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Terfenadine induces thymocyte apoptosis via mitochondrial pathway

Riyo Enomoto[a,b], Tomoe Komai[a], Yukari Yoshida[a], Chiyoko Sugahara[a], Emi Kawaguchi[a], Keiko Okazaki[a], Hiroki Kinoshita[a], Hiroto Komatsu[c], Yasuo Konishi[c], Eibai Lee[a,b,*]

[a] *Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Japan*
[b] *High Technology Research Center, Kobe Gakuin University, Ikawadani-cho, Nishi, Kobe 651-2180, Japan*
[c] *Biotechnology Research Institution, National Research Council Canada, 6100 Royalmount Avenue, Montreal, Quebec, Canada H4P 2R2*

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Abstract

The treatment of rat thymocytes with 10 μM terfenadine resulted in a significant increase in DNA fragmentation. The DNA fragmentation induced by terfenadine was dependent on its concentration and incubation time. In terfenadine-treated cells, the translocation of phosphatidylserine from the inside of plasma membrane to the outside, an early event of the apoptotic process, and chromatin condensation, the morphological characterization of apoptotic cell death, were observed. Terfenadine stimulated caspase-8, -9 and -3-like activities in an incubation time-dependent manner in thymocytes. The active forms of caspase-3 and -9 were detected in the extract from terfenadine-treated cells by immunoblotting analysis using specific antibodies to caspases, but active caspase-8 was not found in this fraction. Decrease in mitochondrial membrane potential and the release of cytochrome *c* from mitochondria to cytosol were observed in terfenadine-treated thymocytes. These results suggest that terfenadine induces apoptosis in rat thymocytes via mitochondrial pathway.
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Keywords: Terfenadine; Apoptosis; Thymocyte; Mitochondria; Fexofenadine; Voltage-dependent K^+ channel

1. Introduction

Apoptosis is the characteristic process of cell death and is thought to play an important role in many physiological phenomena such as cell growth, differentiation and tissue development (Arends and Wyllie, 1991; Thompson, 1998). Since apoptosis is also relevant to several diseases including cancer (Antonsson and Martinou, 2000; Arends and Wyllie, 1991) or ischemic vascular diseases (Krijnen et al., 2002; Snider et al., 1999), the regulation of apoptosis can be applied to cure of these diseases.

In fact, induction of apoptosis is one of key mechanism of the radiation treatment and the chemotherapy for cancer (Arends and Wyllie, 1991; Mesner et al., 1997; Vaux, 2002) because many cells including cancer cells undergo apoptosis when cells are exposed to ionizing radiation or chemotherapeutic agents. On the other hand, many of the side effects of chemotherapy appear in normal cells in the bone marrow or gut because chemotherapeutic agents easily induce apoptotic cell death in these cells (Vaux, 2002). It is well known that glucocorticoids such as dexamethasone induce thymocyte apoptosis (Nieto et al., 1992; Wyllie and Wyllie, 1980). Also, apoptotic cell death concerns immune suppression by the environmental pollutants such as dioxins (McConkey et al., 1988) or triorganotins (Raffray et al., 1993). Thus it is possible that apoptosis may involve in the adverse reaction of drugs or the toxic action of chemical compounds. In our preliminary study, we have examined whether apoptosis is induced by various drug medicine using rat thymocytes and have found that terfenadine, an antiallergic agent, induces apoptosis.

Terfenadine is classified into the second-generation histamine H_1 receptor antagonist in conjunction with inhibitory effect of chemical mediator release. Second-generation histamine H_1 receptor antagonists such as terfenadine and astemizole have been developed to overcome the antimuscarinic and the sedative properties displayed by first-generation antihistamines such as

* Corresponding author. Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Ikawadani-cho, Nishi, Kobe 651-2180, Japan. Tel.: +81-78-974-4429; fax: +81-78-974-5689.
E-mail address: elee@pharm.kobegakuin.ac.jp (E. Lee).

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diphenhydramine and promethazine (Slater et al., 1999). However, it is known that the second-generation antihistamines show rare but life-threatening side effect on circulatory system. At present, the mechanism of the prolongation of the QT interval and the ventricular arrhythmia by terfenadine has been recognized as blockade of the cardiac voltage-dependent K^+ (K_v) channels involved with action potential repolarization (Roy et al., 1996). Recently, it has been shown that K^+ extrudes from the cell during apoptotic process (Bortner et al., 1997). K^+ efflux accompanies the increase in PS externalization, cell shrinkage and loss of mitochondrial membrane potential ($\Delta\psi$) during the induction of apoptosis (Bortner et al., 1997; Bortner and Cidlowski, 1999; Dallaporta et al., 1998). Thus, K_v channels may assume important roles in induction of apoptotic cell death. This paper describes terfenadine induced thymocyte apoptosis via mitochondrial dysfunction and the role of K_v channels in apoptosis induced by this drug is also discussed.

2. Materials and methods

2.1. Materials

Terfenadine, astemizole, ketoconazole, bretylium, bepridil, amiodarone, *dl*-sotalol and quinidine were from Sigma (St. Louis, MO). Pepstatin A and leupeptin were obtained from Wako (Osaka, Japan). (L-3 *trans*-ethoxycarbonyloxirane-2-carbonyl)-L-leucine(3-methylbutyl)amide (E-64-d), acetyl-Ile-Glu-Thr-Asp α-(4-methyl-cumaryl-7-amide) (Ac-IETD-MCA), acetyl-Leu-Glu-His-Asp α-(4-methyl-cumaryl-7-amide) (Ac-LEHD-MCA) and acetyl-Asp-Glu-Val-Asp α-(4-methyl-cumaryl-7-amide) (Ac-DEVD-MCA) were purchased from Peptide Institute (Osaka, Japan). carbobenzoxy-Asp(OCH_3)-Gln-Met-Asp(OCH_3)-fluoromethane (Z-DQMD-FMK) and E-4031 were obtained from Calbiochem, EMD Bioscience, (Darmstadt, Germany). Mouse anti-cytochrome *c* monoclonal antibody was purchased from BD sciences (San Diego, CA). Rabbit anti-caspase-8 polyclonal antibody was from Chemicon International, (Temecula, CA). Rabbit anti-caspase-9 polyclonal antibody was from StressGen Biotechnologies (Victoria, Canada). Rabbit anti-caspase-3 polyclonal antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Goat anti-rabbit immunoglobulin (Ig) G (H + L)-AP conjugate and goat anti-mouse IgG (H + L)-AP conjugate were from Bio-Rad Laboratories (Hercules, CA). Annexin V-EGFP Apoptosis Detection Kit was from Medical and Biological Laboratories (Nagoya, Japan). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and Hoechst 33342 were from Molecular Probes (Eugene, OR). RPMI 1640 medium and BCIP-NBT Solution Kit was obtained from Nacalai Tesque (Kyoto, Japan). Fexofenadine hydrochloride was extracted from the tablets of Allegra® (Aventis Pharma, Tokyo, Japan) using methanol, purified by high-pressure liquid chromatography (HPLC) and re-crystallization from methanol-water. This compound was identified as fexofenadine by use of mass spectrometry and nuclear magnetic resonance (NMR) analysis. All other reagents were the analytical grade available. All experiments were performed according to the guiding principles for the care and use of laboratory animals approved by the Japanese Pharmacological Society.

2.2. Cell culture of thymocytes

Since thymocytes are used to study on the molecular mechanism of apoptosis and many researchers including us have accumulated the experimental data about this type of cell death, all of experiments were carried out using rat thymocytes. Sprague–Dawley rats (5 weeks) were obtained from Charles River Japan (Kanagawa, Japan). Isolated thymocytes from rats (1.3×10^9 – 1.5×10^9 cells/rat) were incubated with RPMI 1640 medium supplemented with 10% fetal calf serum at a density of 10×10^6 cells/ml under 5% CO_2 in air immediately after the isolation (Lee et al., 1994).

2.3. DNA fragmentation

After the incubation of thymocytes in the absence or presence of the compounds, the cells were collected by centrifugation and then the supernatant was removed. The cells were washed twice with phosphate buffered saline (PBS), and were lysed by 10 mM Tris–HCl (pH 7.4) including 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 mM 2-mercaptoethanol and 0.1% Triton X-100. Then the cell lysates were centrifuged to separate the supernatants (fragmented DNA) from the pellets (intact DNA). The intact and fragmented DNA in the cells was assayed by the diphenylamine method (Lee et al., 1994). DNA fragmentation was expressed as the percentage of fragmented DNA in the total DNA (intact plus fragmented DNA).

2.4. Annexin V-apoptosis detection

The translocation of phosphatidylserine from the inside to the outside of plasma membrane is accentuated in the early stage of apoptosis. Annexin V staining was carried out to determine the translocation of phosphatidylserine at the surface of the cell (Koopman et al., 1994) using Annexin V-EGFP Apoptosis Detection Kit. We have reported that thymocyte apoptosis can be measured by annexin V-EGFP and propidium iodide double staining using a laser-scanning microscope (Lee et al., 2002). According to its protocol the cells were stained after the incubation with terfenadine. A laser scanning-microscope (LSM GB200, Olympus Optical, Tokyo, Japan) equipped with argon laser and a 40×, water immersion objective was used to visualize annexin V-positive (green fluores-

cence) and propidium iodide-positive (red fluorescence) cells (Lee et al., 2002).

2.5. Hoechst 33342 staining

The nuclear structure in thymocytes treated with various drugs was analyzed according previous report (Lee et al., 1996). Briefly, after the treatment of thymocytes with the drugs, the cells were fixed by formaldehyde solution and stained by 6.15 μg/ml Hoechst 33342 for 10 min at room temperature. The cells were washed and resuspended with PBS and observed nuclei with a laser-scanning microscope.

2.6. Mitochondrial membrane potential determination

Mitochondrial membrane potential ($\Delta\psi$) was measured by the use of JC-1. JC-1 is cationic dye that exhibits potential-dependent accumulation in mitochondria and indicates a fluorescence emission shift from green to red. Cells were incubated with 10 μg/ml JC-1 for 20 min at 37 °C in the dark. The cells were collected and were washed with ice-cold PBS including 1 mg/ml bovine serum albumin (Di Lisa et al., 1995). The cells were resuspended in adequate same solution and analyzed by a laser-scanning microscope.

2.7. Determinations of caspase-like protease activities

The activities of caspase-8, -9 and -3 were determined by fluorescent substrates as described previously (Yamashita et al., 1999). After the incubation of thymocytes in the absence or presence of 10 μM terfenadine, cell extracts were prepared by repeated freezing and thawing of the cells (100×10^6 cells) in 50 μl extracting buffer containing 50 mM KCl, 50 mM PIPES–NaOH (pH 7.4), 5 mM EGTA, 2 mM $MgCl_2$, 20 μM cytochalasin B, 1 mM phenylmethylsulfonyl fluoride, 1 mM (±)-dithiothreitol, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 2.83 μg/ml E-64-d. 10 μl of all of the cell extracts (caspase preparation) were then diluted with 90 μl of assay buffer containing 100 mM HEPES–KOH (pH 7.5), 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-propansulfonic acid (CHAPS), 10 mM (±)-dithiothreitol and 0.1 mg/ml ovalbumin to become about 0.5 mg/ml of protein concentration. These dilutions were incubated at room temperature with 100 μM Ac-IETD-MCA, Ac-LEHD-MCA and Ac-DEVD-MCA as the substrates of caspase-8, -9 and -3, respectively.

2.8. Immunoblotting analysis

Immunoblotting analysis of caspases cleavage were performed as follows. After the incubation of thymocytes in the absence or presence of 10 μM terfenadine, the caspase preparations were prepared as described above. Proteins (30 μg/lane) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% acrylamide and transferred to an Immun-Blot™ PVDF Membrane (Bio-Rad Laboratories, Hercules, CA) using a semidry blotting apparatus. After the transfer, the blots were washed three times in Tris-buffered saline (TBS) and then saturated for 1 h in TBS including 1% skim milk. The membranes were incubated overnight at 4 °C with anti-caspase-3 (1:500), -8 (1:200) or -9 (1:250) antibodies, respectively. Blots were washed five times with TBS including 0.05% Tween 20 and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000) for 1 h at room temperature and specific protein antibody interactions were detected using BCIP-NBT Solution Kit.

Release of cytochrome *c* to cytosol from mitochondria was analyzed by immunoblotting analysis. After the incubation of thymocytes in the absence or presence of 10 μM terfenadine,

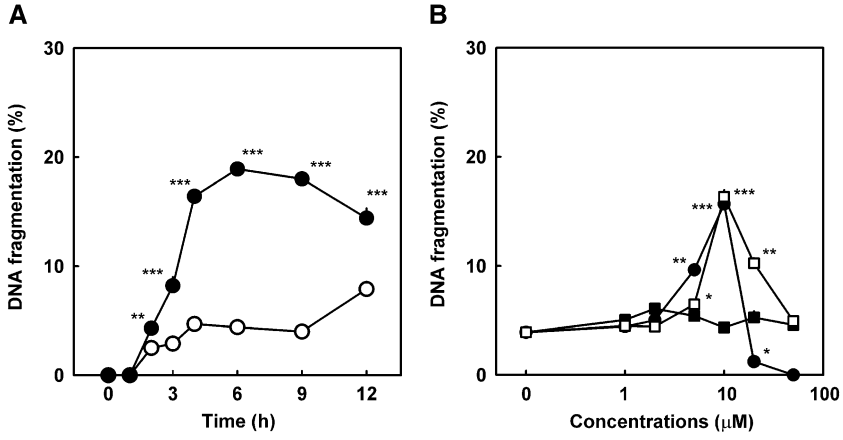

Fig. 1. The effect of terfenadine on DNA fragmentation in rat thymocytes. (A) The thymocytes were incubated with 10 μM terfenadine for indicated times. (B) The thymocytes were treated with various concentrations of terfenadine, astemizole and fexofenadine for 6 h. After the incubation, the cells were collected and DNA fragmentation was determined. Values are means ± S.E.M of four to eight separate experiments. ○, control; ●, terfenadine; □, astemizole; ■, fexofenadine. *$P<0.05$, **$P<0.01$ and ***$P<0.001$, significant from control (Student's *t*-test).

cytosolic fractions were prepared as follows. Briefly, cells (300×10^6 cells) were collected and washed twice with PBS. The cells were resuspended in 300 μl of ice-cold buffer containing 20 mM HEPES-KOH (pH 7.5), 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM (±)-dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride, and the suspension was left on ice for 15 min. The cells were disrupted by POLYTRON homogenizer (PT 1200, KINEMATICA AG, Lucerne, Switzerland) with dial 4 for 30 s. The homogenates were centrifuged at $15,000 \times g$ for 20 min and then the resulting supernatants and pellets were analyzed by SDS-PAGE with 15% acrylamide (30 μg protein/lane). Immunoblotting analysis was carried out as described above by use of anti-cytochrome *c* antibody (1:500). Alkaline phosphatase-conjugated goat anti-mouse IgG was used as secondary antibody (1:3000).

2.9. Statistical analysis

Statistical analysis of the experimental data was carried out by the Student's *t*-test using a software package (Microsoft Excel 2000) for Windows 2000.

3. Results

Fig. 1 shows the effects of terfenadine on DNA fragmentation, a biochemical hallmark of apoptosis, in rat

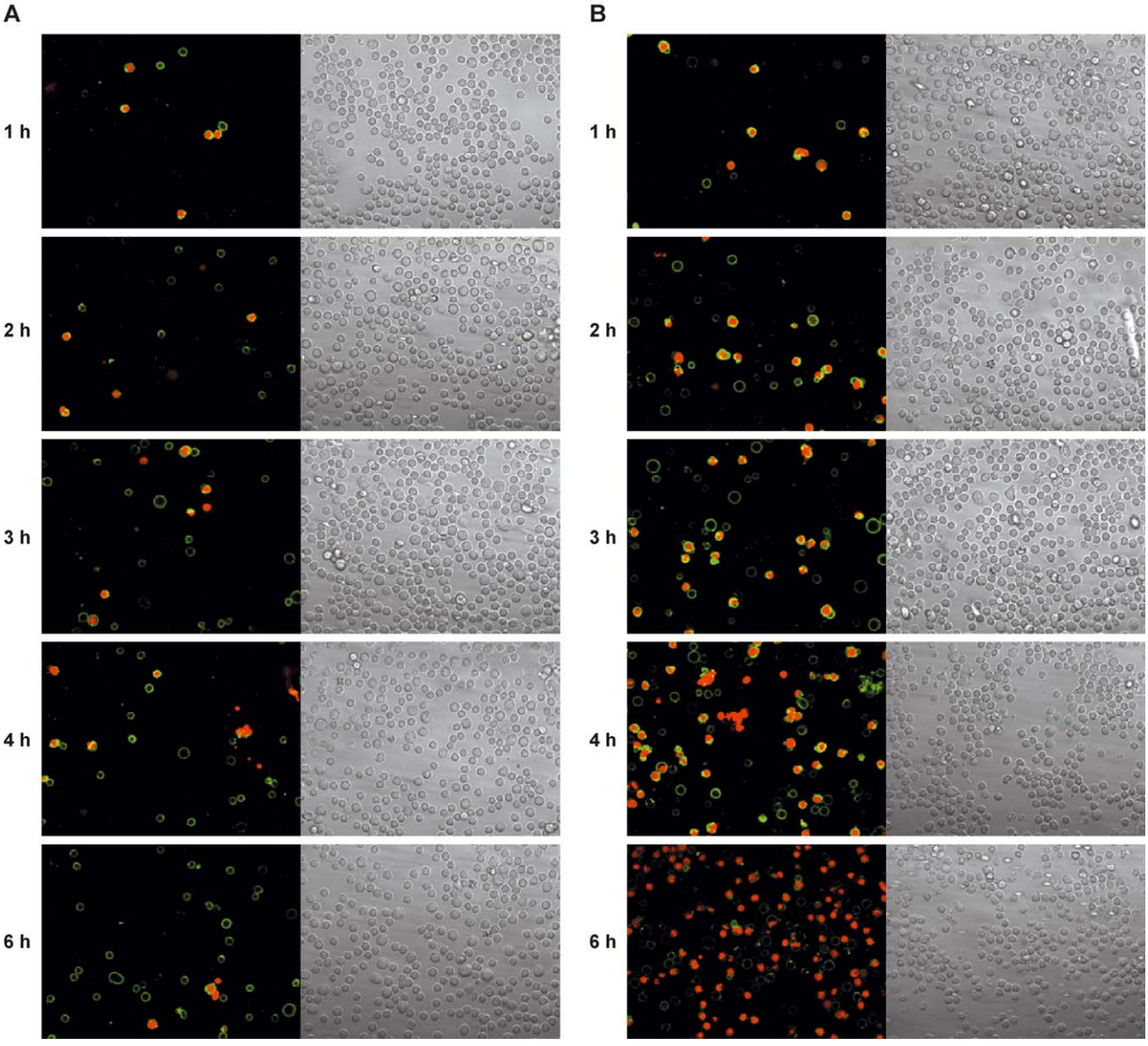

Fig. 2. Annexin V-EGFP and propidium iodide staining of untreated and terfenadine-treated thymocytes. The cells were incubated with 10 μM terfenadine for indicated time, then the cells were stained with annexin V-EGFP (green) and propidium iodide (red) without fixation. The right panels indicated the dioptric images of the same field of vision; (A) control, (B) terfenadine-treated.

Table 1
The ratio of the number of annexin V-positive or annexin V and propidium iodide (PI) double positive cells for the number of total cells

	Control		10 μM Terfenadine-treated	
	Annexin V^{+}/PI^{-}	Annexin V^{+}/PI^{+}	Annexin V^{+}/PI^{-}	Annexin V^{+}/PI^{+}
1 h	1.6	1.9	1.6	2.7
2 h	3.9	2.3	7.8	5.2
3 h	6.8	1.9	8.7	4.8
4 h	7.6	1.8	14.7	13.8
6 h	13.0	1.1	19.0	32.4

Values in the table show the percentage of the number of stained cells for the number of total cells in each viewing field of Fig. 2.

thymocytes. Terfenadine increased DNA fragmentation of rat thymocytes in the incubation time- and the concentration-dependent manner. Since DNA fragmentation by terfenadine was induced by the treatment for over 2 h, it seems to be necessary for the lag time of more than 2 h to increase DNA fragmentation (Fig. 1A). The maximum effect of DNA fragmentation was observed with 10 μM terfenadine-treated thymocytes and more than 30 μM of terfenadine made DNA fragmentation reversely decrease (Fig. 1B). Astemizole, an analogous drug of terfenadine, also increased the DNA fragmentation in rat thymocytes but fexofenadine, a carboxylic metabolite of terfenadine, did not induce DNA fragmentation in this cells (Fig. 1B). In order to clarify whether terfenadine induces thymocyte apoptosis, annexin V-EGFP staining in the cells treated with terfenadine was examined. The typical data provided by three independent experiments were shown in Fig. 2. A significant increase of phosphatidylserine exposure was

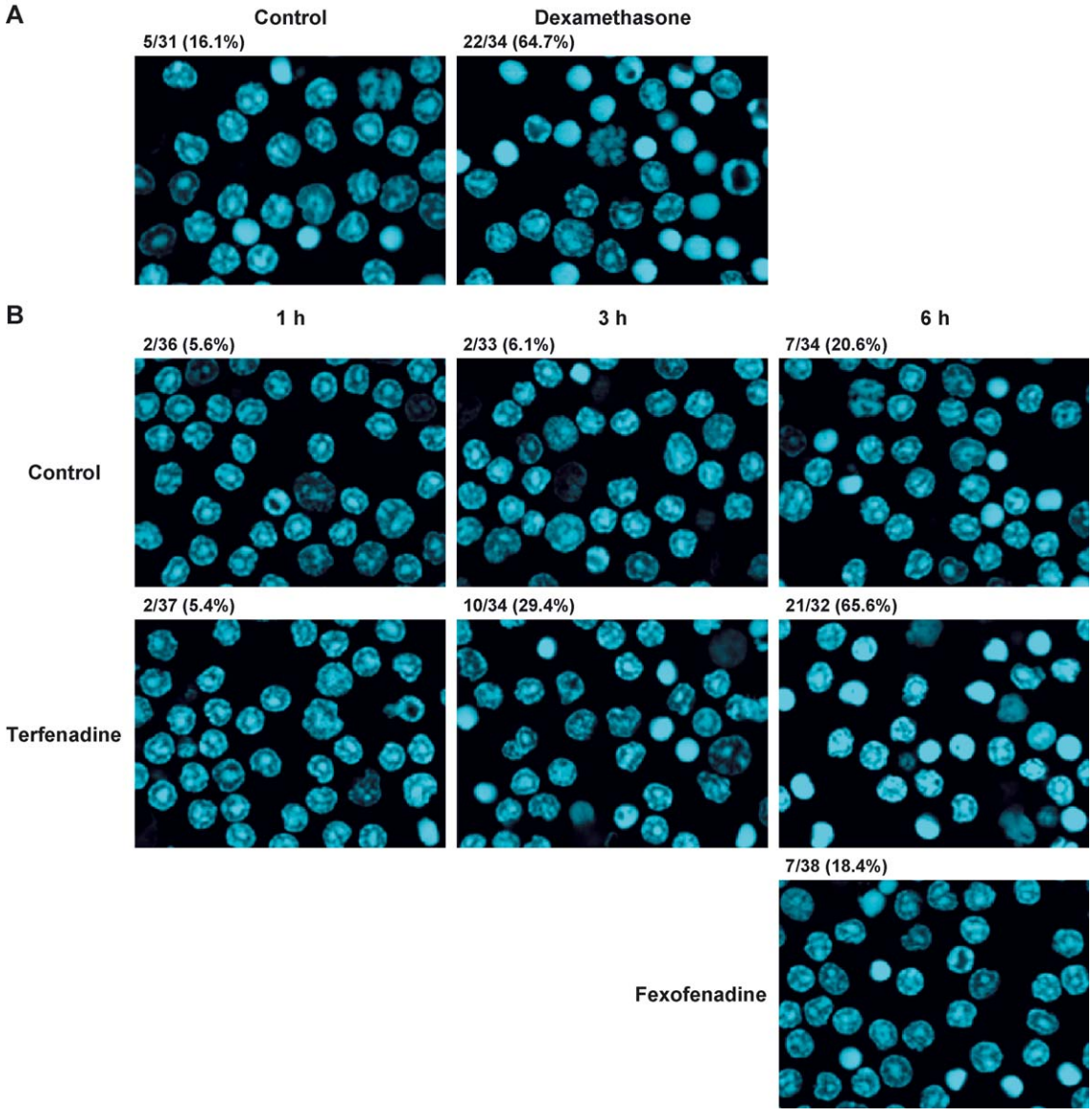

Fig. 3. Laser-scanning microscopic images of thymocytes treated with terfenadine. (A) The thymocytes were treated with or without 0.1 μM dexamethasone (positive control) for 6 h. (B) The cells were treated with 10 μM terfenadine or 100 μM fexofenadine for indicated times. The numbers in the figure indicated the condensed nuclei per total nuclei in viewing field. The values in the parentheses show the percentage of condensed nuclei.

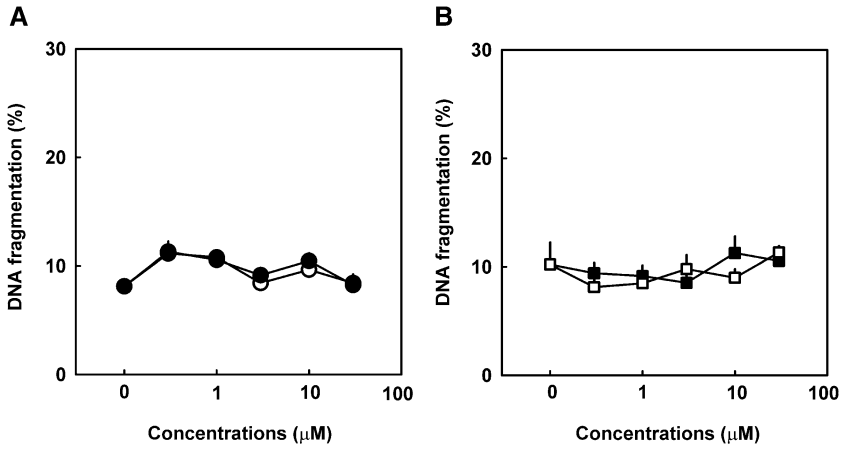

Fig. 4. The effects of other antiallergic agents on DNA fragmentation in rat thymocytes. The thymocytes were incubated with various concentrations of diphenhydramine (A, ○), cromolyn (A, ●), chlorpheniramine (B, □) or ketotifen (B, ■) for 6 h. After the incubation, the cells were collected and DNA fragmentation was determined. Values are means ± S.E.M of four separate experiments.

observed 2 h after the treatment with terfenadine. These results represents the translocation of phosphatidylserine from the inside to the outside of the plasma membrane, an early events of the apoptotic process, precedes DNA fragmentation. The apoptotic cells of early stage are indicated as annexin V-positive cells and the cells of advanced stage of apoptosis are shown as annexin V and propidium iodide double positive cells. The number of annexin V-positive cells and annexin V and propidium iodide double positive cells were increased by the treatment of the cells with terfenadine in the incubation time-dependent manner. The percentage of annexin V-positive cells and annexin V and propidium iodide double positive cells for the total cells in the viewing field was twofold increased by terfenadine treatment of the cells for 2 h (Table 1). The morphological study on the nuclear structure by use of a laser scanning-microscope was shown in Fig. 3. The typical data derived in three independent experiments were shown in this figure. The laser scanning-microscopic image of control thymocytes showed a core-like structure and unequal contours of fluorescent intensity in most cells. In contrast, the cells treated with dexamethasone which is a typical inducer of thymocyte apoptosis for 6 h revealed a peculiar feature of nuclei having an equal distribution much more intense fluorescence and lacking of core-like structure (positive control, Fig. 3A). Similar apoptotic nuclei were seen by the treatment of the cells with terfenadine (Fig. 3B). The formation of this type of the nuclei was dependent on the incubation time and paralleled the increase in DNA fragmentation (Fig. 1) and annexin V staining (Fig. 2). The morphological change of nuclei was also observed in

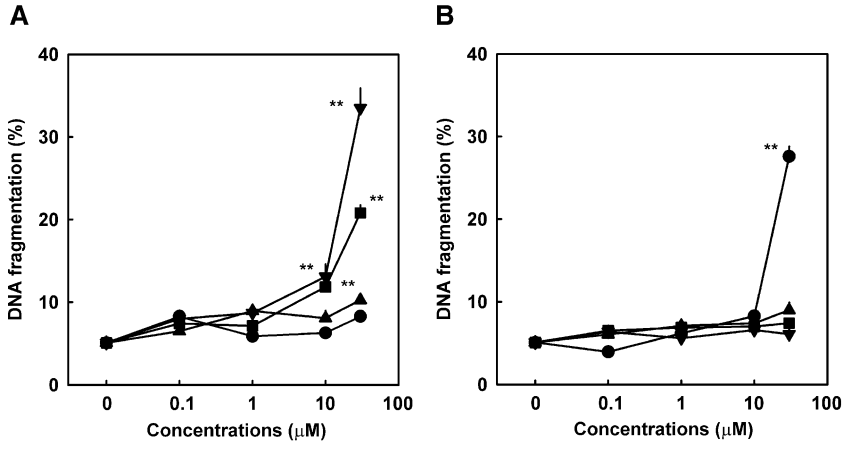

Fig. 5. The effects of various drugs on DNA fragmentation in rat thymocytes. The thymocytes were incubated with various concentrations of drugs for 6 h. After the incubation, the cells were collected and DNA fragmentation was determined. (A) ▼, chlorpromazine; ■, bepridil; ▲, ketoconazole; ●, quinidine. (B) ●, amiodarone; ▲, bretylium; ■, *dl*-sotalol; ▼, E-4031. Values are means ± S.E.M of four separate experiments. $^{**}P < 0.01$, significant from control (Student's *t*-test).

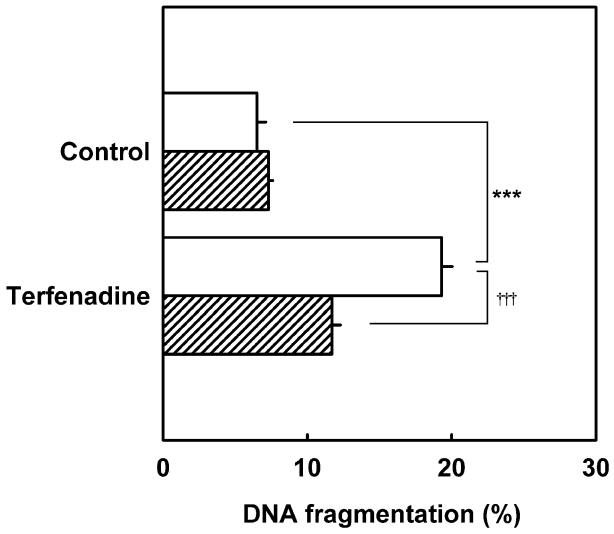

Fig. 6. The effect of caspase inhibitors on DNA fragmentation induced by terfenadine. The cells were preincubated in the absence or presence of 100 μM Z-DQMD-FMK for 1 h. Then, 10 μM of terfenadine was added to the cells, and was incubated for 6 h. Open column, none; hatched column; Z-DQMD-FMK-pretreated. Values are means ± S.E.M of 4 separate experiments. ***$P<0.001$, significant from control, $^{\dagger\dagger\dagger}P<0.001$ vs. Z-DQMD-FMK-pretreated (Student's *t*- test).

astemizole-treated cells (data not shown) but the nuclear structure of fexofenadine-treated cells was not different from that of control cells (Fig. 3B). These biochemical and morphological findings prove that terfenadine induces thymocyte apoptosis. However, other antiallergic agents such as diphenhydramine, cromolyn or ketotifen did not affect DNA fragmentation in rat thymocytes (Fig. 4). To evaluate the mechanism of terfenadine-induced apoptosis, the relationship between pro-apoptotic action and the side effect of terfenadine such as QT prolongation was examined. Fig. 5 shows the effect of various drugs, which are known to prolong the QT interval, on DNA fragmentation. DNA fragmentation was increased in the treatment of the cells with amiodarone, bepridil and chlorpromazine. Other drugs hardly affected DNA fragmentation in the cells.

Since caspases play a crucial role in apoptotic process, the effects of the inhibitor of the effector caspase on terfenadine-induced DNA fragmentation were examined. As shown in Fig. 6, Z-DQMD-FMK, a specific inhibitor of caspase-3, prevented DNA fragmentation in the cells treated with terfenadine. Furthermore, the activities of caspases were determined in rat thymocytes treated with terfenadine. Fig. 7 shows that the incubation of thymocytes with terfenadine increased caspase-8, -9 and -3-like activities in an incubation time-dependent manner. As shown in Fig. 8A, the activation of caspase-8 in terfenadine-treated cells was analyzed by immunoblotting using a specific polyclonal antibody against the 55 (procaspase-8), 43 (active caspase-8) and 25 kD fragments of this enzyme. Terfenadine did not induce the activation of caspase-8. The active form of caspase-9 (35 kD fragment) increased by the treatment of the cells with terfenadine for 1 h. Following the stimulation of caspase-9, caspase-3 was activated in terfenadine-treated cells (17 and 12 kD fragments) (Fig. 8A). Thus, the caspase cascade is likely to be involved in terfenadine-induced apoptotic cell death.

To clarify whether terfenadine induces thymocyte apoptosis via mitochondria/caspase-9 activation pathway, release of cytochrome *c* to cytosol from mitochondria was analyzed (Fig. 8B). Furthermore, $\Delta\psi$ was determined by laser-scanning microscope using JC-1 dye. JC-1 has been used for measurements of $\Delta\psi$ because of its dual emission characteristics that are sensitive to membrane potential. JC-1 exists either as a green-fluorescent monomer at depolarized membrane potentials or as a red-fluorescent J-aggregate at hyper-

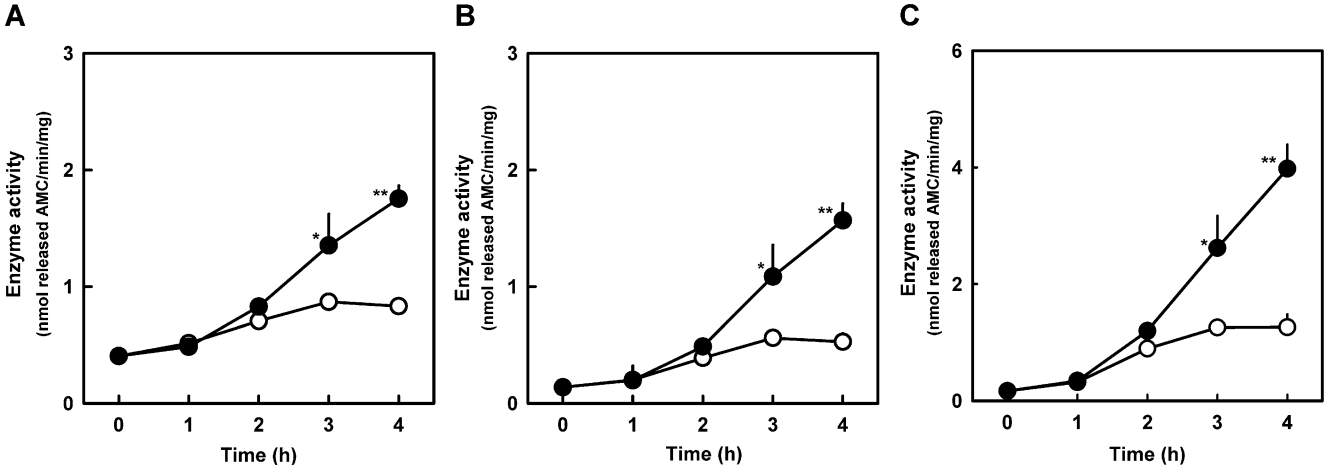

Fig. 7. Caspases activation in terfenadine-treated thymocyte. Thymocytes were incubated in the absence or presence of 10 μM terfenadine for indicated times, and then the extracts from the cells were prepared. The resulting extracts were used as the enzyme preparations. The activities of caspase-8 (A), -9 (B) and -3 (C) were determined using the substrates Ac-IETD-MCA, Ac-LEHD-MCA and Ac-DEVD-MCA, respectively. ○, control; ●, terfenadine-treated. Results are representative of four independent experiments and are expressed as mean values ± S.E.M. (error bars are smaller than the symbols in many data points). *$P<0.05$, significant from control (Student's *t*-test).

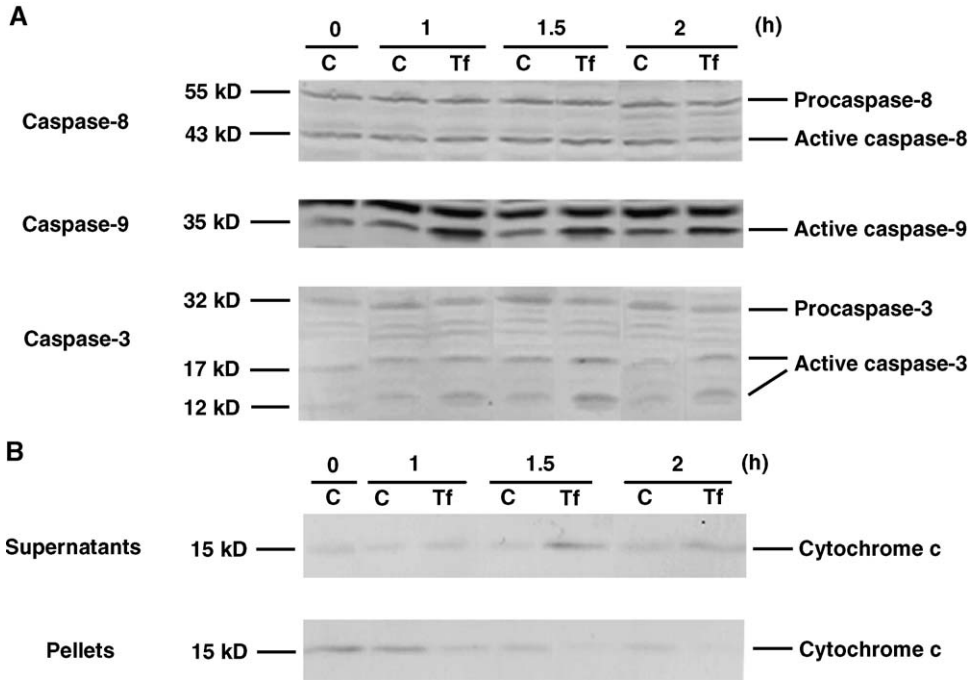

Fig. 8. Immunoblotting analysis for caspases processing and cytochrome *c* release induced by terfenadine treatment. Thymocytes were incubated in the absence or presence of 10 μM terfenadine for indicated times. (A) The caspase preparation was extracted from the cells. Precursors or active caspase-8, -9 and -3 were detected by use of each anti-caspase antibody. (B) Cells were disrupted and these homogenates were distributed cytosolic and residual fraction including mitochondria. Release of cytochrome *c* from mitochondria to cytosol was analyzed using anti-cytochrome *c* antibody. C, control; Tf, terfenadine-treated.

polarized membrane potentials. The treatment of the cells with terfenadine resulted in an increase in green fluorescence and a decrease in red fluorescence (Fig. 9). The typical data derived in two independent experiments were shown in this figure. Thus, terfenadine induced a decrease in $\Delta\psi$.

4. Discussion

Generally, it is necessary for evaluation of apoptotic cell death to demonstrate the biochemical and morphological changes in the cells. The biochemical and morphological studies shows that terfenadine induced apoptotic cell death in rat thymocytes. However, terfenadine used at high concentrations resulted in a decrease of DNA fragmentation. The fragmented DNA probably leaked to the culture medium by the damage of plasma membrane in these cells. These results suggest the lysis of the cells or the intensive damage of plasma membrane. Liu et al. (2003) have reported that terfenadine induced G_0/G_1 cell-cycle arrest and apoptosis for various human cancer cells. Other antiallergic agents did not affect DNA fragmentation in rat thymocytes, and these results accorded with the report by Wang et al. (2002). Thus, the blockade of histamine H_1 receptor and the inhibition of chemical mediator release are unlikely to be involved in the induction of thymocyte apoptosis.

The caspase-3 inhibitor prevented terfenadine-induced DNA fragmentation, and terfenadine stimulated caspase-8, -9 and -3-like activities in the cells. However, the determination of caspase activities by fluorescent substrates has a problem with substrate specificity. In the preliminary study, we have found that the activity of Ac-IETDase is inhibited by not only caspase-8 inhibitor but also other caspases inhibitor such as caspase-9 and -3. Similar inhibitions are seen in the activities of Ac-LEHDase and Ac-DEVDase. Therefore, the activation of caspases should be evaluated using immunoblotting analysis together with the measurement of the enzyme activity. Immunoblotting analysis revealed that the active forms of caspase-9 and -3 but not caspase-8 were detected in terfenadine treated cells. Terfenadine treatment of the cells caused the loss of $\Delta\psi$ and the release of cytochrome *c* to cytosol from mitochondria. These results suggest that terfenadine induces thymocyte apoptosis via mitochondria/caspase-9 activation pathway. Ho et al. described that the cellular responses to terfenadine-induced apoptosis were demonstrated to be associated with the p53-signaling pathway, including induction of p53, p21/Cip1, p27/Kip1, bax protein expression and inhibition of bcl-2 protein expression (Wang et al., 2002; Liu et al., 2003). Since these proteins exist upstream of mitochondria signaling pathway, subset of their results are consistent with our results.

Although astemizole also increased the DNA fragmentation in rat thymocytes, fexofenadine did not induce the DNA fragmentation in these cells. Terfenadine and astemizole have inhibitory effects on I_{Kr} current (Salata et al., 1995) encoded by human *ether-a-go-go-related* gene (HERG) type of the K_v channel in cardiac cells and prolongs the QT interval. Furthermore, it is reported that terfenadine blocks I_{Ks} (Salata

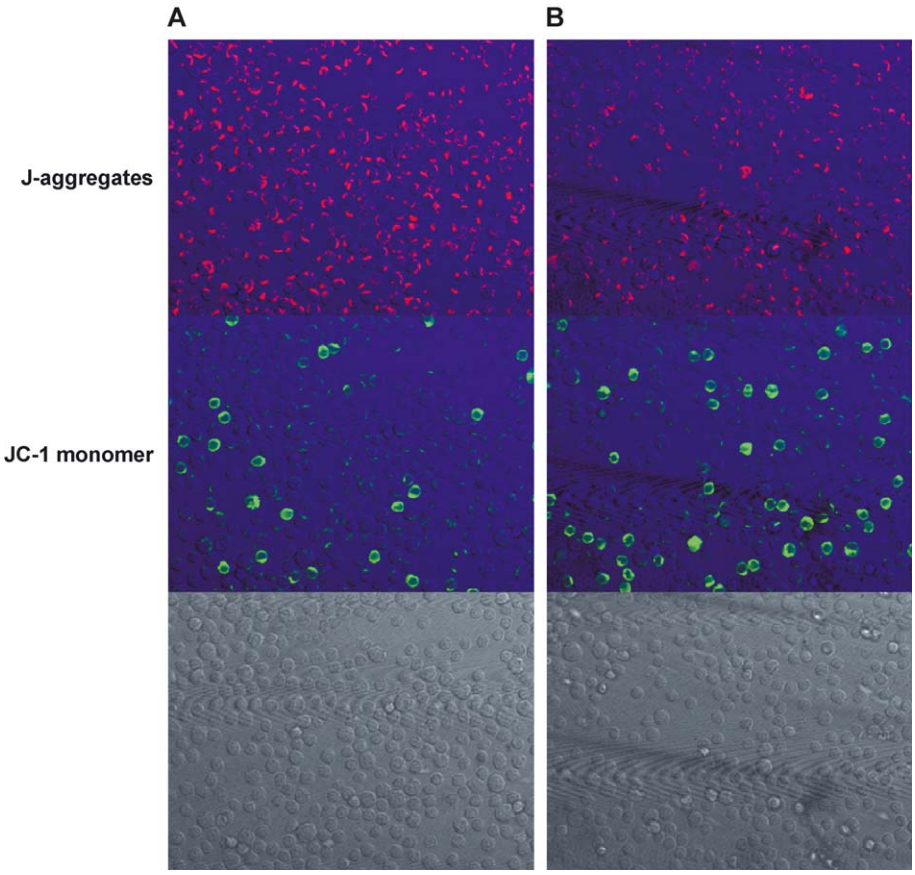

Fig. 9. Change in the mitochondrial membrane potential by the treatment of thymocytes with terfenadine. Thymocytes were treated with 10 μM terfenadine for 30 min. A total of 10 μg/ml JC-1 was added into incubation medium 20 min before the end of terfenadine treatment. Cells were washed and analyzed by a laser-scanning microscope. The lower panels indicated the dioptric images of the same field of vision; (A) control, (B) terfenadine-treated.

et al., 1995) encoded by K_vLQT1 (KCNQ1) and I_{Kur} (Roy et al., 1996) encoded by K_v1.5 currents in cardiac cells. On the other hand, fexofenadine does not block HERG channel in vitro (Roy et al., 1996), and clinical trials did not reveal any effect of fexofenadine on QTc time (Pratt et al., 1999). We examined DNA fragmentation in the cells treated with various agents which prolong the QT interval to clarify the role of the K_v channel in apoptosis induction. Some antiarrhythmic, antipsychotic or antifungal agents prolong QT interval by antagonism of the K_v and/or the voltage-dependent Na^+ channels. Among the agents we examined, amiodarone, bepridil and chlorpromazine increased DNA fragmentation. Both amiodarone and bepridil are antiarrhythmic drugs, and the former and the latter are classified into the class III and IV, respectively. Amiodarone strongly blocks the various types of K^+ current such as I_{Kur}, I_{Kr} and I_{Ks}, and also inhibits the Na^+ and the Ca^{2+} currents in ventricular heart cells (Kodama et al., 1999). Bepridil is the blocker of the L-type Ca^{2+} channel and also blocks the I_{Ks} and the Na^+ currents in the cells (Wang et al., 1999). DNA fragmentation was also increased in the treatment of chlorpromazine, a phenothiazine neuroleptic. This agent also blocks a variety of K^+ channels and Na^+ channel in heart muscle cells (Kon et al., 1994). On the other hand, E-4031 and DL-sotalol which are classified into the class III antiarrhythmic drugs and selectively inhibit I_{Kr} (Lynch et al., 1995; Wang et al., 1999) current did not affect DNA fragmentation. Therefore, we speculated that the inhibition of HERG type of K_v channel was related to pro-apoptotic action of terfenadine, but this channel is unlikely to be related in apoptosis induced by this drug.

Quinidine, bretylium and ketoconazole had no effect on DNA fragmentation in the cells. Quinidine and bretylium are concurrently classified into antiarrhythmic drugs, and the former and the latter respectively belong to the class Ia and III. Quinidine blocks the K^+ currents including I_{Kr} and the Na^+ current in cardiac cells (Po et al., 1999). Bretylium also inhibits Na^+ and K^+ currents (Bkaily et al., 1988). Ketoconazole is an antifungal agent and blocks HERG and K_v1.5 channels (Dumaine et al., 1998). Because little is known about the specific inhibitors of each K_v channel, it is difficult to identify which type of K_v channel participates in pro-apoptotic action. Since common action of the drugs causing apoptotic cell death is only the blockade of K_v channel, some types of the K_v channels are likely to play a role in the induction of apoptotic cell death.

In immune cells, K_v channel plays a role in proliferation (Lewis et al., 1995), cytotoxicity (Schlichter et al.,

1986), volume regulation (Deutsch and Chen, 1993) and the respiratory burst (Khanna et al., 2001). At least three types of K_v channels have been identified in murine T lymphocytes. K_v channel isoform $K_v1.3$ is expressed by both immature and mature thymocytes whereas $K_v1.1$ and $K_v3.1$ have been only found in immature $CD4^-CD8^-$ and mature $CD4^-CD8^+$ thymocytes, respectively (Freedman et al., 1995). These K_v channels contribute to the development and the maturation of thymocytes. The role of K_v channel in lymphocytes is to maintain the driving force for Ca^{2+} entry to trigger a cascade of these events (Smith et al., 2002). K_v channel is also likely to relate in the induction of apoptotic cell death. Fas receptor-mediated inhibition of $K_v1.3$ channel may influence apoptosis in the human leukemic T-cell line Jurkat (Szabo et al., 1996). Moreover, two apoptotic proteins that are named as Reaper and Grim seem to promote apoptosis by stably blocking of K_v channels (Avdonin et al., 1998). These studies conclude that inhibition of K_v channel depolarizes the cells and initiates a caspase-dependent apoptotic pathway. At present it is unclear about the inhibition of $K_v1.3$ by terfenadine. On the other hand, some of K_v channel blockers prevent apoptotic processes of the cells. Tetrapentylammonium, a broad inhibitor of K_v channels, prevented thymocyte apoptosis induced by various inducers such as dexamethasone, etoposide and γ-irradiation but this inhibitor did not affect Fas-induced apoptosis (Dallaporta et al., 1999). In CTLL-2 T-lymphocytes, actinomycin D-induced apoptotic alternations such as DNA fragmentation, depolarization of mitochondria and release of cytochrome *c* were dependent on $K_v1.3$ (Bock et al., 2002). The difference of cellular response to K_v channel blockers may depend on the complement of ion channels and pumps expressed in the plasma membrane (Manikkam et al., 2002; Mann et al., 2001). Thus, the role of K_v channel in induction of apoptosis is still uncertain. Since sustained membrane depolarization has been recognized recently as a general component of the apoptotic process (Bortner et al., 2001; Mann et al., 2001), K_v channels may be involved in the induction of apoptotic cell death. The role of K_v channel in the induction of apoptotic cell death remains to be elucidated. Terfenadine may be a useful compound to study on the role of the K_v channel in apoptosis induction.

Although terfenadine and astemizole induced apoptotic cell death in rat thymocytes, fexofenadine has no effect on the apoptosis induction. Fexofenadine was developed as the antiallergic agent without arrhythmic side effect, and our data give support that this is a good agent in terms of apoptosis induction.

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References

Antonsson, B., Martinou, J.-C., 2000. The bcl-2 protein family. Exp. Cell Res. 256, 50–57.

Arends, M.J., Wyllie, A.H., 1991. Apoptosis: mechanism and roles in pathology. Int. Rev. Exp. Pathol. 32, 223–254.

Avdonin, V., Kasuya, J., Ciorba, M.A., Kaplan, B., Hoshi, T., Iverson, L., 1998. Apoptotic proteins Reaper and Grim induce stable inactivation in voltage-gated K^+ channels. Proc. Natl. Acad. Sci. U. S. A. 95, 11703–11708.

Bkaily, G., Payet, M.D., Benabderrazik, M., Sauve, R., Renaud, J.F., Bacaner, M., Sperelakis, N., 1988. Intracellular bretylium blocks Na^+ and K^+ currents in heart cells. Eur. J. Pharmacol. 151, 389–397.

Bock, J., Szabo, I., Jekle, A., Gulbins, E., 2002. Actinomycin D-induced apoptosis involves the potassium channel $K_v1.3$. Biochem. Biophys. Res. Commun. 295, 526–531.

Bortner, C.D., Cidlowski, J.A., 1999. Caspase independent/dependent regulation of K^+, cell shrinkage, and mitochondrial membrane potential during lymphocyte apoptosis. J. Biol. Chem. 274, 21953–21962.

Bortner, C.D., Hughes Jr., F.M., Cidlowski, J.A., 1997. A primary role for K^+ and Na^+ efflux in the activation of apoptosis. J. Biol. Chem. 272, 32436–32442.

Bortner, C.D., Gomez-Angelats, M., Cidlowski, J.A., 2001. Plasma membrane depolarization without repolarization is an early molecular event in anti-Fas-induced apoptosis. J. Biol. Chem. 276, 4304–4314.

Dallaporta, B., Hirsch, T., Susin, S.A., Zamzami, N., Larochette, N., Brenner, C., Marzo, I., Kroemer, G., 1998. Potassium leakage during the apoptotic degradation phase. J. Immunol. 160, 5605–5615.

Dallaporta, B., Marchetti, P., de Pablo, M.A., Maisse, C., Duc, H.T., Metivier, D., Zamzami, N., Geuskens, M., Kroemer, G., 1999. Plasma membrane potential in thymocyte apoptosis. J. Immunol. 162, 6534–6542.

Deutsch, C., Chen, L.Q., 1993. Heterologous expression of specific K^+ channels in T lymphocytes: functional consequences for volume regulation. Proc. Natl. Acad. Sci. U. S. A. 90, 10036–10040.

Di Lisa, F., Blank, P.S., Colonna, R., Gambassi, G., Silverman, H.S., Stern, M.D., Hansford, R.G., 1995. Mitochondrial membrane potential in single living adult rat cardiac myocytes exposed to anoxia or metabolic inhibition. J. Physiol. 486 (Pt 1), 1–13.

Dumaine, R., Roy, M.L., Brown, A.M., 1998. Blockade of HERG and Kv1.5 by ketoconazole. J. Pharmacol. Exp. Ther. 286, 727–735.

Freedman, B.D., Fleischmann, B.K., Punt, J.A., Gaulton, G., Hashimoto, Y., Kotlikoff, M.I., 1995. Identification of Kv1.1 expression by murine $CD4^-CD8^-$ thymocytes. A role for voltage-dependent K^+ channels in murine thymocyte development. J. Biol. Chem. 270, 22406–22411.

Khanna, R., Roy, L., Zhu, X., Schlichter, L.C., 2001. K^+ channels and the microglial respiratory burst. Am. J. Physiol., Cell Physiol. 280, C796–C806.

Kodama, I., Kamiya, K., Toyama, J., 1999. Amiodarone: ionic and cellular mechanisms of action of the most promising class III agent. Am. J. Cardiol. 84, 20R–28R.

Kon, K., Krause, E., Gogelein, H., 1994. Inhibition of K^+ channels by chlorpromazine in rat ventricular myocytes. J. Pharmacol. Exp. Ther. 271, 632–637.

Koopman, G., Reutelingsperger, C.P., Kuijten, G.A., Keehnen, R.M., Pals, S.T., van Oers, M.H., 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84, 1415–1420.

Krijnen, P.A., Nijmeijer, R., Meijer, C.J., Visser, C.A., Hack, C.E., Niessen, H.W., 2002. Apoptosis in myocardial ischaemia and infarction. J. Clin. Pathol. 55, 801–811.

Lee, E., Miura, M., Yoshinari, M., Iwai, H., Kariya, K., Lee, E., Miura, M., Yoshinari, M., Iwai, H., Kariya, K., 1994. Selective inhibition of dexamethasone-induced apoptosis in rat thymocytes by herbimycin A. Biochem. Biophys. Res. Commun. 202, 128–134.

Lee, E., Miyaguchi, F., Inoue, M., Kariya, K., Sasaki, H., 1996. A novel DNA cleaving agent, 2,2′-bis(2-aminoethyl)-4,4′-bithiazole, induces thymocyte apoptosis. Biochem. Mol. Biol. Int. 40, 151–157.

Lee, E., Enomoto, R., Takemura, K., Tsuda, Y., Okada, Y., 2002. A selective plasmin inhibitor, trans-aminomethylcyclohexanecarbonyl-L-(*O*-picolyl)tyrosine-octylamide (YO-2), induces thymocyte apoptosis. Biochem. Pharmacol. 63, 1315–1323.

Lewis, R.S., Cahalan, M.D., Lewis, R.S., Cahalan, M.D., 1995. Potassium and calcium channels in lymphocytes. Annu. Rev. Immunol. 13, 623–653.

Liu, J.D., Wang, Y.J., Chen, C.H., Yu, C.F., Chen, L.C., Lin, J.K., Liang, Y.C., Lin, S.Y., Ho, Y.S., 2003. Molecular mechanisms of G_0/G_1 cell-cycle arrest and apoptosis induced by terfenadine in human cancer cells. Mol. Carcinog. 37, 39–50.

Lynch Jr., J.J., Baskin, E.P., Nutt, E.M. , Guinosso Jr., P.J., Hamill, T., Salata, J.J., Woods, C.M. 1995. Comparison of binding to rapidly activating delayed rectifier K^+ channel, I_{Kr}, and effects on myocardial refractoriness for class III antiarrhythmic agents. J. Cardiovasc. Pharmacol. 25, 336–340.

Manikkam, M., Li, Y., Mitchell, B.M., Mason, D.E., Freeman, L.C., 2002. Potassium channel antagonists influence porcine granulosa cell proliferation, differentiation, and apoptosis. Biol. Reprod. 67, 88–98.

Mann, C.L., Bortner, C.D., Jewell, C.M., Cidlowski, J.A., 2001. Glucocorticoid-induced plasma membrane depolarization during thymocyte apoptosis: association with cell shrinkage and degradation of the Na^+/K^+-adenosine triphosphatase. Endocrinology 142, 5059–5068.

McConkey, D.J., Hartzell, P., Duddy, S.K., Hakansson, H., Orrenius, S., 1988. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin kills immature thymocytes by Ca^{2+}-mediated endonuclease activation. Science 242, 256–259.

Mesner, P., Budiharadjo, I., Kaufmann, S.H., 1997. Chemotherapy-induced apoptosis. Adv. Pharmacol. 41, 461–499.

Nieto, M.A., Gonzalez, A., Gambon, F., Diaz-Espada, F., Lopez-Rivas, A., 1992. Apoptosis in human thymocytes after treatment with glucocorticoids. Clin. Exp. Immunol. 88, 341–344.

Po, S.S., Wang, D.W., Yang, I.C., Johnson Jr., J.P., Nie, L., Bennett, P.B., 1999. Modulation of HERG potassium channels by extracellular magnesium and quinidine. J. Cardiovasc. Pharmacol. 33, 181–185.

Pratt, C.M., Mason, J., Russell, T., Reynolds, R., Ahlbrandt, R., 1999. Cardiovascular safety of fexofenadine HCl. Am. J. Cardiol. 83, 1451–1454.

Raffray, M., McCarthy, D., Snowden, R.T., Cohen, G.M., 1993. Apoptosis as a mechanism of tributyltin cytotoxicity to thymocytes: relationship of apoptotic markers to biochemical and cellular effects. Toxicol. Appl. Pharmacol. 119, 122–130.

Roy, M., Dumaine, R., Brown, A.M., 1996. HERG, a primary human ventricular target of the nonsedating antihistamine terfenadine. Circulation 94, 817–823.

Salata, J.J., Jurkiewicz, N.K., Wallace, A.A., Stupienski III, R.F., Guinosso Jr., P.J., Lynch Jr., J.J., 1995. Cardiac electrophysiological actions of the histamine H1-receptor antagonists astemizole and terfenadine compared with chlorpheniramine and pyrilamine. Circ. Res. 76, 110–119.

Schlichter, L., Sidell, N., Hagiwara, S., 1986. Potassium channels mediate killing by human natural killer cells. Proc. Natl. Acad. Sci. U. S. A. 83, 451–455.

Slater, J.W., Zechnich, A.D., Haxby, D.G., 1999. Second-generation antihistamines: a comparative review. Drugs 57, 31–47.

Smith, G.A., Tsui, H.W., Newell, E.W., Jiang, X., Zhu, X.P., Tsui, F.W., Schlichter, L.C., 2002. Functional up-regulation of HERG K^+ channels in neoplastic hematopoietic cells. J. Biol. Chem. 277, 18528–18534.

Snider, B.J., Gottron, F.J., Choi, D.W., 1999. Apoptosis and necrosis in cerebrovascular disease. Ann. N.Y. Acad. Sci. 893, 243–253.

Szabo, I., Gulbins, E., Apfel, H., Zhang, X., Barth, P., Busch, A.E., Schlottmann, K., Pongs, O., Lang, F., 1996. Tyrosine phosphorylation-dependent suppression of a voltage-gated K^+ channel in T lymphocytes upon Fas stimulation. J. Biol. Chem. 271, 20465–20469.

Thompson, E.B., 1998. Special topic: apoptosis. Annu. Rev. Physiol. 60, 525–532.

Vaux, D.L., 2002. Apoptosis and toxicology–what relevance? Toxicology 181–182, 3–7.

Wang, J.C., Kiyosue, T., Kiriyama, K., Arita, M., 1999. Bepridil differentially inhibits two delayed rectifier K^+ currents, I_{Kr} and I_{Ks}, in guinea-pig ventricular myocytes. Br. J. Pharmacol. 128, 1733–1738.

Wang, Y.J., Yu, C.F., Chen, L.C., Chen, C.H., Lin, J.K., Liang, Y.C., Lin, C.H., Lin, S.Y., Chen, C.F., Ho, Y.S., 2002. Ketoconazole potentiates terfenadine-induced apoptosis in human Hep G_2 cells through inhibition of cytochrome p450 3A4 activity. J. Cell. Biochem. 87, 147–159.

Wyllie, A.H., Wyllie, A.H., 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284, 555–556.

Yamashita, K., Takahashi, A., Kobayashi, S., Hirata, H., Mesner, P.W., Kaufmann, S.H., Yonehara, S., Yamamoto, K., Uchiyama, T., Sasada, M., 1999. Caspases mediate tumor necrosis factor-alpha-induced neutrophil apoptosis and downreguration of reactive oxygen production. Blood 93, 674–685.