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Inhibitory effect of M50054, a novel inhibitor of apoptosis, on anti-Fas-antibody-induced hepatitis and chemotherapy-induced alopecia

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Abstract

M50054, 2,2′-methylenebis (1,3-cyclohexanedione), was identified as a novel inhibitor of apoptosis (programmed cell death) using an in vitro cell death assay system induced in human Fas-expressing WC8 cells by soluble human Fas ligand. Furthermore, M50054 inhibited the apoptotic cell death of U937, a human monocytic leukemic cell line, induced by anticancer agents such as etoposide; it was also confirmed that M50054 inhibited apoptotic features such as DNA fragmentation and phosphatidylserine exposure in these cells. These anti-apoptotic effects were attributable to inhibition of caspase-3 activation. Additionally, M50054 significantly inhibited anti-Fas-antibody-induced elevation of plasma alanine aminotransferase and aspartate aminotransferase. Alopecia (hair loss) symptoms were also significantly improved with topical treatment with M50054. In conclusion, M50054 inhibits apoptosis induced by a variety of stimuli via inhibition of caspase-3 activation, and may thus be effective for hepatitis and chemotherapy-induced alopecia.

Keywords: Apoptosis; Fas; Etoposide; Caspase-3; Hepatitis; Alopecia

1. Introduction

Apoptosis is a form of cell death (Cohen, 1991), so-called ''programmed cell death'', which is quite distinct from necrosis. In necrotic cell death, cellular membranes are ruptured and intracellular substances have exuded out of cells due to injuries or extracellular stimuli, resulting in adverse effects on surrounding cells. In contrast, apoptosis is characterized by condensation of chromatin and cytoplasm or DNA fragmentation, and apoptotic cells are phagocytosed by other cells, including neighboring macrophages and neutrophils, without adverse effects on surrounding cells (Wyllie et al., 1980). Therefore, apoptosis is believed to be a physiological mechanism of cell death responsible for removal of unnecessary cells or abnormal cells from the body, and programmed cell death is considered necessary for growth as well as homeostasis of cells throughout the body (Ellis et al., 1991; Raff, 1992). Emerging evidence suggests that a variety of disorders may occur if this apoptotic mechanism is dysregulated (Thompson, 1995).

Fas and Fas ligand are well-known molecules (Nagata and Golstein, 1995; Nagata, 1999) identified as initiators of apoptosis, and the Fas/Fas ligand system contributes to development of some disorders such as hepatitis (Galle et al., 1995), graft versus host disease (GVHD) (Hattori et al., 1998; Ueno et al., 2000), T-cell death in acquired immune deficiency syndrome (AIDS) patients (Estaquier et al., 1996) and ischemic heart disease (Tanaka et al., 1994). In particular, Fas/Fas ligand-system-mediated hepatocyte apoptosis is observed in hepatitis C virus- or hepatitis B virus-associated chronic liver disease (Chisari, 1997; Galle et al., 1995; Hiramatsu et al., 1994; Mita et al., 1994). Therefore, in the development of a treatment of liver failure, inhibition of Fas-mediated signaling should have value in managing progression of virus-induced hepatitis, though effective treatments for this have yet to be found.

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On the other hand, etoposide, an anti-cancer agent used to inhibit topoisomerase II, has also been reported to induce DNA fragmentation and to cause apoptotic cell death (Kaufmann, 1989). A variety of chemotherapeutic agents have since been shown to induce extensive apoptosis (Hannun, 1997), and it has been found that chemotherapy-induced alopecia (hair loss) occurs as a result of increased apoptotic cell death of hair follicles rather than decreased cell proliferation, i.e., imbalance between cell proliferation and death (Cece et al., 1996; Schilli et al., 1998; Takahata et al., 1999). Since chemotherapy-induced alopecia causes distress in cancer patients, it is considered very important to alleviate this disorder during the course of anti-cancer chemotherapy. Although it has been reported that intrafollicular apoptosis in chemotherapy-induced alopecia is reduced in animal models by calcitriol-analogs (Schilli et al., 1998) or docosahexaenoic acid (Takahata et al., 1999), the precise effects of these drugs against apoptosis are still unknown, and clinically effective treatments have not yet been found for chemotherapy-induced alopecia.

In this study, we detected the inhibitory activity of M50054, 2,2′-methylenebis (1,3-cyclohexanedione), in an in vitro cell death random screening assay system using human Fas-expressing transformant through addition of soluble human Fas ligand, and evaluated the inhibitory activity in human myeloid leukemia U937 cells through addition of anticancer agents such as etoposide. Furthermore, we examined whether M50054 inhibits apoptotic features such as DNA fragmentation and phosphatidylserine exposure. In addition to these in vitro studies, we have examined whether M50054 improves anti-Fas antibody-induced hepatitis in mice by oral administration and chemotherapy-induced alopecia in newborn rats by topical administration.

2. Materials and methods

2.1. Materials

M50054, 2,2′-methylenebis (1,3-cyclohexanedione), was synthesized by Mochida Pharmaceutical. Etoposide (VP-16) was purchased from Sigma. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), 2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-terazolium monosodium salt (WST-1) and 1-methoxy-5-methyl phenazinium methylsulfate (1-Methoxy PMS) were purchased from Dojindo. Agonistic antimouse Fas monoclonal antibody (hamster, clone Jo2) was purchased from Pharmingen.

Female BALB/c mice were obtained from Charles River Japan. Pregnant female Sprague–Dawley rats (day 14 of gestation) were purchased from the Charles River Japan, and newborn rats were used for the experiment. The animals were kept in an air-conditioned room and pathogen-free room with temperature of 23 ± 2 °C and humidity of 55 ± 10% on a regulated 12-h light/dark cycle. They were given standard laboratory chow (CE-2; Clea Japan) and tap water ad libitum. All experimental procedures mentioned below were approved by the Institutional Animal Use Committee of our laboratory.

2.2. Plasmid construction

The extracellular region of human Fas ligand was cloned by polymerase chain reaction (PCR) using a sense primer (CACCTG-CAGAAGGAGCTGGCAGAA) containing the extracellular region of human Fas ligand and *Pst*I site, and antisense primer (AATAAGCTTGGTACCCTATTA-GAGCTTATATAA) containing the termination codon, *Hin*dIII site and *Kpn*I site. The PCR product was digested with *Pst*I and *Hin*dIII, and subcloned into pUC118. The signal sequence of human Fas was amplified by PCR using a sense primer (TGCGAATTCACCATGCTGGGCATCTGG) carrying the 5′ terminus of the signal sequence of the human Fas with *Eco*RI site, and antisense primer (AACCTGCAG GTGGAAGAGCTGAGCAACAGACGTAAG) carrying the 3′ terminus of the signal sequence of human Fas, the N terminus of the extracellular region of the human Fas ligand and *Pst*I site. After double digestion with *Eco*RI and *Pst*I, the PCR product was ligated to the plasmid produced as described above. For expression in mammalian cells, the DNA fragment containing the signal sequence of human Fas and the extracellular region of human Fas ligand was digested with *Eco*RI and *Kpn*I, and inserted into the expression vector pEF-BOS (Mizushima and Nagata, 1990).

2.3. Transfection of COS cells

Monkey kidney fibroblast COS-1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. COS-1 cells in 150 cm^2 Roux flasks that had grown to their semiconfluent stage were transfected with 8.1 μg of plasmid DNA using DEAE-dextran, and cultured in medium containing 10% fetal calf serum. After 72-h incubation at 37 °C, the culture supernatant was collected for the cell death assay. The concentration of soluble human Fas ligand in the COS-1 culture supernatant was 702 ng/ml, as determined by enzyme-linked immunosorbent assay (ELISA) (Kanda et al., 1998).

2.4. Cell death assay of WC8 induced by soluble human Fas ligand

Human Fas-expressing WC8 cells (Itoh et al., 1993) were maintained in RPMI1640 supplemented with 10% fetal calf serum and 200 μg of G418 (Gibco BRL) per milliliter in a humidified atmosphere of 5% CO_2 and 95% air. This experiment was performed using a 96-well microtiter plate, and RPMI1640 containing 10% fetal calf serum was used as a culture medium. M50054 was dissolved in dimethylsulfoxide (DMSO) and diluted with

the culture medium so that the final concentration of DMSO was below 0.3%. After 2.5×10^4 cells of WC8 cells per well were cultured in the presence of M50054 for 30 min, COS-1 culture supernatant containing 702 ng of soluble human Fas ligand per milliliter was added to each well at a final concentration of 3% (21 ng of soluble human Fas ligand/ml). After culturing for 18 h at 37 °C with 5% CO_2, cell survival was determined by formazan production using the tetrazolium salt (MTT) assay, and anti-cell death activity of M50054 was calculated. IC_{50} value was designated as the amount of M50054 needed to inhibit cell death by 50% compared with control.

We used MTT assay and WST-1 assay to measure "cell death" in our experiment, since we could confirm that MTT assay and WST-1 assay under our culture conditions correlated well with the cell death determined with trypan blue exclusion test.

2.5. Cell death assay of U937 induced by chemotherapeutic agent

The human myeloid leukemia U937 cell line was maintained in RPMI1640 supplemented with 10% fetal calf serum and 100 μg of kanamycin per milliliter in a humidified atmosphere of 5% CO_2 and 95% air. This experiment was performed using a 96-well microtiter plate, and RPMI1640 containing 10% fetal calf serum was used as a culture medium. M50054 was dissolved in DMSO and diluted with the culture medium so that the final concentration of DMSO was below 0.3%. After 2.5×10^4 cells per well were cultured in the presence of M50054 for 30 min, etoposide was added to each well at a final concentration of 10 μg/ml. After culturing for 12 h at 37 °C with 5% CO_2, cell survival was determined by highly water-soluble formazan production using the sulfonated tetrazolium salt (WST-1) assay, and anti-cell death activity of M50054 was calculated. IC_{50} value was designated as the amount of M50054 needed to inhibit cell death by 50% compared with control.

2.6. DNA fragmentation assay of U937 induced by chemotherapeutic agent

U937 cells were cultured in RPMI1640 containing 10% FCS. Etoposide (10 μg/ml) was added to U937 cells cultured in the presence of M50054 (2.5×10^5 cells/tube). After culturing U937 cells for 4 h at 37 °C with 5% CO_2, cells were recovered via centrifugation. Anti-apoptotic activity of M50054 was determined using a Cell Death Detection ELISA kit (Boehringer Mannheim) based on fixation of DNA fragments by anti-histone antibody immobilized on microtiter wells and peroxidase-labeled anti-DNA detection antibody, according to the kit protocol. IC_{50} value was designated as the amount of M50054 needed to inhibit DNA fragment by 50% compared with control.

2.7. Phosphatidylserine exposure of U937 induced by chemotherapeutic agent

This experiment was performed using a 48-well plate. U937 cells were cultured in RPMI1640 containing 10% fetal calf serum. Etoposide (10 μg/ml) was added to U937 cells cultured in the presence of M50054 (2.5×10^5 cells/well). After culturing U937 cells for 4 h at 37 °C with 5% CO_2, cells were recovered via centrifugation. Phosphatidylserine exposure was measured by the binding of annexin V-FITC according to the protocol outlined by the manufacturers in the MEBCYTO-Apoptosis kit (MBL). Cells were also stained with propidium iodide, before analysis with the FACSCalibur flow cytometer (Becton Dickinson).

2.8. In vitro assay of caspase-3 activity

The effect of M50054 on caspase-3 activity was examined in vitro. This experiment was performed using a 48-well plate. U937 cells were cultured in RPMI1640 containing 10% fetal calf serum. Etoposide (10 μg/ml) was added to U937 cells cultured in the presence of M50054 (2.5×10^5 cells/well). After culturing U937 cells for 3 h at 37 °C with 5% CO_2, cells were recovered via centrifugation. The caspase-3 activity was measured using a commercially available kit (Caspase-3/CPP32 Fluorometric Protease Assay Kit, MBL). Briefly, 2.5×10^5 cells were lysed and a fluorometrically labeled substrate, DEVD-*p*-nitroanilide, was incubated with the cell lysate at 37 °C for 30 min. Subsequently, fluorescence intensity was measured using a fluorometer ($ARVO_{sx}$, Amersham Pharmacia Biotech) (excitation wavelength: 405 nm, fluorescence wavelength: 535 nm). IC_{50} value was designated as the amount of M50054 needed to inhibit caspase-3 activation by 50% compared with control. In the measurement of direct inhibition of the enzymatic activity of caspase-3, recombinant human caspase-3 activity (host: *E. coli*, the Medical and Biological Research Foundation) was determined with use of the same commercially available kit.

2.9. Anti-Fas-antibody-induced hepatitis in mice

The inhibitory effect of M50054 on anti-Fas-antibody-induced hepatitis in mice was examined by the modified Ogasawara's method (Ogasawara et al., 1993). Briefly, the mice were divided into five groups (10 mice per group) based on body weight. M50054 was suspended in 0.5% (w/v) Tween 80 and orally administered at 10, 30, 100 or 300 mg/kg in a volume of 10 ml/kg. Control mice were administrated 0.5% (w/v) Tween 80 only. The agonistic anti-Fas antibody (3 μg) was injected in 100 μl of saline into 5-week-old female BALB/c mice via a tail vein 30 min after oral administration of M50054. Seven hours after administration of the anti-Fas antibody, plasma samples were collected to determine alanine aminotransferase and aspar-

tate aminotransferase using a standard clinical analyzer (DRI-CHEM5000, Fuji film).

2.10. Etoposide-induced alopecia (hair loss) in newborn rats

In this animal model of etoposide-induced alopecia (hair loss), the efficacy of M50054 was investigated by the modified Jimenz's method (Jimenz and Yunis, 1992). Briefly, the newborn rats were divided into two groups (10 rats per group). M50054 was dissolved in acetone (5% w/v) and topically applied to the head and back of 5-day-old rats once daily for 10 days. Acetone was applied to age-matched control rats in a similar manner. Hair loss was induced by repeated intraperitoneal administrations of etoposide (1 mg/kg/day), i.e., etoposide was injected once daily for 3 days, starting on day 6 after initiation of M50054 therapy. Thereafter, rats were observed for hair loss for 8 days after the last administration of etoposide, and the degree of hair loss was scored. On day 8 after etoposide treatment, photographs were taken and inhibitory effects of M50054 on hair loss were assessed. Degree of hair loss was scored in accordance with the following criteria: (1) No hair loss was seen at all; (2) Partial hair loss was seen on the head; (3) Hair loss was seen on most of the head as well as on the back (less than 50%); (4) Hair loss was seen on most of the head as well as on the back (more than 50%). (5) Hair loss was seen on most of the head and back (more than 90%).

2.11. Statistical analysis

Parametric data were analyzed as follows. First, variances among groups were analyzed using Bartlett's test. When they were homogeneous, comparisons between the control group and each treatment group were made using the parametric multiple comparison test (Dunnett's method). Otherwise, the nonparametric multiple comparison test (Dunnett's method) was used. Comparisons between two groups were made using Wilcoxon's test. For all analyses, P values less than 0.05 were considered significant.

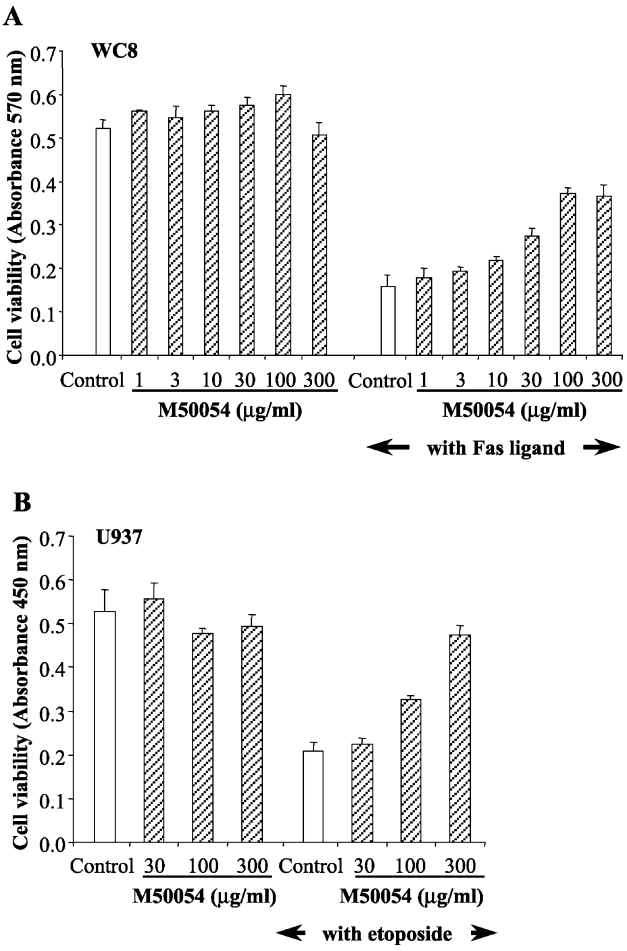

Fig. 1. Effects of M50054 on cell death induced by (A) Fas/Fas ligand system or (B) etoposide. (A) WC8 cells (2.5×10^4 cells) were pre-incubated for 30 min with M50054. Cells in 0.1 ml of RPMI1640 medium containing 10% fetal calf serum were then incubated at 37 °C for 18 h in the presence of 3% of the supernatant from the human Fas ligand-transfected cells (COS-1/pM1070) (21 ng/ml of Fas ligand). Cell viability was measured by MTT assay. Each bar represents mean ± S.D. for four wells in one reproducible experiment. The results of a representative cell culture are shown. (B) U937 cells (2.5×10^4 cells) were pre-incubated for 30 min with M50054. Cells in 0.1 ml of RPMI1640 medium containing 10% fetal calf serum were then incubated at 37 °C for 12 h in the presence of 10 μg/ml of etoposide. Cell viability was measured by the WST-1 assay. Each bar represents mean ± S.D. for five wells in one reproducible experiment.

3. Results

3.1. Effect of M50054 on apoptotic cell death induced by human Fas ligand or a chemotherapeutic agent

Initial experiments were aimed at determining the effect of M50054 on death of human Fas-expressing WC8 cells induced by soluble human Fas ligand. When WC8 cells were treated for 18 h with COS-1 culture supernatant containing human Fas ligand (21 ng/ml), approximately 70% of them died. As shown in Fig. 1A, M50054 inhibited soluble human Fas ligand-induced cell death of human Fas-expressing WC8 cells in a dose-dependent manner, and the IC_{50} of M50054 was 67 μg/ml.

In addition, the inhibitory effects of M50054 on death of human monocytic leukemia U937 cells induced by etoposide, a chemotherapeutic agent, were determined. In this assay, 60% of U937 cells were killed by addition of etoposide (10 μg/ml) with 12-h incubation. As shown in Fig. 1B, M50054 inhibited the death of U937 cells induced by etoposide in a dose-dependent manner, and the IC_{50} value of M50054 against etoposide-induced cell death of U937 cells was 130 μg/ml.

To determine the anti-apoptotic activity of M50054, the inhibitory effects of M50054 on major features of apoptosis, DNA fragmentation and phosphatidylserine exposure,

were evaluated. In this assay, DNA fragmentation and phosphatidylserine exposure of U937 cells occurred within 4 h after treatment with etoposide (10 μg/ml). M50054 inhibited DNA fragmentation of U937 cells induced by etoposide in a dose-dependent manner; this was confirmed by ELISA based on fixation of DNA fragments by anti-histone antibody immobilized on microtiter wells and peroxidase-labeled anti-DNA detection antibody (Fig. 2A). The IC_{50} value of M50054 against etoposide-induced DNA fragmentation of U937 cells was 54 μg/ml, and slightly less than that against death of U937 cells. M50054 also inhibited DNA fragmentation of WC8 cells induced by soluble human Fas ligand (data not shown). Furthermore, M50054 inhibited phosphatidylserine exposure induced by etoposide, which was confirmed by annexin V-propidium iodide binding assay (Fig. 2B). Annexin V binds to early apoptotic as well as late apoptotic/necrotic cells; propidium iodide binds to late apoptotic/necrotic cells. These results thus suggest that M50054 actually inhibits cell death via inhibition of DNA fragmentation and phosphatidylserine exposure.

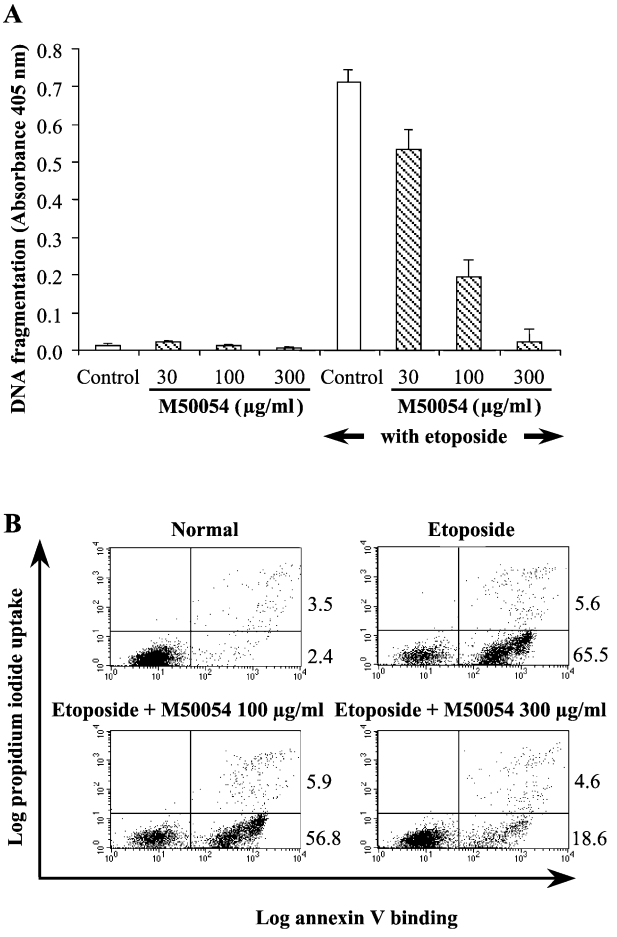

Fig. 2. Effects of M50054 on apoptosis of U937 induced by etoposide. (A) Effect of M50054 on DNA fragmentation of U937 induced by etoposide. U937 cells (2.5×10^5 cells) were pre-incubated for 30 min with M50054. Cells in 0.5 ml of RPMI1640 medium containing 10% fetal calf serum were then incubated at 37 °C for 4 h in the presence of etoposide (10 μg/ml). The anti-apoptotic activity was measured by Cell Death Detection ELISA (Boehringer Mannheim). Each bar represents mean ± S.D. for five cultures in one reproducible experiment. The results of a representative assay are shown. (B) Effects of M50054 on phosphatidylserine exposure of U937 induced by etoposide. U937 cells (2.5×10^5 cells) were pre-incubated for 30 min with M50054. Cells in 0.5 ml of RPMI1640 medium containing 10% fetal calf serum were then incubated at 37 °C for 4 h in the presence of etoposide (10 μg/ml). The phosphatidylserine exposure of U937 induced by etoposide was measured by the degree of Annexin V and propidium iodide binding using a MEBCYTO Apoptosis Kit (MBL). Annexin V binds to early apoptotic and late apoptotic/necrotic cells. Propidium iodide binds to late apoptotic/necrotic cells. Percentages of cells in the upper and lower right quadrants are given. The results of a representative assay are shown.

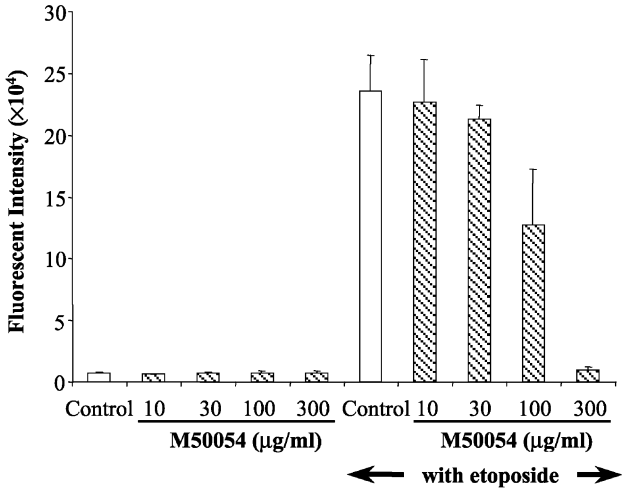

Fig. 3. Effect of M50054 on caspase-3 activation in vitro. U937 cells (2.5×10^5 cells) were pre-incubated for 30 min with M50054. Cells in 0.5 ml of RPMI1640 medium containing 10% fetal calf serum were then incubated at 37 °C for 3 h in the presence of etoposide (10 μg/ml). Caspase-3 activity was measured using a Caspase-3/CPP32 Fluorometric Protease Assay Kit (MBL). The values shown are the mean ± S.D. of triplicate cultures.

3.2. Effect of M50054 on caspase-3 activation in vitro

M50054 inhibited the apoptotic cell death induced by both Fas/Fas ligand and a chemotherapeutic agent, as described above. Therefore, M50054 might exert pharmacological effects via inhibition of cellular factors common to the Fas/Fas ligand- and chemotherapeutic agent-induced apoptotic pathways. We therefore examined the inhibitory effects of M50054 on caspase-3 activation of U937 cells. In this assay, caspase-3 activation of U937 cells occurred within 3 h after addition of etoposide (10 μg/ml). M50054 inhibited the caspase-3 activation by etoposide in a dose-dependent manner (Fig. 3). The IC_{50} value of M50054 against etoposide-induced caspase-3 activation of U937 cells was 79 μg/ml, and slightly more than the IC_{50} value of M50054 against etoposide-induced DNA fragmentation of U937 cells. However, M50054 did not directly inhibit the enzymatic activity of caspase-3 at all concentrations up to

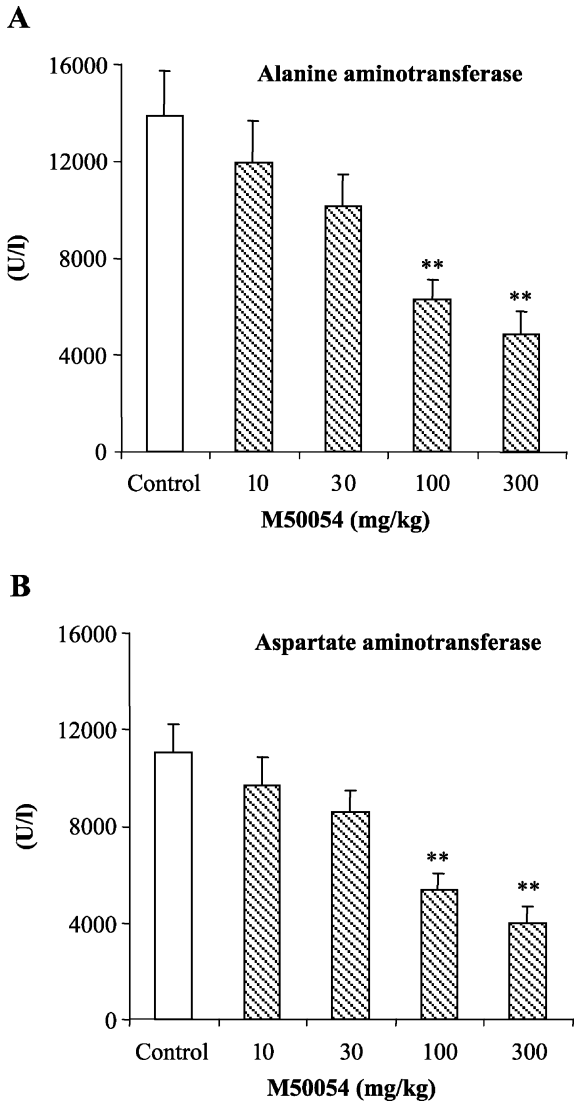

Fig. 4. Effects of M50054 on Anti-Fas-induced hepatitis in mice. Anti-Fas monoclonal antibody (Jo2) (3 μg) in 100 μl of saline was injected into 5-week-old female BALB/c mice intravenously 30 min after oral administration of M50054. Seven hours after Jo2 injection, plasma samples were collected, and plasma alanine aminotransferase and aspartate aminotransferase levels were quantified. Values are mean ± S.E.M. ($n = 10$, * * $P < 0.01$ from the control group determined by Dunnett's method).

1000 μg/ml, a finding confirmed using recombinant human caspase-3 and DEVD-*p*-nitroanilide as a substrate (data not shown). M50054 thus appears to protect against apoptotic cell death via inhibition of caspase-3 activation.

3.3. Effect of M50054 on anti-Fas-induced hepatitis in mice

Fas-system-mediated apoptosis is known to play an important role in the liver cell injury induced by hepatitis B virus or hepatitis C virus infection, which often leads to chronic hepatitis. We therefore investigated the effects of oral M50054 on anti-Fas-antibody-induced hepatitis in mice. In this model, mice were intravenously treated with anti-Fas antibody (3 μg/mouse), and plasma levels of alanine aminotransferase and aspartate aminotransferase in the anti-Fas-antibody-treated mice increased by about 300 times above basal level within 7 h after treatment with the antibody. Mice were pretreated with M50054 by oral administration 30 min before anti-Fas antibody injection (i.v.). M50054 inhibited the elevation of alanine aminotransferase and aspartate aminotransferase in a dose-dependent manner; at doses of 100 mg/kg and 300 mg/kg, it significantly improved these parameters ($P < 0.01$) (Fig. 4).

3.4. Effects of M50054 on chemotherapy-induced alopecia in newborn rats

Alopecia (hair loss) was induced in newborn rats (day 11) following repeated intraperitoneal administration of 1 mg/kg etoposide once daily for 3 days. At day 11, all newborn rats had a nearly full coat of hair at the start of injection of etoposide. Alopecia began over the head 3 days after the last etoposide injection, and rapidly progressed to the entire body. Alopecia score reached peak level 7 days after the last etoposide injection (Fig. 5). The experimental group received 5% (w/v) M50054 in acetone, and the control group received acetone only. Treatment was given daily beginning on day 5 after birth and ending on day 14. In the M50054-treated group, alopecia scores significantly decreased throughout the entire observation period for all rats treated with M50054 compared with the control group (treated with acetone alone) ($P < 0.01$) (Fig. 5). This finding

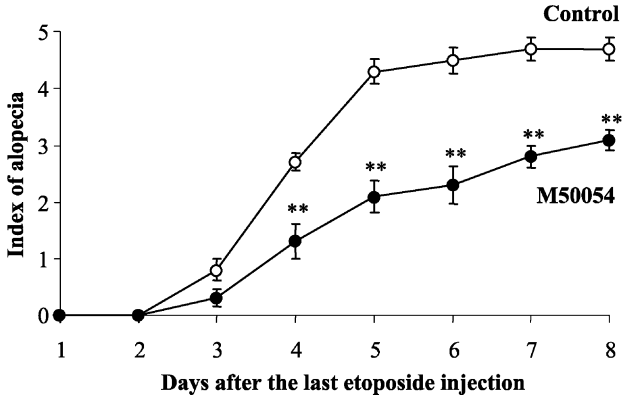

Fig. 5. Effects of M50054 on etoposide-induced alopecia in rats. Five-day-old SD rats were randomly divided into two groups of equal numbers. The experimental group of rats received 5% (w/v) solution of M50054 in acetone daily over the head and back for 10 days. Control rats were similarly treated with same volume of acetone. Six days after the first topical treatment, etoposide (1 mg/kg) was given intraperitoneally to each rat for 3 consecutive days. The pattern of hair loss was evaluated subjectively using a grading system: No hair loss, 0; mild head alopecia, 1; severe head alopecia, 2; severe head alopecia and back alopecia with less than 50% hair loss, 3; severe head alopecia and back alopecia with more than 50% hair loss, 4; and virtually total hair loss (>90%), 5. Values are mean ± S.E.M. ($n = 10$, * * $P < 0.01$ from the control group determined by Wilcoxon test).

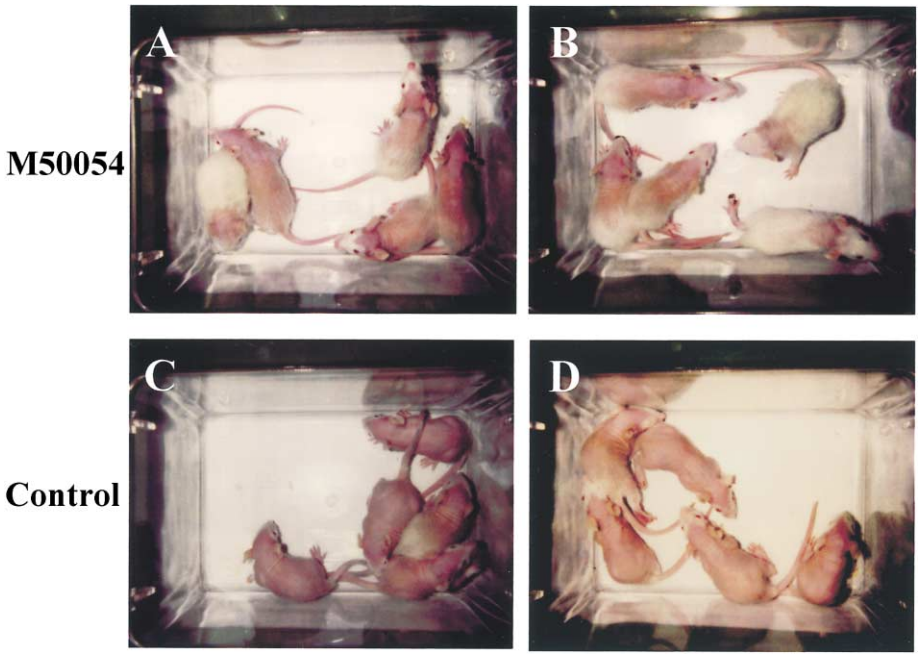

Fig. 6. Effects of M50054 on etoposide-induced alopecia in newborn rats. Five-day-old rats were divided into two groups of 10 rats each. The experimental group of rats (A and B) received 5% w/v M50054 in acetone to the head and back once daily for 10 days. Control rats (C and D) were similarly treated with acetone. These photographs were taken 8 days after the last etoposide treatment.

was also confirmed in photographs taken 8 days after etoposide administration (Fig. 6).

4. Discussion

M50054, 2,2′-methylenebis (1,3-cyclohexanedione), inhibited soluble human Fas ligand-induced cell death of human Fas-expressing WC8 cells and etoposide-induced death of human myeloid leukemia U937 cells in vitro. Moreover, we confirmed that M50054 inhibited the characteristics of apoptosis, i.e., DNA fragmentation and phosphatidylserine exposure. These findings indicate that M50054 exerts pharmacological effects via inhibition of cellular factors common to the Fas/Fas ligand pathway and etoposide-induced apoptotic pathways. Although the details of the mechanisms of etoposide-induced apoptosis remain unclear, the caspase-3 activation resulting from postmitochondrial activation is thought to be a common pathway of the Fas/Fas ligand system and chemotherapeutic agents such as etoposide (Dubrez et al., 1996; Sun et al., 1999). Therefore, we examined the effect of M50054 on caspase-3 activation, which is an intracellular common signal responsible for apoptosis. In this study, M50054 inhibited caspase-3 activation, but did not directly inhibit enzymatic activity of caspase-3 at all (data not shown). Based on these results, we predict that the site of action of M50054 is located upstream of caspase-3 activation; M50054 might inhibit mitochondorial function related to the progression of apoptosis, but further studies will be needed to determine whether this is the case.

Patients suffering from hepatitis are often infected with hepatitis B virus or hepatitis C virus. It is generally assumed that the hepatitis occurring during viral hepatitis is mediated by cytotoxic T lymphocytes (Berke, 1995). Cytotoxic T lymphocytes are involved in the immune clearance of hepatitis B virus- or hepatitis C virus-infected hepatocytes and in the pathogenesis of these chronic viral liver diseases (Chisari, 1997; Galle et al., 1995; Hiramatsu et al., 1994; Mita et al., 1994). The cytotoxic T lymphocyte clone specific for hepatitis B surface antigen caused liver diseases in hepatitis B surface antigen-transgenic mice in a Fas-dependent manner (Kondo et al., 1997). Indeed expression of Fas and Fas ligand was elevated in the liver tissue in hepatitis B virus-related failure (Galle et al., 1995), and Fas-system-mediated apoptosis plays an important role in liver cell injury by hepatitis B virus or hepatitis C virus infection (Hayashi and Mita, 1999; Nasir et al., 2000). In vivo treatment of mice with anti-Fas monoclonal agonistic antibody has been confirmed to induce early massive apoptosis of hepatocytes (Ogasawara et al., 1993) in a sequence of pathological changes similar to those found in liver failure due to hepatitis viruses. It has been reported that some compounds, such as peptide inhibitor of caspase (Rodriguez et al., 1996; Rouquet et al., 1996), antisense oligonucleotide targeting Fas (Zhang et al., 2000), glycyrrhizin (Okamoto, 2000) and aminoguanidine (Okamoto and Okabe, 2000) inhibit anti-Fas-antibody-induced hepatitis in mice. However, these compounds were administered by the intravenous or intraperitoneal route, and orally active compound has not been reported. In this study, we have reported for the first time that a novel inhibitor of apoptosis, M50054,

inhibited anti-Fas-antibody-induced hepatitis in mice on oral administration. Since various types of hepatitis appear to occur as a result of mechanisms similar to Fas-mediated apoptosis, M50054 may be useful as a therapeutic drug for various types of hepatitis.

Chemotherapy-induced alopecia (hair loss) is a common side effect of many chemotherapeutic drugs used for the treatment of cancer. Several methods currently utilized to prevent chemotherapy-induced alopecia are reported to be unsatisfactory. Although the mechanism responsible for chemotherapy-induced alopecia is still unknown in detail, it has been suggested that chemotherapy-induced apoptosis of hair follicle cells leads to hair loss (Cece et al., 1996; Müller-Röver et al., 1999, 2000; Schilli et al., 1998; Takahata et al., 1999). Because hair follicle regression is characterized by substantial apoptosis, modulating the balance of hair follicular cell proliferation and apoptosis may be a key strategy for the regulation of hair regression, and may lead to more effective therapies of hair diseases. In this study, we have examined whether chemotherapy-induced hair loss is prevented by topical application of M50054, an inhibitor of caspase-3 activation, using a newborn rat model with etoposide-induced hair loss; this model is thought to be a good model with a high degree of reproducibility of visible hair loss after chemotherapeutic treatment (Cece et al., 1996; Hussein et al., 1990; Jimenz and Yunis, 1992). M50054 was topically applied for potential applicability to the clinical setting, since systemic administration of anti-apoptotic agents might inhibit the effect of anti-cancer drug related to apoptosis. Moreover, we have confirmed that the plasma concentration of M50054 after topical application of it (5% w/v) to rats are less than 0.1 μg/ml (data not shown) and they are insufficient to systemically inhibit apoptotic cell death. Symptomatic improvement was clearly achieved by topical administration of M50054, whereas hairs in the parts that had not been treated with M50054 almost fell out. This is the first demonstration that an inhibitor of caspase-3 activation can protect against chemotherapy-induced alopecia, suggesting that caspase-3 might be an important factor in chemotherapy-induced alopecia. All chemotherapeutic agents with potent anticancer activity are known to induce severe alopecia as an adverse effect; among them, etoposide appears to induce it with an extremely high incidence (Peckham et al., 1985). Since alopecia is unavoidable but not fatal in cancer chemotherapy, chemotherapy-induced alopecia tends to be ignored. However, from the quality of life point of view, the importance of management of chemotherapy-induced alopecia has recently been emphasized. Therefore, a novel inhibitor of caspase-3 activation, M50054, might provide one possible solution, alleviating alopecia-related symptoms and reducing patient distress.

We conclude that M50054 inhibits apoptosis induced by a variety of apoptotic stimuli such as the Fas/Fas ligand system and etoposide, and that these anti-apoptotic effects are caused by affecting upstream of caspase-3 activation in the apoptotic pathway. M50054 might thus be effective for hepatitis when administered orally and chemotherapy-induced alopecia when administered topically.

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