

Induction of Apoptosis in Hepatocellular Carcinoma Cell Lines by Emodin

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Previous experiments have shown that emodin is highly active in suppressing the proliferation of several tumor cell lines. However, it is not clear that emodin can induce growth inhibition of hepatoma cells. We have found that emodin induces apoptotic responses in the human hepatocellular carcinoma cell lines (HCC) Mahlavu, PLC/PRF/5 and HepG2. The addition of emodin to these three cell lines led to inhibition of growth in a time- and dose-dependent manner. Emodin generated reactive oxygen species (ROS) in these cells which brought about a reduction of the intracellular mitochondrial transmembrane potential ($\Delta\psi_m$), followed by the activation of caspase-9 and caspase-3, leading to DNA fragmentation and apoptosis. Our findings demonstrate that ROS and the resulting oxidative stress play a pivotal role in apoptosis. Preincubation of hepatoma cell lines with the hydrogen peroxide-scavenging enzyme, catalase (CAT) and cyclosporin A (CsA), partially inhibited apoptosis. These results demonstrate that enhancement of generation of ROS, $\Delta\psi_m$ disruption and caspase activation may be involved in the apoptotic pathway induced by emodin.

Key words: Emodin — Apoptosis — Reactive oxygen species — Mitochondrial transmembrane potential — Caspase

The anthraquinone derivative, emodin (1,3,8-trihydroxy-6-methylanthraquinone), is an orange-red crystalline compound that is one of the active components in many traditional Chinese medicinal herbs, especially from the Rhizoma and Radix families. It has been shown to have a number of biological properties, including antiviral, antimicrobial, immunosuppressive, hepatoprotective, anti-inflammatory, and antiulcerogenic^{1,2} activities, and to suppress topoisomerase II activity and the proliferation of various tumor cell lines.³ Nevertheless, whether emodin can induce growth inhibition of hepatoma cells remains largely unknown.

The process of programmed cell death, apoptosis, is fundamental in the developmental and homeostatic maintenance of complex biological systems.^{4–6} Dysregulation or failure of normal apoptotic mechanisms will contribute to transformation of cells and provide a growth advantage to cancer cells.⁷ Apoptosis is a response to physiological and pathological stresses that disrupt the balance between the rates of cell division and elimination. Agents that promote or suppress apoptosis can alter the rates of cell division and death, influencing the anomalous accumulation of neoplastic cells.⁸ Reactive oxygen species (ROS) may induce mitochondrial depolarization and consequently induce apoptosis in cells.^{9,10} It has been reported that oxidant or oxidant-promoting agents cause necrosis at high concentrations and apoptosis at lower concentrations.¹¹ Recent studies have demonstrated that ROS and the result-

ing oxidative stress play a pivotal role in apoptosis. Antioxidants, such as catalase (CAT), can block or delay apoptosis.¹² To evaluate whether the growth-inhibitory effect of emodin is associated with apoptosis, this study investigated emodin-induced cytotoxicity and sought evidence of apoptosis, including DNA fragmentation and double-staining with annexin V and propidium iodide (PI), in hepatocellular carcinoma cell lines. The mechanisms of emodin-induced apoptosis were also studied by evaluating ROS, mitochondrial damage and the activities of caspase-9 and -3 after emodin treatment. Our results suggest that mitochondria are the primary target for emodin-induced cell apoptosis and that free radical generation is an important and early event conveying cell death signals.

MATERIALS AND METHODS

Drug Emodin was purchased from the Chinese Medicine Center in Beijing, dissolved in dimethylsulfoxide (DMSO) at a concentration of 2 mg/ml, and stored at -20°C . The compound was diluted in the appropriate medium to 2.5, 5, 10 and 20 $\mu\text{g}/\text{ml}$ immediately before use. The final concentration of DMSO was $<0.1\%$. 2',7'-Dichlorofluorescein diacetate (DCFH-DA), dihydroethidium (HE) and 3,3-dihexyloxycarbocyanine iodide (DIOC₆) were purchased from Molecular Probes (Eugene, OR). CAT and cyclosporin A (CsA) were purchased from Wako (Osaka). **Cell culture** The human hepatocellular carcinoma cell lines, Mahlavu, PLC/PRF/5 and HepG2 were cultured in Dulbecco's modified Eagle's medium (DMEM) (ICN Biomedical, Inc., Aurora, MI) supplemented with 10% fetal

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calf serum (FCS), 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin, at 37°C under 5% $\text{CO}_2/95\%$ air.

Cell growth assay To assess the viability of Mahlavu, PLC/PRF/5 and HepG2 cells, cell numbers were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (Sigma Chemicals Co., St. Louis, MO), performed according to the method of Gerlier and Thomasset.¹³⁾ Briefly, cells were plated at a density of 2×10^4 cells/well in 96-well microtiter plates and each plate was incubated at 37°C in 5% $\text{CO}_2/95\%$ air. After 24 h, the medium was changed and the replacement medium was supplemented with various concentrations of emodin. All measurements were carried out using three separate wells for each treatment. After treatment, 50 μl of MTT solution (2 mg/ml in phosphate-buffered saline (PBS)) was added to each well and the plates were incubated for another 4 h at 37°C. The supernatant was discarded and 50 μl of DMSO was added to each well. Reduced MTT was then measured spectrophotometrically in a dual-beam microtiter plate reader at 570 nm with a 650 nm reference.

Analysis of oligonucleosomal DNA fragmentation Mahlavu, PLC/PRF/5 and HepG2 cells at a density of 1×10^6 cells per 100 mm culture dish were treated with various concentrations of emodin for the indicated periods and then floating and adherent cells were collected by centrifugation at 2500g for 5 min. The DNA was isolated using a Nucleic Acid Extractant Kit (TaKaRa, Otu) by the acid guanidinium thiocyanate-phenol-chloroform method according to manufacturer's instructions. The fragmented DNA in the supernatant was precipitated overnight at -20°C with 70% ethanol, resuspended in 30 μl Tris/EDTA buffer, and treated with ribonuclease A (100 $\mu\text{g/ml}$) for 2 h at 37°C. Electrophoresis was performed in 1.5% agarose gels in Tris-borate buffer at about 50 V/cm for approximately 30 min. After electrophoresis, the gels were stained with ethidium bromide (1 mg/ml), and photographed under UV light.

Cell cycle analysis The proportions of cells in G_0 - G_1 , S, and G_2 -M were determined by flow-cytometric analysis of DNA content according to a published method.¹⁴⁾ Cell cycle distribution in Mahlavu cells was measured after 24 h of treatment with emodin. Briefly, a cell suspension was prepared by trypsinization, and 1×10^6 cells/ml were washed twice with PBS. The cells were fixed overnight in 10 ml of 70% (-20°C) ethanol at 4°C. Then the cells were washed three times with PBS, incubated with RNase at a concentration of 0.25 mg/ml at 37°C for 1 h, and treated with PI (50 $\mu\text{g/ml}$ in PBS), then incubated for 30 min at 4°C in the dark. Before flow cytometry, the samples were syringed through a 25-gauge needle to prevent nuclear clumping. DNA histograms were analyzed using Lysis-II software to evaluate cell cycle compartments. PI was excited at 488 nm, and fluorescence was analyzed at 620 nm.

Annexin V FITC labeling Phosphatidylserine (PS) expression on the external surface of cells was measured in terms of the binding of FITC-labeled annexin V according to the protocol outlined by the manufacturer of the annexin V-FITC reagent.¹⁵⁾ Briefly, Mahlavu cells were collected by trypsinization after 6-h treatment with various concentrations of emodin, and the number of cells was adjusted to 1×10^6 cells/ml. Cells were washed once with PBS (4°C) and centrifuged to collect the cell pellet. The cell pellet was resuspended in cold (4°C) binding buffer and annexin V-FITC (10 $\mu\text{l/ml}$) and PI (10 $\mu\text{l/ml}$) solutions were added to the cell suspension and mixed gently. The tube was then incubated for 15 min in the dark prior to flow cytometry.

Measurement of intracellular ROS formation HE is a specific O_2^- dye, and DCFH-DA has been used frequently to monitor H_2O_2 levels in cells.^{8,16)} HE or DCFH-DA was dissolved in DMSO and diluted with PBS to the final concentrations of 2.5, 5, 10 and 20 $\mu\text{g/ml}$, respectively. Briefly, cells were seeded in 6-well plates and cultured until 90% confluence. The cells were then treated with various concentrations of emodin for the indicated periods and loaded with 5 μM DCFH-DA and HE (Molecular Probes) for 30 min in culture medium at 37°C. Cells were washed twice with PBS, harvested and the fluorescence intensity of the cells was determined using flow cytometry, with excitation and emission settings of 488 and 530 nm, respectively. The principle of this test is that endogenous esterases hydrolyze the DCFH-DA to free dichlorofluorescein inside the cells. ROS, predominantly hydroperoxides, convert nonfluorescent dichlorofluorescein to highly fluorescent DCF.⁹⁾

Measurement of mitochondrial membrane potential Induction of mitochondrial damage is a key step for apoptosis in many experimental systems. Changes of mitochondrial transmembrane potential ($\Delta\psi_m$) have been considered to be an indicator of mitochondrial damage. DIOC₆ is a mitochondrion-specific fluorescent dye widely used for determination of $\Delta\psi_m$.^{8,16,17)} The dye was dissolved in DMSO and diluted with PBS to a final concentration of 100 nM. The cells were seeded in a 6-well plate and cultured to 90% confluence. The cells were then treated with various concentrations of emodin for 2 h. DIOC₆ was applied to the cells and incubated for 30 min at 37°C. The cells were washed twice with PBS and harvested for analysis by flow cytometry. DIOC₆ was excited at 488 nm and detected at 525 nm.

Caspase-3 and caspase-9 assay Caspase-3 and caspase-9 activities were assayed using a Colorimetric Protease Assay Kit according to the manufacturer's instructions (MBL, Nagoya). Briefly, emodin-stimulated cells were suspended in 50 μl of the lysis buffer, kept on ice for 10 min, and then centrifuged (10 000g, 3 min at 4°C). The supernatants were collected for the assay. The assays of

activation of caspase-9 and caspase-3 are based on spectrophotometric detection of the chromophore *p*-nitroanilide (pNA) after cleavage from the labeled substrate, LEHD-pNA or DEVD-pNA, respectively. Enzyme reaction was performed in a buffer containing supernatant proteins (100 μ g/sample) and caspase substrate LEHD-pNA or DEVD-pNA at 37°C for 2 h, followed by colorimetric detection of pNA at a wavelength of 405 nm using a microplate reader (Bio-Rad, Hercules, CA).

RESULTS

Growth inhibition Supplementation of HepG2, PLC/PRF/5, and Mahlavu cell culture medium with 2.5, 5, 10

and 20 μ g/ml emodin for 24, 48 and 72 h reduced cell growth. Emodin exhibited a statistically significant time- and dose-dependent growth-inhibitory effect on the Mahlavu, PLC/PRF/5 and HepG2 cells evaluated in this study (Fig. 1).

DNA fragmentation The hepatoma cell lines Mahlavu, PLC/PRF/5 and HepG2, were used to study emodin-induced apoptosis. Cleavage of DNA by endonucleases is a typical feature of apoptosis. The DNA of Mahlavu, PLC/PRF/5 and HepG2 cells treated with emodin was extracted and analyzed by electrophoresis on agarose gels. The gel presented in Fig. 2 shows unambiguously that the genomic DNA of the drug-treated cells was severely fragmented, even at a concentration of emodin as low as 5 μ g/

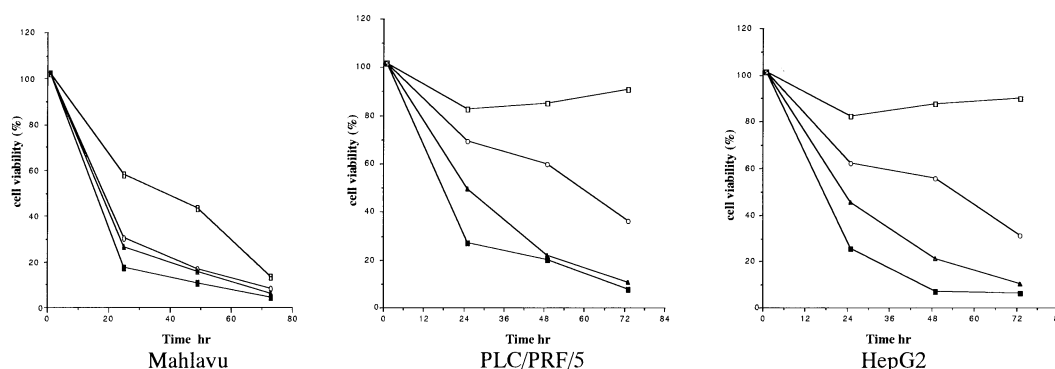


Fig. 1. Growth-inhibitory effect of emodin on Mahlavu (A), PLC/PRF/5 (B) and HepG2 (C) cell viability assessed by MTT. A time- and concentration-dependent growth inhibitory effect of emodin on Mahlavu, PLC/PRF/5 and HepG2 cells was noted. Representative data from three independent experiments are shown. \square 5 μ g/ml, \circ 10 μ g/ml, \blacktriangle 20 μ g/ml, \blacksquare 40 μ g/ml.

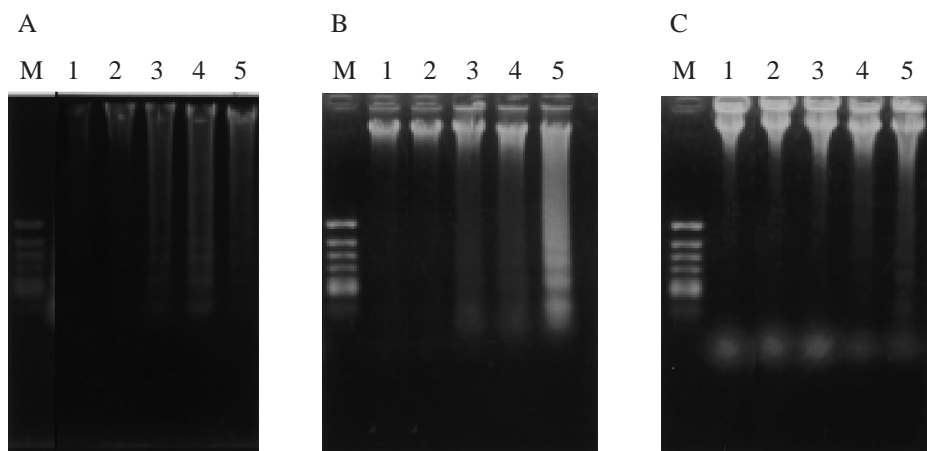


Fig. 2. DNA laddering after treatment with emodin. DNA fragments were isolated from emodin-treated Mahlavu (A), PLC/PRF/5 (B) and HepG2 (C) cells at the indicated time points and run on a 1.5% agarose gel. Treatment of cells with emodin resulted in detectable DNA laddering after 24 h. M, 100-base-pair marker. Lane 1, medium control; lanes 2, 3, 4 and 5, emodin (5, 10, 20, and 40 μ g/ml) treatment groups.

ml. The degradation of DNA to oligonucleosomal fragments is a late event in apoptosis.¹⁸⁾ The apoptosis increased in parallel with emodin concentration from 5 to 40 $\mu\text{g/ml}$. Mahlavu cells were more sensitive to emodin than HepG2 and PLC/PRF/5 cells, with apoptosis saturating at a dose above 20 $\mu\text{g/ml}$, and decreasing slightly at 40 $\mu\text{g/ml}$.

Effect of emodin on the cell cycle distribution of Mahlavu cells In order to assess the extent of emodin-induced apoptosis and to observe whether this process was selective to any phase of the cell cycle, we studied the cell cycle distribution of Mahlavu cells treated with emodin. A sub- G_1 peak could be observed after the addition of 5 $\mu\text{g/ml}$ emodin for 24 h. Treatment of Mahlavu cells with increasing concentrations of emodin for 24 h led to profound changes of the cell cycle profiles (Fig. 3). The induction of apoptosis is cell cycle-dependent. Similar results were observed for the other two cell lines, HepG2 and PLC/PRF/5 (data not shown).

Detection of apoptosis with annexin V To evaluate whether the growth-inhibitory effect of emodin was associated with apoptosis, we used a method that allows one to detect concurrently viable, necrotic, early apoptotic and

late apoptotic cells based on distinct double-staining patterns with a combination of FITC-conjugated annexin V and PI. Annexin V is a calcium- and phospholipid-binding protein, with high affinity for PS.¹⁵⁾ Annexin V-FITC in concert with flow cytometry is a sensitive method to detect cells that are in an early phase of apoptosis (Fig. 4). Samples were labeled simultaneously with annexin V-FITC and PI. The viable cells depicted in Fig. 4 (box 3) are double-negative (FITC⁻/PI⁻). Cells at an early stage of apoptosis are positive for FITC, but negative for PI (FITC⁺/PI⁻; box 4). Cells treated with emodin showed a population of cells that was apoptotic, and stained double-positive (FITC⁺/PI⁺, box 2). These cells were in the later stages of apoptosis and showed evidence of necrosis, because they were permeable to PI. Cells treated without or with 2.5, 5, 10 and 20 $\mu\text{g/ml}$ emodin for 6 h were found to be positive for PS in the outer leaflet (0.06%, 12.61%, 19.87%, 33.11% and 44.42% of total cells, respectively). Emodin treatment of the Mahlavu cells increased the number of annexin V-positive cells in a dose-dependent fashion.

Induction of ROS generation by emodin To understand in more detail the mechanism of sub- G_0/G_1 apoptosis and

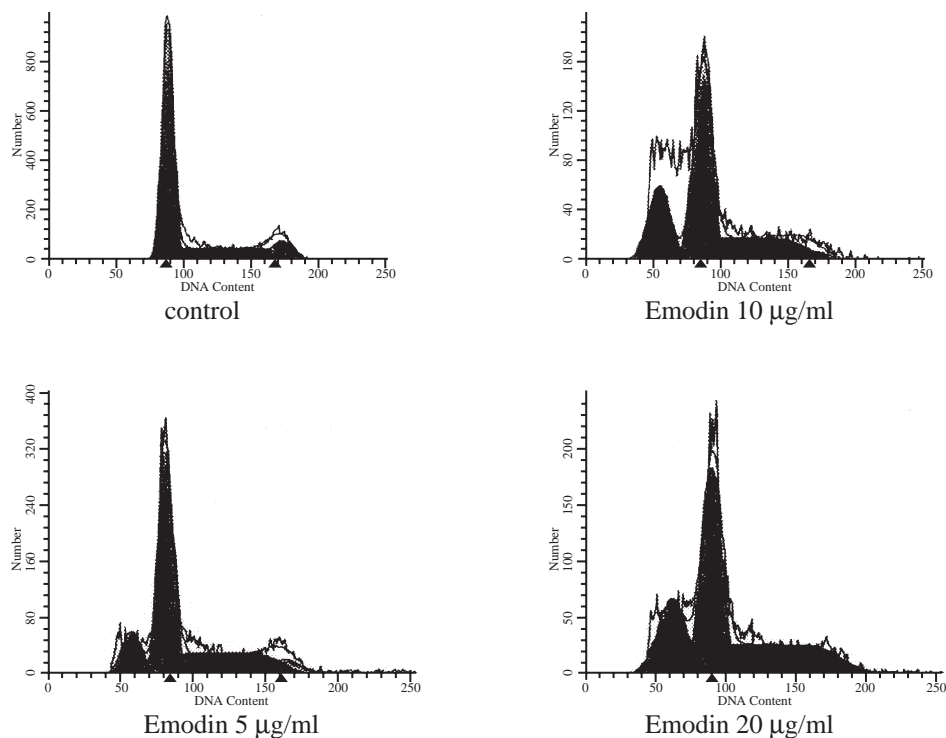


Fig. 3. Flow-cytometric analysis of Mahlavu cells by PI staining. PI-stained Mahlavu cells were analyzed by flow cytometry after addition of 5, 10 or 20 $\mu\text{g/ml}$ emodin for 24 h. Because of loss of internucleosomal DNA cleavage fragments, cells with a subdiploid amount of DNA appeared on the left side of the G_1 peak, indicating apoptosis.

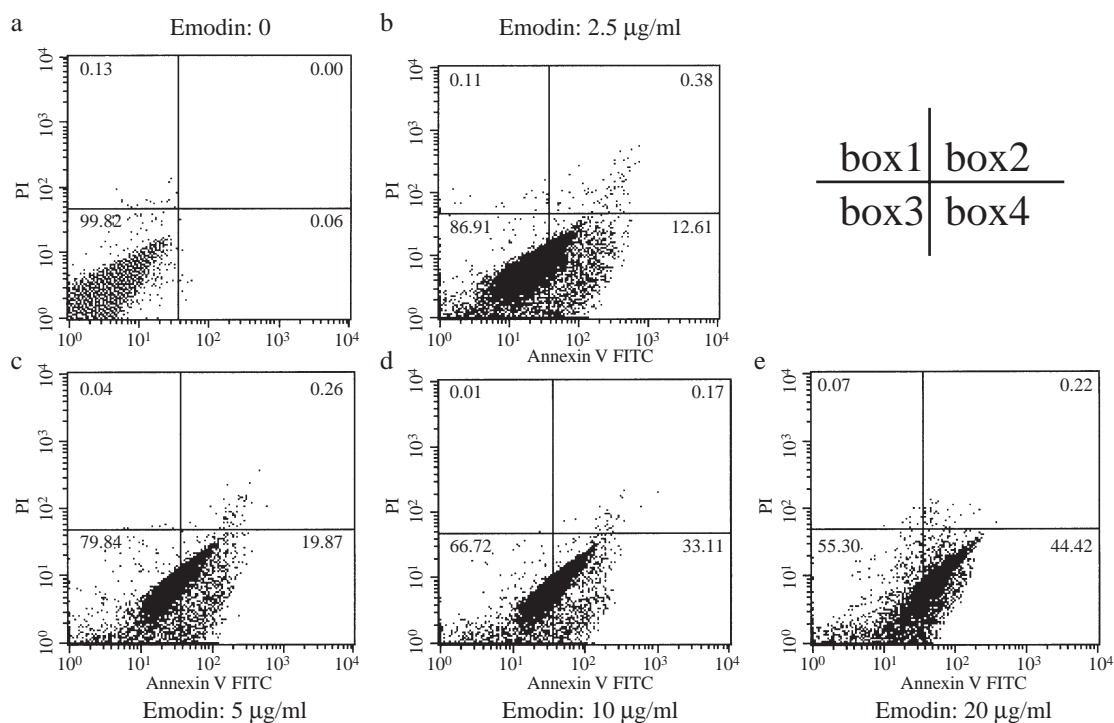


Fig. 4. Effect of emodin on Mahlavu cell apoptosis. The percentage of apoptotic cells was determined after 2.5–20 µg/ml emodin treatment of Mahlavu cells for 6 h. Boxes 2 to 4, necrotic cells, viable cells, and apoptotic cells, respectively. The percentages of apoptotic cells after 0, 2.5, 5, 10, and 20 µg/ml emodin treatment were 0.06%, 12.61%, 19.87%, 33.11% and 44.42%, respectively.

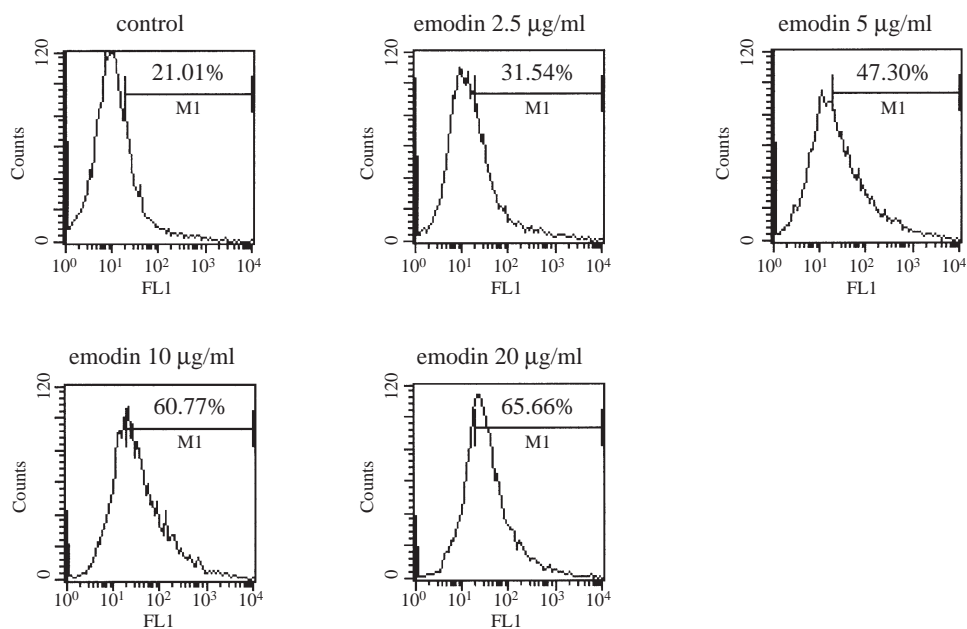


Fig. 5. Determination of intracellular O₂⁻ by HE staining. Mahlavu cells were seeded in 6-well plates and cultured to 90% confluence. The cells were then treated with or without emodin for 2 h. HE was applied to the cells and incubation was continued for another 30 min at 37°C. The cells were washed twice with PBS and harvested for analysis by flow cytometry. A right-shift of the HE peak was observed.

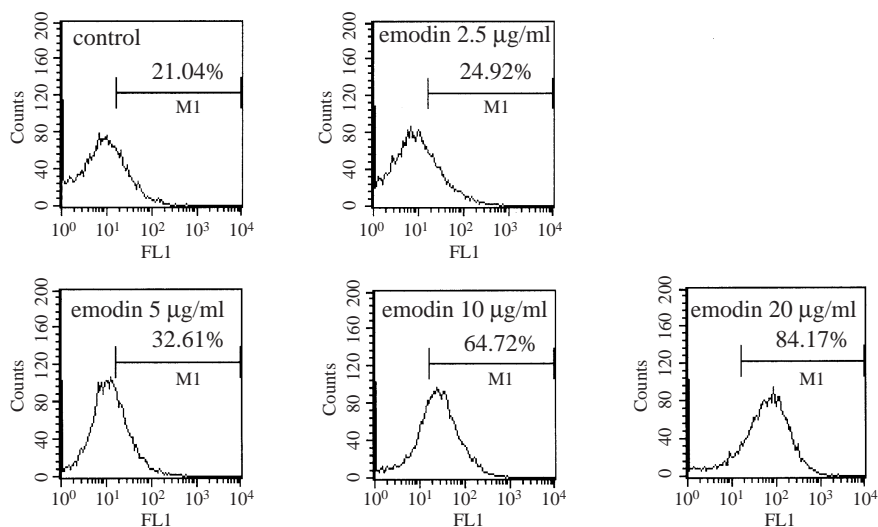


Fig. 6. Determination of intracellular H_2O_2 by DCFH-DA staining. Mahlavu cells were seeded in 6-well plates and cultured to 90% confluence. The cells were then treated with or without emodin for 2 h. DCFH-DA was applied to the cells and incubation was continued for another 30 min at 37°C . The cells were washed twice with PBS and harvested for analysis by flow cytometry. A right-shift of the DCF peak was observed.

the relationship between ROS generation and apoptosis, emodin-induced ROS production was determined in Mahlavu cells by flow cytometry. Measurements using HE and DCFH-DA demonstrate that incubation of cells with various concentrations of emodin for 2 h led to a significant increase in the generation of both O_2^- (right-shift of the HE peak) and H_2O_2 (right-shift of the DCF of peak) (Figs. 5, 6). This shift represented a dose-dependent increase in intracellular O_2^- and H_2O_2 , as demonstrated by an increase in fluorescence due to oxidation of HE and DCFH-DA.

To investigate the possible role of ROS in apoptosis by emodin, CAT (2500 U/ml) and CsA ($5\ \mu\text{M}$) were added to the cells. As shown in Fig. 7, CAT and CsA pretreatment could partially prevent emodin-induced apoptosis. The effect of these agents indicated that ROS could be involved in emodin-induced apoptosis. These data support the hypothesis that ROS generation by emodin is required for the induction of apoptosis in Mahlavu cells.

Induction of mitochondrial damage by emodin Mitochondria are the limiting factor in the apoptotic pathway in many experimental systems and are a major physiological source of ROS. Mahlavu cells were treated with emodin and then analyzed by flow cytometry after DIOC_6 labeling.¹⁹⁾ The result showed that $\Delta\psi_m$ was significantly reduced on cellular uptake of the fluorochrome (Fig. 8). This shift was dose-dependent and decreased linearly in the range between 2.5 and 20 $\mu\text{g}/\text{ml}$ emodin, confirming damage at the mitochondrial level, which may be related to the apoptotic response of the cells to emodin. The

decrease of fluorescence intensity measured with the DIOC_6 probe reflects the collapse of $\Delta\psi_m$, which is a sign of the opening of mitochondrial megachannels.²⁰⁾ Emodin treatment caused a dose-dependent loss of $\Delta\psi_m$. Inhibition of permeability transition leads to prevention of $\Delta\psi_m$ disruption.

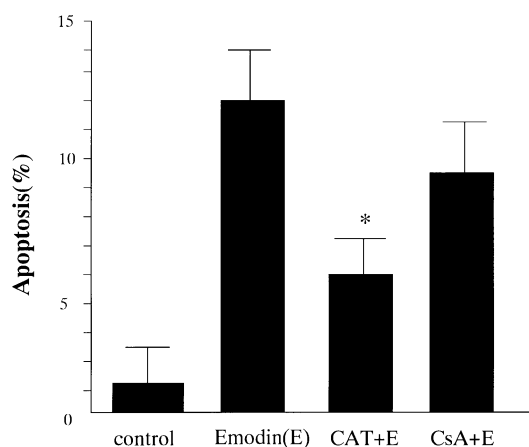


Fig. 7. Effects of CAT and CsA on emodin-induced apoptosis. CAT (2500 U/ml) and CsA ($5\ \mu\text{M}$) were examined as inhibitors of emodin-induced apoptosis in Mahlavu cells. The results showed that CAT and CsA pretreatment can partially prevent emodin-induced apoptosis. Data represent means \pm SD from three independent experiments. * $P < 0.05$.

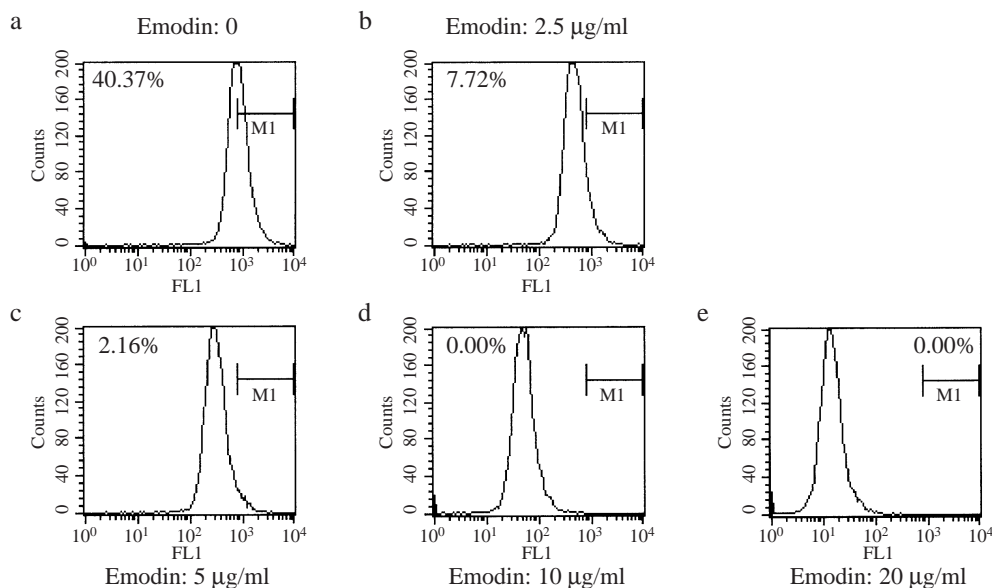


Fig. 8. Effect of emodin on mitochondrial membrane potential. Mahlavu cells were seeded in 6-well plates and cultured to 90% confluence. The cells were then treated with or without emodin for 2 h. DIOC₆ was applied to the cells and incubation was continued for another 30 min at 37°C. The cells were washed twice with PBS and harvested for analysis by flow cytometry. A left-shift of fluorescent intensity was observed.

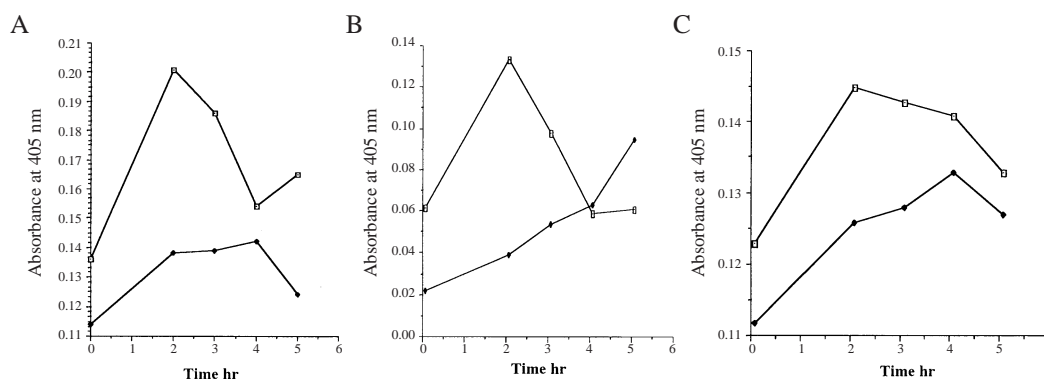


Fig. 9. Effects of emodin on caspase-9 and -3 activities in Mahlavu, PLC/PRF/5 and HepG2 cells. Cells were collected after treated with emodin (2.5 µg/ml) for 2, 3, 4, 5 h. Caspase-9 and -3 activities were measured as described in "Materials and Methods." Both caspases were up-regulated after treatment with emodin. □ caspase-9, ◆ caspase-3.

Caspase-9 and caspase-3 activity Caspases are cysteine proteases that have a central role in apoptosis, because other caspases predominantly are activated in turn by the degradation of cellular proteins by the activated caspase.²¹⁾ Several caspases have been shown to be key executors of apoptosis mediated by various inducers, including anti-tumor agents.²²⁾ More than 10 caspases have been found and caspase-3 is the most widely functioning caspase.²¹⁾ In many cell types, overexpression of caspases induces apo-

ptosis, whereas inhibition of caspase activity has the opposite effect, showing that these enzymes are important mediators of apoptosis.²³⁾ The activation of the effector caspase-3 is catalyzed by the initiator caspase-9. Caspase-9 and caspase-3 activities were rapidly up-regulated when the cells were treated with emodin. The activation of caspase-9 reached a maximum within 2 h of treatment with emodin and caspase-3 was subsequently activated within 4 to 5 h (Fig. 9).

DISCUSSION

This study demonstrated that the emodin exerts its growth-inhibitory effect on human hepatocellular carcinoma cell lines via the induction of apoptosis. Using flow cytometry, we found that less DNA content than in G_0/G_1 cells (sub- G_1) accumulated in Mahlavu cells after emodin treatment. Annexin V-FITC staining and DNA ladder formation confirmed the above results and suggested again that the emodin-induced cytotoxicity was the result of apoptosis in hepatocellular carcinoma cells. Our finding is consistent with the conclusion of a recent report about the effect of emodin in human lung squamous cell carcinoma cell lines.²⁴⁾

Mitochondria are now considered to be major players in the apoptotic process of mammalian cells. Activators of apoptosis, such as caspase-9, caspase-3, cytochrome *c* (as an activator of caspases), and apoptosis-inducing factor, are all found in the mitochondria.²⁵⁾ Disruption of $\Delta\psi_m$ has been established to be an indicator of mitochondrial damage and generally is defined as an early but irreversible stage of apoptosis, preceding caspase-3 activation.^{26–28)} A reduction in $\Delta\psi_m$ is believed to be mediated by the opening of the mitochondrial permeability transition (MPT) pore, a multi-protein complex.²⁹⁾ In normal circumstances, most of the mitochondrial pores are closed. Opening of the pores has dramatic consequences for mitochondrial physiology, including $\Delta\psi_m$ collapse, uncoupling of the respiratory chain, and efflux of small molecules and some proteins from the mitochondria (e.g., apoptosis-inducing factor, cytochrome *c*, calcium and so on). Fluorescence measurement with the DIOC₆ probes showed that emodin causes the pores to open. Current concepts implicate MPT as the cause of hepatocyte necrosis mediated by mitochondrial dysfunction.^{30,31)} MPT is characterized by a rapid increase in the permeability to ions of the inner mitochondrial membrane, associated with mitochondrial swelling and a collapse of $\Delta\psi_m$.³²⁾ The changes in the mitochondrial membrane potential after emodin treatment show that mitochondria are involved in the course of emodin-induced apoptosis.

ROS produced in mitochondria contribute to cell death by acting as apoptotic signaling molecules. We found that emodin induced disruption of MPT and that this induction

was partially blocked by antioxidants, suggesting that it is induced by ROS. This conclusion is based on the observations that exposure of cells to emodin resulted in generation of ROS and a decrease in $\Delta\psi_m$, as well as in apoptosis. In terms of reduction of emodin-induced H_2O_2 by CAT and CsA, treatment of Mahlavu cells with CAT and CsA conferred partial protection against emodin-induced apoptosis, whereas SOD did not affect it (data not shown). One of the possible causes for mitochondrial depolarization and apoptosis in cells is ROS. The induction of apoptosis by cytotoxic drugs appears to be mediated by an ROS-dependent pathway. The results presented here indicate a mixed action of emodin in inducing apoptosis. It is clear that antioxidants can protect cells under these conditions.³³⁾ However, the limited protective effects of CAT and CsA suggests that emodin-induced apoptosis may involve other effects of emodin than inducing oxidative stress, i.e. damage to proteins and to DNA.

Caspase-9 can activate downstream caspases, such as caspase-3. Recently, caspase-3 has been specifically implicated as the effector caspase responsible for cleavage of the human DNA fragmentation factor (DFF) and an inhibitor of murine caspase-activated DNase (ICAD). Cleavage of DFF/ICAD then activates the DNA endonuclease required for formation of the apoptotic DNA ladder. Caspase-3 has also been shown to be necessary for the typical morphologies associated with apoptosis. Although caspase-3 is usually activated via proteolytic cleavage of procaspase-3 by caspase-9, caspase-3 and caspase-9 are formed by autocatalytic cleavage of procaspase-9, thereby further amplifying the apoptotic signal.²⁶⁾ Our results showed that caspase-9 and caspase-3 activities were up-regulated by emodin, and this is consistent with the result of western blotting in human lung squamous cell carcinoma.²⁴⁾ In summary, the pathway of emodin-induced apoptosis in hepatocellular carcinoma cell lines may be described as follows: induction of ROS generation, down-regulation of mitochondrial membrane potential, up-regulation of the activities of caspase-9 and caspase-3 and subsequent DNA fragmentation.

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