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Coordinate Involvement of Cell Cycle Arrest and Apoptosis Strengthen the Effect of FTY720

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A novel reagent, FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride), has been shown to induce a significant decrease of lymphocytes and lymphoma cells and is expected to be a potent immunosuppressant and anti-tumor drug. The decrease in lymphocytes and lymphoma cells is mainly the result of FTY720-induced apoptosis. FTY720 directly affects mitochondria and induces cell death. Moreover, FTY720 activates protein phosphatase (PP) 2A and affects anti-apoptotic intracellular signal transduction proteins to attenuate the anti-apoptotic effect. In this study, we examined the relationship between FTY720-induced apoptosis and cell cycle regulation. FTY720 induced apoptosis significantly at the G0/G1 phase and caused G0/G1 cell cycle arrest of the human lymphoma cell lines HL-60RG and Jurkat. Simultaneously, retinoblastoma protein (pRB) was dephosphorylated, suggesting that dephosphorylation of pRB was related to FTY720-induced G0/G1 cell cycle arrest. Because this dephosphorylation was completely blocked by a specific PP1/2A inhibitor, okadaic acid, it appears that FTY720-activated PP2A is essential for FTY720-induced cell cycle arrest. FTY720-induced apoptosis was inhibited by Bcl-2 overexpression in Jurkat cells, but this did not prevent FTY720-induced cell cycle arrest, suggesting that the mechanism of FTY720-induced cell cycle arrest is independent of the mechanism of FTY720-induced apoptosis. These two independent pathways strengthen the effect of FTY720.

Key words: FTY720 — Cell cycle arrest — Apoptosis — Retinoblastoma protein — Lymphoma cells

Cells, including tumor cells, exist in an ongoing balance among cell growth, cell proliferation, and cell death. Cell growth is tightly regulated in tandem with the cell cycle by the mutual relationships of numerous proteins, including cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors, and tumor suppressors. Upon occurrence of an event critical to cells, e.g., DNA damage or some other stressor, CDK inhibitors are activated and ultimately induce cell cycle arrest or, in many cases, apoptosis. One of the tumor suppressor proteins, pRB, regulates cell cycle progression from the G1 to S phase.[1,2] pRB has the ability to bind with the S-phase-promoting transcription factors E2F and c-Abl.[3] In late G1, pRB is phosphorylated by CDK-cyclin complexes, such as CDK4/cyclin D1, CDK6/cyclin D, CDK2/cyclin A, and CDK2/cyclin E. This phosphorylation induces conformational changes of pRB and releases E2F transcription factors and c-Abl.[4–6] Released E2F and c-Abl activate their transcriptional targets and promote S-phase entry. Without this phosphorylation, pRB prevents cell cycle progression into S phase and finally induces G0/G1 arrest.[7] Another tumor suppressor protein, p53, is activated in response to DNA damage. This activation leads to activation of one of the CDK inhibitors, p21, and inhibits pRB phosphorylation.[7] p27, another CDK inhibitor, also inhibits pRB phosphorylation. In this case, p27 is activated by contact inhibition, serum deprivation, and suppression of various growth signals.[8,9] In addition, pRB has been shown to play a role in apoptosis.[10] Caspase-3 cleaves pRB, and this cleavage attenuates anti-apoptotic mechanisms in some types of cells. p53, cyclins, CDKs, and CDK inhibitors have also been linked to apoptosis.[11–17] Thus, cell cycle arrest and apoptosis are closely related.

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*.[18,19] FTY720 is thought to suppress graft rejection via a significant decrease in the number of blood lymphocytes, particularly T cells.[20] We have previously demonstrated that this decrease is mainly a result of FTY720-induced apoptosis.[21] FTY720 has also been shown to induce a significant rate of apoptosis in various tumor cells, including lymphoma (HL-60 and Jurkat) and prostate (DU145) cells,[22–24] suggesting its potential as an antitumor drug. The mechanism of FTY720-induced cell death has been studied, and it has been shown that FTY720 directly perturbs mitochondrial function from an early stage (<1 h), resulting in a decrease of mitochondrial

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transmembrane potential and release of cytochrome *c*, followed by activation of caspase-3. Inhibition of mitochondrial permeability transition as mentioned above by overexpression of the anti-apoptotic endogenous molecule Bcl-2 prevented all FTY720-induced apoptotic events in intact cells and in a cell-free system with isolated mitochondria, suggesting that the mitochondria mediated apoptotic pathway is the main pathway for FTY720-induced apoptosis.[25] However, little is known about the effect of FTY720 on cell growth, i.e., on cell cycle regulation.

In this study, we examined the involvement of FTY720 in cell cycle regulation, and the relationship between this involvement and FTY720-induced apoptosis. We investigated what is involved in FTY720-induced cell cycle regulation.

MATERIALS AND METHODS

Cells and drugs The human myelogenous leukemia cell line HL-60RG was provided by the Human Science Research Resources Bank (Osaka). The human lymphoid T cell line, Jurkat, stably transfected with a human bcl-2 expression plasmid (bcl-2) or the neomycin resistance vector (neo) was provided by Dr. T. Miyashita of the National Children's Medical Research Center (Tokyo). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and 75 mg/liter kanamycin, and maintained at 37°C in a humidified chamber under an atmosphere of 95% air and 5% CO_2. All cells were washed, suspended at a density of 2×10^5 cells/ml in fresh culture medium and incubated with FTY720. FTY720 was synthesized and supplied in powder form by Taito (Tokyo), in cooperation with WelFide (Osaka). FTY720 was dissolved in saline (1 m*M*).

Cell proliferation assay Cells (4×10^5) were incubated in dish culture with or without FTY720. After incubation, 5 μM Hoechst 33342 was added and the number of cells was counted by fluorescence microscopy.

DNA fragmentation Apoptosis was determined by assay of DNA fragmentation, by means of agarose gel electrophoresis. Cells (2×10^6) were rinsed once with 10 m*M* Tris-HCl buffer, pH 8.7, containing 3 m*M* $MgCl_2$ and 2 m*M* 2-mercaptoethanol and dissolved in 50 m*M* Tris-HCl buffer, pH 7.8, containing 10 m*M* EDTA, 0.5% sodium lauryl sarcocinate, and 1 mg/ml proteinase K. After incubation at 50°C for 30 min, RNase A was added at a concentration of 0.5 mg/ml and the mixture was further incubated at 50°C for 15 min. Lysates were mixed with an equal volume of loading buffer containing TBE buffer (89 m*M* Tris, pH 8.4, 2.5 m*M* EDTA, and 89 m*M* boric acid), 20% glycerol, and 0.01% bromophenol blue. Samples were electrophoresed on 1.8% agarose gels in TBE containing 0.5 mg/ml ethidium bromide.

Cell cycle analysis Cells (1×10^6) were pulse-labeled by adding 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 30 min, and fixed with 70% ethanol. Fixed cells were incubated with 0.1 mg/ml RNase A at 37°C for 20 min. DNA was made single-stranded by incubating in 4 *N* HCl at room temperature for 30 min, and neutralized by rinsing with 0.1 *M* $Na_2B_4O_7$. Thereafter, cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-BrdU monoclonal antibody (mAb) (PharMingen, San Diego, CA) and 5 μg/ml propidium iodide (PI). Analysis was performed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) using CELLQuest software. Debris and damaged cells were excluded from the analysis by gating. A known concentration of polystyrene beads (6 μm "Polybead," Polysciences, Warrington, PA) was added to cell samples just prior to flow cytometric analysis to determine cell concentration.

"APO-BRDU" method Cell cycle-dependent apoptosis was detected by APO-BRDU, a method of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling using BrdU, followed by flow cytometric analysis.[26, 27] The APO-BRDU kit was purchased from Phoenix Flow Systems (San Diego, CA). Experimental procedures were carried out in accordance with the manufacturer's instructions. Briefly, cells (5×10^5) were fixed with 1% paraformaldehyde and 70% ethanol. The fixed cells were resuspended in DNA-labeling solution including BrdU triphosphate (BrdUTP) and TdT. After incubation for 3 h at 37°C, the cells were resuspended in antibody solution including FITC-labeled anti-BrdU mAb and incubated for 30 min at room temperature. Cell suspensions were mixed with PBS containing 2 μg/ml PI and 50 μg/ml RNase A, and then analyzed by flow cytometry.

Western blots Cells were lysed with lysis buffer (50 m*M* Tris (pH 7.5), 1% Nonidet P-40, 150 m*M* NaCl, 50 m*M* NaF, 1 m*M* EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 m*M* phenylmethylsulfonyl fluoride, and 0.1 m*M* sodium orthovanadate). Cell lysates (15–30 μg) were mixed in an equal volume of sodium dodecylsulfate (SDS) sample buffer (4% SDS, 125 m*M* Tris (pH 6.8), 10% glycerol, 0.02 mg/ml bromophenol blue, 10% 2-mercaptoethanol) and heated at 100°C for 3 min. Proteins were separated by 4–20% gradient polyacrylamide gel SDS-electrophoresis and electrically transferred to a polyvinylydene difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking of the membrane with 3% skimmed milk, pRB, p21, and p27 were immunodetected using rabbit anti-phosphorylated-pRB specific (Ser780, Ser795, and Ser807/811) and nonspecific mAb (1:1000; New England Biolabs, Beverly, MA), mouse anti-p21 polyclonal Ab (1:500; Transduction Laboratories, Lexington, KY), or mouse anti-p27 polyclonal Ab (1:2500; Transduction Laboratories). Thereafter, horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG was applied as a second Ab and positive bands were detected by enhanced chemi-

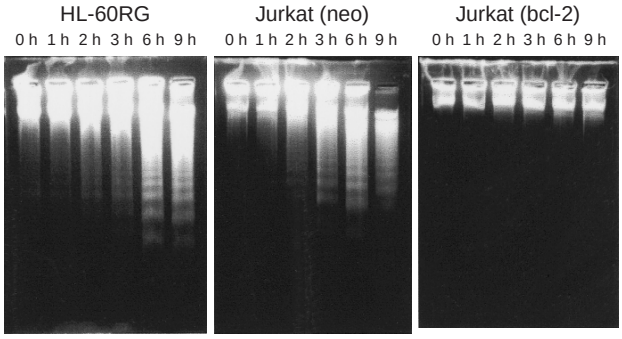

Fig. 1. DNA fragmentation of FTY720-treated cells on agarose gel electrophoresis. Lanes 1, 2, 3, 4, 5, and 6 are DNA from the cells incubated for 0, 1, 2, 3, 6, and 9 h, respectively, with 6 μM FTY720 for HL-60RG cells and 8 μM FTY720 for Jurkat (neo) and Jurkat (bcl-2) cells.

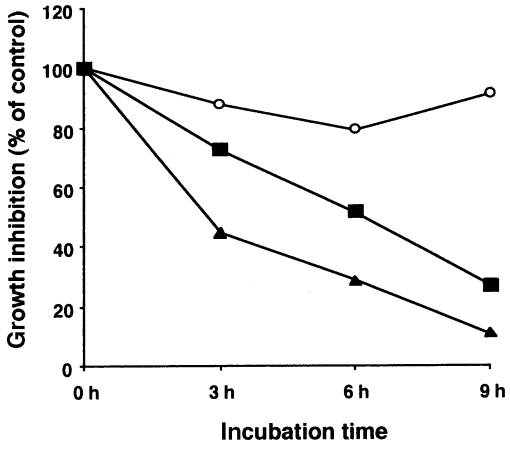

Fig. 2. Change in cell number by FTY720. Cells (4×10^5) were incubated for 0, 1, 2, 3, 6, and 9 h, respectively, with 6 μM FTY720 for HL-60RG cells (▲) and 8 μM FTY720 for Jurkat (neo) (■) and Jurkat (bcl-2) (○) cells. At the times indicated, 5 μM Hoechst 33342 was added, and the number of cells was counted by fluorescence microscopy.

luminescence (Amersham Life Science, Buckinghamshire, UK) and quantified by using NIH Image software (National Institutes of Health, Bethesda, MA).

Immunoprecipitation and histone H1 kinase assay Cell lysates (100 μg) were immunoprecipitated with rabbit anti-CDK2 polyclonal Ab (2 μg; Santa Cruz Biotechnology, Santa Cruz, CA), followed by protein A-Sepharose. The immunoprecipitates were mixed with 20 μl of kinase buffer (50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 μM ATP, 2.5 μCi [γ-^{32}P]ATP, 0.1 mg/ml histone H1), and incubated at 37°C for 15 min. Reactions were terminated by addition of 20 μl of SDS sample buffer followed by heating at 100°C for 3 min. Proteins were separated by 4–20% gradient polyacrylamide gel SDS-electrophoresis and electrically transferred to a PVDF membrane. The phosphorylation of histone H1 was detected and quantified by an imaging analyzer (BAS 2000, Fuji Film, Tokyo) using BAStation software.

RESULTS

Effect of FTY720 on cell proliferation and apoptotic cell death HL-60RG and Jurkat cells were treated with 6 μM and 8 μM FTY720, respectively. DNA fragmentation, detected as ladder formation in agarose gel electrophoresis, was observed within 2 h of treatment in HL-60RG cells and Jurkat (neo) cells. However, it was barely seen (even after 9 h of treatment) in Jurkat (bcl-2) cells, which overexpress Bcl-2 (Fig. 1). This indicates that FTY720 has a strong apoptotic effect on leukemia cells, and overexpression of Bcl-2 blocks FTY720-induced apoptosis. The effect of FTY720 on cell proliferation was determined by counting Hoechst 33342-stained cells. Only blue-stained cells were counted, and cells with bubbled polynuclei were considered apoptotic cells and therefore excluded. As shown in Fig. 2, FTY720 induced a marked decrease in the number of HL-60RG and Jurkat (neo) cells in a time-dependent manner. In comparison with the cell numbers in Fig. 1, it would appear that the decreases in these two cell lines were the result of apoptotic cell death. However, Jurkat (bcl-2) cells showed no significant increase or decrease in number within 9 h of incubation with FTY720. In addition, 24-h incubation of Jurkat (bcl-2) cells with FTY720 resulted in an approximately 60% decrease in cell number. Therefore, these results indicate that Jurkat (bcl-2) cells are able to abrogate almost entirely the FTY720-induced cell death, but lack the ability to proliferate in the presence of FTY720.

G0/G1 phase arrest by FTY720 Based on the above-mentioned studies, we hypothesized that FTY720 induces cell cycle arrest. To estimate the effect of FTY720 on cell cycle regulation, we utilized a flow cytometric technique with BrdU. The actual number of cells in each of the cell phases was calculated by analyzing a known concentration of polystyrene beads together with samples. As shown in Fig. 3A, FTY720 increased the percentage of HL-60RG cells in G0/G1 phase (from 20.8% to 39.9% after 9 h of treatment) and induced an equally marked reduction of the percentage of cells in S phase (from 65.6% to 49.0% after 9 h of treatment), with both effects occurring in a time-dependent manner. The percentage of cells in G2/M phase appeared to remain constant throughout FTY720 treatment. Similar results were obtained from Jurkat (neo) (the G0/G1 population increased from 44.9% to 54.0% after 9 h of treatment) and Jurkat (bcl-2) (the G0/G1 population increased from 63.3% to 86.2% after 24 h of treatment)

cells, indicating that FTY720 induced G0/G1 cell cycle arrest in these cells. In Fig. 3B, the actual number of cells in each cell cycle phase was analyzed using HL-60RG cells. A significant reduction of the total number of cells was observed, with a particularly large reduction in S phase, presumably because cells are destined to die before S phase and thus the number of cells is radically decreased at this phase. However, G2/M phase cells and even G0/G1 phase cells also decreased, suggesting that FTY720-treated cells underwent cell death throughout the entire cell cycle.

Cell cycle arrest and apoptosis induced by FTY720 In order to clarify whether cell-phase-specific reductions in cell number were the result of FTY720-induced apoptosis, we carried out an APO-BRDU assay. This assay uses flow cytometry to detect apoptotic DNA strand breaks and total cellular DNA. Apoptotic DNA strand breaks were endolabeled with BrdUTP, catalyzed by TdT. The amount of incorporated BrdU can be estimated from the binding reaction of FITC-labeled anti-BrdU mAb. Total cellular DNA can be analyzed by staining with PI.[26,27] Two-dimensional contour plots were developed as shown in Fig. 4A, where DNA contents with PI were plotted on the X-axis and apoptotic ratio with binding to FITC-labeled anti-BrdU mAb was plotted on the Y-axis. Apoptotic cells were observed at G0/G1 phase to a significant extent within 2 h of treatment of HL-60RG cells with 6 μM FTY720 (Fig. 4B). This result was consistent with that of our previous study (Fig. 3), in which the numbers of S-phase cells were greatly reduced because of the G0/G1-specific apoptosis. Moreover, a longer treatment time with FTY720 made the number of apoptotic cells increase not only at the G0/G1 phase, but also throughout the entire cell cycle (Fig. 4A), just as in our previous study (Fig. 3).

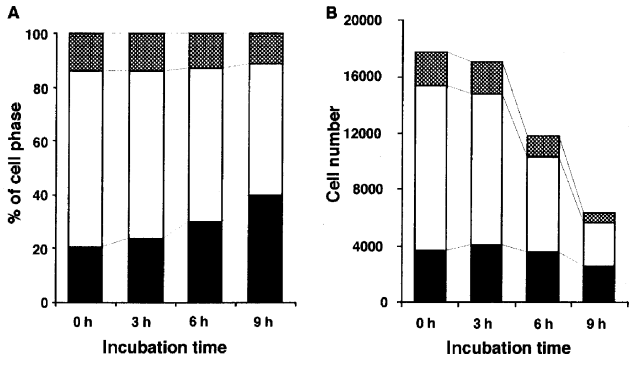

Fig. 3. G0/G1 cell cycle arrest by FTY720. (A) HL-60 cells (1×10^6) were incubated with 6 μM FTY720 for 0, 3, 6, and 9 h, respectively. Percentages of cells in each cell cycle phase were measured as described in "Materials and Methods." (B) The number of cells in each cell cycle phase in (A) was counted. ■ G0/G1, □ S, □ G2/M.

Thus, it is concluded that FTY720 induced both G0/G1 cell cycle arrest and G0/G1-specific apoptotic cell death.

Downregulation of phosphorylated pRB To confirm further that FTY720 induced G0/G1 cell cycle arrest, we conducted western blot analysis using specific Abs to

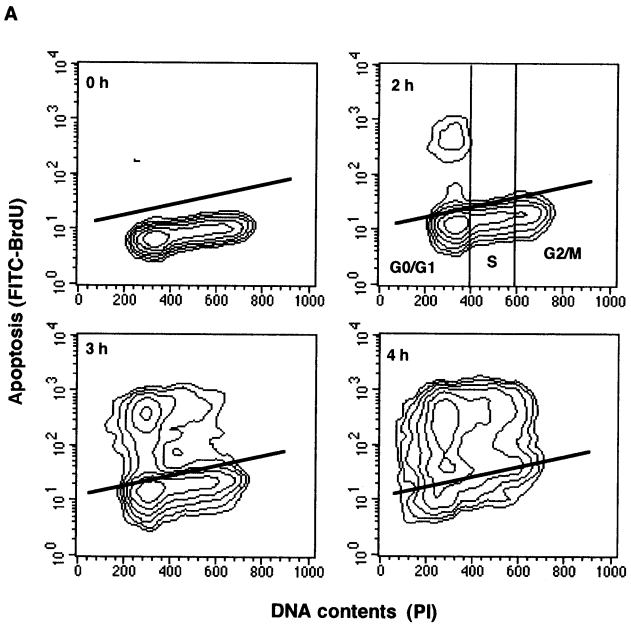

Fig. 4. FTY720 induced apoptosis specifically in G0/G1-phase cells. (A) HL-60 cells (5×10^5) were incubated with 6 μM FTY720 for 0, 2, 3, and 4 h, respectively. Thereafter, an APO-BRDU assay was performed as described in "Materials and Methods." Bold lines indicate baselines of apoptotic cells/non-apoptotic cells. (B) The number of apoptotic cells/non-apoptotic cells in each cell cycle phase (divided by lines) was counted with 2 h treatment of FTY720 shown in (A). Results are presented as an apoptotic rate calculated according to the following equation: [apoptotic rate (%)]=[apoptotic cells]/[total cells]×100.

phosphorylated pRB. These Abs specifically recognize the pRB phosphorylation sites at Ser780, Ser795, or Ser807/811, respectively, and do not cross-react with other phosphorylation sites or non-phosphorylated forms of pRB. Two (Ser780 and Ser807/811) phosphorylation sites of pRB were dephosphorylated in the presence of FTY720 in a time-dependent manner in Jurkat (neo) cells and Jurkat (bcl-2) cells, suggesting that pRB function is related to FTY720-induced G0/G1 cell cycle arrest (Fig. 5). However, only the Ser780 phosphorylation site of pRB was dephosphorylated in HL-60RG. Consistent with the results of FTY720-induced G0/G1 cell cycle arrest (not all of the cells were arrested in G0/G1 phase), phosphorylated pRB was partially dephosphorylated. Moreover, concordant reduction of phosphorylated pRB was observed between Jurkat (neo) cells and Jurkat (bcl-2) cells. This result indicates that FTY720-induced apoptotic pathways are not involved in FTY720-induced pRB dephosphorylation.

We next investigated the upstream events of FTY720-induced pRB dephosphorylation. First, we measured the expression of the CDK inhibitors, p21 and p27. Once p21 or p27 is activated, the CDK reaction of phosphorylating pRB is inhibited. As shown in Fig. 6A, p27 in HL-60RG and both types of Jurkat cells was increased during 2–3 h of treatment with FTY720, but the p21 level in both types of Jurkat cells was not increased, and in fact decreased after 6 h of treatment with FTY720. This may have been the result of caspase-3-mediated cleavage, since FTY720 has been shown to activate caspase-3 within 3 h.[25, 28] We could not detect p21 in HL-60RG cells, in agreement with the previous report.[29] Further confirmation of the FTY720-induced p27 increase to CDKs kinase activity was sought. One of the CDKs, CDK2, was immunoprecipitated and its

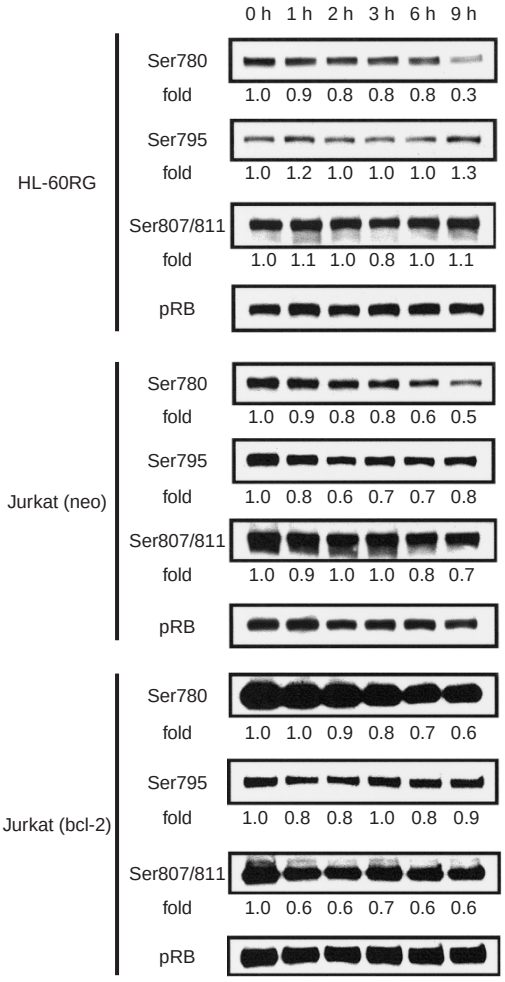

Fig. 5. FTY720 decreased phosphorylation of pRB. Cells (2×10^6) were incubated for 0, 1, 2, 3, 6, and 9 h, respectively, with 6 μM FTY720 for HL-60RG cells and 8 μM FTY720 for Jurkat (neo) and Jurkat (bcl-2) cells. At the indicated times, the cells were lysed, and phosphorylated pRB (Ser780, Ser795, and Ser807/811) was detected by western blotting. An Ab that reacts with both non-phosphorylated and phosphorylated pRB was used as a control for equal volumes of sample loading.

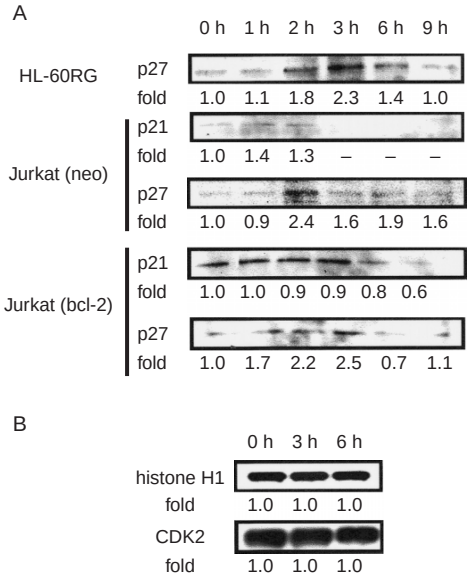

Fig. 6. FTY720 increased p27 but did not inhibit CDK activity. (A) Cells (2×10^6) were incubated for 0, 1, 2, 3, 6, and 9 h, respectively, with 6 μM FTY720 for HL-60RG cells and 8 μM FTY720 for Jurkat (neo) and Jurkat (bcl-2) cells. At the indicated times, the cells were lysed, and p21 and p27 were detected by western blotting. (B) Jurkat (neo) cells (2×10^6) was incubated for 0, 3, and 6 h, respectively, with 8 μM FTY720. At the indicated times, the cells were lysed and subjected to immunoprecipitation with CDK2 antibody. Kinase activity of CDK2 was detected using histone H1 as described in "Materials and Methods." CDK2 was detected by western blotting.

kinase activity was determined using histone H1 as a substrate. However, the kinase activity was unchanged up to 6 h FTY720 treatment (Fig. 6B). Also, FTY720 treatment caused no change in the levels of CDK2. As p27 increase was observed within 2–3 h treatment of FTY720, the resulting FTY720-induced p27 increase was not sufficient to inhibit the activity of CDK and thereby to cause dephosphorylation of pRB. The other possibility is that FTY720 enhances PP2A activity and thereby dephosphorylates pRB. We have previously found that FTY720 activates PP2A both *in vivo* and *in vitro* (unpublished results). PP1 and PP2A can dephosphorylate pRB, and microinjection of these PPs into cells inhibits cell cycle progression into S phase.[30] To determine the relationship between PP2A and FTY720-induced pRB dephosphorylation, we used a specific PP1/2A inhibitor, okadaic acid (OA). After 30-min pre-incubation with OA, FTY720-induced pRB dephosphorylation was completely blocked, indicating that FTY720-induced PP2A activation is a major factor in dephosphorylating pRB (Fig. 7).

DISCUSSION

In the present study, we demonstrated that FTY720 induces tumor cells to arrest in G0/G1 phase in addition to provoking apoptosis. pRB plays a role in this cell cycle arrest, since FTY720 dephosphorylated pRB. FTY720-induced pRB dephosphorylation is presumed to be induced by FTY720-activated PP2A. Even Bcl-2-overexpressing Jurkat cells, which were FTY720-induced apoptosis inhibitable, arrested to G0/G1 phase and pRB was dephosphorylated. Thus, FTY720-induced cell cycle arrest and apoptosis are complex, and together strengthen the effect of this drug.

Human lymphoid cells, such as HL-60RG and Jurkat cells, tend to be affected by FTY720. FTY720 induced a significant decrease in the number of these cells, and this decrease was the result of apoptotic cell death (Figs. 1 and 2). In our previous study, we showed that overexpression of Bcl-2 blocked FTY720-induced apoptosis and that Bcl-2 was related to the FTY720-induced apoptotic pathway.[23]

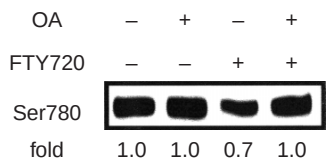

Fig. 7. OA blocked FTY720-induced pRB dephosphorylation. Jurkat (neo) cells (2×10^6) were incubated with or without 500 n*M* OA for 30 min followed by 8 μM FTY720 for 3 h. Thereafter, the cells were lysed, and phosphorylated pRB (Ser780) was detected by western blotting.

In the present study, Bcl-2-overexpressing Jurkat cells blocked FTY720-induced apoptosis, just as in our previous study, and surprisingly, cell proliferation was stopped. This reminded us of another effect of FTY720, cell cycle arrest. Normal lymphoid cells as well as Bcl-2-overexpressing cells arrested in G0/G1 phase. Because no difference was observed between normal lymphoid cells and Bcl-2-overexpressing cells in the ability to arrest in G0/G1 phase, we can say that the Bcl-2 irrelevant FTY720-induced cell cycle arrest mechanism is independent of the Bcl-2 relevant FTY720-induced apoptosis mechanism.

The cell cycle regulatory protein pRB was dephosphorylated by FTY720. In Jurkat cells, pRB was dephosphorylated as well as in Bcl-2-overexpressing cells at two out of three sites, but only one (Ser780) site was dephosphorylated in HL-60RG cells. These phosphorylation sites have distinct abilities to interact with various pRB-related binding proteins. Phosphorylation of Ser780 results in the loss of the ability of pRB to bind to E2F,[6] and phosphorylation of Ser807/811 is required to release c-Abl from pRB.[5] In addition, phosphorylation of Ser795 inactivates pRB-mediated growth suppression.[4] Because the Ser780 site has been identified as the most susceptible phosphorylated site, it may be that only the Ser780 site is dephosphorylated in HL-60RG cells. Phosphorylation of the pRB Ser780 site is related to E2F-1 release, assuming that to keep E2F-1 bound to pRB is a sufficient condition for FTY720-induced G0/G1 cell cycle arrest.

Previous studies revealed that FTY720 activated PP2A and dephosphorylated various intracellular signal transduction kinases, such as Akt, Bad, and $p70^{s6k}$ (unpublished results). In the present study, we showed that FTY720-induced pRB dephosphorylation also occurred mainly via the direct effect of FTY720-activated PP2A, since the specific PP1/2A inhibitor, OA, completely blocked FTY720-induced pRB dephosphorylation (Fig. 7). Moreover, the CDK inhibitor, p27, was increased by FTY720. Recent studies have shown that phosphorylated Akt blocks increase of p27 by activating Bcr-Abl.[31, 32] Once Akt is dephosphorylated, Bcr-Abl is attenuated and p27 is increased. As mentioned above, FTY720 dephosphorylates Akt by activating PP2A, and this may cause a subsidiary p27 increase. Indeed, we further found that p27 increase was blocked by OA (unpublished results). However, this p27 increase seems not to participate in cell cycle arrest. FTY720-activated PP2A might induce not only cell cycle arrest by dephosphorylating pRB, but also induce apoptosis by dephosphorylating Bad. Dephosphorylated Bad activates its pro-apoptotic function, induces mitochondrial permeability transition, and promotes apoptotic events such as caspase activation. We have found that pre-incubation with OA slightly inhibited FTY720-induced caspase-3 activation (unpublished results). This indicates that the main apoptotic pathway for FTY720-induced apoptosis is

direct perturbation of mitochondria, but a secondary pathway involving PP2A also exist. We are currently engaged with this issue.

In addition to pRB, other mechanisms may take part in FTY720-induced G0/G1 arrest. $p70^{s6k}$, which is a downstream protein of Akt, is known to play a role in cell cycle progression.[33–35] Phosphorylation of $p70^{s6k}$ results in a progression from G1 phase to S phase. Previous studies revealed that $p70^{s6k}$ was dephosphorylated by FTY720 (unpublished results). The Akt-$p70^{s6k}$ pathway may also provoke cell cycle arrest.

In HL-60 and Jurkat cells, FTY720-induced apoptotic cell death was observed throughout the entire cell cycle. This can be explained by our previous finding that FTY720 perturbs mitochondria directly and thereby initiates the process of apoptosis.[25] In the present study, however, particularly after early incubation with FTY720 (2–3 h), cell death occurred specifically in the G0/G1 phase, and G2/M phase cells seemed to be protected against the action of FTY720. It has been demonstrated that Bcl-2 protein can be phosphorylated, resulting in a change of its function. Phosphorylation of Bcl-2 by protein kinase C or CDC2 activates the anti-apoptotic activity of Bcl-2.[36, 37] In addition, in normal cell progression, CDC2-mediated Bcl-2 phosphorylation occurs during the G2 to M phases.[38] Since FTY720-induced apoptosis was related to Bcl-2, FTY720-induced G0/G1 specific apoptosis may be described by the phosphorylation state of Bcl-2. However, other groups have favored the opposite hypothesis, i.e., that Bcl-2 phosphorylation inactivates Bcl-2 function.[39] Although further studies are needed in regard to this phenomenon, the present study provides substantial support for the former point of view, i.e., that phosphorylation of Bcl-2 activates its anti-apoptotic function.

FTY720 has a potent effect on proliferation of lymphocytes, based on our present finding that FTY720 induced G0/G1 phase cell cycle arrest and G0/G1 susceptible apoptosis in lymphoma cells. Because all lymphocytes permanently remain in G0 phase, FTY720 induces only apoptosis in these cells. In lymphoma cells, is likely that the G0/G1 cell cycle arrest pathway and apoptotic pathway both play a role in the actions of FTY720. Further investigations on FTY720 will be needed to clarify not only the drug's potential uses, but also cell cycle-dependent apoptosis generally.

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