specific antibody as described in Methods. The results are presented as averages of the median fluorescence intensity. Each bar denotes standard deviation ($n=4$). *$P<0.05$ compared to T cells.

Jurkat cells that overexpressed Bcl-2, Jurkat (bcl-2), which abrogated FTY720-induced apoptosis in our previous study. Another anti-apoptotic Bcl-2 family protein, Bcl-x_L, was also expressed more highly in BALL-1 than in Jurkat, suggesting that BALL-1 cells seem to be less susceptible to mitochondria-involved apoptosis (Figure 6a).

We also determined the Bcl-2 expression level in rat peripheral T cells and B cells. Flow cytometric analysis revealed that peripheral B cells have higher levels of Bcl-2 than do peripheral T cells (Figure 6b).

FTY720 did not affect BALL-1 mitochondria

The FTY720-insensitive BALL-1 mechanism was further examined. Isolated mitochondria from Jurkat cells reduced $\Delta\Psi_m$ and released cytochrome *c* as a result of FTY720 treatment. Overexpression of Bcl-2 completely blocked these phenomena (Nagahara *et al*., 2000b). Because BALL-1 mitochondria expressed abundant Bcl-2, we considered the possibility that they may inhibit FTY720-induced mitochondria perturbation. Actually, cytochrome *c* release was inhibited when isolated BALL-1 mitochondria were treated with FTY720 (Figure 7a). The same results were obtained with isolated Jurkat (bcl-2) mitochondria, but mitochondria isolated from Jurkat cells that do not overexpress Bcl-2, Jurkat (neo), released cytochrome *c* directly after FTY720 treatment. Moreover, isolated BALL-1 mitochondria did not reduce $\Delta\Psi_m$ and neither did isolated Jurkat (bcl-2) mitochondria, but $\Delta\Psi_m$ was greatly decreased in isolated Jurkat (neo) mitochondria (Figure 7b).

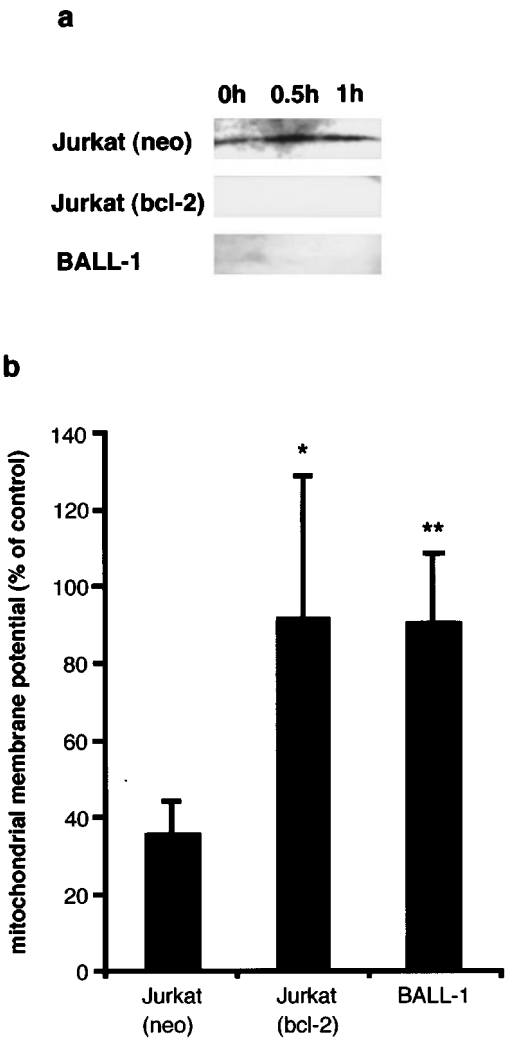

Figure 7 FTY720 did not affect isolated Bcl-2 rich mitochondria. (a) Mitochondria from Jurkat (neo), Jurkat (bcl-2), and BALL-1 cells (100 μg/100 μl) were prepared as described in Methods. Mitochondria were incubated with no additives and 10 μM FTY720 for 0, 30, and 60 min at 37°C. Mitochondrial supernatant was prepared and cytochrome *c* was detected by Western blotting. (b) Mitochondria from Jurkat (neo), Jurkat (bcl-2), and BALL-1 cells (25 μg/100 μl) were prepared as described in Methods. After incubation with or without drugs for 30 min at 37°C, the mitochondrial suspensions were further incubated for 15 min with 50 nM $DiOC_6$ (3). Mitochondrial $\Delta\Psi_m$ was examined by flow cytometry. The results are presented as averages of the median fluorescence intensity (FTY720-treated per control). Each bar denotes standard deviation ($n=3$). $^*P<0.05$, $^{**}P<0.01$ compared to isolated mitochondria from Jurkat (neo) cell groups.

Discussion

In the present study, we demonstrated that T lymphoma is more susceptible to FTY720-induced apoptosis than B lymphoma is. Viability loss, DNA fragmentation, and PS exposure to the outer membrane occurred commonly in Jurkat (neo) cells but not significantly often in BALL-1 cells. Cytochrome *c* was released by FTY720 in intact cells and even in isolated mitochondria in Jurkat (neo) cells but not in BALL-1 cells. Bcl-2 overexpression cells, Jurkat (bcl-2), inhibit all FTY720-induced apoptotic effects (Nagahara *et al*., 2000a; Suzuki *et al*., 1996b). BALL-1 cells highly expressed Bcl-2 identically as Jurkat (bcl-2), Bcl-2 seemed to be regulating the FTY720-induced apoptotic effect and the sensitivity to this drug. The same results were obtained using peripheral blood T cells and B cells.

PS is ordinarily sequestered in the plasma membrane inner leaflet. Upon induction of apoptosis, PS becomes exposed to outer membrane. The exposed PS triggers recognition and phagocytosis of the apoptotic cell. How PS becomes exposed is not yet defined, though downregulation of Ca^{2+}-dependent aminophospholipid translocase activity and the activation of phospholipid scramblase seem to mediate this activity (Savill & Fadok, 2000). Bratton *et al*. (1997) revealed that only the loss of aminophospholipid translocase activity is insufficient to exposure PS. On the other hand, overexpression of phospholipid scramblase induces movement of PS to the cell surface (Zhao *et al*., 1998), and phospholipid scramblase activity is augmented by phosphorylation by protein kinase Cδ (Frasch *et al*., 2000). As caspase-3 cleaves and activates protein kinase C, caspase-dependent PS exposure is thought to be one of the PS-exposing pathways. In the present study, FTY720 induced PS exposure in Jurkat cells, but not in BALL-1 cells (Figure 3b). Since FTY720 activated caspase-3 only in Jurkat cells (Figure 4), FTY720 may expose PS in a caspase-dependent fashion.

In the present study, FTY720 activated caspase-9, caspase-3, and slightly activated caspase-8 in Jurkat cells. A previous study revealed that FTY720 did not activate caspase-1 in HL-60 cells. Fujino *et al*. (2001) observed the activation of other diverse caspases by FTY720 in Jurkat cells. Those researchers

indicated that caspases-2, -3, -6, -8, -9, and -10 were activated, whereas caspases-1 and -5 were not activated by FTY720. Also, in the presence of a caspase-3 inhibitor, Ac-DEVD-CHO, or a caspase-9 inhibitor, Ac-LEHD-CHO, FTY720-induced caspase-8 activation was completely down-regulated. Activated caspase-3 cleaves caspases-2 and -6. Activated caspase-6 in turn activates caspases-8 and -10 (Slee *et al*., 1999). The activation of caspases-2, -6, -8, -10 by FTY720 is suggested to be a result of the mitochondria-mediated apoptotic pathway.

Bcl-2 blocks the PTPC opening and does not trigger PT nor release cytochrome *c* from mitochondria (Zamzami *et al*., 1998). In the previous studies, FTY720 triggered PT and released cytochrome *c*, which was completely inhibited by the overexpression of Bcl-2 (Nagahara *et al*., 2000a). The present study shows that Bcl-2-weakly-expressed Jurkat (neo) cells are more susceptible to FTY720-apoptotic effects than Bcl-2-highly-expressed BALL-1 cells, as shown in previous studies. T cell lymphomas are likely to express lower levels of Bcl-2 in contrast to B cell lymphomas (Akagi *et al*., 1994; Zutter *et al*., 1991). This difference can be explained by the fact that Bcl-2 protein was highly expressed when bearing the t(14;18) chromosomal translation. Human B cell lymphomas tend to have this translation (Ngan *et al*., 1988), although this is not always the case, and there are many B cell lymphomas without the t(14;18) chromosomal translation that also express Bcl-2 (Akagi *et al*., 1994; Kondo *et al*., 1992). Some other mechanisms might exist to regulate Bcl-2 expression, and these may have caused the difference in Bcl-2 expression between T cell lymphomas and B cell lymphomas. In lymphocyte differentiation, Bcl-2 expression was highly involved in the clonal deletion of autoreactive or low-affinity lymphocytes (Akbar *et al*., 1993; Tamaru *et al*., 1993). In human peripheral blood T cells, Bcl-2 was expressed less than B cells (Ohta *et al*., 1995). In the present study, FTY720 induced about a 2 fold increase of PS exposure in rat peripheral T cells in comparison with B cells (Figure 1). Coincidentally, Bcl-2 was expressed about 2 fold in peripheral B cells in comparison with T cells (Figure 6b). These results suggest that the expression of Bcl-2 regulates the FTY720 susceptibility between T cells/T cell lymphomas and B cells/B cell lymphomas. As the FTY720 immunosuppressant mechanism induces apoptosis in peripheral blood lymphocytes, T cells are the main target of FTY720 for inducing apoptosis and for immunosuppression.

FTY720 specifically induces apoptosis in T lymphocytes and lymphomas, but other cell types have resistance to it. FTY720 induces apoptosis in human prostate cell line DU145 at doses over 40 μM (Wang *et al*., 1999), while IC_{50} for normal human lung cells is about 150 μM (Sonoda *et al*., 2001). These doses are very high, since 4 μM FTY720 can induce apoptosis in human peripheral lymphocytes (Suzuki *et al*., 1996b). Similarly, human uterine cell line HeLa cells have resistance to FTY720. However, mitochondria isolated from HeLa cells were as susceptible (with reduced $\Delta\Psi_m$ and released cytochrome *c*) to those from Jurkat cells (data not shown). Therefore, we considered that intracellular FTY720 concentration differs among cell types in which FTY720 has the potential to pass through cell membranes at different doses. Also, mitochondria Bcl-2 and Bcl-x_L levels regulate FTY720-susceptibility, as described throughout this study. In addition to the above experiment, Bcl-2 expression levels of HeLa mitochondria and Jurkat mitochondria were almost the same (data not shown). These mitochondria anti-apoptotic protein expression levels and intracellular concentration level of FTY720 assumed to regulate the FTY720-susceptibility. FTY720 is a potent immunosuppressant that prolongs allografts of various models of animal organ transplantation (Furukawa *et al*., 2000; Nikolova *et al*., 2000; Ueda *et al*., 2000). The combination of other immunosuppressants, e.g., cyclosporine A, with FTY720 significantly increases its immunosuppressive activity (Suzuki *et al*., 1996a; Tamura *et al*., 2000). This finding can be explained by the fact that FTY720 and other immunosuppressants utilize distinct immunosuppressive mechanisms. With FTY720-induced T-cell selective apoptosis, this immunosuppressant can be a promising drug in transplantation and autoimmunity treatments.

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