Harmol induces apoptosis by caspase-8 activation independently on Fas/Fas ligand interaction in human lung carcinoma H596 cells

Akihisa Abe[a] and Hiroyuki Yamada[b]

The β-carboline alkaloids are naturally existing plant substances. It is known that these alkaloids have a wide spectrum of neuropharmacological, psychopharmacological, and antitumor effects. Therefore, they have been traditionally used in oriental medicine for the treatment of various diseases including cancers and malaria. In this study, harmol and harmalol, which are β-carboline alkaloids, were examined for their antitumor effect on human lung carcinoma cell lines, and structure–activity relationship was also investigated. H596, H226, and A549 cells were treated with harmol and harmalol, respectively. Apoptosis was induced by harmol only in H596 cells. In contrast, harmalol had negligible cytotoxicity in three cell lines. Harmol induced caspase-3, caspase-8, and caspase-9 activities and caspase-3 activities accompanied by cleavage of poly-(ADP-ribose)-polymerase. Furthermore, harmol treatment decreased the native Bid protein, and induced the release of cytochrome c from mitochondria to cytosol. The apoptosis induced by harmol was completely inhibited by caspase-8 inhibitor and partially inhibited by caspase-9 inhibitor. The antagonistic antibody ZB4 blocked Fas ligand-induced apoptosis, but had no effect on harmol-induced apoptosis. Harmol had no significant effect on the expression of Fas. In conclusion, our results showed that the harmol could cause apoptosis-inducing effects in human lung H596 cells through caspase-8-dependent pathway but independent of Fas/Fas ligand interaction. ***Anti-Cancer Drugs*** **20:373–381 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Lung cancer is the leading cause of cancer death in the world for both men and women. It is known that non-small cell lung carcinoma (NSCLC) has a low cure rate because it is relatively insensitive to chemotherapeutic agents [1,2]. Furthermore, the high mortality of this disease is because of the difficulty of early diagnosis and its high potential to invade locally and metastasize to distant organs. Therefore, there is an immediate need to discover effective novel diagnostic, therapies, and prophylactic methods, against lung cancer. Recently, there is an increasing interest in the discovery of novel antitumor agents from natural resources [3]. Beta-carboline alkaloids are present in some medicinal plants, including *Peganum harmala*, *Passiflora edulis*, *Passiflora incarnate*, and *Bansteriopsis caapi* [4,5]. These plants have been used in traditional medicine to treat asthma, jaundice, lumbago, and other human ailments [5–7]. The β-carboline alkaloids are also found in common plants (e.g. wheat, rice, soybean, grapes, mushrooms) and plant-derived drinks (e.g. wine, beer, grape juice, whisky, brandy) [8]. These compounds are normal constituents of the human body [9,10] and some are formed under special conditions such as after alcohol intake [11–13]. Recently, it is reported that certain β-carbolines and their related compounds have cytotoxic effects on cancer cells [14–16] and cerebellar granule neurons [17]. However, these alkaloids, especially harmine and harmaline, have a wide spectrum of pharmacological actions in the central nervous system such as tremorogenesis [18,19], hypothermia [20], hallucinogenesis [21,22], monoamine oxidase (MAO) inhibition [23,24], and convulsive or anticonvulsive actions [25]. Therefore, it is difficult to consider these compounds as anticancer drugs. Chemotherapeutic agents need to have potent antitumor activity and low toxicity. In contrast, it was reported that β-carbolines, which have a hydroxyl group at the C-7 position, such as harmol, have slightly or no MAO-inhibiting effect [24,26] and do not induce neuropharmacological effects, such as convulsions [27]. Therefore, it is considered that harmol has low toxicity for humans and animals (Fig. 1). In this study, the apoptotic effect of harmol and harmalol were studied in three lung carcinoma cell lines to closely investigate the signal transduction pathway underlying harmol-induced apoptosis.

Materials and methods

Chemicals

Harmol and harmalol were purchased from Aldrich Chemicals (Milwaukee, Wisconsin, USA). Proteinase K,

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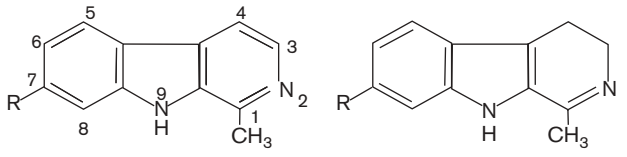
Fig. 1



Chemical structures of β-carbolines.

ribonuclease A, and dimethylsulfoxide were purchased from Wako Pure Chemical (Osaka, Japan). Caspase substrates Ac-DEVD-AFC (caspase-3), Ac-IETD-AFC (caspase-8), Ac-LEHD-AFC (caspase-9), caspase-8 inhibitor z-IETD-FMK, and caspase-9 inhibitor z-LEHD-FMK were obtained from MBL (Nagoya, Japan). The mitochondrial membrane potential assessment dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) was from Wako. SuperFas ligand was purchased from Alexis Corporation (San Diego, California, USA).

Cell lines and culture condition

Human NSCLC A549 (adenocarcinoma), H226 (squamous carcinoma), and H596 (adenosquamous carcinoma) cells were obtained from American Type Culture Collection (Rockville, Maryland, USA). These cell lines were cultured in modified Eagle's medium (Wako) with L-glutamine, nonessential amino acids, 70 mg/l kanamycin sulfate (Wako) and heat-inactivated fetal bovine serum (Biowest, Miami, Florida, USA) and maintained at 37°C in an incubator containing 95% air and 5% CO_2.

Cell viability

Cell viability was assessed by the WST-8 reagent [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, salt] method, using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Briefly, the tumor cells (6×10^4 cells/well) were precultured in a 48-well flat-bottomed microtiter plate for 24 h at 37°C in a 5% CO_2 humidified chamber. Then, various concentrations of harmol or harmalol were added and incubated for 1–24 h. After incubation, 40 µl of WST-8 reagents (Cell Counting Kit-8) was added to each well and incubation was continued for 1.5 h. After the incubation, the medium of each well was analyzed using a Powerscan HT microplate reader (DS Pharma Biomedical Co., Osaka, Japan) at a wavelength of 450 nm. Cell viability was determined by referring to the absorbance of nontreated cells.

DNA analysis by flow cytometry

Apoptosis was monitored by measuring hypodiploid DNA content. After 50 µmol/l of harmol treatment, cells were harvested by centrifugation, washed twice with ice-cold phosphate-buffered saline (PBS), and fixed with 70% ethanol for 1 h. Fixed cells were then washed with ice-cold PBS and stained with 20 µg/ml propidium iodide (Sigma, St. Louis, Missouri, USA) containing 10 µg/ml RNase A (Wako). After incubation in the dark for at least 30 min, the DNA content of cell was determined by a flow cytometer (PAS-I, Paltec, Münster, Germany).

Assay for caspase activity

Caspase activity was measured using fluorogenic peptide substrates. Briefly, both untreated control cells and cells after 60 µmol/l harmol treatment were washed with ice-cold PBS and suspended in lysis buffer [100 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 1% Nonidet p-40, 1 mmol/l EDTA, 1 mmol/l dithiothreitol (DTT), 1 mmol/l phenylmethylsulfonyl fluoride, 10 µmol/l leupeptin, and 1 µmol/l pepstatin] for 30 min on ice followed by centrifugation at 12 000*g* for 10 min at 4°C. In total, 50 µg protein in 40 µl buffer solution was mixed with 10 µl of 15 mmol/l fluorogenic report substrate specific for each caspase: Ac-DEVD-AFC for caspase-3, Ac-IETD-AFC for caspase-8, and Ac-LEHD-AFC for caspase-9. The reaction mixture was added to 2950 µl of assay buffer [20 mmol/l HEPES (pH 7.4), 0.1 mol/l NaCl, 5 mmol/l DTT, 0.1% Nonidet p-40] and incubated at 37°C for 1 h. The 7-amino-4-trifluoro-methyl coumarin (AFC) released from the substrate was excited at 400 nm to measure the emission at 505 nm. Untreated cells were used as a control.

Mitochondrial membrane potential assessment using JC-1

To assess the mitochondrial membrane potential, JC-1 assays were performed. The cells (1×10^6 cells/dish) were incubated in the culture medias overnight. Harmol, dissolved in dimethylsulfoxide, was added to the culture media at a final concentration of 60 µmol/l for 3–6 h. After treatment, 25 µl JC-1 was added to each dishes and further incubated for 20 min at 37°C. After incubation, cells were trypsinized and washed twice with PBS. These cells were resuspended in PBS and analyzed on a flow cytometer (PAS-I, Partec). This dye exists as aggregates in mitochondria that maintain a high membrane potential ($\Delta\Psi_m$). In contrast, in mitochondria presenting membrane depolarization, the dye accumulates as a monomer. Upon excitation at 488 nm, aggregates have a red fluorescence (approximately 590 nm), whereas monomers have a green fluorescence (approximately 525 nm) [28]. After excitation at 488 nm, emission in FL1 corresponds to JC-1 monomer and in FL3 to JC-1 aggregates.

Western blot analysis

H596 cells were seeded at 2×10^6 cells/ml in 90-mm cell culture dishes and incubated overnight. Then, 60 µmol/l harmol was added to the media and incubated for 3–9 h. After treatment, the cells were washed with ice-cold PBS. These cells were centrifuged at 500*g* for 5 min and the

pellet was lysed in a buffer containing 25 mmol/l HEPES (pH 7.4), 150 mmol/l NaCl, 0.5% Triton X-100, 10% glycerol, 1 mmol/l DTT, 1 mmol/l sodium orthovanadate, 25 mmol/l β-glycerophosphate, 1 mmol/l NaF, and 5 μl/ml protease inhibitor cocktail (Wako). The protein content of each lysate was determined using the BCA protein assay kit (Pierce, Rockford, Illinois, USA). Protein lysates were then mixed with an equal volume of gel loading buffer (20% glycerol, 4% SDS, 100 mmol/l Tris, 5% β-mercaptethanol, and 0.01% bromophenol blue) before being boiled for 5 min. After boiling, 30 μg of protein was subjected to SDS–polyacrylamide gel electrophoresis. Proteins were then transferred on a PVDF membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Blots were then blocked for 1 h at room temperature in 5% nonfat dry milk diluted in Tris–buffered saline supplemented with 0.1% Tween 20 (TBS-T; ICN Biomedicals Inc., Aurora, Ohio, USA). The following primary antibodies were incubated for 1 h at room temperature in TBS-T as follows: mouse monoclonal anti-PARP [poly-(ADP-ribose) polymerase] (BD Biosciences, Franklin Lakes, New Jersey, USA, 1 : 4000), rabbit polyclonal anti-Bid (Cell Signalling Technology, Danver, Massachusetts, USA, 1 : 2000), mouse monoclonal anti-cytochrome c (BD Biosciences, 1 : 4000), mouse monoclonal anti-Fas (BD Biosciences, 1 : 3000), mouse monoclonal anti-Fas ligand (BD Biosciences, 1 : 3000), mouse monoclonal anti-Hsp60 (BD Biosciences, 1 : 3000), and mouse monoclonal anti-β-actin (Sigma, 1 : 5000). Peroxidase-conjugated secondary antibodies were incubated for 1 h at room temperature as follows: sheep anti-mouse IgG (GE Healthcare, 1 : 7500). Blots were then washed with TBS-T and developed using enhanced chemoluminescence western blotting kit (GE Healthcare).

Statistical analysis

The results were presented as means ± SD. The data were analyzed by the nonpaired Student's *t*-test and a *P* value of less than 0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxic effects of harmol and harmalol

To determine the effects of harmol and harmalol on cell viability, the viability of the treated A549, H226, and H596 cells was examined after 24 h in culture by the Cell Counting Kit-8 assay (Fig. 2a–c). In these three cell lines, harmalol had no cytotoxic effects. In contrast, in A549 and H226 cells, harmol had slight cytotoxicity as the viability after treatment with 100 μmol/l harmol for 24 h was 85.6 and 84.7%, respectively. Interestingly, in H596 cells, harmol showed strong cytotoxicity and this effect was dose dependent. When H596 cells were exposed to harmol at various concentrations (25, 50, 75, and 100 μmol/l) in triplicate for 24 h, cell viability was 58.4, 21.2, 13.8, and 1.9%, respectively. When the H596 cells were exposed to harmol at a concentration of 50 μmol/l for 8, 16, and 24 h, the decrease in cell viability was time dependent (Fig. 2d).

Detection of harmol-induced DNA fragmentation on H596 cells

DNA fragmentation is a characteristic feature of apoptosis [29]. We performed fluorescence-activated cell sorter analysis of propidium iodide-stained nuclei to assess the cells for the presence of a hypodiploid or sub-G1 fraction resulting from DNA fragmentation. Figure 3 shows that the number of H596 cells in the sub-G1 fraction increased in a time-dependent manner in response to harmol.

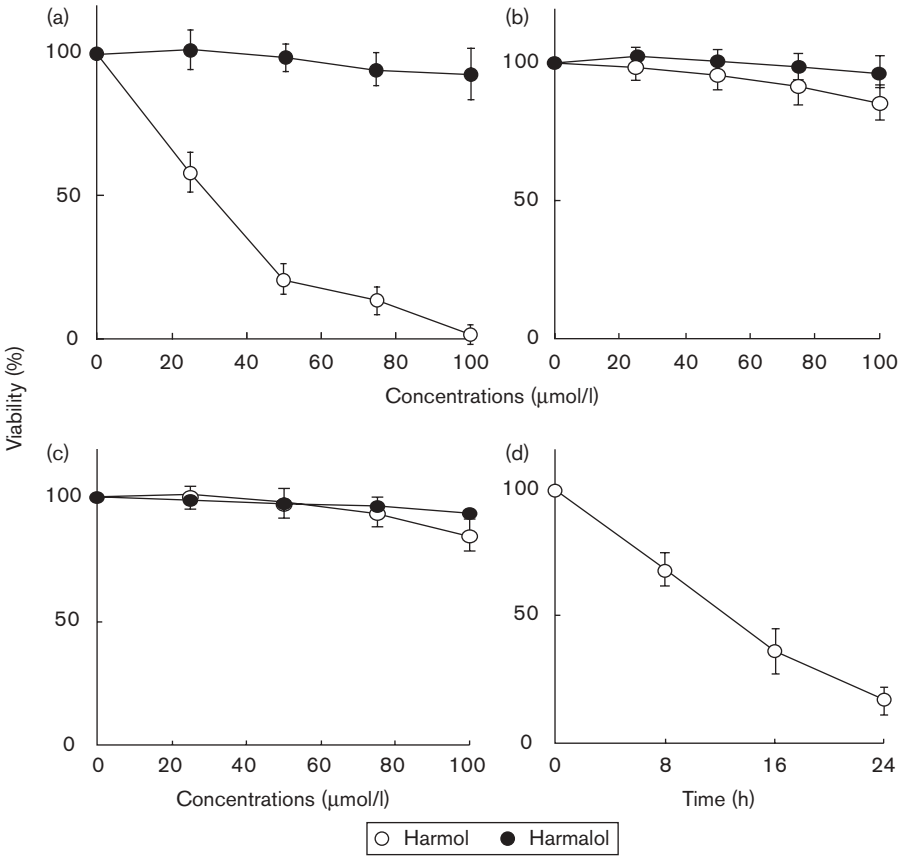
Activation of caspases during harmol-induced apoptosis and role of caspase-8 and caspase-9 in harmol-activated cascade in H596 cells

The involvement of caspases in harmol-induced apoptosis was determined by measuring the activities of three types of caspase using the peptide substrates Ac-DEVD-AFC, Ac-IETD-AFC, and Ac-LEHD-AFC, respectively. After treatment with 60 μmol/l harmol for 3 h, the activity of caspase-3 increased 566% compared with control. This also increased the activities of caspase-8 by 334%, and those of caspase-9 by 476% within 6 h, respectively (Fig. 4). To investigate which caspase dominates the harmol-activated caspase cascade, we used two inhibitors, z-IETD-FMK and z-LEHD-FMK, against caspase-8 and caspase-9, respectively, and measured the activities of the other caspases. Pretreatment of H596 cells with 50 μmol/l of z-IETD-FMK for 1 h completely suppressed the increase in the activities of all caspases (caspase-3, caspase-8, and caspase-9) to the control levels. In contrast, z-LEHD-FMK also partially decreased the caspase activities but did not completely suppress them (Fig. 4). These results indicated that caspase-8 was present upstream from caspase-9. In the case of caspase-3, z-IETD-FMK reduced the activity of caspase-3 to the control level, whereas z-LEHD-FMK did not decrease completely, suggesting that caspase-3 was activated mainly by the caspase-8-mediated pathway and partly by the caspase-9-mediated pathway.

Cleavage of PARP

It is well known that PARP, the zinc-dependent DNA binding protein that specifically recognizes DNA strand breaks, is cleaved by caspase-3, and the cleavage of PARP is regarded as a specific marker of apoptosis [30]. To confirm the activation of caspases, a western blot was performed to examine the cleavage of PARP by harmol. After treating the cells with 60 μmol/l harmol, the content of the cleaved form (85 kDa) of PARP increased time-dependently (Fig. 5, upper panel). These results indicated that caspase-3 activation (Fig. 4) was accompanied by the cleavage of PARP (116 kDa) into an 85 kDa C-terminal fragment. No changes could be identified when the samples were blotted by anti-β-actin (Fig. 5, lower panel).

Fig. 2

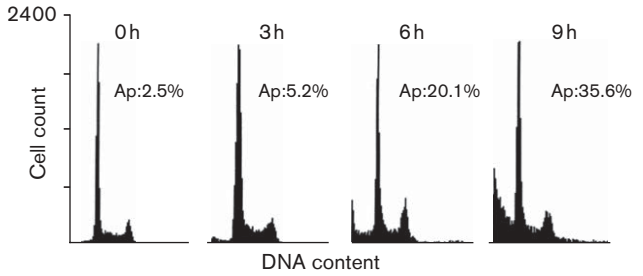


Effect of harmol and harmalol treatment in human lung H596, A549, and H226 cells. These three cell lines were treated with various concentrations of harmol and harmalol for 24 h. (a) H596 cells, (b) A549 cells, and (c) H226 cells. Control cells were treated with the same volume of dimethylsulfoxide (DMSO) as vehicle alone (final concentration of DMSO was below 0.1%). After culture, cell viability was measured by a Cell Counting Kit-8 assay as described in the 'Materials and methods' and then calculated as percentage of viability of the control cells. The results of harmol and harmalol treatment assay for each figure are shown as mean ± SD from three independent experiments. ○, harmol; ●, harmalol. Effect of harmol treatment on H596 cells in terms of period of treatment. (d) The H596 cells were treated with 50 μmol/l harmol for the indicated times.

Activation of the mitochondrial pathway by Bid mediation during apoptosis

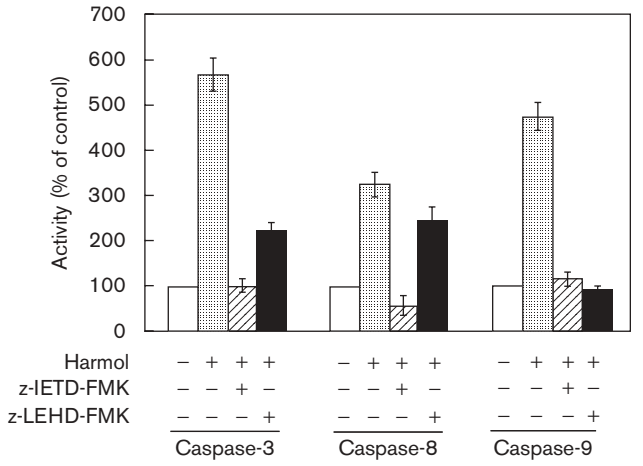
As harmol induced the activation of both initiator caspases, that is, caspase-8 and caspase-9 (Fig. 4), it is thought that harmol activates the mitochondrial apoptotic pathway through caspase-8-mediated Bid cleavage, which results in cytochrome c release and caspase-9 activation. To examine this idea, we tested the status of truncated-Bid (t-Bid), the active form of Bid protein, during harmol-induced apoptosis. The level of its protein increased within 3 h of harmol treatment (Fig. 6a). This closely matched the activation of caspase-8 (Fig. 4). To assess whether proteolytic cleavage by caspase-8 is really responsible for the truncation of Bid, we investigated the effects of various caspase inhibitors on Bid cleavage. When the cells were pretreated with the specific caspase-8 inhibitor z-IETD-FMK before harmol treatment, the increased level of t-Bid in harmol-treated cells was recovered to near that of the untreated control cells. However, pretreatment with the specific caspase-9 inhibitor z-LEHD-FMK did not prevent the increase of t-Bid protein (Fig. 6b). These results indicate that Bid protein is cleaved by caspase-8, which is activated by harmol treatment, and that the activation of caspase-9 may be a downstream event of this caspase-8-mediated Bid cleavage. We then investigated whether the cleavage of Bid by caspase-8 leads to the activation of the mitochondrial pathway. We investigated the loss of mitochondrial inner membrane potential ($\Delta\Psi_m$) and release of cytochrome c into cytosol. Loss of $\Delta\Psi_m$ has been shown to be implicated in the execution of apoptosis, because of alterations in permeability transition pores located between the inner and outer membranes [31]. When $\Delta\Psi_m$ was measured by staining harmol-treated cells with the $\Delta\Psi_m$ probe, JC-1, the loss of $\Delta\Psi_m$ increased until 3 h after treatment and then rapidly increased after 6 h (Fig. 6c), which correlated with the status of t-Bid protein (Fig. 6a). Furthermore, mitochondrial cytochrome c was released into the cytosol from mitochondria (Fig. 6d).

Fig. 3



Effect of harmol on apoptotic DNA contents in H596 cells. At the indicated time points after treatment with 50 μmol/l harmol, cells were fixed with 70% ethanol, stained with propidium iodide, and then subjected to flow cytometric analysis. The percentage of apoptotic cells (Ap) was determined based on the DNA content histograms. Results are representative of at least three individual experiments.

Fig. 4

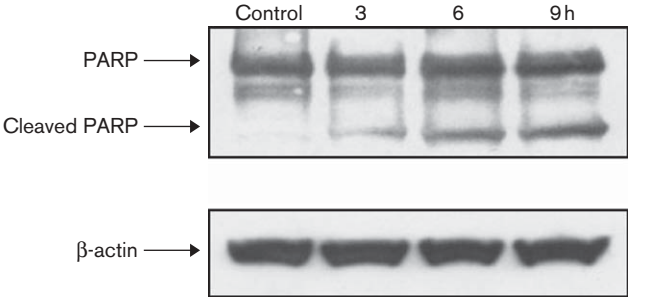


Harmol-induced caspase activity and effect of inhibitors of the initiator caspase on the harmol-activated cascade in H596 cells. The activities of caspase-3, caspase-8, and caspase-9 in the cells were measured as described in the 'Materials and methods'. H596 cells were pretreated with 50 μmol/l z-IETD-FMK (caspase-8 inhibitor) or z-LEHD-FMK (caspase-9 inhibitor) for 1 h, and then treated with 60 μ mol/l harmol or dimethylsulfoxide as a control for 3 h.

Effect of antagonistic Fas antibody on harmol-induced apoptosis

To elucidate which pathway was predominant for harmol-induced apoptosis, we used the antagonistic Fas antibody ZB4. As caspase-8 is closely linked to a signal transduction pathway initiated by interaction of Fas/Fas ligand, we examined whether activation of Fas would be important in mediating the effect of harmol. As shown in Fig. 7, pretreatment with ZB4 did not have any inhibitory effect on harmol-induced apoptosis, although it completely blocked the apoptosis induced by Fas ligand. We then further studied whether the expression of Fas could be increased by harmol. As shown in the upper panel of Fig. 8, stimulating the cells with harmol at 60 μmolo/l for

Fig. 5



Cleavage of poly-(ADP-ribose) polymerase, PARP, in harmol-treated H596 cells. H596 cells were treated with 60 μmol/l harmol for 3–9 h. The 116 kDa full length and 85 kDa fragment of PARP were detected.

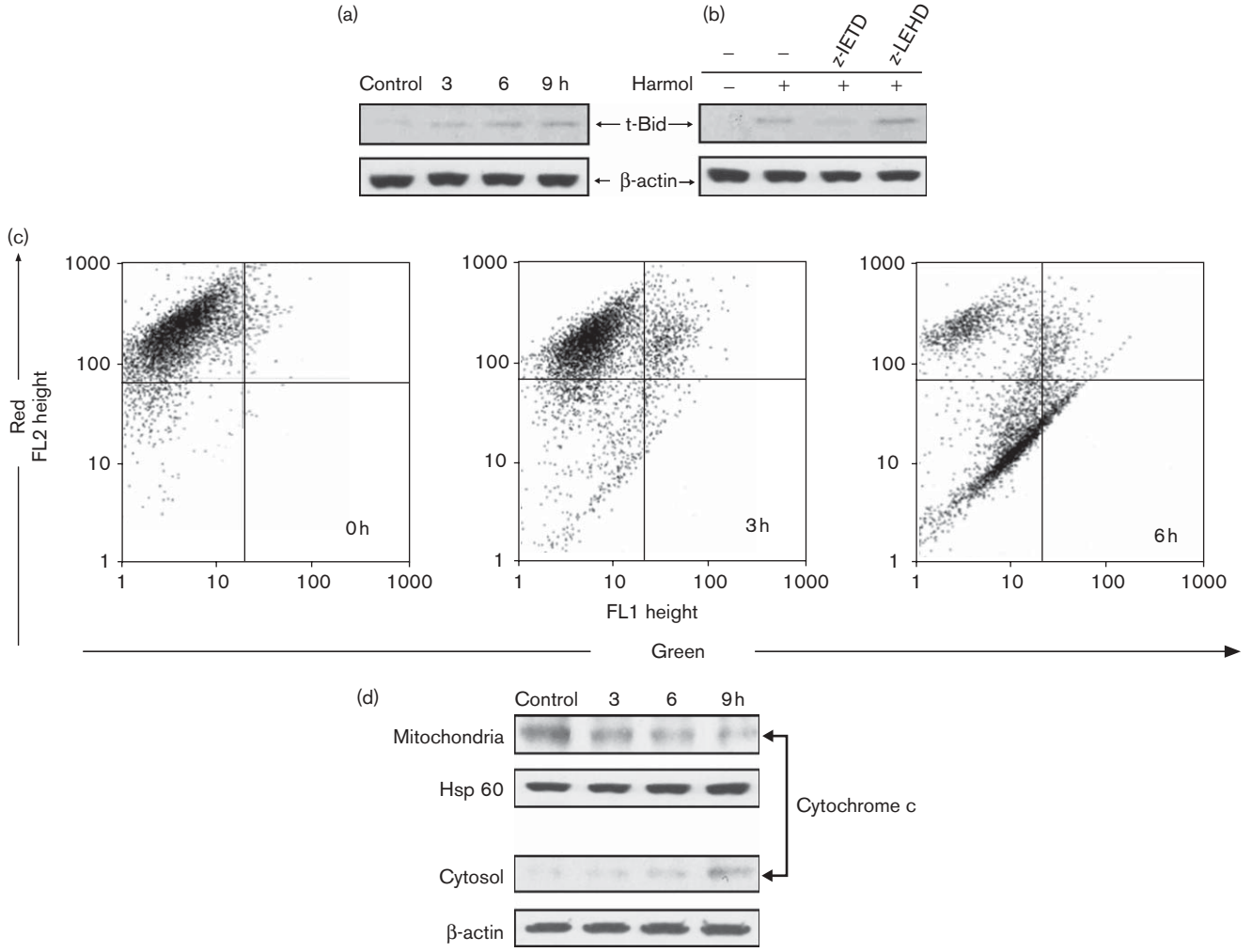
3–9 h had no significant effect on the levels of Fas in the cells. This negative effect was strengthened by similar levels of β-actin in the samples, as shown in the lower panel of Fig. 8.

Discussion

We set out to determine whether Fas/Fas ligand interaction is involved in harmol-induced apoptosis and the role of caspase-8 and caspase-9 in harmol-induced and Fas-induced apoptosis in lung carcinoma.

Lung carcinoma is one of the leading causes of cancer death not only in Japan, but also throughout the world. These carcinomas are characterized by an exceptionally low cure rate because of their resistance to chemotherapy, except for small cell lung carcinoma [1,2]. Therefore, there is a great demand for the development of new approaches, including chemotherapy against lung carcinoma. In this study, we found that harmol induced apoptosis in a human lung adenosquamous carcinoma cell line H596. The apoptotic effect of harmol was demonstrated by the formation of hypodiploid DNA fragments (Fig. 3) and this effect was dose dependent and time dependent (Fig. 2a, d and Fig. 3). Furthermore, harmol was found to induce caspase-3, caspase-8, and caspase-9 (Fig. 4) activations in H596 cells. In contrast, harmalol had negligible cytotoxicity in these three cell lines (Fig. 2a–c). Despite the similarity of the chemical structures, the degree of cell death-inducing action between harmol and harmalol in H596 cells were very different. The reason for this phenomenon is unclear. However, from the results of this study, it is likely that a double bond at the 3,4-position in the pyridine ring of harmol is very important for the apoptosis-inducing effect (Fig. 1). In this study, harmol-induced apoptosis occurred only in H596 cells, but not in A549 and H226 cells. The adenosquamous carcinoma cell line H596 is a NSCLC, and the adenocarcinoma cell line A549 and squamous carcinoma cell line H226 are also NSCLCs. From these

Fig. 6

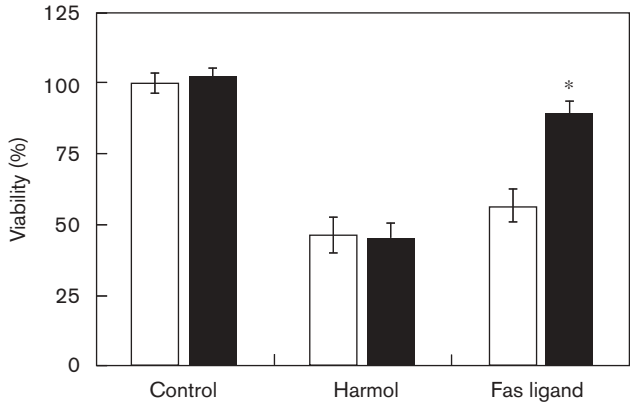


Activation of the mitochondrial pathway by caspase-8-mediated Bid cleavage. (a) Bid cleavage during harmol-induced apoptosis. H596 cells were treated with 60 μmol/l harmol for the indicated times. Equal amounts of whole-cell extracts were separated by SDS–polyacrylamide gel electrophoresis, and the level of truncated-Bid (t-Bid) protein was determined by western blotting with anti-t-Bid antibody. The amount of protein loaded in each lane was assessed by stripping and reprobing with β-actin antibody. (b) Effect of caspase inhibitors on Bid cleavage. Cells were treated for 6 h with or without 60 μmol/l harmol in the presence of 50 μmol/l of various caspase inhibitors. (c) The loss of mitochondrial inner membrane potential ($\Delta\Psi_m$) during harmol-induced apoptosis. Cells were treated with 60 μmol/l harmol for the indicated times and then stained with the mitochondrial membrane potential probe JC-1 (10 μg/ml). Fluorescence intensity was measured by flow cytometry. The data shown are representative of experiments run more than three times. Emission in FL1 (abscissae) corresponds to JC-1 monomers and in FL2 (ordinates) to aggregates. (d) Cytochrome c release from mitochondria to the cytosol in harmol cells. After treatment with 60 μmol/l harmol for the indicated time, mitochondria was separated from the cytosol, and the level of cytochrome c was determined by the western blotting.

results, it is thought that the apoptosis-inducing effect of harmol is not related to NSCLC-type features.

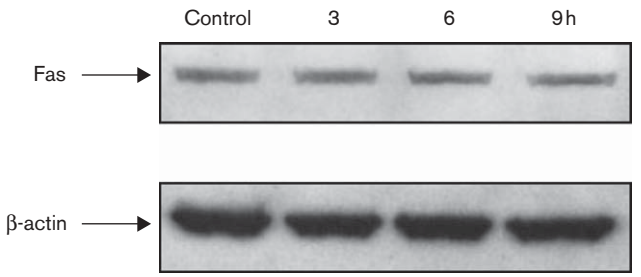
The apoptotic effect is considered to be an important property of many anticancer drugs. In principle, apoptosis occurs by two major different activation pathways (intrinsic and extrinsic pathways) [31,32]. In the intrinsic pathway, diverse stimuli that provoke cell stress or damage typically activate one or more members of the BH3-only protein family, leading to the release of cytochrome c. Cytochrome c then binds to the apoptosis-activating factor 1 and procaspase-9, resulting in the activation of caspase-9 by proteolytic cleavage. The extrinsic pathway starts with death receptor ligation or Fas/Fas ligand interaction, followed by oligomerization of the receptor, use of Fas-associated death domain (FADD) protein, and activation of caspase-8 [33]. However, significant crossover can occur, leading to amplification of the apoptotic process. For example, caspase-8 can participate in either pathway. Caspase-8, activated by the extrinsic pathway through a death receptor, may directly activate caspase-3 or may activate Bid, leading to activation of the intrinsic mitochondrial pathway [34]. Furthermore, once the intrinsic pathway has been

Fig. 7



Effect of antagonistic Fas antibody ZB4 on harmol-induced apoptosis. H596 cells were pretreated with 0.5 μg/ml ZB4 for 1 h (black bar). Harmol or Fas ligand was then added, to a final concentration of 60 μmol/l or 0.5 μg/ml, respectively. Experiments were performed in triplicate in three different experiments and results are presented as means ± SD. White bar indicated the nontreated cells and black bar indicated the ZB4 pretreatment cells. *$P < 0.005$ compared with effect of Fas ligand.

Fig. 8



Effect of harmol on Fas expression. The cells were treated with 60 μmol/l harmol for 3–9 h. Cell extract was subjected to 12.5% SDS–polyacrylamide gel electrophoresis, and the level of Fas protein was determined by western blotting with anti-Fas antibody. The amount of protein loaded in each lane was assessed by stripping and reprobing with β-actin antibody.

activated, caspase-8 may be activated by a caspase-3-dependent system through caspase-6 [35]. However, caspase-8 and caspase-9 are generally defined as initiator caspases and can in turn activate caspase-3, the executor of apoptosis [36,37]. In this study, we demonstrate for the first time that the activities of caspase-3, caspase-8, and caspase-9 were activated by harmol, indicating that both death receptor-related apoptotic pathway and the mitochondria-related pathway were activated (Fig. 6). When specific inhibitors were used, we found that specific caspase-8 inhibitor completely blocked the harmol-induced apoptosis, but the caspase-9 inhibitor only partially inhibited it (Fig. 4). These findings indicated that apoptosis induced by harmol in H596 cells is initiated by a pathway involving the activation of caspase-8, leading to the activation of caspase-3, and the cleavage of PARP. The activation of caspase-9 is probably a secondary consequence of the activation of caspase-8 through the cross-communication between the two apoptotic pathways. Caspase-8 has been shown to be a classical initiator caspase of the Fas pathway. The Fas pathway has also been shown to contribute to chemotherapeutic agent-induced apoptosis in various cellular systems. In this study, we found that Fas/Fas ligand interaction was not critical in harmol-induced apoptosis, because antagonistic Fas antibody ZB4 failed to inhibit harmol-induced apoptosis at a dose that completely blocked the apoptosis induced by Fas ligand (Fig. 7). Further, harmol did not affect the expression level of Fas in H596 cells on immunoblotting (Fig. 8). These data show for the first time that harmol activates a key element of the Fas signaling pathway independently of Fas/FADD activation. These findings suggest that harmol-induced apoptosis in H596 cells is mediated by mechanisms downstream of the Fas/Fas ligand interaction, such as Fas clustering, use of FADD, or enhanced cleavage of procaspase-8. These possibilities have been recently indicated in studies with several other anticancer drugs [38–40].

The β-carbolines occur naturally as harmala alkaloids in *Peganum harmala Linné*, which are also distributed widely in other medicinal plants [4,5] and found endogenously in mammalian tissues [9,10]. Harmala alkaloids have been used in hallucinogenic preparations of South America and African tribes [41]. Further, from ancient days, plants containing harmala alkaloids were used in traditional medicine to treat asthma, jaundice, lumbago, and other ailments [5–7]. It is known that certain β-carboline alkaloids have a wide spectrum of neuropharmacological and psychopharmacological actions in the central nervous system such as tremorogenesis [18,19], hypothermia [20], hallucinogenesis [21,22], MAO inhibition [23,24], convulsive or anticonvulsive actions [25], and binding to various receptors including 5-hydroxytryptamine receptors and the benzodiazepine binding site of γ-aminobutyric $acid_A$ receptors [42–44]. In addition, these compounds possess antioxidative [45] and radical scavenging properties [46], inhibition of platelet aggregation [47], and inhibition of DNA topoisomerase I [14]. The central nervous effects by β-carbolines mainly depend on β-carbolines, which have a methoxyl group at the C-7 position in the structures. Therefore, β-carbolines, which have a methoxyl group at the C-7 position, such as harmine and harmaline, are not suitable for chemotherapeutic agents. It is necessary for a chemotherapeutic agent to have potent antitumor activity and low toxicity. It was reported that β-carbolines, which have a hydroxyl group at the C-7 position, such as harmol has slightly or no MAO-inhibiting effect [24,26], and neuro-

pharmacological effect, such as convulsion [27]. Furthermore, it is reported that the metabolic pathway for harmol is conjugated with sulfate and excreted in urine and bile, and the rate of human urinary excretion after intravenous administration is fast [48]. Therefore, it is considered that harmol has low toxicity with regard to humans and animals.

In this study, after the treatment of harmol, only H596 cells caused conspicuous cell death. However, in A549 and H226 cells, when over 80 μmol/l of harmol was given, some cell deaths occurred. From this, moreover, by extending the time of harmol treatment or increasing the concentration, it was thought possible to induce cell death.

We investigated the effects of harmol only in NSCLC, but henceforth we should examine other kinds of cancer. However, as mentioned above, (i.e. the harmol has low toxicity) it is thought to be one of the candidates for a chemotherapeutic agent in cancer treatment. One of the obvious limitations of the study is that it was an in-vitro study and therefore, the results must be confirmed in an in-vivo model.

In conclusion, our data indicate that Fas/Fas ligand interaction is not involved in harmol-induced apoptosis and that caspase-8-dependent and caspase-9-dependent apoptoses play an important role in harmol-induced as well as Fas-induced apoptosis. The specific mechanisms by which harmol initiates the apoptosis signaling pathway is currently being investigated.

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