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Apoptotic related biochemical changes in human amnion cells induced by tributyltin

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Abstract

Tributyltin (TBT) is one of the environmental pollutants, which is mostly accumulated in marine animals. The toxic effects of TBT have been extensively documented in several types of cells, but the molecular mechanisms responsible for TBT-induced cell damage are still not fully elucidated. The present study was undertaken to evaluate the apoptotic related biochemical changes in human amnion cells induced by TBT. After cells were exposed to TBT at the concentrations of 1–4 μM for 2 h, the results suggested that TBT could induce an early and typical apoptosis, moreover caspase-3, the modifications of cytoskeletal structure and the Bcl-2 family were involved in this process. The results will deepen our understanding about the toxic mechanism of TBT on human amnion cells.

Keywords: Tributyltin; Apoptosis; Cytoskeleton; Caspase; Bcl-2; Bax; p53

1. Introduction

Organotin compounds, particularly tributyltin (TBT), have been widely used in agriculture as a biocide, in anti-fouling painting and fabrics, as well as in industry as a disinfectant of circulating cooling water and slime control in paper mills. TBT has polluted the marine environment and bioaccumulated in some aquatic organisms due to its release from the anti-fouling painting of ships. Human exposure mostly arises from consumption of those TBT-contaminated products (Goldberg, 1986; Kannan and Falandyz, 1997). Extensive studies have shown that TBT is toxic to the respiratory system, digestive system, neuronal system, immune system and reproductive system of human beings (World Health Organization, 1990; Tsukazaki et al., 2004). It has been indicated that many cellular events are involved in the mechanism of TBT intoxication (Gray et al., 1987; Snoeij et al., 1986; Tiano et al., 2003; Kawanishi et al., 2001). The induction of apoptosis is believed to play an important role in TBT induced toxicity, however the exact mechanism of TBT induced apoptosis are still unknown (Jurkiewicz et al., 2004; Gennari et al., 2000; Milena et al., 2002).

Microfilaments, intermediate filaments and microtubules, three major components of cytoskeleton which

Abbreviations: TBT-Cl, tri-*n*-butyltin chloride; FL cell, human amnion cell

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distribute throughout the cytoplasm are involved in determining the cell shape, cell motility, chromosome movement and intracellular transport (Gourlay and Ayscough, 2005). Cytoskeleton disruption is one of the most important features in apoptosis. Moreover, it has been identified that some cytoskeleton-associated proteins which play a key role in the function of cytoskeleton are caspase substrates in TBT-induced apoptosis (Lavastre and Girard, 2002). It was hypothesized that the modification of the cytoskeleton and caspase activation may result in the TBT-induced apoptosis.

Apoptosis is a complex event regulated by a well-tuned balance of inducer and repressor factors, e.g. the Bcl-2 gene family, which is pivotal integrators of survival and death signal in higher eukaryotes. Both Bcl-2 and Bax belong to the Bcl-2 family, but the former is pro-survival factor that inhibits apoptosis while the latter is pro-apoptotic protein that promotes apoptosis (Borner, 2003). Moreover, the Bcl-2 family functions via the members affecting each other under formation of a complex network of homodimers and heterodimers. It has been proved that the ratio of Bax to Bcl-2 determines survival or death of cells following apoptotic stimulus, cells with higher Bax/Bcl-2 are easier to undergo apoptosis than those lower ones (Oltval et al., 1993). In spite of extensive studies on TBT-mediated cytotoxicity and apoptosis, there is little information concerning about Bcl-2 family, which might have a key function during the process.

p53 is well known as a tumor-suppressor gene, also it is a multi-faceted nuclear phosphoprotein induced in response to cellular stress, functioning as a transcriptional transactivator in DNA repair, cell cycle arrest and apoptosis pathway. Moreover, as a direct transcriptional factor, p53 might influence the apoptosis by changing the levels of Bcl-2 and Bax (Shen and White, 2001). Therefore, the levels of both p53 and Bcl-2 family in TBT-exposed cells will better clarify the TBT-mediated apoptosis.

TBT has been shown to induce apoptosis in various cells and tissues (Nopp et al., 2002; Reader et al., 1999; Stridh et al., 2001; Aw et al., 1990), and it is clearly known that in some cell type, the mechanisms responsible for TBT-induced apoptosis mainly involve the damage to mitochondrial function, the increase of the cytosolic free calcium concentration, the production of ROS, the release of cytochrome C from the mitochondrial membrane into the cytosol and the activation of caspase (Chow et al., 1992; Gennari et al., 2000; Tiano et al., 2003). However, the mechanisms are still not fully elucidated, such as: how are the three apoptotic pathways (mitochondrial pathway, death receptor pathway and ER stress pathway) involved in the TBT-induced apoptosis? What role do the apoptosis-related proteins play in the process? The present study was undertaken to evaluate the apoptotic effect in FL cells induced by TBT. The biochemical changes including levels of phosphatidylserine, caspase-3, cytoskeleton, p53 and Bcl-2 as well as Bax during the process were evaluated. These results can deepen our understanding about the mechanism of TBT-induced apoptosis.

2. Materials and methods

2.1. Materials

Tri-*n*-butyltin chloride was from Acros Company (NJ, USA). Powdered MEM was from Gibco (Scotland, UK). Trypsin was purchased from Serva (Heidelberg, Germany). Fluorescein isothiocyanate (FITC)-conjugated phalloidin (F-PHD) was obtained from Sigma Chemical Company (St. Louis, MO, USA). Annexin V-FITC Kit was purchased from Bender (Vienna, Austria). CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit was purchased from BioVision (Mountain View, CA, USA). Polyclonal goat anti-human Bcl-2 antibodies, monoclonal mouse anti-human Bax antibodies, polyclonal goat anti-human p53 antibodies and ECL chemiluminescence detection kit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nitrocellulose membrane (0.2 μm pore size) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were of the analytical grade available from commercial sources.

2.2. Cell culture and treatment

The human amnion cells FL (ATCC, CCL-62) were grown in MEM supplemented with 10% heat-inactive fetal bovine serum in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. Experimental FL cells were cultured at 1×10^8 cell/L in six-well plate or 25 cm^2 Falcon flasks (Becton Dickinson, Franklin Lakes, NJ). TBT was first dissolved in 95% ethanol at 5 mM and then added to the culture system to achieve the final concentrations of 0–4 μM. The highest concentration of ethanol in the culture was 0.076%, which had no effect on the cells viability. After exposure for 2 h, the cells were washed twice with ice cold phosphate-buffered saline (PBS) and harvested with 0.02% EDTA and 0.025% trypsin, rinsed three times in PBS, then pelleted in microtubes by centrifugation for the subsequent processing.

2.3. Evaluation of live cell undergoing apoptosis

By means of flow cytometric method, Annexin V-FITC and propidium iodide (PI) were used to distinguish intact, dead and apoptotic cells, according to the change of cell membranes,

for the phosphatidylserine (PS) can translocate to the outer leaflet of the cell membrane during the early stage of apoptosis (Nopp et al., 2002). The density of cell suspension was adjusted to 5×10^5 cell/mL and 195 μL of the cell suspension was transferred into the microtubes. Five microliter Annexin V-FITC was added to the cell suspension and incubated in darkness at room temperature for 10 min. After centrifugation at 1000 rpm/min (4 °C) for 5 min, the supernatant was removed and the cells were washed with 1× binding buffer once, then cells were resuspended in 190 μL buffer, after added 10 μL PI, the samples were detected by the flow cytometer (BDLSR, BD, USA).

2.4. Cytoskeletal changes measurement

Cells were grown in six-well plate. After cells were incubated with different concentrations TBT for 2 h, the qualitative analysis of the disruption of F-actin cytoskeleton by the fluorescence assay was performed as follows: cells grown on glass culture covers are washed with phosphate buffered saline and fixed in 3.7% formaldehyde for 5 min. Following washed extensively in PBS, cells are dehydrated with acetone and permeabilized with 0.1% Triton X-100. Then the fixed cells were specifically labeled with FITC-conjugated phalloidin and stained in the dark at room temperature for 40 min. After removing unbound phalloidin, the cells were observed in a fluorescence microscope (Olympus CX-RFL-2, Japan) using an excitation wavelength of 475 nm and an emission wavelength of 515 nm.

2.5. Detection of caspase-3 activation

The activation of caspase-3 in live cells was detected by fluorescent dye labeled specific inhibitor of caspase-3 (FITC-DEVD-FMK). One microliter FITC-DEVD-FMK work solution was added to 300 μL cell suspension (density was 1×10^6 cell/mL), then incubated in dark at 37 °C, 5% CO_2 for 1 h. After centrifugation at 3000 rpm/min (4 °C) for 5 min, the supernatant was removed and the cells were washed with 1× washing buffer twice. At last, cells were resuspended in 150 μL PBS and were added to the 96-well black plate. The fluorescent intensity was detected by the fluorescence spectroscopy (Synergy HT, Bio-Tek, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.6. Western blot analysis of the apoptosis-related proteins

For western blot sample preparation, cell pellet was solubilized in 100 μL lysis buffer (50 mM Tris–Cl, 150 mM NaCl, 15 mM EDTA, 0.1% Triton X-100, pH 8.0) containing protease inhibitors (10 mg/mL Aprotinin, 10 mg/mL Leupeptin and 1 mM PMSF) and put on ice for 30 min. After centrifugation at $12{,}000 \times g$ (4 °C) for 20 min to remove debris, the supernatant was carefully recovered. Protein concentrations were determined by the Bradford assay (Bradford, 1976). All samples were stored at −70 °C prior to electrophoresis.

Aliquots from supernatant containing 50 μg proteins were mixed with equal volume of 2× sample buffer. The samples were boiled for 5 min and subjected to 12% SDS-PAGE. After electrophoresis, the seperated proteins were transferred electrophoretically from the gel to nitrocellulose membrane. The membranes were blocked at room temperature for 3 h in TBS buffer (50 mM Tris–C, 150 mM NaCl, pH 7.6) containing 5% non-fat dry milk to prevent non-specific binding of reagents and then incubated with anti-Bcl-2 (1:500 dilution), anti-Bax (1:200 dilution) or anti-p53 (1:100 dilution) at 4 °C overnight. After that, the membranes were washed in TBST (50 mM Tris–Cl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) for 30 min and incubated with secondary antibody for 2 h at room temperature. Then the membranes were washed three times in TBST and exposed to 2 mL ECL chemiluminescence reagents for 2 min. Blots were exposed to X-ray film for radiographic detection of the bands. The autoradiograms were scanned and the level of Bcl-2, Bax and p53 expression was quantified by densitometry using a computer-based image analysis system (Media Cybernetics, USA).

2.7. Statistical analysis

Results were expressed as mean ± standard deviation (S.D.) from three sets of experiments. Statistical analysis was performed by the one-way analysis of variance (ANOVA). $p < 0.05$ was considered significant.

3. Results

3.1. Induction of apoptosis by TBT on FL cells

After treated by Annexin V-FITC and PI, cells were divided into four groups appearing as quadrant I (left and up), quadrant II (right and up), quadrant III (left and down) and quadrant IV (right and down) (Fig. 1A). Cells exhibiting both FITC and PI fluorescence, only FITC fluorescence and neither PI nor FITC fluorescence were dead cells (quadrant II), apoptotic cells (quadrant IV) and intact cells (quadrant III), respectively. Under control condition, large amount of cells were intact live cells. After TBT exposure for 2 h, especially in 3 and 4 μM TBT group, the population of intact cells decreased, while the apoptotic and dead cells increased obviously. The apoptotic rate that presented by the percentage of the population of apoptotic cells to the total cell population was used to describe the apoptosis induced by TBT. Fig. 1B showed that the apoptotic rate was elevated in each experimental group compared with the control. The results presented that TBT induced apoptosis on FL cells in a dose-dependent manner.

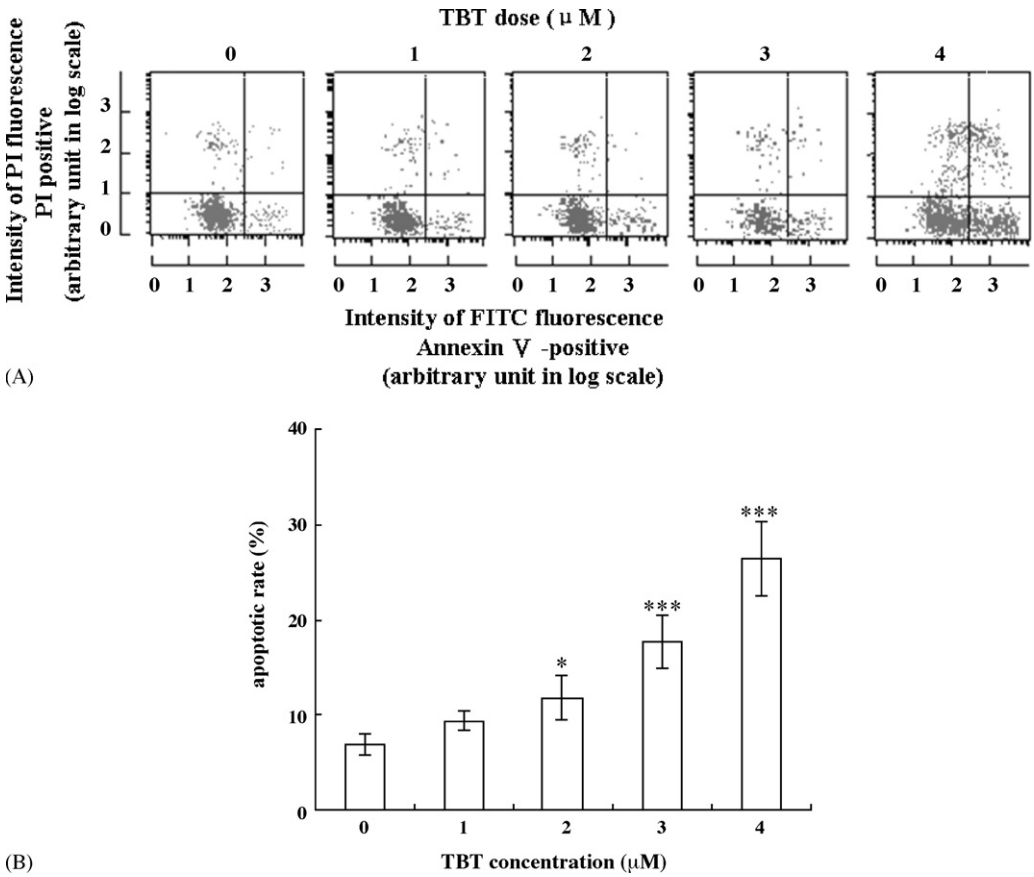

Fig. 1. Dose-course effect of TBT on apoptosis of FL cells. After incubated with 1–4 μM TBT for 2 h, cells were collected and treated with PI and Annexin V-FITC, respectively. Samples were analyzed by the flow cytometer. (A) Contour diagram of PI/Annexin V flow cytometric detection of apoptosis induced by TBT on FL cells. (B) The apoptotic rate of FL cells after TBT exposure. Results were presented by the percentage of the population of apoptotic cells to the total cell population. Each bar indicated mean and S.D. obtained from three independent experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$.

3.2. Cytoskeletal changes

Using a fluorescence microscopy, actin cytoplasmatic distribution in TBT-treated FL cells after labeling with FITC-phalloidin was observed. The normal actin fibers distribution showed that F-actin consisted of an extensive system of cross-linked bundles with higher density and organization, traversing the cell through the entire cytoplasm in control cells (Fig. 2A). After incubated with 2 μM TBT for 2 h, the cell shape and cytoskeleton did not change obviously (Fig. 2B). Treatment with 4 μM TBT for 2 h led to obvious cell shape change. Moreover, cells appeared to lose actin cytoskeleton organization (Fig. 2C), which was characterized by F-actin depolymerization, as evident by breaks along fibers. These findings presented the cytoskeleton change of FL cells exposed to TBT.

3.3. Effect of TBT on the caspase-3 activity

Following the exposure of FL cells to different concentrations of TBT for 2 h, consistent with an induction of apoptosis, there was a statistically significant increase in caspase-3 activity. Data revealed that the caspase-3 activity was significant increased by 1.87-, 2.85-, 3.54- and 6.29-fold, respectively, in each TBT-treated group compared with control (Fig. 3). These results suggested that TBT-induced apoptosis on FL cells was associated with caspase-3 activation.

3.4. Expression of the Bcl-2, Bax and p53 in TBT-treated FL cells

The change of Bcl-2, Bax, p53 and GAPDH expression in FL cells exposed to TBT could be observed

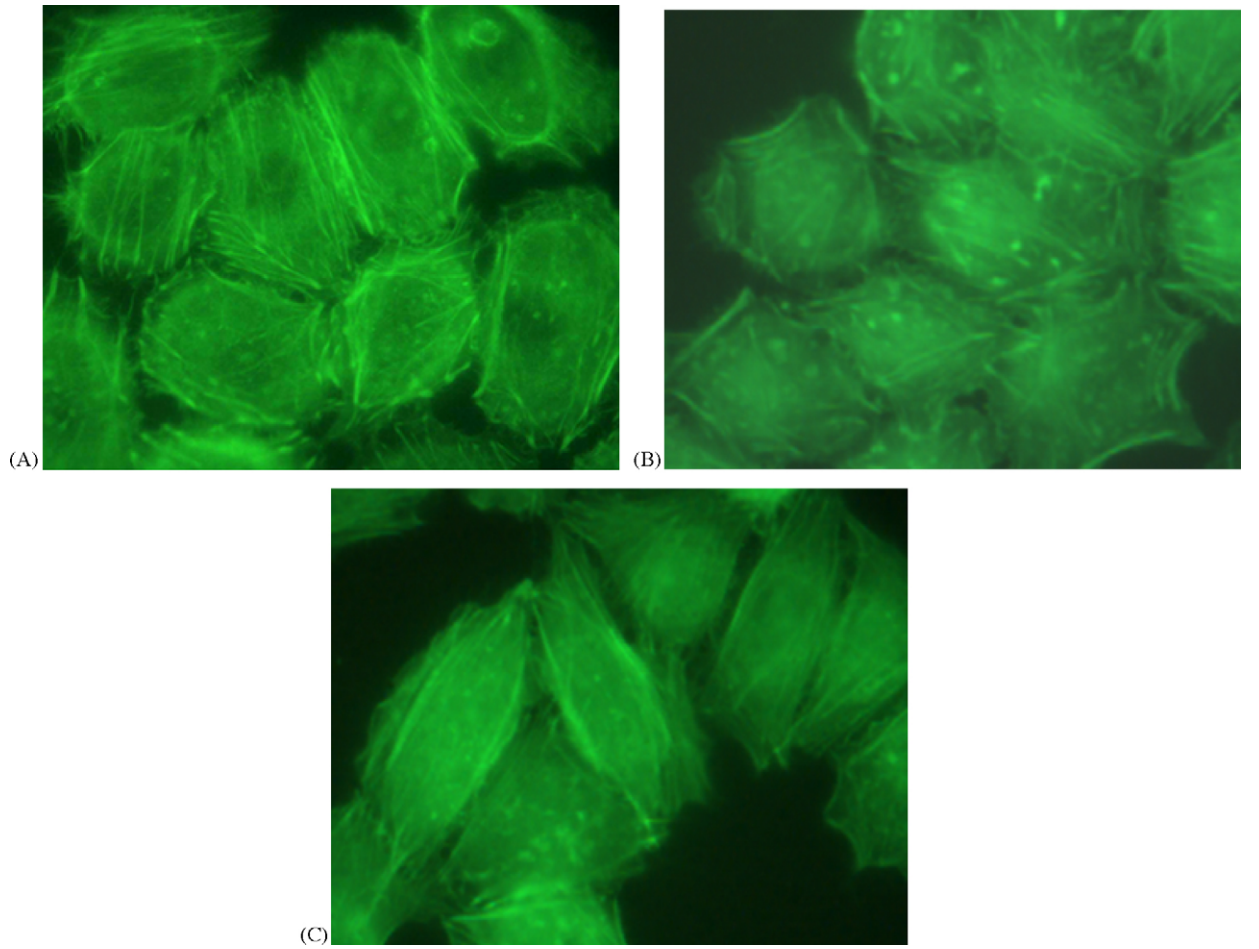

Fig. 2. Fluorescent microscope representative of F-actin distribution in FL cells after incubation with different doses of TBT in the following experimental conditions: control cells (A), FL cells stimulated with 2 μM (B) and 4 μM (C) TBT for 2 h. Three experiments were performed with similar results. Original magnification, ×1000.

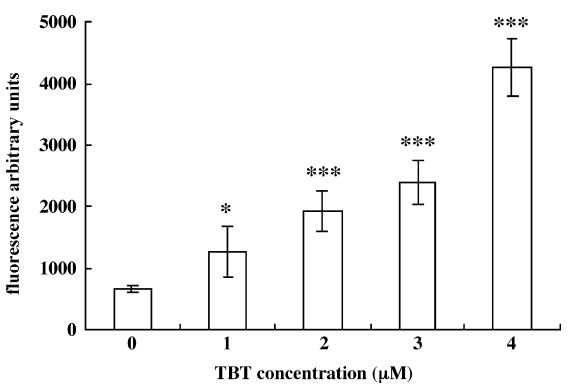

Fig. 3. Caspase-3 activity of FL cells after treated by TBT. Cells were pretreated with 1–4 μM TBT for 2 h and collected, then FITC-DEVD-FMK was used to detect the caspase-3 activity, which was performed as described in Section 2.4. Each bar indicated mean and S.D. obtained from three independent experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$.

directly from the autoradiograph of the western blotting (Fig. 4A). As displayed in Fig. 4B, the Bcl-2 expression gradually increased and reached the highest at 2 μM, then decreased obviously at 3 and 4 μM. Bax expression was increased in a dose-dependent manner and the difference was significant at 4 μM as compared to the control group. With regard to the p53 expression, there was not a distinct change between each TBT-treated group and the control group. The result of Bax/Bcl-2 ratio following TBT treatment was presented in Fig. 4C, there was no obvious change in 1 and 2 μM TBT-treated groups, but the ratio increased significantly in 3 and 4 μM TBT-treated groups over control value, thus shifting the balance toward a preference for apoptosis. These results revealed that some of Bcl-2 family proteins were modulated in TBT-induced apoptosis.

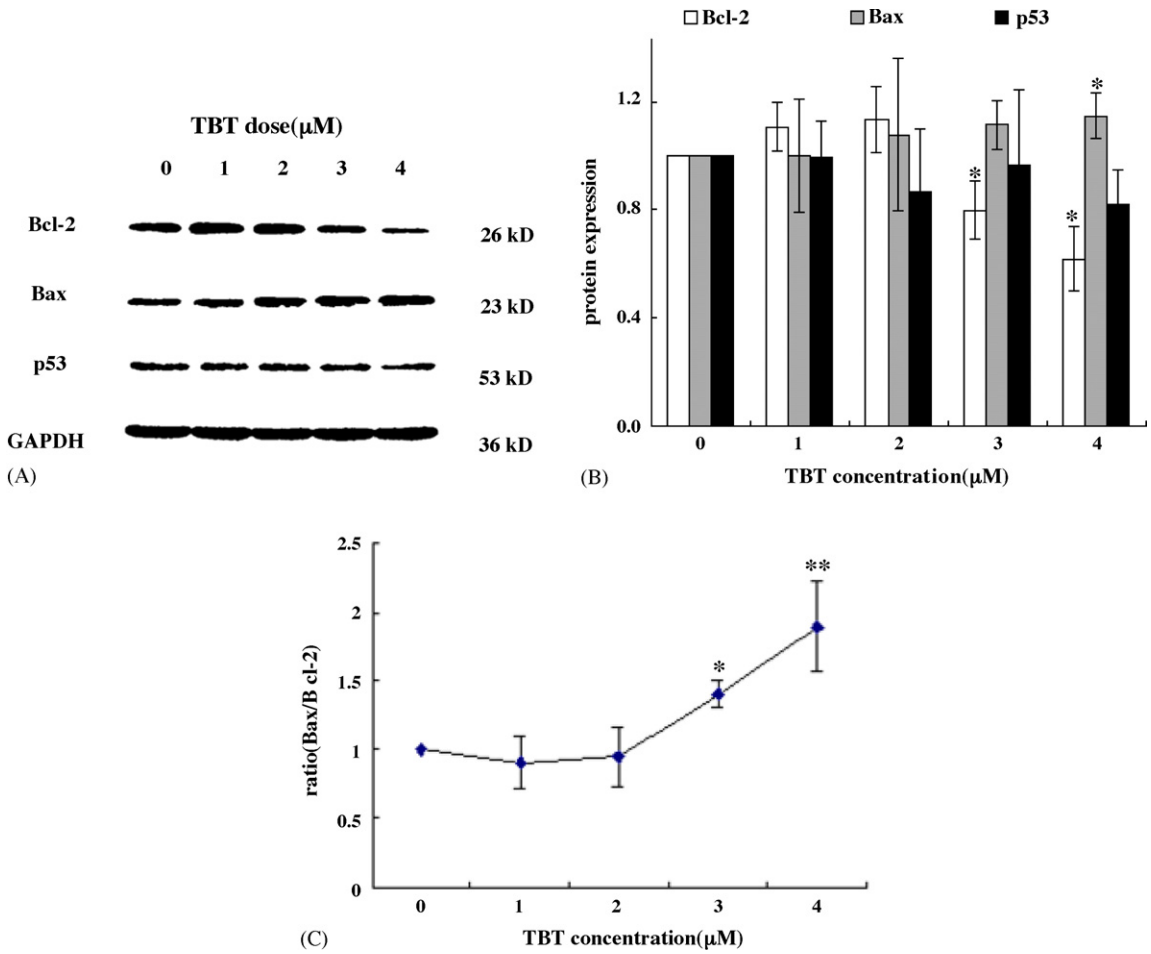

Fig. 4. Effect of TBT upon the Bcl-2, Bax and p53 expression in FL cells. Cells were incubated with 1–4 μM TBT for 2 h and collected. Lysates for SDS-PAGE were prepared and Western blot were performed as described in Section 2.5. (A) A representative autoradiograph of Bcl-2, Bax and p53 expression was shown. (B) Intensities of Bcl-2, Bax and p53 protein bands were quantified by densitometry, respectively. Results were expressed as folds of optical density of target protein and the GAPDH determined in control. The mean protein expression from control lysate was designated as 1 in the graph. (C) Results were expressed as ratio of optical density presents in Bax vs. Bcl-2 band. Data were indicated as mean and S.D. obtained from three independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$.

4. Discussion

Apoptosis is modulated by complex pathways that involve a series of biochemical regulators and molecular interactions. Although the upstream signaling of apoptosis is obscure, the caspase family of proteases has been proved to play a key role in apoptosis. Their functions are involved in the extrinsic death receptor pathway and intrinsic mitochondrial pathway. Activation of both pathways triggers an amplifying cascade of the caspase-3 (Fan et al., 2005), which demonstrates the importance of this effector caspase. Results in the present study showed that caspase-3 was activated by TBT, which was highly consistent with the results of apoptotic rate of the cell exposed to TBT. The correlative changes of both caspase-3 and apoptotic rate implied the importance of caspase-3 in the TBT-induced apoptosis on FL cells.

It is well known that cell shape is governed by the cytoskeleton that acts as a mechanical supporting framework. In the current study, FL cells shape appeared to be affected after TBT exposure and F-actin depolymerization was also observed. The most interesting finding is the co-occurrence of F-actin depolymerization and caspase-3 activation during the apoptosis induced by TBT. Study of Lavastre and Girard (2002) has proved that the two well-known microfilament-associated proteins including gelsolin and paxillin are important substrates of caspase and the degradation of these proteins by caspase is a tightly regulated process in TBT-mediated toxicity. Accordingly it may be considered that the

cytoskeleton disruption in FL cell exposed to TBT is related to the caspase activation.

Part members of Bcl-2 family have been implicated in the regulation of apoptosis. Both Bcl-2 and Bax belong to Bcl-2 family, but they have different functions in the apoptosis process. Bcl-2 can inhibit the transformation of the mitochondrial permeability and interact with pro-apoptosis proteins, such as Bax to invalidate them. While Bax can cause the release of apopotosis-related factors under apoptotic stimulus, then lead to the caspase activation (Tsujimoto, 2003). Yin et al. (1994) had proved that Bcl-2 and Bax could form heterodimers to accelerate apoptosis, which are mainly mediated by the interaction of the Bcl-2 homology 1 and 2 (BH1 and BH2) domains. They initially proposed a model in which the ratio of Bax to Bcl-2 determined survival or death following an apoptotic stimulus (Oltval et al., 1993). In this study, the change of Bax/Bcl-2 was more clear and sensitive than the individual protein level and was significant compared with control at 3 and 4 μM TBT groups. This increased Bax/Bcl-2 ratio was consistent to the dose-dependent elevation of apoptosis rate and activation of caspase. Therefore, these findings hinted that the cooperant effect of *bcl-2* and *bax* gene products via the mitochondria might be responsible for the increase of caspase-3 activation and the modulation of TBT-induced apoptosis in the FL cells. The conclusion also supported the model that the ratio seemed to be more important in determining the sensitivity to apoptotic stimuli than the expression of protein individually.

p53 plays a critical role in apoptosis after treatment with cytotoxic agents or irradiation and it is responsible for regulating cell death through a disruption of Bax/Bcl-2 equilibrium (Mihara et al., 2003). Although p53 had been proved to rise several folds after exposed to other cytotoxic agents in other cell types, this is not the case for all sorts of apoptosis and it remains unclear whether the p53 activation is required for apoptosis (Sakamuro et al., 1995; Moll et al., 2005). Results obtained in this study showed no change in p53 protein level, which appeared to resemble tamoxifen-induced apoptosis in breast cancer cells (Zhang et al., 1999). It illustrated that the alteration of the Bcl-2 and Bax here probably was not attributed to the modulation of p53. Moreover, TBT-induced apoptosis on FL cells may not be mediated by altering p53 levels.

In conclusion, a dose-dependent apoptosis as well as activation of caspase-3 and modification of F-actin demonstrated that cytoskeleton might participate in the process of apoptosis in FL cell induced by TBT. The involvement of Bcl-2 and Bax in apoptosis of FL cell induced by TBT was demonstrated by the conjunct change of Bax/Bcl-2 ratio and apoptotic rate. No obvious change in p53 level may indicate that p53 is not concerned with the regulation of TBT induced apoptosis in FL cell.

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