

# Dexamethasone protects normal human liver cells from apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand by upregulating the expression of P-glycoproteins

BO ZHAO, GUI-JUAN XIE, RUI-FENG LI, QING CHEN and XU-QING ZHANG

Department of Infectious Diseases, Southwest Hospital, Third Military Medical University, Chongqing 400038, P.R. China

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**Abstract.** Glucocorticoids are effective for the treatment of acute-on-chronic pre-liver failure, severe chronic hepatitis B and acute liver failure; however, the mechanism underlying the effects of treatment by glucocorticoids remains to be fully elucidated. The role and detailed mechanism of how glucocorticoids prevent liver disease progression can be elucidated by investigating the apoptosis of hepatocytes following glucocorticoid treatment. P-glycoproteins (P-gps) also confer resistance to apoptosis induced by a diverse range of stimuli. Glucocorticoids, particularly dexamethasone (DEX), upregulate the expression of P-gp in several tissues. In the present study, the normal human L-02 liver cell line was used, and techniques, including immunocytochemistry, western blot analysis, flow cytometry and reverse transcription-quantitative polymerase chain reaction analysis were used for determining the expression levels of P-gps, and for evaluating the effect of DEX pretreatment on the expression of P-gps. DEX (1-10  $\mu$ M) was added to the cell culture media and incubated for 24-72 h. The results revealed that DEX upregulated the mRNA and protein levels of P-gp in a dose- and time-dependent manner. Subsequently, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was used for the induction of apoptosis in the cells, followed by a terminal deoxynucleotidyl transferase dUTP nick end labeling assay to assess the apoptotic stages. The results demonstrated that apoptosis in the group of cells,

which were pre-treated with DEX was significantly lower than that in the control group. Treatment with tariquidar, a P-gp inhibitor, reduced the anti-apoptotic effects of DEX. These results established that DEX protects normal human liver cells from TRAIL-induced apoptosis by upregulating the expression of P-gp. These observations may be useful for elucidating the mechanism of DEX for preventing the progression of liver disease.

## Introduction

Patients with severe acute exacerbation of chronic hepatitis B virus (HBV) infection, including acute-on-chronic pre-liver failure (pre-ACLF), are at risk for potential progression to acute-on-chronic liver failure (ACLF) with high rates of mortality (1,2). Therefore, it is important to investigate therapies, which can prevent the progression of severe acute episodes to liver failure in patients with chronic HBV. The role of antiviral treatments, including nucleoside analogs in preventing the progression of severe HBV to liver failure remains to be elucidated. However, glucocorticoids are effective for the treatment of pre-ACLF, severe and potentially life-threatening exacerbation of chronic HBV and acute liver failure (3,4). Studies have indicated that the mechanisms by which glucocorticoids prevent the progression of liver disease include: (i) Prevention of HBV-induced primary liver injuries by inhibiting excessive immune responses; (ii) prevention of endotoxin-induced secondary liver injuries by inhibiting the production of oxygen-free radicals and cytokines (5); (iii) prevention of cytolysis of ballooned hepatocytes by stabilizing the lysosomal membrane, and inhibiting the production of lysosomal proteases and circulating toxic substances (6); and (iv) improvement in the functional activity of the residual hepatocytes (7). However, the mechanisms by which glucocorticoid treatment alleviates various liver conditions remain to be fully elucidated. Similar to hepatic necrosis, apoptosis of hepatocytes is also a key feature of almost all acute and chronic cases of HBV, including acute liver failure (8). Therefore, investigating the role of glucocorticoids and their underlying mechanism in preventing the apoptosis of hepatocytes is necessary for improved treatment of severe chronic HBV and acute liver failure associated with HBV infection. Dexamethasone (DEX) is a synthetic glucocorticoid, which inhibits apoptosis in certain cells, including human neutrophils, hair cells, human

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*Correspondence to:* Professor Xu-Qing Zhang, Department of Infectious Diseases, Southwest Hospital, Third Military Medical University, 30 Gaotanyan Street, Shapingba, Chongqing 400038, P.R. China

E-mail: xuqing651005@tom.com

**Abbreviations:** DEX, dexamethasone; P-gp, P-glycoprotein; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; pre-ACLF, acute-on-chronic pre-liver failure; ACLF, acute-on-chronic liver failure; NA, nucleoside analog; MDR, multidrug resistance; PXR, pregnane X receptor; TQD, tariquidar

**Key words:** multidrug resistance protein, P-glycoprotein, dexamethasone, L-02 cell, apoptosis

fibroblasts and primary rat hepatocytes; and induces apoptosis in other cells, including thymocytes and lymphocytes (9,10). DEX also enhances trichosanthin-induced apoptosis in the HepG2 hepatoma cell line (11). However, the precise mechanism by which DEX inhibits or induces apoptosis remains to be elucidated.

Multidrug resistant (MDR) P-glycoprotein (P-gp), encoded by the ABCB1 gene, was first discovered in MDR tumor cells, where it reduces the cellular accumulation of chemotherapeutic agents (12,13). In addition to being expressed in cancer cells, P-gps are also expressed in normal tissues, where they have an important protective role in limiting the absorption and/or facilitating the excretion of a wide range of substrates, by actively transporting substrates from the inner to the outer leaflets of the cell membranes (14). In addition to their ability to discharge toxins, P-gps can also inhibit apoptosis, which is induced by a wide array of cell death stimuli that rely on the activation of intracellular caspases for complete functionality (15-17). Therefore, the present study hypothesized that the expression of P-gps in hepatocytes also have an important hepatoprotective role. A number of studies have reported that glucocorticoids, particularly DEX, are able to improve the blood-brain barrier and placental barrier functions by increasing the expression and function of P-gps (18,19). DEX increases the expression of pregnane X receptor (PXR) at the transcriptional level, and PXR mediates the spironolactone-induced expression of P-gp in HepG2 cells (20).

Based on evidence that DEX and P-gps have the ability to prevent the apoptosis induced by a wide array of cell death stimuli, and that DEX alone induces the expression and function of P-gps in several organs and cells; the present study hypothesized that DEX prevents apoptosis in human hepatocytes by upregulating the expression of P-gp. At present, few studies detailing the role of P-gps in the inhibition of hepatocyte apoptosis by DEX have been performed. The present study was performed to determine the anti-apoptotic effects of DEX on the L-02 normal human liver cell line, and to ascertain whether the anti-apoptotic effects are associated with an upregulation in the expression of P-gp. The results from these investigation aim to establish whether DEX protects L-02 normal human liver cells from tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, by upregulating the expression of P-gps.

## Materials and methods

**Materials.** The L-02 normal human liver cell line was purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). RPMI 1640 medium was purchased from Gibco Life Technologies (Carlsbad, CA, USA) and newborn bovine serum was purchased from Lanzhou Minhai Bioengineering Co., Ltd. (Lanzhou, China). Trypsin (0.25%) was purchased from GE Healthcare Life Sciences (Logan, UT, USA) and DEX was purchased from Chongqing Xi'nan Pharmaceutical Group Co., Ltd. (Chongqing, China). Primary mouse anti-human monoclonal P-gp antibody (cat. no. ab3366) was obtained from Abcam (Cambridge, UK). An avidin-free, polymer detection kit [PV-9000; containing a polymer helper (reagent 1) and polyperoxidase anti-mouse/rabbit IgG (reagent 2)]

for immunocytochemical staining and hematoxylin were purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd., (Beijing, China). Hydrogen peroxide was purchased from Chongqing Chuandong Chemical Group Co., Ltd. (Chongqing, China) IC fixation buffer (cat. no. 00-8222) and permeabilization buffer (cat. no. 00-8333) were purchased from eBioscience, Inc. (San Diego, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody was purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd. (cat. no. ZF-0312). Radioimmunoprecipitation assay (RIPA) cell lysis buffer, the bicinchoninic acid (BCA) protein assay kit and horseradish peroxidase-labeled goat anti-mouse secondary antibody (cat. no. A0216) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Polyvinylidene fluoride (PVDF) membranes were purchased from EMD Millipore (Billerica, MA, USA). SDS was purchased from Sigma-Aldrich (St. Louis, MO, USA), and acrylamide was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). The UNIQ-10 TRIzol total RNA extraction kit and paraformaldehyde were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). The QuantScript reverse transcription kit was obtained from Tiangen Biotech Co., Ltd. (Beijing, China). The Mx3000P Real-Time QPCR System was obtained from Agilent Technologies, Inc. (Santa Clara, CA, USA). The FastStart Universal SYBR Green Master kit was obtained from Roche Diagnostics (Basel, Switzerland). The primers for the ABCB1 gene, encoding P-gp, were synthesized according to the method reported by Jigore *et al* (21). The primers for the  $\beta$ -actin gene were synthesized by Sangon Biotech. All the primers used for the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis were purchased from Sangon Biotech Co., Ltd. The sequences of the primer pairs were as follows: ABCB1, forward, 5'-GCCAAAGCCAAAATATCAGC-3' and reverse, 5'-TTCCAATGTGTTCGGCATT-3'; and  $\beta$ -actin, forward 5'-GACGACATGGAGAAAA-3' and reverse 5'-AAGGCTGGAAGAGTGC-3'. Recombinant Human sTRAIL/Apo2 L (TRAIL) was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA). The *in situ* Cell Death Detection kit, POD, was purchased from Roche Diagnostics and tariquidar (TQD) was purchased from Selleck Chemicals (Shanghai, China).

**Human L-02 cell culture and induction with DEX.** The L-02 cells were seeded in 6-well plates or 75-cm<sup>2</sup> culture flasks (Nunc, Roskilde, Denmark) at a density of 5x10<sup>5</sup> cells/ml, and cultured in RPMI 1640 medium supplemented with 10% (v/v) newborn bovine serum at 37°C and 5% CO<sub>2</sub>. Following treatment with varying concentrations (1 and 10  $\mu$ M) of DEX at 37°C for 24-72 h, the cells were harvested for detection of the expression of P-gp. Untreated cells were used as a control group.

**Immunocytochemistry.** Following fixation with 4% paraformaldehyde for 10 min, the cells were processed for immunocytochemistry by staining using a PV-9000 avidin-free polymer detection system. The procedures included the following steps: i) Incubation with 3% hydrogen peroxide for 10 min to inhibit the activity of endogenous peroxidase; ii) incubation with the primary mouse anti-human

monoclonal P-gp antibody (cat. no. ab3366; 1:50 dilution; Abcam) at 4°C overnight, followed by incubation with the polymer helper (reagent 1) at 37°C for 20 min; iii) incubation with polyperoxidase anti-mouse/rabbit IgG (reagent 2) at 37°C for 20 min; iv) development of the peroxidase reaction with 3,3'-Diaminobenzidine (DAB) tetrahydrochloride following three washes with phosphate-buffered saline (PBS); and v) counterstaining of the cells with hematoxylin. In the negative control group, the primary antibody (cat. no. ab3366) was substituted with PBS. The cells were examined and images were captured under an Olympus IX70 microscope (Olympus Corporation, Beijing, China). Image-Pro Plus image analysis software v6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used for quantitatively assessing the optical densities.

**Flow cytometric analysis.** Flow cytometry was performed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Briefly, 10<sup>6</sup> cells were trypsinized in each well of a 6-well plate, washed with PBS and resuspended in PBS. The cells were fixed and permeabilized using the IC fixation and permeabilization buffers, respectively, according to the manufacturer's instructions (eBioscience, Inc.). The cells were incubated with primary mouse anti-human monoclonal P-gp antibody (ab3366; 1:50 dilution; Abcam) overnight at 4°C, followed by incubation with the FITC-conjugated goat anti-mouse secondary antibody (1:50 dilution; cat. no. ZF-0312; Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 20 min in the dark. Following three washes, the samples were analyzed using the flow cytometer and FlowJo software (v 6.4.7; Tree Star, Inc. Ashland, OR, USA).

**Protein extraction and western blot analysis.** Total protein was extracted from the cells using RIPA cell lysis buffer, containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na<sub>3</sub>VO<sub>4</sub> and 0.5 mg/ml leupeptin. A BCA protein assay kit was used for measuring the protein concentrations in the cell extracts. Subsequently, the proteins (20 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 6% gels, and were then transferred onto PVDF membranes by electroelution. The membranes were incubated overnight with the primary mouse anti-human monoclonal P-gp antibody (ab3366; 1:50 dilution; Abcam), followed by incubation with the horseradish peroxidase-labeled goat anti-mouse secondary antibody (cat. no. A0216; 1:1,000 dilution; Beyotime Institute of Biotechnology) for 2 h at room temperature. The bands were visualized using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL, USA) and images were captured using a ChemiDoc XRS+ system (Bio-Rad Laboratories, Inc.). The relative intensities in each band were quantified densitometrically using Quantity One Software (v. 4.4.0.36, Bio-Rad Laboratories, Inc.) and calculated by comparing the band densities with those of the reference protein, β-actin.

**Total RNA purification and RT-qPCR.** Total RNA was extracted from the cells using a UNIQ-10 TRIzol total RNA extraction kit, according to the manufacturer's instructions. The extracted RNA samples were reverse transcribed into cDNA using a QuantScript reverse transcription kit. qPCR

was performed on an Mx3000P Real-Time QPCR System using the FastStart Universal SYBR Green master kit. qPCR was performed in 96-well plates, with each well containing 1X FastStart universal SYBR Green master mix, 2X each of the forward and reverse primers, ddH<sub>2</sub>O and template cDNA, in a 20 µl total reaction volume. The DNA template (1 µl) was pre-incubated at 95°C for 2 min prior to the amplification cycle, followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 40 sec. β-actin was used as the reference gene. The relative mRNA expression levels were calculated using the 2<sup>-ΔΔC<sub>t</sub></sup> method (22). All the experiments were performed in triplicate.

**Effects of DEX and TQD on apoptosis induced by TRAIL/Apo2 L.** It has been reported that P-gps can protect cells from apoptosis induced by a wide range of drugs (23,24). To examine the effects of DEX on apoptosis induced by TRAIL in the present study, the L-02 cells were treated with 20 ng/ml TRAIL for 24 h to induce apoptosis, followed by treatment with either 1 or 10 µM DEX for 24 or 48 h. Untreated cells were used as a control group. A TUNEL assay was performed to analyze the induction of apoptosis by TRAIL in the cells.

For investigating the effects of TQD on apoptosis, the L-02 cells were divided into six groups: i) control group comprising untreated L-02 cells; ii) L-02 cells pretreated with 10 µM DEX for 24 h, followed by incubation with TRAIL for 24 h; iii) L-02 cells pretreated with 10 µM DEX for 24 h, followed by a 24 h incubation with TRAIL and TQD (25 nM); iv) L-02 cells pretreated with 10 µM DEX for 24 h, followed by a 24 h incubation with TRAIL and TQD (50 nM); v) L-02 cells pretreated with 10 µM DEX for 24 h, followed by a 24 h incubation with TRAIL and TQD (100 nM); vi) L-02 cells incubated with TRAIL for 24 h. All the groups were incubated at 37°C, and with the exception of the control, were treated with TRAIL to induce apoptosis. Following the various treatments, all groups reached the same final cell density of ~90% confluence. Apoptosis was evaluated using the TUNEL assay, and the number of apoptotic cells in each group were counted.

The apoptosis status of the L-02 cells was measured using the TUNEL assay, using a POD *In Situ* Cell Death Detection kit, according to the manufacturer's instructions. Briefly, following treatment with 4% paraformaldehyde for 1 h at room temperature and 3% hydrogen peroxide for 10 min at room temperature, the cells (~90% confluence) were washed twice in PBS for 5 min. Following incubation with 0.1% Triton X-100 on ice for 5 min and two washes with PBS for 5 min, the labeling reactions were performed using 50 µl TUNEL reagent for 1 h at 37°C for each group. Following washing with PBS, the cells were incubated with a converter reagent for 30 min at 37°C, developed using DAB for 10 min, and then counterstained with hematoxylin. Following dehydration in ethanol and clearing in xylene, the cells were mounted on slides and examined under a light microscope (Olympus IX70; Olympus Corporation). Apoptotic cells were quantified by counting the number of TUNEL-positive cells in ten random microscopic fields (magnification, x200).

**Statistical analysis.** The data are presented as the mean ± standard deviation. One-way analysis of variance was used for determining significant differences. All analyses were



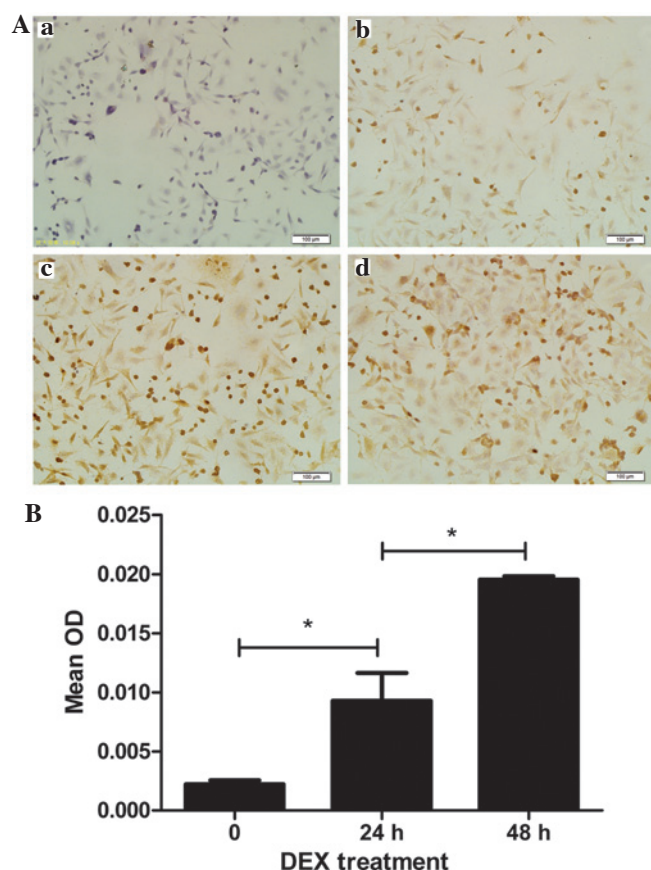


Figure 1. Detection of the expression of P-gp in L-02 cells using immunocytochemistry. (A) Representative immunocytochemical staining of P-gp in the L-02 cells. (a) Negative control; (b) untreated L-02 cells; (c) L-02 cells incubated with 10  $\mu$ M DEX for 24 h; (d) L-02 cells incubated with 10  $\mu$ M DEX for 48 h. The marked positive staining (brown) represents P-gps in the L-02 cells. (B) Quantitative analysis of the immunocytochemical staining. Data are presented as the mean  $\pm$  standard deviation. \* $P$ <0.01. Magnification,  $\times$ 200. P-gp, P-glycoprotein; Dex, dexamethasone; OD, optical density.

performed using the SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA).  $P$ <0.05 was considered to indicate a statistically significant difference.

## Results

**DEX increases the mRNA and protein expression levels of P-gp in the L-02 cell line.** The results from the immunocytochemistry assays demonstrated definitive expression of P-gp in the L-02 normal human liver cell lines, compared with the negative control (Fig. 1Aa and b). Treatment of the L-02 cells with 10  $\mu$ M DEX for 24 and 48 h resulted in marked P-gp staining in the membrane and in the cytoplasm of the majority of the cells (Fig. 1Ac and d). The protein expression levels of P-gp, presented as mean densitometric units, in the L-02 cells following treatment with 10  $\mu$ M DEX for 24 and 48 h were  $9.284 \times 10^{-3} \pm 0.578 \times 10^{-2}$  and  $19.530 \times 10^{-3} \pm 0.719 \times 10^{-3}$ , respectively, which were significantly higher than in the cells without DEX treatment ( $2.228 \times 10^{-3} \pm 0.770 \times 10^{-3}$ ; Fig. 1B). The cells, which were incubated for 48 h with DEX had higher levels of P-gps, compared with those incubated for 24 h ( $P$ <0.01).

The results of the flow cytometric analysis (Fig. 2) demonstrated that the levels of P-gp were significantly higher in the

L-02 cells treated with 10  $\mu$ M DEX for 48 h, compared with levels in the untreated control group ( $P$ <0.01). Additionally, the levels of P-gp were higher in the cells, which were treated with DEX for 48 h, compared with those treated for 24 h ( $P$ =0.014). The L-02 cells, which were treated with DEX for 24 h exhibited a degree of upregulation in the expression of P-gp, compared with the untreated cells; however, no statistical difference was observed between these two groups ( $P$ =0.054).

The protein expression levels of P-gp in the L-02 cells were also determined by western blot analysis, using a monoclonal antibody against P-gp (JSB-1). As shown in Fig. 3, the results revealed a band at 170 kDa, which corresponded with P-gp. The results were analyzed quantitatively, according to the grayscale values. The expression of P-gp in the group treated with 1  $\mu$ M DEX for 24 h were significantly increased, compared with the control group ( $P$ =0.001). Treatment with 10  $\mu$ M DEX for 24 h resulted in higher expression levels of P-gp than that following 1  $\mu$ M DEX treatment for 48 h ( $P$ =0.006). In addition, the protein expression levels in the cells treated with 10  $\mu$ M DEX for 72 h were markedly upregulated, compared with those in the groups treated with 10  $\mu$ M DEX for 24 and 48 h ( $P$ <0.05). Therefore, it was concluded that the protein expression levels of P-gp increased in a dose-dependent manner, between 1 and 10  $\mu$ M DEX, and a time-dependent manner, between 24 and 72 h DEX stimulation.

RT-qPCR was used for detecting the mRNA expression levels of the ABCB1 gene, encoding P-gp, in the L-02 cells. As shown in Fig. 4, the present study detected for the first time, to the best of our knowledge, transcription of the ABCB1 gene in the L-02 cell lines. In addition, the effects of DEX on the transcription of ABCB1 mRNA were also demonstrated in the L-02 cell line. The results demonstrated that the mRNA levels of the ABCB1 gene increased in response to treatment with 1  $\mu$ M DEX for 24 h, compared with the control ( $P$ =0.004), similar to the results of the protein levels. During the same time interval, treatment with 1  $\mu$ M and 10  $\mu$ M DEX exhibited differences in mRNA levels, which were significantly higher in the 10  $\mu$ M DEX group, compared with the 1  $\mu$ M group ( $P$ <0.05). Additionally, treatment with DEX for 48 h lead to increased mRNA levels, compared with treatment with DEX for 24 h ( $P$ <0.05). These results suggested that treatment with DEX increased the expression of P-gp at the mRNA and protein levels, in a dose- and time-dependent manner.

**Effects of DEX on apoptosis induced by TRAIL.** As shown in Fig. 5, all the nuclei were stained blue with hematoxylin, whereas the brown color indicated the presence of apoptotic nuclei, which were visualized following staining with DAB reagent. The highest degree of apoptosis was observed in the control group, which was incubated with TRAIL in the absence of DEX. By contrast, treatment with 10  $\mu$ M DEX for 48 h resulted in the lowest observed levels of apoptosis in the L-02 cells. In addition, the results demonstrated that the degree of DNA fragmentation decreased with prolonged exposure to DEX, between 24–48 h, and increased concentrations of DEX between 1 and 10  $\mu$ M ( $P$ <0.05).

**Effects of TQD on apoptosis induced by TRAIL.** As shown in Fig. 6, the lowest degree of apoptosis was observed in the

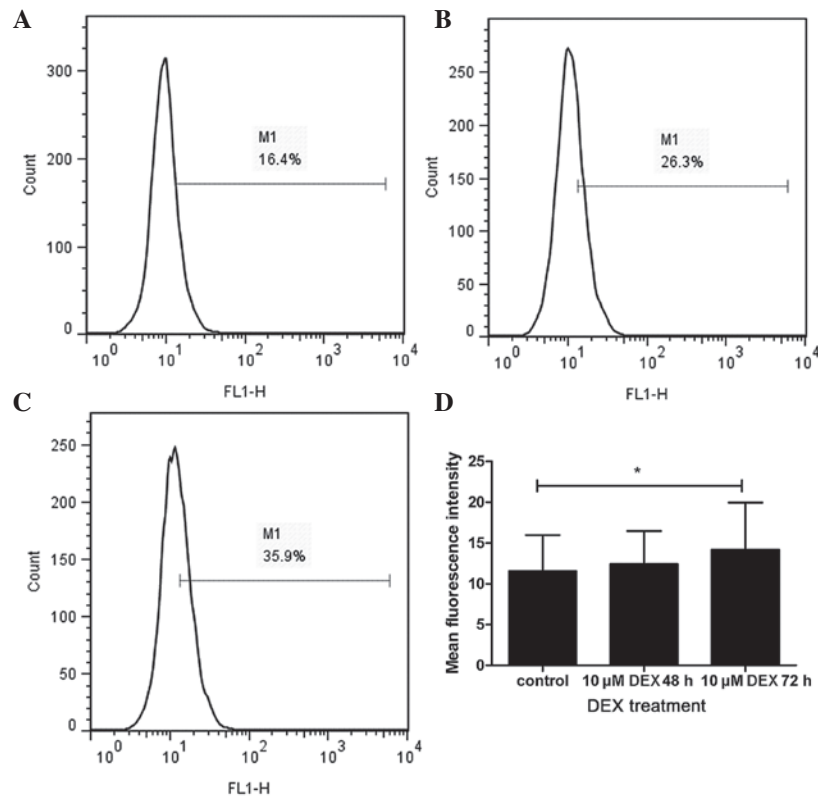


Figure 2. Flow cytometric analysis of the expression of P-gp. The results in each flow cytometry histogram represent one of three independent experiments. (A) Untreated L-02 cells (B) L-02 cells incubated with 10  $\mu$ M DEX for 48 h; (C) L-02 cells incubated with 10  $\mu$ M DEX for 72 h. (D) Quantitative analysis of the flow cytometry data. Data are presented as the mean  $\pm$  standard deviation. \* $P$ <0.01. P-gp, P-glycoprotein; DEX, dexamethasone; M1 region, positive cells.

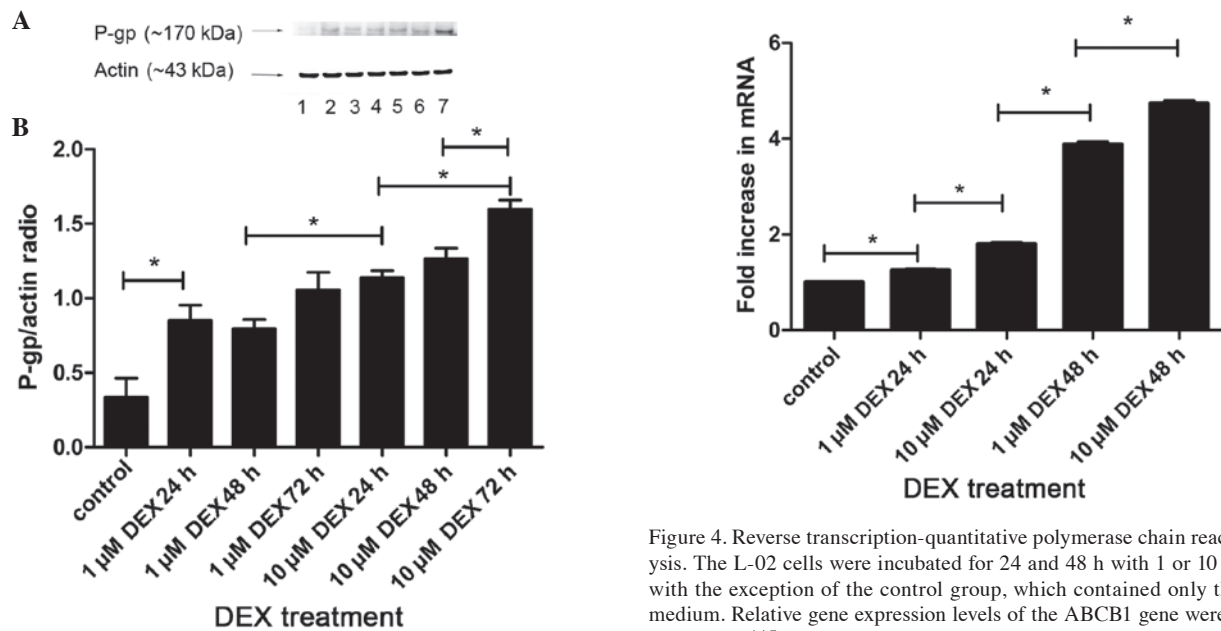


Figure 3. Expression of P-gp, determined using western blot analysis. (A) Representative electrophoretograms of western blots. (1) Untreated L-02 cells, (2) L-02 cells incubated with 1  $\mu$ M DEX for (2) 24, (3) 48 and (4) 72 h, and with 10  $\mu$ M DEX for (5) 24, (6) 48 and (7) 72 h. (B) Quantitative analysis of the western blot protein bands. Data are presented as the mean  $\pm$  standard deviation. \* $P$ <0.05. P-gp, P-glycoprotein; DEX, dexamethasone.

control group, which comprised L-02 cells cultured in media alone with no additional treatments. These results indicated that the levels of apoptosis in the L-02 cells were intrinsically

Figure 4. Reverse transcription-quantitative polymerase chain reaction analysis. The L-02 cells were incubated for 24 and 48 h with 1 or 10  $\mu$ M DEX, with the exception of the control group, which contained only the culture medium. Relative gene expression levels of the ABCB1 gene were analyzed using the  $2^{-\Delta\Delta C_t}$  method. Experiments were performed in triplicate. Data are presented as the mean  $\pm$  standard deviation. \* $P$ <0.05. DEX, dexamethasone.

low. Following exposure to TRAIL, the levels of apoptosis in the L-02 cells increased markedly. By contrast, pretreatment of the cells with DEX inhibited this apoptosis. Notably, the addition of TQD, which is a P-gp inhibitor, increased the levels of apoptosis. When the concentrations of TQD were increased to 50 and 100 nM, statistical significance in the levels of apoptosis were observed, compared with the group treated



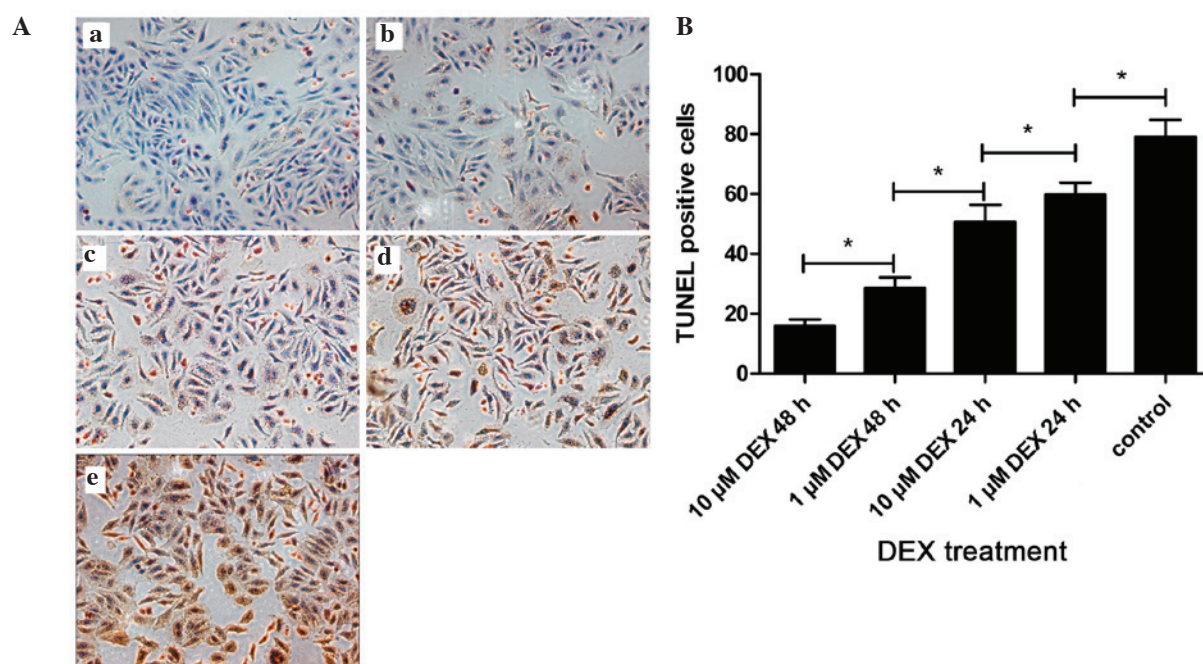


Figure 5. DEX protects L-02 cells from TRAIL-induced apoptosis. Apoptosis was evaluated using a TUNEL assay, and the number of apoptotic cells in each group was counted. (A) Representative photomicrographs of TUNEL-positive cells. L-02 cells were pretreated for 48 h with (a) 10  $\mu$ M or (b) 1  $\mu$ M DEX, and for 24 h with (c) 10  $\mu$ M or (d) 1  $\mu$ M DEX. (e) Control group of untreated L-02 cells. All the cells were treated with TRAIL for the induction of apoptosis. Magnification, x200. (B) Quantitative analysis of the levels of apoptosis. Data are presented as the mean  $\pm$  standard deviation. \* $P$ <0.05. DEX, dexamethasone; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

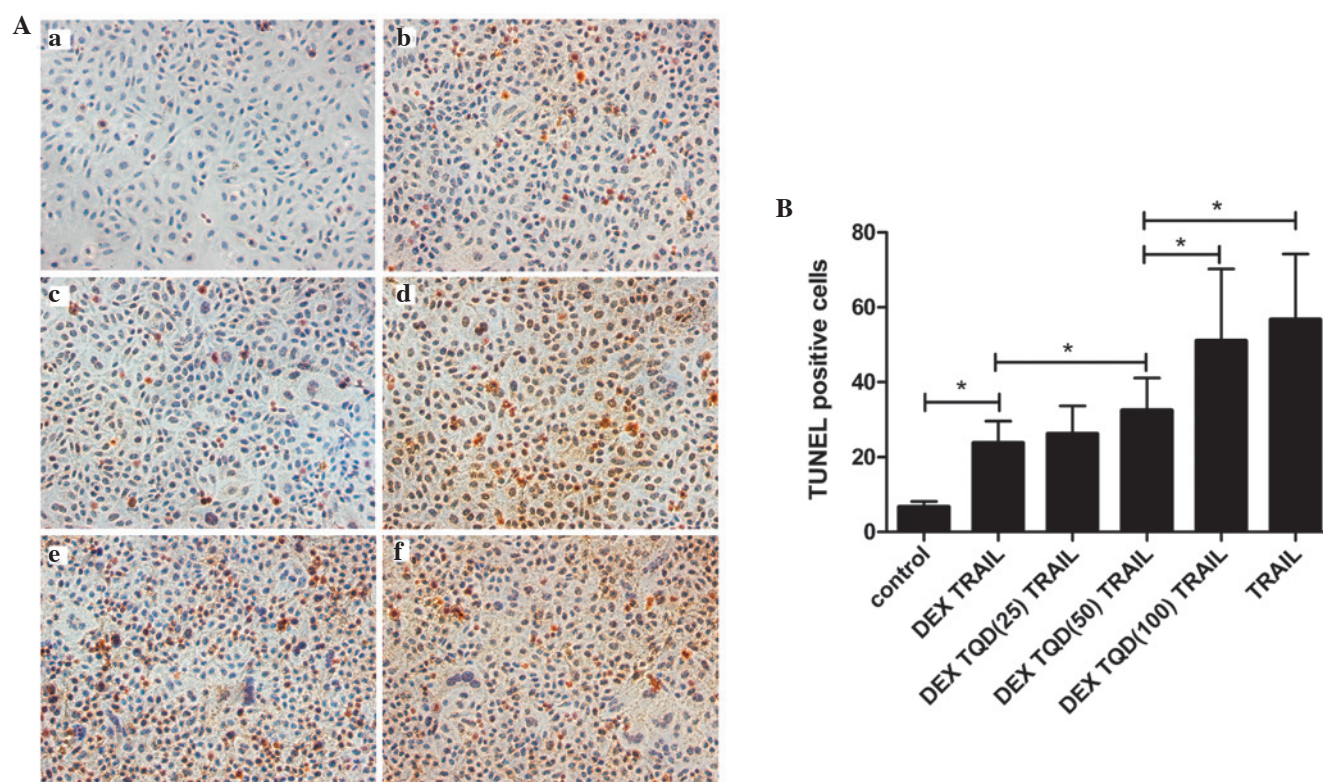


Figure 6. DEX protects L-02 cells from TRAIL-induced apoptosis by upregulating P-gp. Apoptosis was evaluated using a TUNEL assay, and the number of TUNEL-positive cells in each group were counted. (A) Representative photomicrographs of the TUNEL assay. L-02 cells were divided into six groups based on their treatment regimens, (a) control untreated group, (b) pretreated with 10  $\mu$ M DEX for 24 h, followed by incubation with TRAIL for 24 h, (c) pretreatment with 10  $\mu$ M DEX for 24 h, followed by incubation with TRAIL and 25 nM TQD for 24 h, (d) pretreated with 10  $\mu$ M DEX for 24 h, followed by incubation with TRAIL and 50 nM TQD for 24 h, (e) pretreated with 10  $\mu$ M DEX for 24 h, followed by incubation with TRAIL and 100 nM TQD for 24 h, and (f) incubated with TRAIL for 24 h. All the groups were treated with TRAIL for the induction of apoptosis, with the exception of the control group. Magnification, x200. (B) Quantitative analysis of the levels of apoptosis. Data are presented as the mean  $\pm$  standard deviation. \* $P$ <0.05. DEX, dexamethasone; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TQD, tariquidar; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

with DEX and TRAIL ( $P < 0.05$ ). No significant difference was observed between the TRAIL-treated and TQD-treated (100 nM) groups.

## Discussion

Studies have reported that DEX can induce the expression and function of P-gp in the blood-brain barrier, placenta and other tissues (18,19). Therefore, the present study examined whether DEX increases the levels of P-gps in normal human liver cells. The results of the immunocytochemical and flow cytometric analyses revealed that pretreatment of L-02 cells with DEX for 24-72 h significantly upregulated the levels of P-gps (Figs. 1B and 2B). The results of the western blot analysis demonstrated that pretreatment of the cells with DEX increased the expression P-gp in a time- and dose-dependent manner (Fig. 3). In addition, DEX also enhanced the mRNA levels of the ABCB1 gene, similar to the results obtained for the protein levels. These results suggested that pretreatment with DEX induced the expression of P-gp at the mRNA and protein levels in the L-02 cell line.

The results from previous studies reporting the effects of glucocorticoids on the expression of P-gps are inconsistent. In several studies, glucocorticoids, including DEX have been reported to upregulate the expression of P-gps, in agreement with the results of the present study. Salje *et al* reported the ability of DEX to upregulate the expression of P-gps in the liver (25), and the expression of P-gp also increased in the blood-brain barriers of DEX-treated animals in a report by Chan *et al* (19). These results are consistent with those reported by Petropoulos *et al*, which demonstrated the induction of placental P-gp, mediated by DEX (26). By contrast, other studies have demonstrated either no change or decreased expression levels of P-gps upon treatment of cells or animals with DEX. For example, Nishimura *et al* reported that DEX had a tendency to decrease the mRNA levels of the ABCB1 gene in monkey hepatocytes (27). Mark *et al* demonstrated that pre-treatment with glucocorticoids decreases the expression levels of P-gp in the placenta (28). Of note, DEX exerts varied effects on P-gps in different tissues and cell types. Previous studies have demonstrated that DEX pretreatment increases the levels of P-gp in the intestine, but not in the liver (29). Another study reported that DEX rapidly increases the expression levels of P-gps in the liver and lungs, but decreases their expression in kidneys (30). Glucocorticoid treatment exerts differing effects, even in the case of cell lines derived from the same tissue; the expression of P-gp is induced in LS180 cells, but downregulated in Caco-2 cells upon treatment (31). There is no definitive explanation for these contrasting results; however, it is possible that it is due to tissue specificity, as well as the complex regulation of this efflux transporter by glucocorticoids in certain organs.

Notably, incubation with DEX in the present study led to the transcriptional levels of the ABCB1 gene mRNA increasing in parallel with that of P-gp protein levels. This correlation between the ABCB1 gene mRNA and the P-gp protein has been reported in previous studies, which are in good agreement with our studies. Narang *et al* demonstrated that DEX increases the expression of P-gp in primary rat brain microvascular endothelial cells, and an increase in the mRNA expression of the ABCB1 gene results in a change in the protein expression of P-gp (18).

According to Salje *et al*, the mRNA and protein levels of P-gp are significantly induced by DEX in the fetal brain and liver (25). However, there are results contradicting those described above. Katayama *et al* demonstrated that the expression of P-gp can be increased without affecting its mRNA levels (32). Similarly, Micuda *et al* demonstrated that pretreatment with DEX results in an increase in the protein expression of P-gp, but a decrease in the mRNA expression of the ABCB1 gene in the liver (33). The present study hypothesized that these differences may be due to the complex transcriptional regulation of MDR1, which involves several signaling pathways (34).

Several studies have provided evidence suggesting that DEX can protect several cells from apoptosis induced by drugs. Nieuwenhuis *et al* reported that DEX protects human fibroblasts from apoptosis (9). According to a study by Haake *et al*, DEX treatment also protects hair cells against apoptosis (10). Similarly, other studies have demonstrated that P-gps can exert anti-apoptotic effects. Ruefli *et al* reported that P-gps can specifically inhibit Fas-induced caspase activation and apoptosis (15), whereas Tainton *et al* demonstrated that P-gps inhibit apoptotic stimuli in lymphoma cells (16). In acute myeloid leukemia, resistance to apoptosis is associated with the expression of P-gp, as reported by Pallis *et al* (17). In view of these observations, the present study investigated whether DEX had similar anti-apoptotic effects on L-02 cells. The results revealed that pretreatment with DEX for 24-48 h effectively protected the L-02 cells from TRAIL-induced apoptotic cell death. A gradual increase in the protective effect was observed when the duration of treatment was increased between 24 and 48 h, and when the concentration of DEX was increased between 1 and 10  $\mu$ M. Together, these findings indicated that DEX pretreatment suppressed TRAIL-induced apoptosis in the L-02 cell line.

Previous studies have demonstrated that, in addition to their role in MDR, P-gps may also protect cells at two levels, by decreasing the accumulation of toxins in the cells and by inhibiting the apoptotic pathways induced by toxins and other stressors. Ruefli *et al* reported that P-gp can specifically inhibit caspase-8 activation and apoptosis (15). Similarly, Pallis *et al* demonstrated that the expression and activity of P-gps are associated with resistance to apoptosis in acute myeloid leukemia cells (17).

Therefore, the present study subsequently examined the anti-apoptotic effects of DEX on the cells, in which the functions of P-gps were inhibited by treatment with TQD, a P-gp inhibitor. The results revealed that, in addition to inducing the expression of P-gp, DEX alleviated cell apoptosis at increased concentrations and incubation periods. By contrast, treatment with TQD aggravated apoptosis in the L-02 cells, compared with the untreated cells. Additionally, the numbers of apoptotic cells were elevated with increasing concentrations of TQD. Based on these results, it is possible that DEX exerted anti-apoptotic and cell-protective effects through the induction of P-gps in the L-02 cell line.

In conclusion, the present study provided evidence that P-gps exist in L-02 cells, a normal human liver cell line, and the effect of DEX on the expression of P-gps in this cell line were investigated. It was demonstrated that pretreatment with DEX suppressed TRAIL-induced apoptosis in the L-02 cells. In addition, the results indicated that the induction of P-gp is



likely a molecular mechanism underlying the anti-apoptotic effects of DEX. The results also suggest that the L-02 cell line is a suitable cell model for investigating the effects of glucocorticoids on transport proteins, including P-gp. In addition, this model may also assist in understanding the benefits of DEX for the treatment of hepatic failure and other liver diseases. Additional investigations are required for understanding the underlying molecular mechanisms by which DEX regulates the expression of P-gp in the L-02 cell line.

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