Crude Extracts of *Euchresta formosana* Radix Induce Cytotoxicity and Apoptosis in Human Hepatocellular Carcinoma Cell Line (Hep3B)

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Abstract. *In this study, the effects of 95% ethanol extracts of Euchresta formosana radix (EFR) on the cell cycle and apoptosis in human hepatocellular carcinoma (HCC) Hep3B cells were investigated. The results indicated that EFR decreased DNA synthesis and viable Hep3B cell numbers in a concentration-dependent manner. EFR induced a p21- and p27-dependent cell cycle arrest in S-phase and apoptosis of the Hep3B cells. The induction of apoptosis by EFR treatment was also confirmed by DAPI staining. EFR inhibited cyclin-dependent kinase (CDK)-1 and -2 expression and decreased cyclin B1 and E levels, resulting in S-phase arrest. EFR induced reactive oxygen species (ROS) production followed by endoplasmic reticulum (ER) stress that was based on the increase of GADD153 and GRP78 which led to the release of Ca^{2+} in the Hep3B cells. The EFR-promoted apoptosis was associated with increasing activation of caspases 3, 7, and 9 and enhanced poly(ADP-ribose) polymerase cleavage and increased expression of $p21^{CIP1/WAF1}$, $p27^{KIP1}$, Bax and Bad. Furthermore, the levels of Bcl-xl decreased after EFR treatment. Alteration of these key anti- and pro-apoptotic proteins could contribute to the increase in p53-independent apoptosis that was observed in the Hep3B cells.*

Hepatocellular carcinoma (HCC) is a common malignancy affecting approximately one million people every year around the world (1) and it is the third most frequent cause of death from cancer and the eighth most commonly occurring cancer in the world (2). The incidence is low in the occidental world and high in locations such as Southeast Asia and sub-Saharan Africa. HCC primarily affects older people, reaching its highest prevalence among those aged 65 to 69 years (2). Based on reports from the People's Health Bureau of Taiwan, about 8.99 people per 100 thousand die per year of liver cancer in Taiwan. HCC is a multifactorial disease, and the causes include smoking, alcohol, mycotoxins and the human hepatitis virus (3). The current therapy for HCC is surgery, radiotherapy and chemotherapy, however, the results are unsatisfactory.

Apoptosis is regulated through distinct pathways based on the initiating caspases (a family of proteases) (4). These pathways include: the Fas ligand (FasL) binding to its receptor (Fas), which results in activation of caspase-8; chemical and physical stress that triggers the cytoplasmic release of pro-apoptotic mitochondrial proteins leading to activation of caspase-9 and endoplasmic reticulum (ER)-stress, which is attributed to activation of caspase-12 (5) and caspase-4 (6).

Numerous naturally occurring substances are considered to have potential value for improving immune function, preventing cancer and as antioxidants and cancer therapeutic drugs. The plant *Euchresta formosana* has long been used as a herbal medicine in China and the anticancer activity of the crude extract of *Euchresta formosana* radix (EFR) has been examined without reference to cell cycle progression and apoptosis. Therefore, cell cycle arrest and the induction of apoptosis in Hep3B cells after treatment with EFR were investigated in this study.

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Materials and Methods

Plant material and preparation of extracts. The EFR were collected from Taitung Dawu located in the east part of Taiwan in November 2003 and specimens (CMU EF 0612) were deposited in the School of Chinese Medicine Resources, China Medical University, Taichung, Taiwan. Following repeated extraction of the radices (400 g) with 95% EtOH at room temperature, the combined extracts were filtered and evaporated under reduced pressure to yield a brownish viscous residue (37.96 g). For the present experiments, the crude extracts were dissolved in dimethyl sulfoxide (DMSO) to give final concentration of 50, 75, 100, 125 and 150 μg /ml.

Chemicals and reagents. Propidium iodide (PI), potassium phosphates, triton X-100, Tris-HCl, trypan blue and ribonuclease-A were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DMSO and TE buffer was purchased from Merck Co. (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) (90%) with 2 mM L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY, USA).

Human hepatocellular carcinoma Hep3B cell line. The Hep3B cell line, a human HCC cell line, was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The Hep3B cells were placed in 75 cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 atmosphere in 90% DMEM with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 10% FBS and 2% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin)

Morphological changes and cell viability by flow cytometric analysis. The Hep3B cells, at a density of approximately $2x10^5$, were plated into 12-well plates for the various EFR treatments. After 24 h incubation in DMEM + 10% FBS, EFR was added to give a final concentration of 50, 75, 100, 125 or 150 μg/ml (unless otherwise stated). DMSO (solvent) alone was added to the controls. The cells were then grown at 37˚C, 5% CO_2 and 95% air for various lengths of time. Cell viability was determined by flow cytometry (Becton-Dickinson, San Jose, CA, USA) as described previously (7, 8).

Cell cycle and apoptosis examined by flow cytometric analysis. After incubation for 24, 48 or 72 h the cells were harvested by centrifugation and were fixed gently (drop by drop) with 70% ethanol (in PBS). After storage at 4˚C overnight the cells were re-suspended in PBS containing 40 μg/mL PI, 0.1 mg/mL RNase and 0.1% Triton X-100 in a dark room. After 30 minutes at 37˚C, the cell cycle was analyzed with a flow cytometer equipped with an argon ion laser at 488 nm wavelength (7, 8). Annexin V-FITC and a PI double staining kit from PharMingen (San Diego, CA, USA) were used for apoptotic cell quantification (7, 8).

Apoptosis examined by DAPI staining. After 48 h incubation, the cells were stained with DAPI before being photographed under a fluorescence microscope as described previously (9).

DNA fragmentation of the Hep3B cells examined by DNA gel electrophoresis. After 48 h incubation the cells were harvested by centrifugation and the DNA was isolated and separated by gel electrophoresis before being photographed under fluorescence light as described previously (10).

Caspase-3 activity determination by flow cytometric analysis. EFR (125 μg/ml) for 0, 12, 24 or 48 h with or without z-VAD-fmk (caspase inhibitor) was added to the samples before incubation. The cells were harvested by centrifugation and the media were removed. Then, 50 μl of 10 μM substrate solution (Phiphiliux green (OncoImmunin, Gaithersburg, MD, USA) is a unique class of substrates for caspase-3) was added to the cell pellet ($1x10^5$ cells per sample). The cells then were incubated at 37˚C for 60 min and then washed once by adding 1 ml of ice-cold PBS and re-suspended in 1 ml fresh PBS. The cells were analyzed with a flow cytometer (Becton-Dickinson) equipped with an argon ion laser at 488 nm wavelength (7-9).

Detection of reactive oxygen species (ROS) by flow cytometric analysis. The Hep3B cells were treated with 125 μg/ml of EFR and were incubated for 0, 0.25, 0.5, 1, 3, 6, 12 or 24 h to detect the changes of ROS. The cells were harvested and washed with 1X PBS twice, re-suspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (10 μM) and incubated at 37˚C for 30 min and analyzed by flow cytometry (7-9).

Detection of Ca^{2+} concentrations by flow cytometric analysis. The Hep3B cells were treated with 125 μg/ml of EFR and were incubated for 0, 6, 12, 24, 48 h. The cells were harvested and washed with 1X PBS twice, once for apoptosis analysis and the other re-suspensed in Indo 1/AM (Calbiochem, La Jolla, CA, USA) (3 μg/ml) and incubated at 37˚C for 30 min before the Ca^{2+} concentration was analyzed by flow cytometry (7-9).

Detection of mitochondrial membrane potential ($\Delta\Psi_m$) by flow cytometric analysis. The Hep3B cells were incubated for 0, 6, 12, 18, 24, 48 h with 125 μg/ml of EFR to detect the changes of $\Delta\Psi_m$. The cells were harvested and washed in 1X PBS twice, re-suspended in 500 μl of $DiOC_6$ (Molecular Probes) (4 mol/l) and incubated at 37˚C for 30 min then analyzed by flow cytometry (7-9).

Western blotting for protein examination. The cytosolic total proteins were collected from Hep3B cells treated with or without 125 mg/ml of EFR for 6, 12, 24 or 48 h. The Cdc25a, cyclin A, cyclin B1, cyclin E, CDK1, CDK2, E2F-1, P-pRb, p21, p27, Bax, Bad, Bcl-xl, Bcl-xs, cytochrome c, cytosolic cytochrome c, AIF, Endo G, caspase-3, -7, -8, -9 and 12, PARP, Fas, FasL, Bid, GRP78 and GADD153 were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described previously (7-9).

Statistical analysis. The Student's *t*-test was used for the statistical analysis between the EFR-treated and control groups.

Results

Effect of EFR on morphological changes and cell viability. The results of morphological examinations under a phase-contrast microscope and PI staining experiments indicated that EFR induced morphological changes (cell death and shrink) in both a dose- (Figure 1A) and time-dependent (Figure 1B) manner. The percentage of viable cells was significantly different between the EFR-treated groups and controls (Figure 1C).

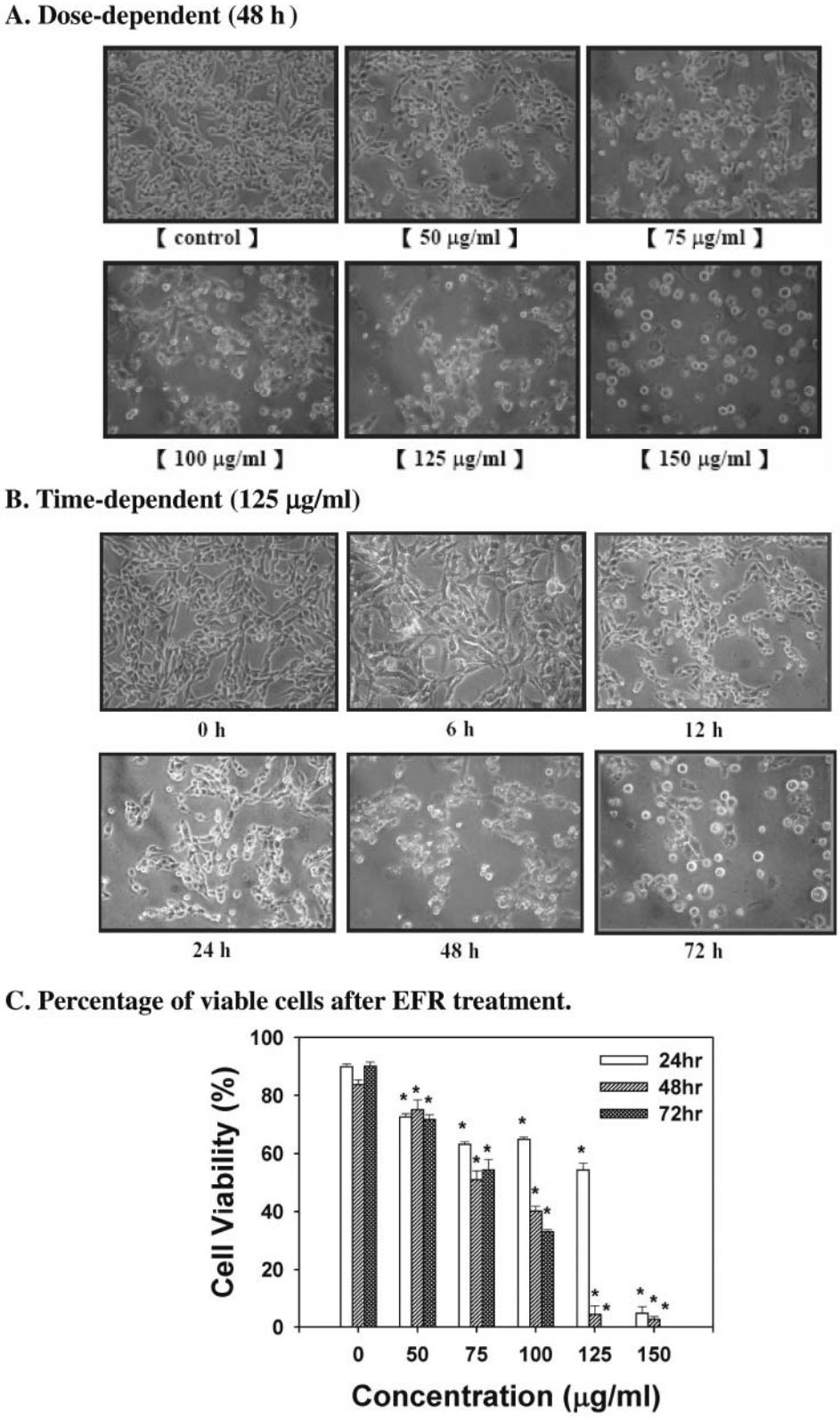

Figure 1. *Viability of human hepatocellular carcinoma Hep3B cells after EFR treatment. The cells were photographed under a phase-contrast microscope x200. Morphological changes: A: dose-dependent; B: time-dependent. The viable cells were determined by flow cytometry as described in Materials and Methods; panel C: Each point is mean±SD of three experiments (*p<0.05).*

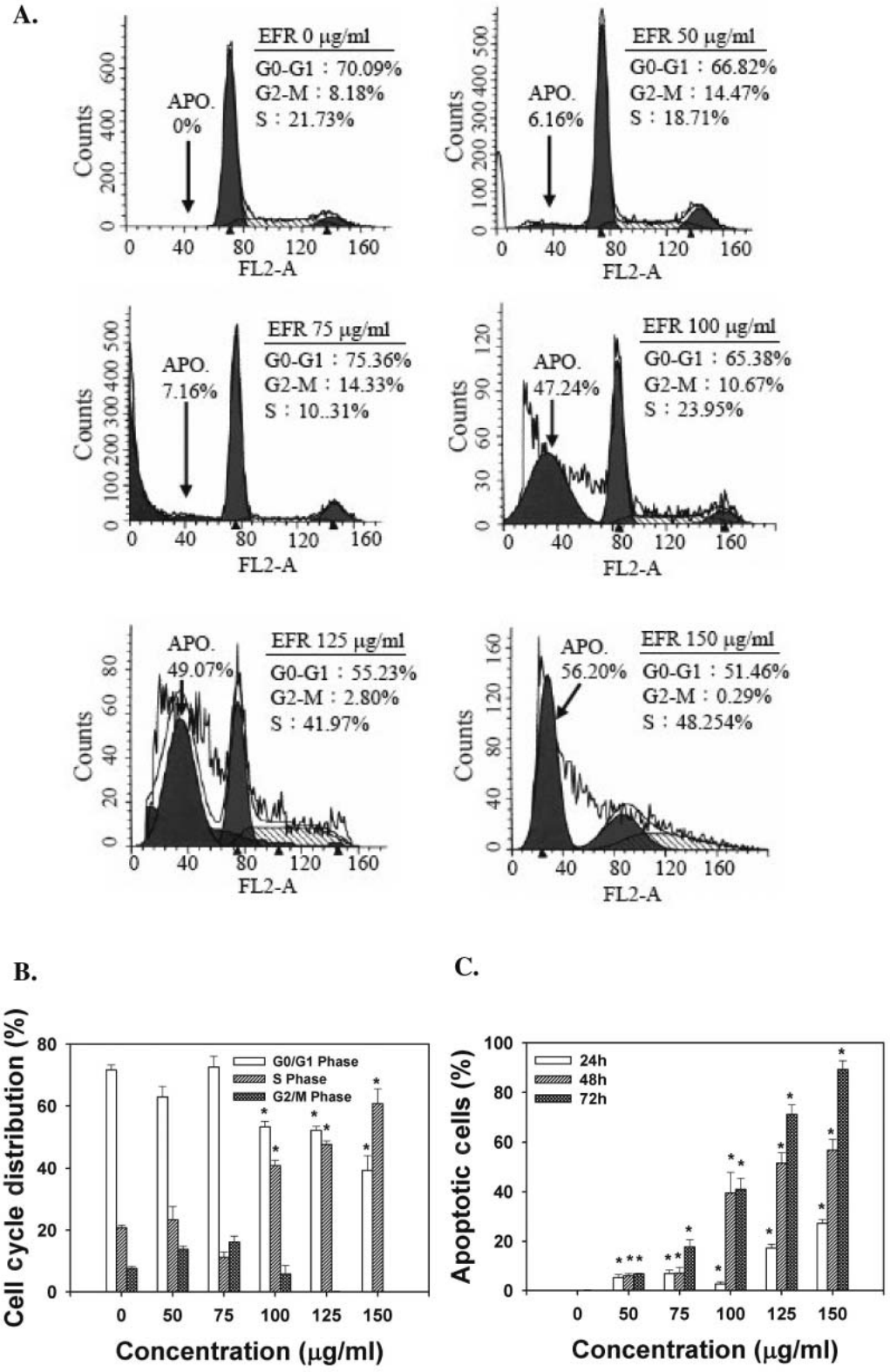

Figure 2. *The effects of EFR on the cell cycle and apoptosis in hepatocellular carcinoma Hep3B cells. The Hep3B cells were incubated with various concentrations of EFR and cell cycle and apoptosis were determined by flow cytometric analysis as described in Materials and Methods. A: representative profile after 48 h incubation; B: percentage of cells in different phases after 48 h incubation; C: percentage of apoptotic cells. Data represents mean±SD of three experiments (*p<0.05).*

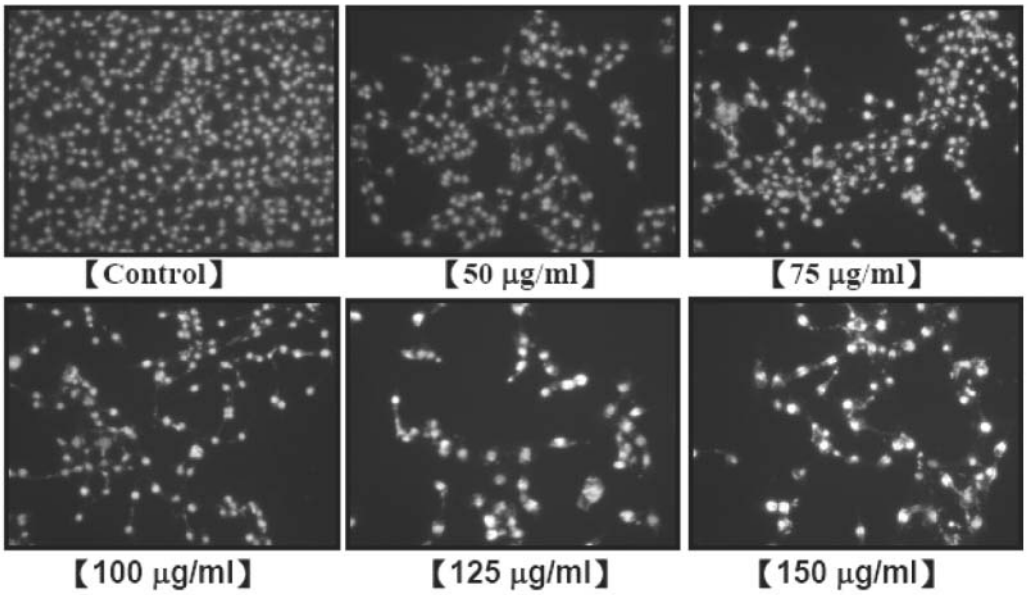

Figure 3. *Apoptosis of Hep3B cells treated with EFR examined by DAPI staining. The Hep3B cells were incubated with various concentrations of EFR for 48 h and apoptosis was determined by DAPI staining and fluorescence microscopy as described in Materials and Methods.*

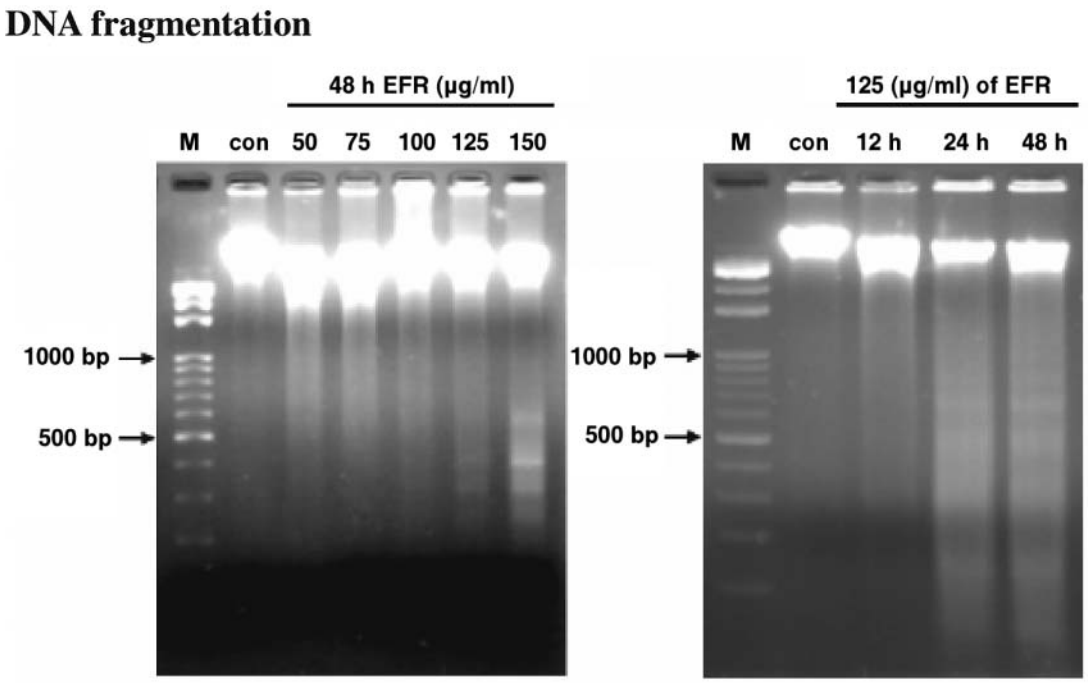

Figure 4. *DNA fragmentation of Hep3B cells treated with EFR examined by DNA gel electrophoresis. The Hep3B cells were incubated with various concentrations of EFR for 48 h or treated with 125 μg/ml of EFR for various times. DNA was extracted before undergoing DNA gel electrophoresis and then photographed under fluorescence microscopy as described in Materials and Methods.*

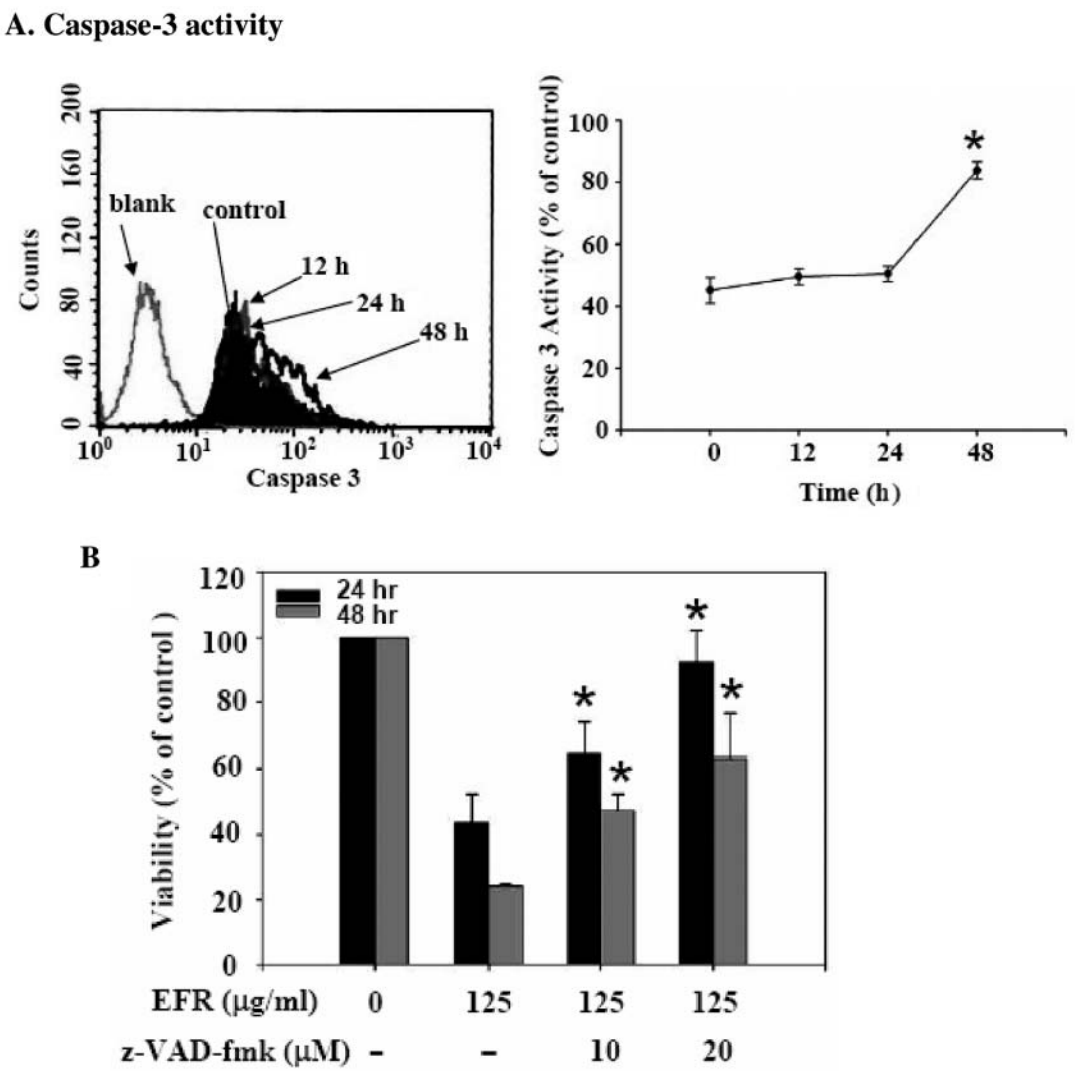

Figure 5. *Flow cytometric assays of the effects of EFR on caspase-3 activity. The Hep3B cells were incubated with 125 μg/ml of EFR for various times before caspase-3 activity determination (A). The effect of pretreatment with the caspase inhibitor (z-VAD-fmk) is shown in B. Data represents mean±SD of three experiments. *p<0.05.*

Effect of EFR on the cell cycle and apoptosis. Apoptosis was detected by a PI staining method after 48 hours of continuous exposure to EFR before being examined by using flow cytometry analysis. As shown in Figure 2A, B and C, EFR induced S-phase arrest and apoptosis in a concentration-dependent manner.

Effect of EFR on induction of apoptosis examined by DAPI staining. As shown in Figure 3, EFR induced apoptosis in a concentration-dependent manner.

Effect of EFR on DNA fragmentation. As shown in Figure 4, EFR induced DNA fragmentation in both a dose- and time-dependent manner.

Effect of EFR on caspase-3 activity. The caspase-3 activity increased more in the EFR-treated group than in the control group and the increase was enhanced persistently from 0 to 48 hours (Figure 5A). The z-VAD-fmk did increase the viability in examined cells (Figure 5B)

Effect of EFR on reactive oxygen species (ROS), cytoplasmic Ca^{2+} levels and the levels of mitochondrial membrane potential. The levels of ROS increased more in the EFR-treated group than that in the control group and peaked at 6 hours of exposure to EFR at 125 μg/ml, then subsequently decreased. However, the levels of ROS were still higher in the EFR-treated group than in the control group for at least 24 hours (Figure 6A). The results from flow cytometric analysis

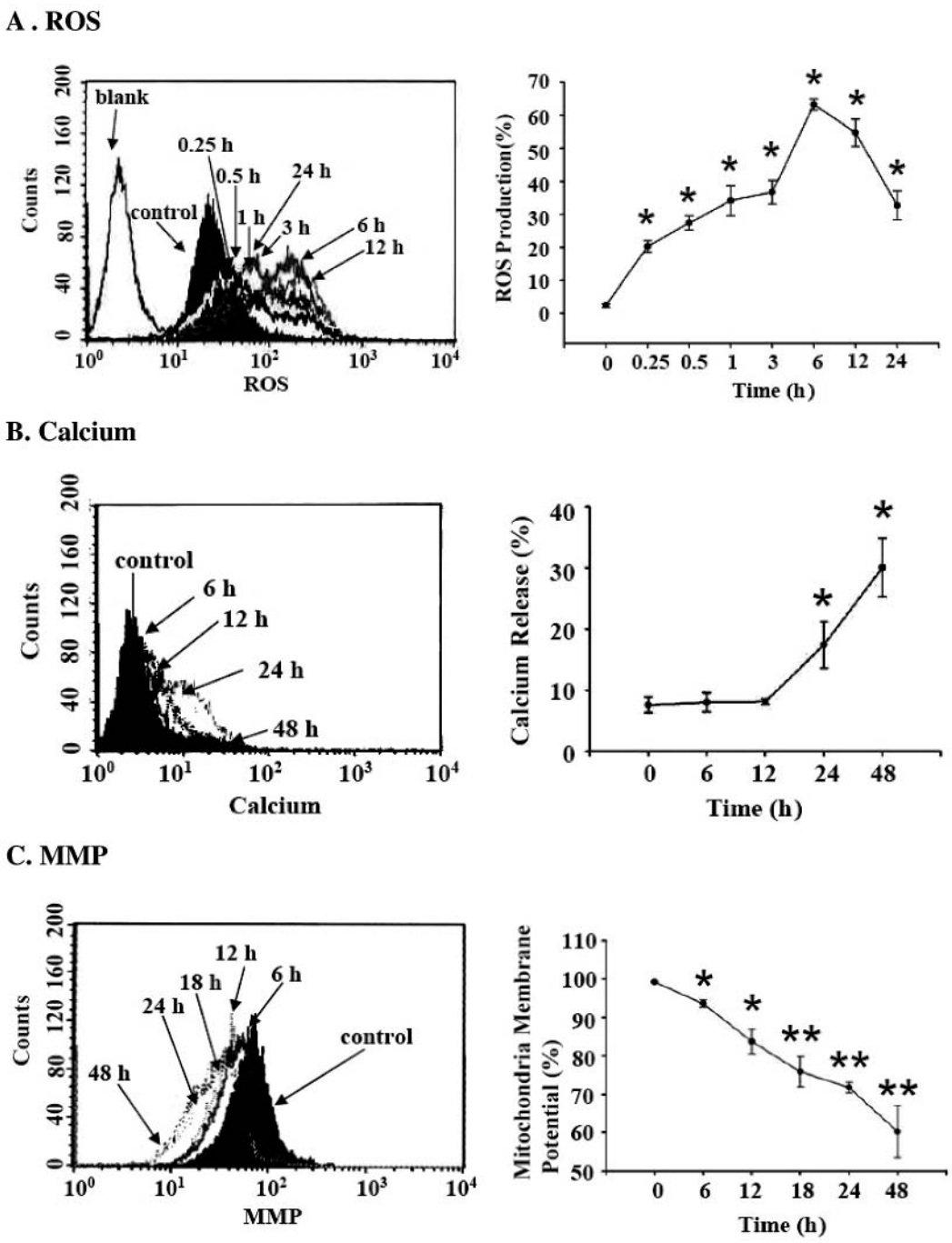

Figure 6. *Flow cytometric analysis of reactive oxygen species (ROS), Ca^{2+} and $\Delta\Psi_m$ in human Hep3B cells treated with EFR for 24 or 48 hours. The Hep3B cells ($2x10^5$ cells/ml) were treated with 125 μg/ml of EFR (control: 0 μg EFR) for 24 or 48 hours to detect the changes of ROS, Ca^{2+} and $\Delta\Psi_m$. A: the percentage of cells that were stained by DCFH-DA dye for ROS; B: stained by Indo-1/AM dye for Ca^{2+} and C: stained by $DiOC_6$ dye for $\Delta\Psi_m$. *Significantly different between EFR and control $p<0.05$.*

indicated that cytoplasmic Ca^{2+} levels increased more in the EFR-treated group than in the control group. On exposure to EFR at 125 μg/ml concentration, the cytoplasmic Ca^{2+} increased gradually from 6 to 24 hours, and then reached its peak at 48 hours (Figure 6B). The results from flow cytometric analysis indicated that mitochondrial membrane potential (MMP) decreased in the EFR-treated group compared to the control group. The decrease of the MMP

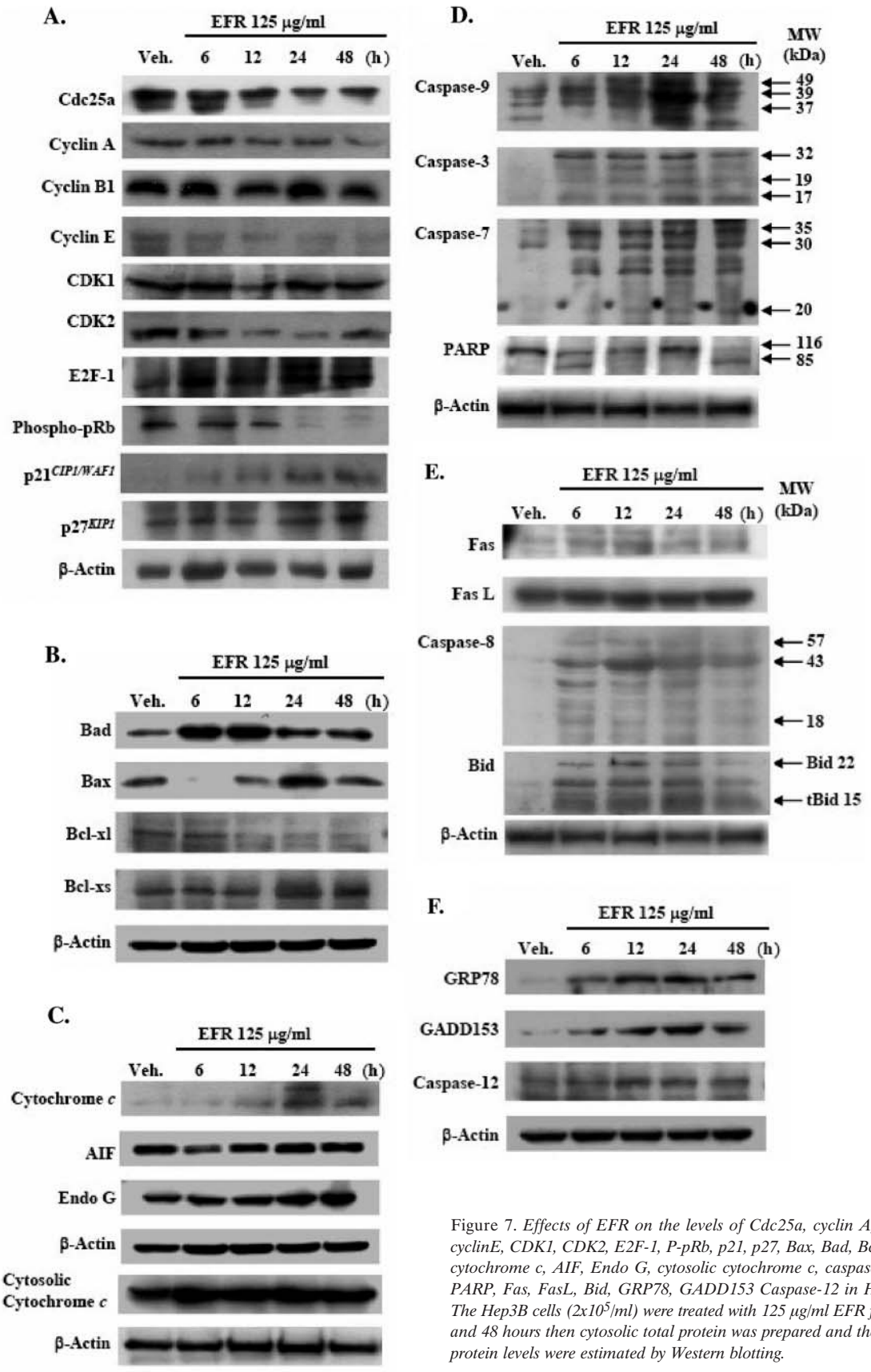

Figure 7. *Effects of EFR on the levels of Cdc25a, cyclin A, cyclin B1, cyclinE, CDK1, CDK2, E2F-1, P-pRb, p21, p27, Bax, Bad, Bcl-xl, Bcl-xs, cytochrome c, AIF, Endo G, cytosolic cytochrome c, caspase-3, 7, 8, 9, PARP, Fas, FasL, Bid, GRP78, GADD153 Caspase-12 in Hep3B cells. The Hep3B cells ($2x10^5$/ml) were treated with 125 μg/ml EFR for 6, 12, 24 and 48 hours then cytosolic total protein was prepared and the individual protein levels were estimated by Western blotting.*

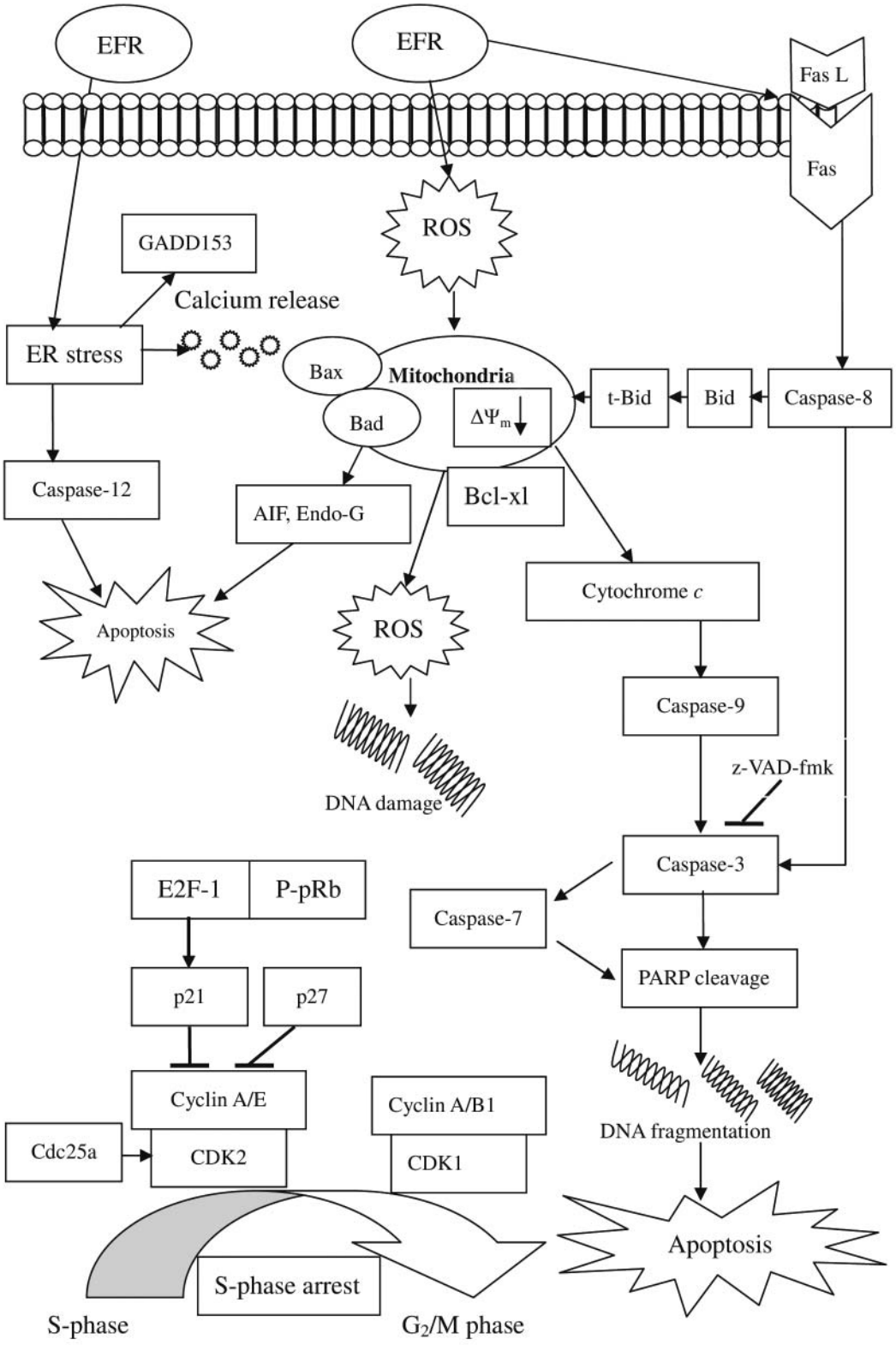

Figure 8. *Proposed model of EFR mechanism of action for apoptosis in Hep3B cells. EFR induced ROS, ER stress and GADD153 expression leading to* Ca^{2+} *release, and decreased the levels of* $\Delta\Psi_m$ *leading to cytochrome c release, caspase-9 activation and caspase-3 activity before causing apoptosis in the Hep3B cells.*

was proportional to EFR treatment duration from 0 to 48 hours, revealing a time-dependent effect (Figure 6C).

Western blot. The results of Western blotting are shown in Figure 7. The increase of the expressions of p21, p27, cytochrome *c*, Bax, tBid, caspases-9, -3, -7, -8 and -12, and the decrease of the expression of Bcl-xl may contribute to the occurrence of apoptosis. The value beside the figures represent the change in protein expression of the bands normalized to β-actin.

Discussion

The present results have demonstrated that EFR reduced the percentage of viable cells and also induced apoptosis in the Hep3B cells both time and dosage dependently.

The effects of EFR on the Hep3B cells were associated with an increase in S-phase arrest of the cell cycle that is known to be controlled by CDK, CDKI and cyclins. Western blot analysis in this study showed that the EFR-induced S-phase cell cycle arrest was mediated through the increased expression of the CDKI proteins ($p21^{CIP1/WAF1}$ and $p27^{KIP1}$), a simultaneous decrease in CDK1, CDK2 and cyclins A, B1, and E and enhanced CDKI-CDK binding. Uncontrolled cell division depends on the activation of cyclins, which bind to CDK to induce cell cycle progression towards the S-phase. CDK activity is one of the major causes of cancer progression and their functions are tightly regulated by CDKI, such as, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ proteins. $p21^{CIP1/WAF1}$ is a universal inhibitor of CDK(s) and $p27^{KIP1}$ is commonly up-regulated in response to antiproliferative signals (10). The increased expression of S-phase cyclins in cancer cells provides an uncontrolled growth advantage because most of these cells either lack CDKI, harbor nonfunctional CDKI, or CDKI expression is not at a sufficient level to control CDK-cyclin activity (11, 12). Our results also showed that EFR could increase the expression of CDK inhibitor $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ proteins and could down-regulate the expression of CDK1 and CDK2. Activation of $p21^{WAF1/CIP1}$ induced cell cycle block by a p53-independent pathway. In addition, EFR also enhanced pro-apoptotic molecule expression (Bax, Bad, Bcl-xs, Fas/APO-1).

It is well documented that Bcl-2 family proteins play a critical role in the induction of apoptosis, in particular the interaction between pro- and anti-apoptotic proteins of the Bcl-2 family which integrate the diverse death and survival signals to control the fate of the cell (16, 19, 20). Many studies have shown that anti-apoptotic Bcl-2 family proteins such Bcl-xl form heterodimers with Bax which might neutralize its pro-apoptotic effects leading to a decrease or cessation of apoptosis (21-23). In addition, anti-apoptotic Bcl-2 family proteins are also known to prevent the release of caspases, especially in the mitochondria (22), and to regulate the release of cytochrome c from the mitochondria into the cytosol (24, 25). The overexpression of anti-apoptotic Bcl-2 family proteins, such as Bcl-xl, has been shown to block the release of cytochrome c in response to a variety of apoptotic signals (24). Bcl-2/Bax also has an important effect on the mitochondrial membrane potential that controls the release of cytochrome c in the apoptotic cells.

On the contrary, the pro-apoptotic members of the Bcl-2 family, such as Bax protein, promote cytochrome c release from the mitochondria (24, 25). Released cytochrome c activates initiator caspase-9 to activate a sequential cascade of caspases, especially caspase-3. The proteolytic activation of executioners, such as caspase-3, results in the proteolysis of death substrates and subsequent DNA degradation and apoptotic death (16, 19, 20, 26, 27).

In this study, the EFR treatment resulted in a significant increase in the pro-apoptotic Bax protein and decrease in the levels of anti-apoptotic Bcl-xl protein, thus shifting the Bax/Bcl-xl ratio in favor of apoptosis (Figure 7B). Furthermore, pronounced induction of the execution protease of apoptosis, caspase-3 (Figure 7D), was demonstrated.

Our experiment clearly demonstrated the typical pattern of apoptosis as DNA laddering on gel electrophoresis in the Hep3B cell line. EFR also first increased the levels of ROS which then caused Ca^{2+} release from the ER. It is well known that ROS may lead to ER stress leading to Ca^{2+} release. It was also shown that EFR increased the levels of GADD153, a modulator of ER stress, and GRP78, a sensor of ER stress. Recently, ROS have been found to play an important role in the induction of apoptosis (28, 29). A proposed model of the EFR mechanism of action in the Hep3B cells is shown in Figure 8.

In conclusion, our results demonstrated that EFR inhibited the growth of Hep3B cells. EFR decreased the levels of Bcl-xl, but increased the levels of Bax, meaning that EFR increased the Bax/Bcl-xl ratio. EFR was also able to decrease the mitochondrial membrane potential, trigger the release of cytochrome c to the cytosol, and subsequently induce the processing of procaspase-9 and procaspase-3, which led to DNA fragmentation. EFR inhibited the Hep3B cells not only by cell cycle arrest at S-phase but also by apoptosis (Figure 8).

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