*Anti-Cancer Drugs 2000,* **11,** pp. 747–756

## Preclinical report

# Differential regulation of P53, c-Myc, Bcl-2, Bax and AFP protein expression, and caspase activity during 10-hydroxycamptothecin-induced apoptosis in Hep G2 cells

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**10-Hydroxycamptothecin (HCPT), a DNA topoisomerase I (Topo I) inhibitor, exhibited a remarkable apoptosis-inducing effect on human hepatoma Hep G2 cells. We studied the effect of HCPT upon the expression of P53, c-Myc, Bcl-2, Bax and α-fetoprotein (AFP) proteins, and caspase (caspase-1 and caspase-3) activity of Hep G2 cells. It showed that HCPT at a dose of 0.1 μg/ml increased the expression of P53, c-Myc and Bax protein, and decreased the expression of Bcl-2 and AFP. The increase of P53, which was remarkable after only 3 h incubation with HCPT, occurred much earlier than the changes of other proteins, suggesting that the increase of P53 expression may be the upstream event in the apoptosis of Hep G2 cells induced by HCPT. Both caspase-1 and caspase-3 were activated in Hep G2 cells by HCPT treatment, suggesting that caspase-1 and caspase-3 are involved in the process of apoptosis in Hep G2 cells, and may be the main effectors of the apoptosis. [© 2000 Lippincott Williams & Wilkins.]**

*Key words*: Apoptosis, caspases, gene expression, Hep G2 cell line, 10-hydroxycamptothecin.

## Introduction

10-Hydroxycamptothecin (HCPT) is a naturally occurring compound isolated from *Camptotheca acuminata*, which is native to China. It was found that HCPT could inhibit the activity of DNA topoisomerase I (Topo I),[1] and was an effective anticancer agent in experimental research and clinical study.[2] Chemotherapeutic drugs cause DNA damage and kill cancer cells mainly through an apoptotic mechanism.[3,4] It was found that HCPT could inhibit the growth of human hepatoma Hep G2 cells and the growth inhibition was accompanied with evidence of apoptotic changes in cells, such as nuclear condensation and DNA fragmentation.[5] Unlike necrosis, apoptosis involves an active energy-dependent mechanism in which cells participate in their own destruction. An increasing number of genes and their proteins were reported to be involved in the process of apoptosis. Previous studies suggested that *p53* may be required for apoptosis in cancer cells treated with chemotherapeutic drugs.[4,6] In addition the c-*myc*, *bcl*-2, *bax* and *caspase* families were also described as important factors in the process of apoptosis. Recent investigation of the *bcl*-2 gene family shows a complex network regulating apoptosis. Of these genes, *bcl*-2 and *bcl*-$x_L$ are antiapoptotic, whereas *bax*, *bcl*-$x_S$, *bad*, *bak* and *bik* are proapoptotic.[7–10] Thus, investigation of the expression of the members of Bcl-2 family of proteins may provide insight to the mechanisms of apoptosis induction in hepatoma cells by therapeutic agents. Overexpression of the c-*myc* gene is strongly associated with many human carcinomas. However, it is not surprising that under adverse culture conditions overexpression of c-Myc can accelerate cell death via apoptosis since the death signaling domain of c-Myc is identical to that for proliferation.[11–13] Furthermore, apoptosis induced by c-Myc overexpression has been found to be *p53*-dependent,[14,15] but cells can be reduced by Bcl-2 protein.[12,16] Thus, the expression of p53, c-Myc and Bcl-2 family proteins during apoptosis could be concomitantly regulated in different ways and war-

This study was supported by grant no. 39570824 from the National Natural Science Foundation of China.

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rants investigation. The executive effects of the apoptosis have been identified as present in the mammalian genome, collectively called caspases (for cystein–aspartate-specific tease). The members of the caspase family are clearly central to mammalian apoptosis—they are activated during apoptosis, death is forestalled by specific inhibitors and germ-line knockout of their genes produces abnormal development with retention of cells which normally are deleted.[17] More than 10 members of the family have been found.

In this work, we investigated the effect of HCPT on the expression of P53, c-Myc, Bcl-2 and Bax proteins, and caspase (caspase-1 and caspase-3) activities in Hep G2 cells. The expression of α-fetoprotein (AFP), a hepatoma-promoting factor produced by Hep G2 cells, was also studied in HCPT-treated Hep G2 cells.

## Materials and methods

### Chemicals and antibodies

HCPT injection, 5 mg in 2 ml sterilized water, was obtained from Huanshi Feiyun Pharmaceutical (Hubei, China). Ac-YVAD-MCA and Ac-DEVD-MCA were obtained from Calbiochem-Novabiochem (San Francisco, CA). 7-Amino-4-methylcoumarin (AMC) was purchased from Sigma (St Louis, MO). Fluorescent isothiocyanate (FITC)-conjugated rabbit anti-mouse and swine anti-rabbit immunoglobulins, and monoclonal mouse anti-human P53, Bcl-2 and rabbit anti-human AFP antibodies were from Dako (Glostrup, Denmark). Monoclonal mouse anti-human c-Myc antibody and a rabbit polyclonal antibody against Bax were purchased from Santa Cruz, Biotechnology (Santa Cruz, CA). ABC (avidin–biotin–peroxidase complex) kit was prepared by Shanghai-American Company (Shanghai, China).

### Cell culture and treatment

The human hepatoma Hep G2 cell line was obtained from ATCC (Rockville, MD). The cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 Nutrient Mixture (Gibco/BRL Life Technologies, Grand Island, NY) containing 15% calf serum, HEPES 15 mmol/l, sodium bicarbonate 2.0 g/l, penicillin $1.0 \times 10^5$ U/l, streptomycin 100.0 mg/l and L-glutamine 2.0 mmol/l, in an incubator with a humidified 5% $CO_2$+95% air atmosphere at 37°C.[18] For all experiments, cells were treated with 0.1 μg/ml HCPT at 24 h after plating. Analysis was performed at different time intervals during culture of the cells.

### Immunocytochemical assay

The immunoreactivity of P53, Bcl-2, Bax or AFP was detected by using the ABC method. Hep G2 cells were plated into 96-well plates precoated with 50 μg/ml poly-L-lysine (Sigma) at a concentration of $5 \times 10^3$/well. After HCPT treatment, the culture medium was removed. The cells were washed with PBS 3 times and fixed with 0.1 M PBS (pH 7.4) containing 4% paraformaldehyde (Merck, Darmstadt, Germany) for 30 min at room temperature. Following three rinses with TBS (0.05 M, pH 7.4), cells were incubated for 30 min with methanol containing 0.3% $H_2O_2$ to block endogenous peroxidase staining. After three rinses, cells were permeabilized by a 15 min exposure to 0.4% Triton X-100 in TBS at room temperature. After preincubation with horse serum for 30 min at 37°C, the cells were incubated at 4°C in a moist chamber overnight with the primary antibodies for P53 (1:100 dilution), AFP (1:300 dilution), Bcl-2 (1:160 dilution) or Bax (1:80 dilution), respectively. After three rinses, the cells were incubated with biotinylated rabbit anti-mouse IgG (for P53 and Bcl-staining, using 1:100 dilution) or biotinylated goat anti-rabbit IgG (for AFP and Bax staining, using 1:80 dilution) at 37°C for 1 h. Following three rinses, the cells were incubated with avidin-linked peroxidase complex at 37°C for 1 h. The peroxidase activity was made visible with 0.5 mg/ml 3,3′-diaminobenzidine (Sigma) in the presence of 0.03% $H_2O_2$ for 4 min.

### Indirect immunofluorescence assay

The levels of P53, c-Myc, Bcl-2, Bax and AFP proteins were measured by flow cytometry[19,20] with minor modifications. Briefly, after the culture medium was removed, Hep G2 cells were collected from dishes and fixed with 2% paraformaldehyde for 20 min at room temperature. Before labeling, the cells incubated with a solution containing 0.5% Triton X-100 and 1% bovine serum albumin for 10 min at room temperature to permeabilize cell membranes and prevent non-specific protein binding. The fixed cells were then incubated with primary antibodies for P53 (1:50 dilution), AFT (1:300 dilution), c-Myc (1:50 dilution), Bcl-2 (1:50 dilution) or Bax (1:50 dilution), respectively. After 45 min at room temperature, the cells were washed 3 times with PBS and incubated with the corresponding FITC-conjugated secondary antibodies (1:50 dilution) for 30 min at room temperature in dark. After three more washes, the antigen density was measured by using a Becton Dickson FACStar Plus flow cytometer and the percentage of positive cells was determined.

### Analysis of caspase activities

Caspase activities were measured as described.[21,22] The assay for caspase-1-like or caspase-3-like protease activity was performed with Ac-YVAD-MCA or Ac-DEVD-MCA as the enzyme substrate, respectively. Collected cells were suspended in 50 mM Tris–HCl (pH 7.4) containing 1 mM EDTA and 10 mM EGTA, then incubated with 10 $\mu$M digitonin (Sigma) at 37°C for 10 min. Lysates were clarified by centrifugation at 15 000 *g* for 3 min and the supernatant was used for the enzyme assay. Protein concentration was determined using the method of Bradford.[23] The cleared lysates containing 50 $\mu$g protein were incubated with 50 $\mu$M of enzyme substrate at 37°C for 1 h. Levels of released AMC were measured using a spectrofluorometer (Hitachi F-3000) with excitation at 380 nm and emission at

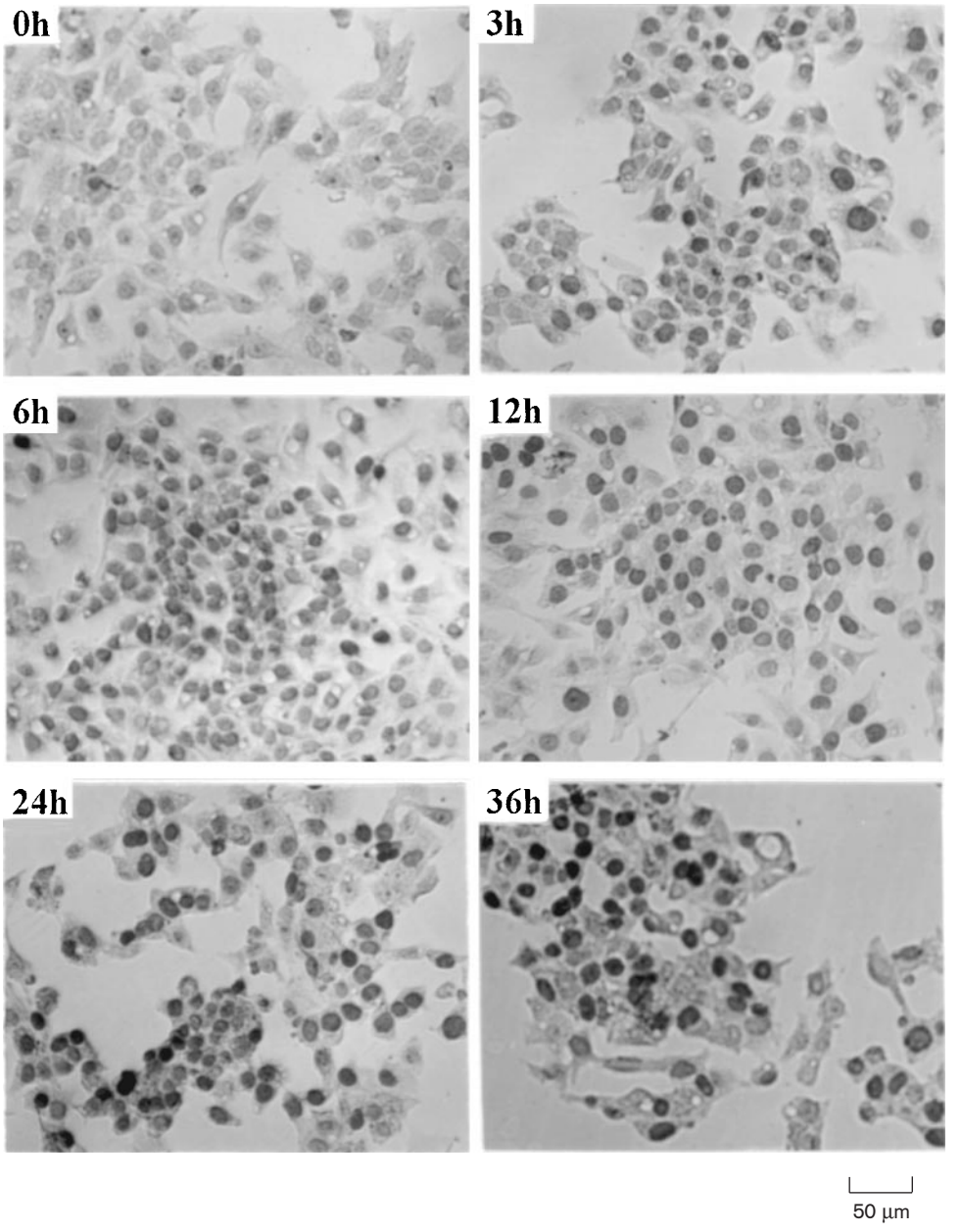


**Figure 1.** Immunocytochemical detection of P53 expression in human hepatoma Hep G2 cells treated with 0.1 $\mu$g/ml HCPT for the indicated times. The immunoreactivity of P53 was detected by using the ABC method, as described in Materials and methods. Photomicrographs are representative of experiments performed at least 3 times. (Magnification $\times$200.)

460 nm. One unit was defined as the amount of enzyme required to release 0.22 nM AMC/min at 37°C.

### Statistical analysis

Data were presented as mean ± SD and analyzed by *t*-test. The criterion for significant difference was $p < 0.05$.

## Results

### Immunostaining of P53, Bcl-2, Bax and AFP protein expression in Hep G2 cells

The results of immunocytochemical staining showed that HCPT at a dose of 0.1 μg/ml could significantly increase the expression of P53 and Bax, and decrease the expression of Bcl-2 and AFP. P53 was expressed in

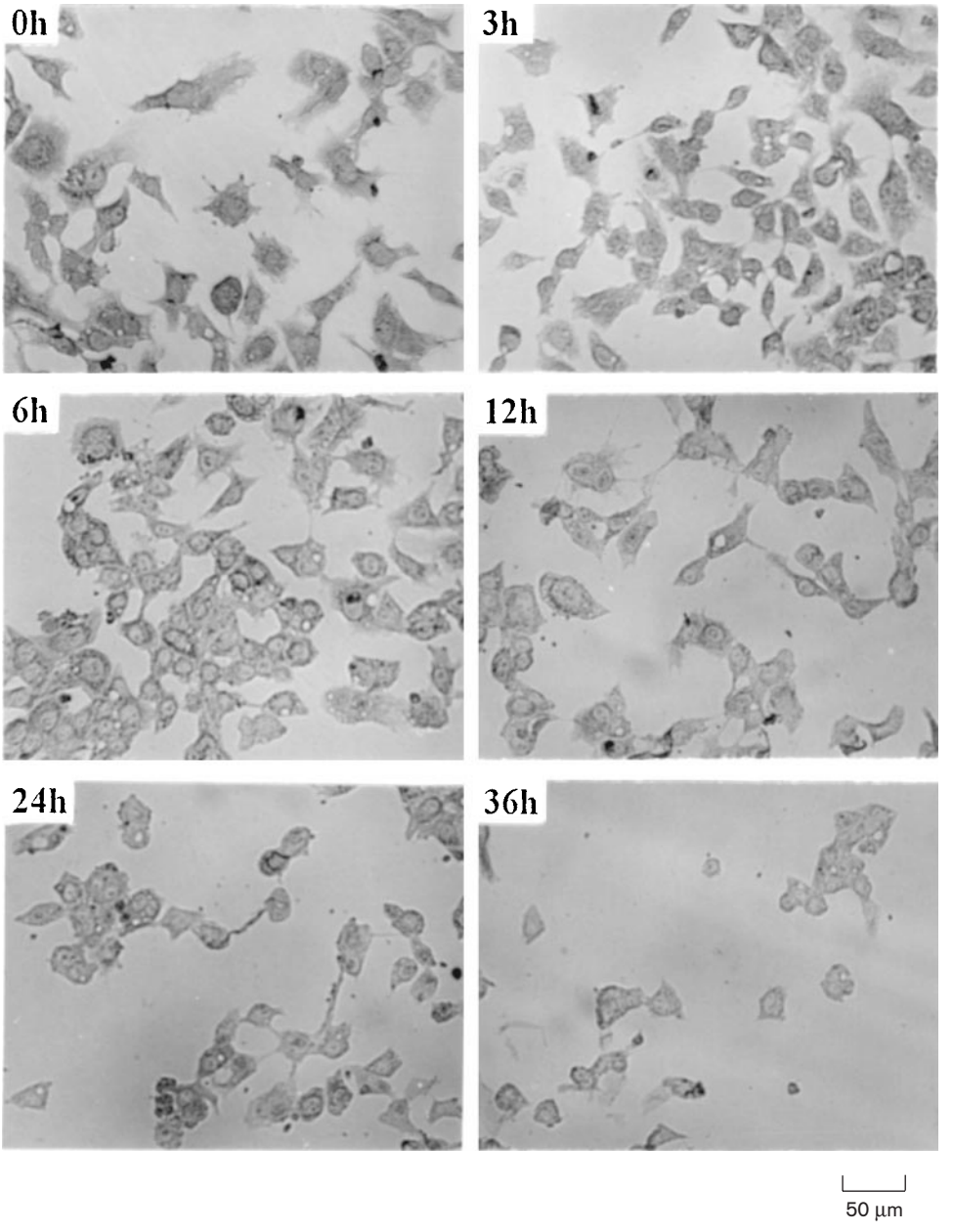


**Figure 2.** Immunocytochemical detection of Bcl-2 expression in human hepatoma Hep G2 cells treated with 0.1 μg/ml HCPT for the indicated times. The immunoreactivity of Bcl-2 was detected by using the ABC method, as described in Materials and methods. Photomicrographs are representative of experiments performed at least 3 times. (Magnification ×200.)

the nuclei of Hep G2 cells. In control cells, the expression of P53 was at a low level. In HCPT-treated cells, the expression of P53 markedly increased after only 3 h incubation and was maintained at high level over 6–36 h (Figure 1). Bcl-2 was expressed in the cytoplasm and nuclear membrane of Hep G2 cells. In control cells, the expression of Bcl-2 was at a high level. The expression of Bcl-2 protein was significantly decreased after 12 h treatment of HCPT and reached a maximum after 36 h incubation (Figure 2). The distribution of Bax was similar to that of Bcl-2 (Figure 3). In control cells, the expression of Bax was at a low level. The expression of Bax remarkably increased after 12 h treatment of HCPT and reached a maximum after 36 h incubation. The AFP was expressed in the cytoplasm of Hep G2 cells and the expression of AFP was at high level in the control group. In HCPT-treated cells, the expression of AFP gradually decreased during 6–36 h incubation and reached a maximum after 36 h incubation (Figure 4).

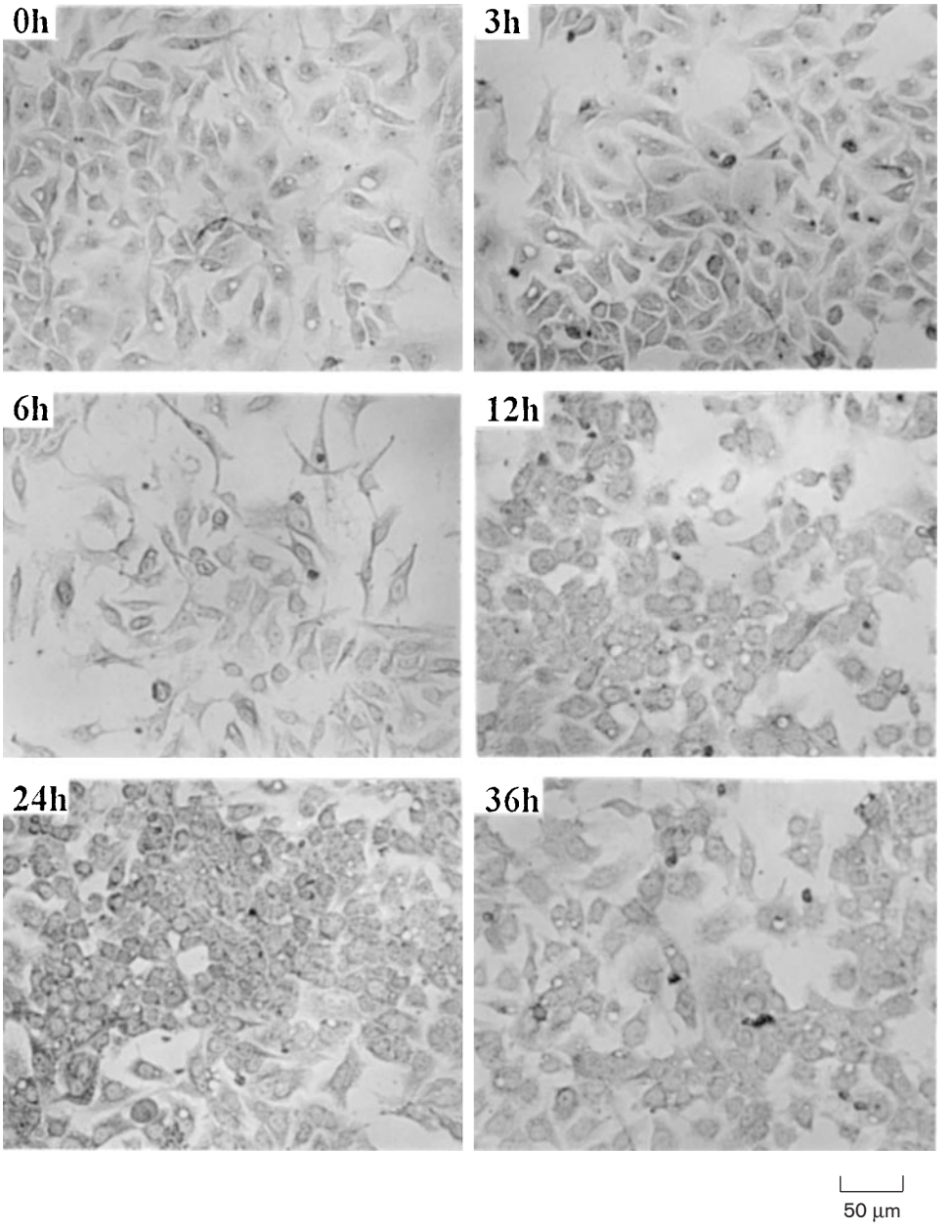


**Figure 3.** Immunocytochemical detection of Bax expression in human hepatoma Hep G2 cells treated with 0.1 $\mu$g/ml HCPT for the indicated times. The immunoreactivity of Bax was detected by using the ABC method, as described in Materials and methods. Photomicrographs are representative of experiments performed at least 3 times. (Magnification $\times$200.)

### Flow cytometric analysis of P53, c-Myc, Bcl-2, Bax and AFP expression in Hep G2 cells

The results of flow cytometric analysis are shown in Figure 5. HCPT at a dose of 0.1 μg/ml could significantly increase the expression of P53, c-Myc and Bax, and decrease the expression of Bcl-2 and AFP. The P53-positive and c-Myc-positive cell rate of HepG2 cells were significantly increased after 6 h incubation with HCPT, and were maintained at a high level afterwards. After treatment with HCPT, the percentage of Bcl-2-positive cell was significantly decreased after 12 h incubation and reached a maximum after 36 h incubation. Under the same experimental conditions, the Bax-positive cell rate was significantly increased after 12 h incubation with HCPT and the effect reached a maximum after 36 h incubation. It was also found that under the influence of HCPT, the AFP-positive cell rate

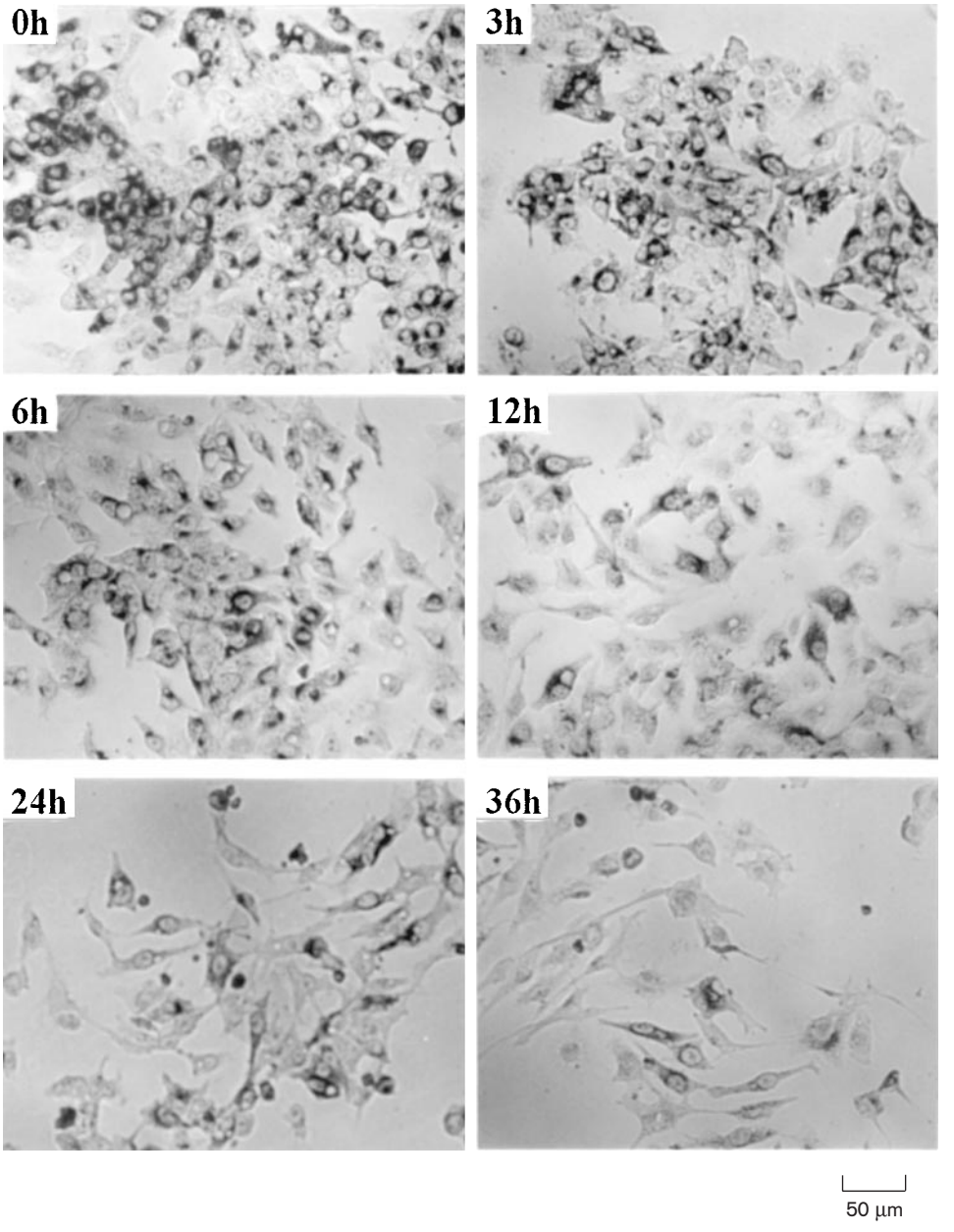


**Figure 4.** Immunocytochemcial detection of AFP expression in human hepatoma Hep G2 cells treated with 0.1 μg/ml HCPT for the indicated times. The immunoreactivity of AFP was detected by using the ABV method, as described in Materials and methods. Photomicrographs are representative of experiments performed at least 3 times. (Magnification ×200.)

was significantly decreased after 12–48 h incubation.

### Caspase-1 and caspase-3 activities in Hep G2 cells

Caspase-1 and caspase-3 activities were significantly increased after 12–36 h incubation with 0.1 μg/ml HCPT and decreased subsequently (Figure 6).

## Discussion

From the above-mentioned data, the results of the immunostaining assay were in good agreement with the results of flow cytometric analysis. It indicated that *p53*, c-*myc*, *bcl*-2, *bax*, *AFP* and *caspases* (*1* and *3*) were involved in the process of apoptosis in Hep G2 cells induced by HCPT.

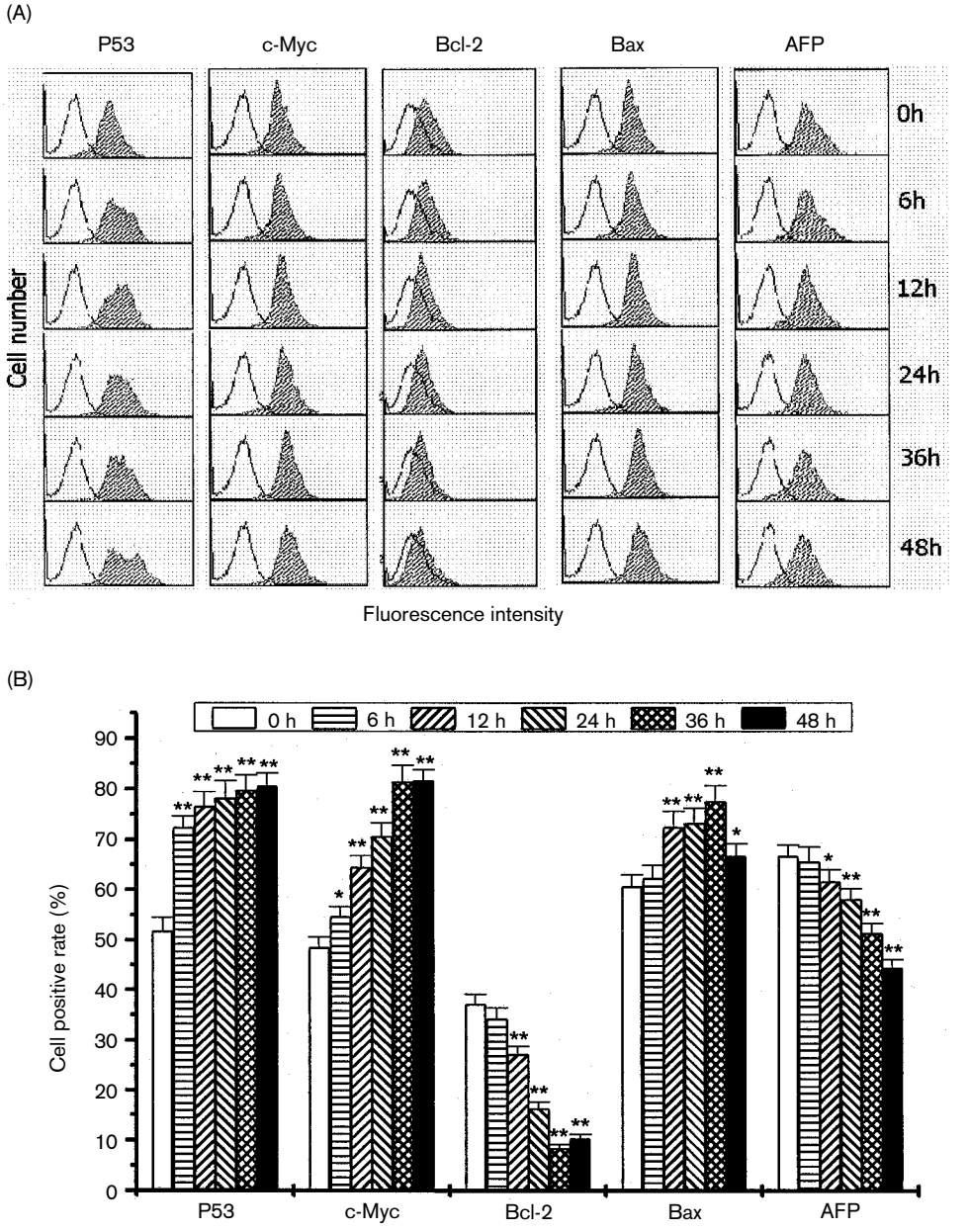


**Figure 5.** Flow cytometric analysis of P53, c-Myc, Bcl-2, Bax and AFP expression in Hep G2 cells treated with 0.1 μg/ml HCPT for the indicated times. (A) Representative data. Shaded profiles, cells incubated with primary antibody as well as secondary antibody. Open profiles, cells incubated with secondary antibody alone. (B) Results of triplicate samples. Mean ± SD. *$p<0.05$, **$p<0.01$ versus control (0 h).

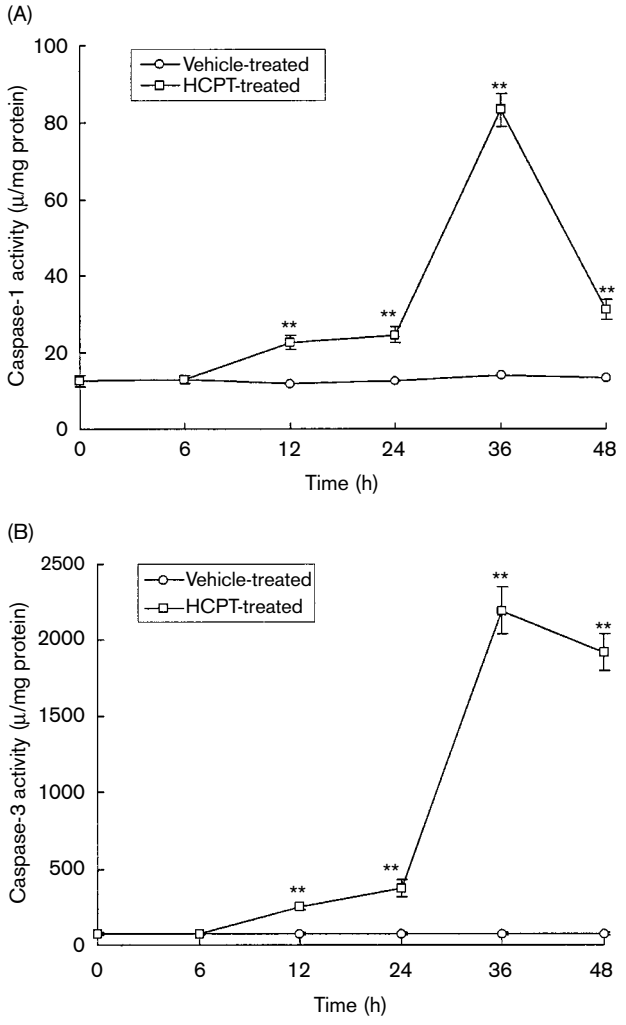


**Figure 6.** Caspase-1 (A) and caspase-3 (B) activities in human hepatoma Hep G2 cells treated with 0.1 μg/ml HCPT for 0–48 h. Caspases activities were determined with the enzyme substrate assay, as described in Materials and methods. Data are mean ± SD. of three independent experiments. $^{**}p < 0.01$ versus control.

Like camptothecin (CPT), HCPT is a potent Topo I inhibitor.[1] Topo I inhibitors can cause cell damage by creating protein-linked DNA single-stand breaks.[24–26] DNA injury can initiate apoptosis by a powerful, early-activated mechanism dependent on the nuclear phosphoprotein *p53*.[27,28] After CPT treatment, wild-type expressing human cancer cells exhibited induction of both P53 and P21 protein levels.[4,29] Only wild-type P53 but not mutant P53 could be expressed in Hep G2 cells.[30] Our results also indicated that P53 expression was induced after HCPT treatment. The P53 expression of HCPT-treated Hep G2 cells increased markedly after only 3 h incubation. It is suggested that the p53 may be the pivotal factor in the initiation of apoptosis in Hep G2 cells induced by HCPT.

Among its diverse functions, the P53 protein acts as a transcription factor that up-regulates or represses the activity of a number of genes, including several genes modulating apoptosis, such as c-*myc* and *bax*.[31,32] Transcriptional activation of c-Myc is considered as initiating a state of susceptibility to both apoptosis and proliferation.[33] The c-Myc expression in HCPT-treated Hep G2 cells increased remarkably after 6 h incubation. The increase of c-Myc expression may reflect the active state of Hep G2 cells and make the cells sensitive to an apoptosis-inducing trigger.

It was not surprising that Bax expression increased in HCPT-treated Hep G2 cells. Wild-type *p53* is known to be an upstream regulator of the *bax* gene promoter which contains *p53* binding sites and can be directly activated by wild-type *p53*.[34] The increase of Bax expression in Hep G2 cells after 12 h incubation of HCPT may be the result of the increase of P53 expression which occurred much earlier (after 3 h incubation). Both Bax and Bcl-2 belong to the Bcl-2 family of proteins. The Bcl-2 family members are key regulators of apoptosis.[6–9] It has been shown that Bcl-2 can physically interact with several of these homologous proteins to form heterotypic dimers. Dimerization of Bcl-2 with Bax seems to be a critical interaction. The cells continue to survive if Bcl-2 predominates over Bax. On the contrary, a higher concentration of Bax, compared with Bcl-2, enhances cell susceptibility to apoptosis.[35,36] In the present study, Bcl-2 protein expression in Hep G2 cells was obviously decreased after incubation with HCPT. The Bcl-2-positive cell rate decreased from 37.2% before incubation to 8.4% after 36 h incubation. These results indicated that Bcl-2 was an important factor in the process of apoptosis induced by HCPT.

AFP has been widely used as a marker for primary hepatocellular carcinoma. AFP can also behave as a growth regulator under certain circumstances.[37] In addition, it has been found that AFP inhibits cell apoptosis, which may be related to its tumor-proliferating effect.[38] HCPT decreased the AFP expression in Hep G2 cells. The down-regulation of the level of the growth-proliferating factor might also contribute to the apoptosis.

Once apoptosis is triggered, downstream events feed into a common effector pathway of apoptosis.[27] Caspase activation was also detected in the process of apoptosis induced by HCPT in HepG2 cells. Both caspase-1 and caspase-3 in HepG2 cells were activated by HCPT incubation. The caspase activation may be responsible for driving all the structural change in the nucleus that accompanied apoptosis.

In summary, our results suggest that HCPT elevates the expression of P53 protein caused by DNA damage through Topo I inhibition. The upregulation of c-Myc and Bax expression induced by HCPT may be related to the increased susceptibility of cells. The decreased expression of both Bcl-2 and growth-proliferating factor (AFP) might also contribute to the apoptosis of Hep G2 cells. Caspase-1 and caspase-3 were involved in the process of apoptosis, and considered as the executive effectors.

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*(Received 15 June 2000; accepted 20 July 2000)*