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ORIGINAL ARTICLE

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Involvement of oxidative stress and caspase activation in paclitaxel-induced apoptosis of primary effusion lymphoma cells

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Abstract Paclitaxel has significant antitumor activity in several human tumors, including Kaposi's sarcoma (KS). Human herpesvirus 8 (HHV-8) is implicated in all forms of Kaposi's sarcoma, primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD), indicating that it is a DNA tumor virus. Since it is difficult to culture cell lines derived from KS patients, we used a cell line derived from PEL (BCBL-1) to investigate whether oxidative stress is involved in the cytotoxicity of paclitaxel on the HHV-8-related tumors. We found that the generation of reactive oxygen species (ROS) in the BCBL-1 cells was increased by paclitaxel treatment, and the increase in ROS production was suppressed by antioxidants, including catalase and ascorbic acid. Moreover, ascorbic acid also attenuated the cytotoxicity induced by paclitaxel. Upon paclitaxel treatment, caspase-2, caspase-3, and caspase-8 were activated in BCBL-1 cells. Cotreatment with antioxidants did not affect caspase-2, caspase-3 or caspase-8 activation. Paclitaxel-induced apoptosis was also accompanied by an increase in the protein levels of Bax, and this effect was attenuated by antioxidants. Paclitaxel slightly decreased the expression of Bcl-2 protein, but antioxidants induced Bcl-2 protein. These results suggest that oxidative stress is only partially involved in the cytotoxicity of paclitaxel in BCBL-1 cells, and that paclitaxel-induced apoptosis of BCBL-1 cells is primarily mediated by the caspase activation pathway.

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Introduction

Paclitaxel, also known as Taxol, was isolated from the bark of *Taxus brevifolia*, and has potent antiproliferative action against tumor cells in vitro and in vivo [25]. These effects relate to its ability to bind tubulin, promote microtubule assembly, and stabilize microtubules by bundle formation [15, 26, 27]. It has also been observed that paclitaxel arrests cells in the G_2/M phase of the cell cycle [12, 13]. Recently, paclitaxel has been recognized as an active agent for the treatment of advanced Kaposi's sarcoma (KS) [3, 20]. However, the mechanism of action of paclitaxel in KS remains unclear.

Human herpesvirus 8 (HHV-8), a gamma-herpesvirus, is closely linked to all forms of KS, primary effusion lymphomas (PEL), and Castleman's disease [2, 4, 7, 21, 28, 29]. Since it is difficult to culture cell lines from KS lesions, we used a cell line derived from PEL (BCBL-1) to investigate the effects of paclitaxel on HHV-8-related tumors.

It is now believed that apoptotic pathways contribute to the cytotoxicity of most chemotherapeutic drugs [18]. Anticancer drugs can induce apoptosis via pathways dependent on or independent of the death receptor (DR) [6, 14]. The DR-dependent pathway can activate caspase-8, which turns on the caspase activation cascade by proteolytic activation of procaspase-3, the principal effector caspase. Alternatively, the pathway involves caspase-8-mediated cleavage of the Bid protein, resulting in truncation of Bid (tBid) and the release of cytochrome *c* from mitochondria. In addition, chemotherapy can elicit cellular stress which leads to a DR-independent pathway. In this latter paradigm, the mitochondrial dysfunction is regulated by the Bcl-2 family. Studies in a variety of cell lines have indicated that cancer chemotherapy drugs induce tumor cell apoptosis partially by

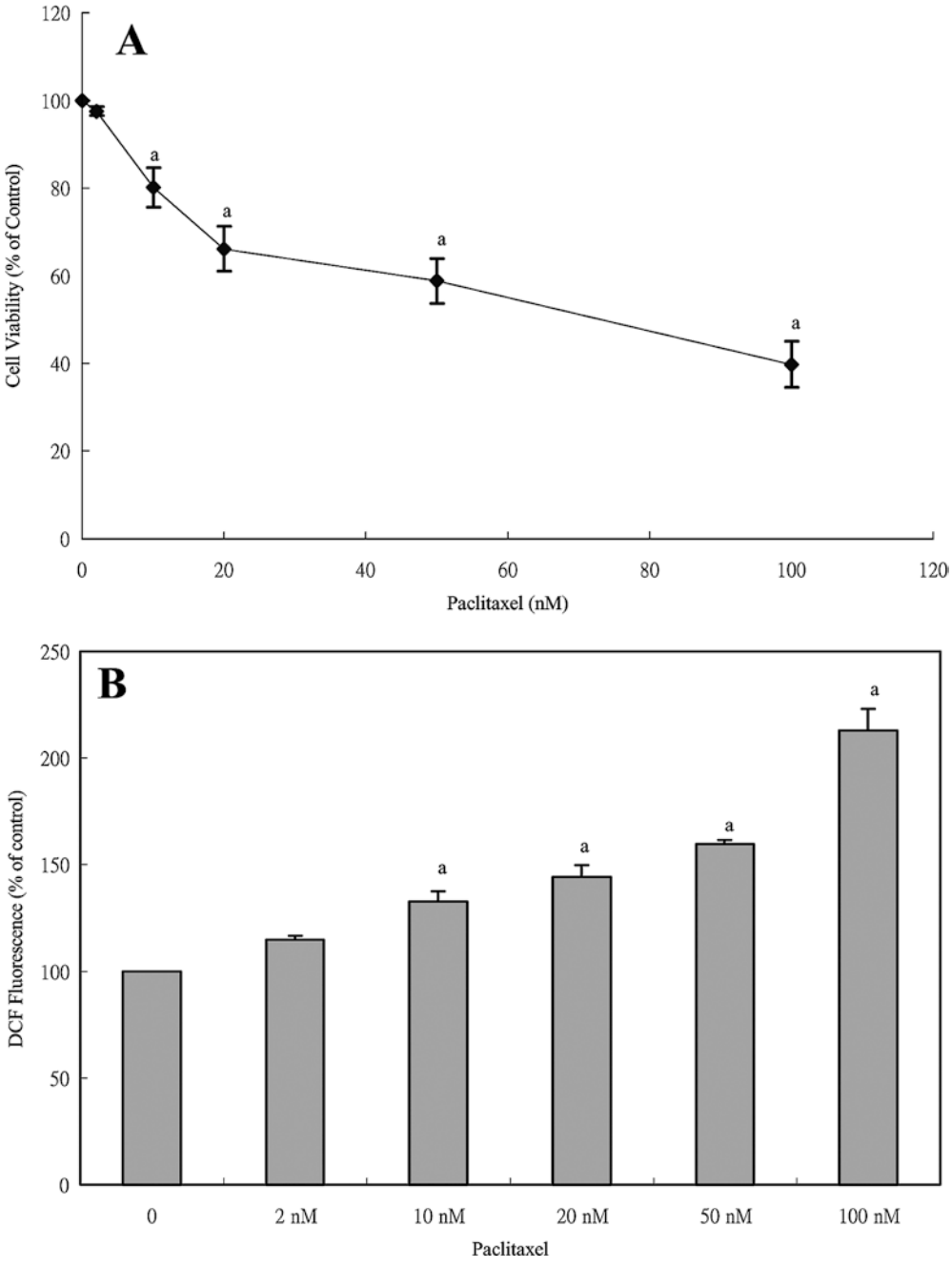

Fig. 1 Oxidative stress and cell viability in paclitaxel-treated BCBL-1 cells. BCBL-1 cells were treated with various concentrations of paclitaxel for 18 h: **A** cell viability was determined by trypan blue exclusion assay; **B** oxidative stress was detected by staining with DCF-DA. BCBL-1 cells in supplemented medium were used as control. The data shown are the means ± SD from three independent experiments. [a] $P < 0.05$ vs control (untreated BCBL-1 cells)

inducing oxidative stress [5]. It has been reported that paclitaxel induces the formation of reactive oxygen species (ROS) and alters mitochondrial permeability [30]. However, whether paclitaxel affects ROS in HHV-8-harboring cells (BCBL-1 cells) is still unknown.

In this study, we investigated whether oxidative stress is involved in the cytotoxicity of paclitaxel on BCBL-1 cells and whether antioxidants affect the cytotoxicity of paclitaxel. We investigated the production of ROS in paclitaxel treated BCBL-1 cells by detection of dichlorofluorescein (DCF) fluorescence. Paclitaxel induced the production of ROS while cell viability decreased in the BCBL-1 cells. Treatment of BCBL-1 cells with paclitaxel also activated caspase-2, caspase-3 and caspase-8, and altered the protein levels of Bax and Bcl-2. Different antioxidants showed different effects on paclitaxel-induced cytotoxicity and oxidative stress, and had no effects on caspase activation.

Materials and methods

Cells and reagents

Paclitaxel was obtained from Bristol-Myers Squibb (Princeton, N.J.). All other chemicals and reagents were from Sigma Aldrich (St Louis, Mo.). BCBL-1 cells were grown in RPMI 1640 (Gibco BRL) supplemented with

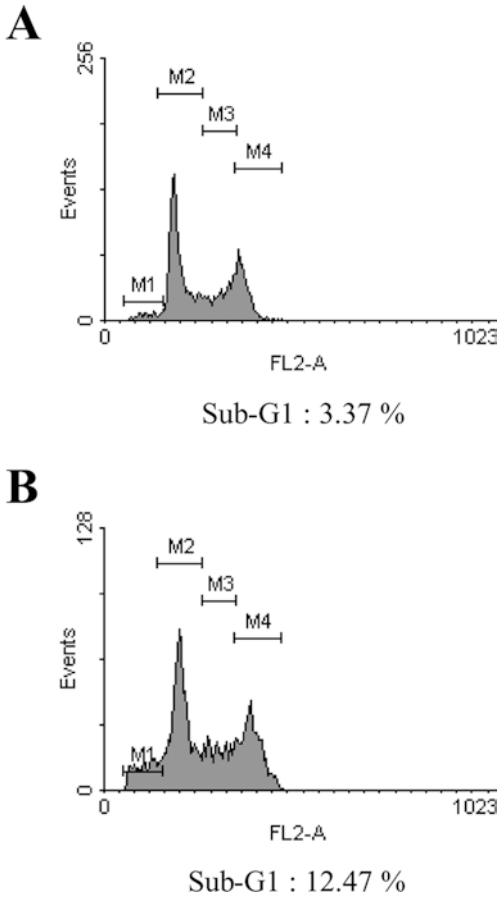

Fig. 2 Paclitaxel-induced apoptosis of BCBL-1 cells as determined by flow cytometry. BCBL-1 cells were untreated (**A**), or treated with 50 n*M* paclitaxel for 18 h (**B**), and apoptotic death of the cells was determined by flow cytometric analysis as described in Methods. The percentages of sub-G_1 cells are shown. Results shown are representative data from three similar experiments

10% fetal calf serum, 1 m*M* glutamine, 10 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere containing 5% CO_2. Cells were seeded the day prior to treatment at a density of about 3×10^5 cells/ml and treated with various concentrations of paclitaxel. Alternatively, the cells were treated with 50 n*M* paclitaxel in the absence or presence of various antioxidants including 100 U/ml superoxide dismutase (SOD), 50 U/ml catalase, 25 μ*M* ascorbic acid and 250 μ*M* glutathione (GSH). Cells were then harvested after 18 h of treatment for determination of cell viability, the production of ROS, caspase-3 activity, and Western blot analysis.

Cell viability assay

Cell viability was determined by Trypan blue exclusion. After treatment, the cells were harvested, and 50 μl 0.4% solution of trypan blue was added to 50 μl treated cells. The suspension was then applied to a hemocytometer. Both viable and nonviable cells were counted. A minimum of 200 cells was counted for each data point in a total of eight microscopic fields. BCBL-1 cells in supplemented medium were used as control. Cell viability is expressed as percent of control.

Measurement of intracellular ROS generation

The principle of this assay is based on nonpolar 2,7-dichlorofluorescein diacetate (DCF-dAc) diffusing into cells through the cell membrane. DCF-dAc is then hydrolyzed into nonfluorescent dichlorofluorescein (DCFH) [8]. ROS led to oxidation of DCFH generating a measurable fluorescent product, dichlorofluorescein (DCF) [16]. BCBL-1 cells were treated with paclitaxel or cotreated with different antioxidants for 18 h. The treated cells were then incubated with DCF-dAC (25 μ*M*) for 1 h at 37°C, washed with PBS twice, and suspended in PBS to give 1×10^6 cells/ml for detection of fluorescence (excitation 502 nm, emission 522 nm). BCBL-1 cells in supplemented medium were used as control. DCF fluorescence is expressed as percent of control.

Flow cytometric analysis

Cell cycle distribution was analyzed by flow cytometry as follows. Cells were harvested by centrifugation, washed three times with cold PBS, and fixed with 70% ethanol at −20°C for 18 h. After washing twice with cold PBS, 10^6 cells were resuspended in 1 ml staining solution (1× PBS, 1 mg/ml RNase A DNase-free, 1 mg/ml propidium iodide) at room temperature for 30 min. The cells were then subjected to flow cytometric analysis using a FACSCalibur (Becton Dickinson).

Caspase-3-like activity assay

Caspase-3-like activity was assessed using the colorimetric CaspACE assay system (Promega) according to the manufacturer's instructions. Briefly, after treatment with paclitaxel (50 n *M*) in the absence and in the presence of caspase inhibitor z-VAD-fmk (50 μ*M*) or cotreatment of paclitaxel with different antioxidants for 18 h, the cells were lysed in cold lysis buffer provided by the manufacturer. Total cell lysate (20 μg) was incubated with the caspase-3 substrate Ac-DEVD-pNA at 37°C for 4 h. The chromophore *p*-nitroaniline (pNA) was released from the substrate upon cleavage by caspase-3, and free pNA was monitored by a spectrophotometer at 405 nm.

Western blotting

The cell lysates were extracted by RIPA buffer (50 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 0.1% SDS, 0.5% NP-40, and 0.1% sodium deoxycholate) containing protease inhibitors (Roche). Protein concentrations were determined using the method of Bradford. Equal

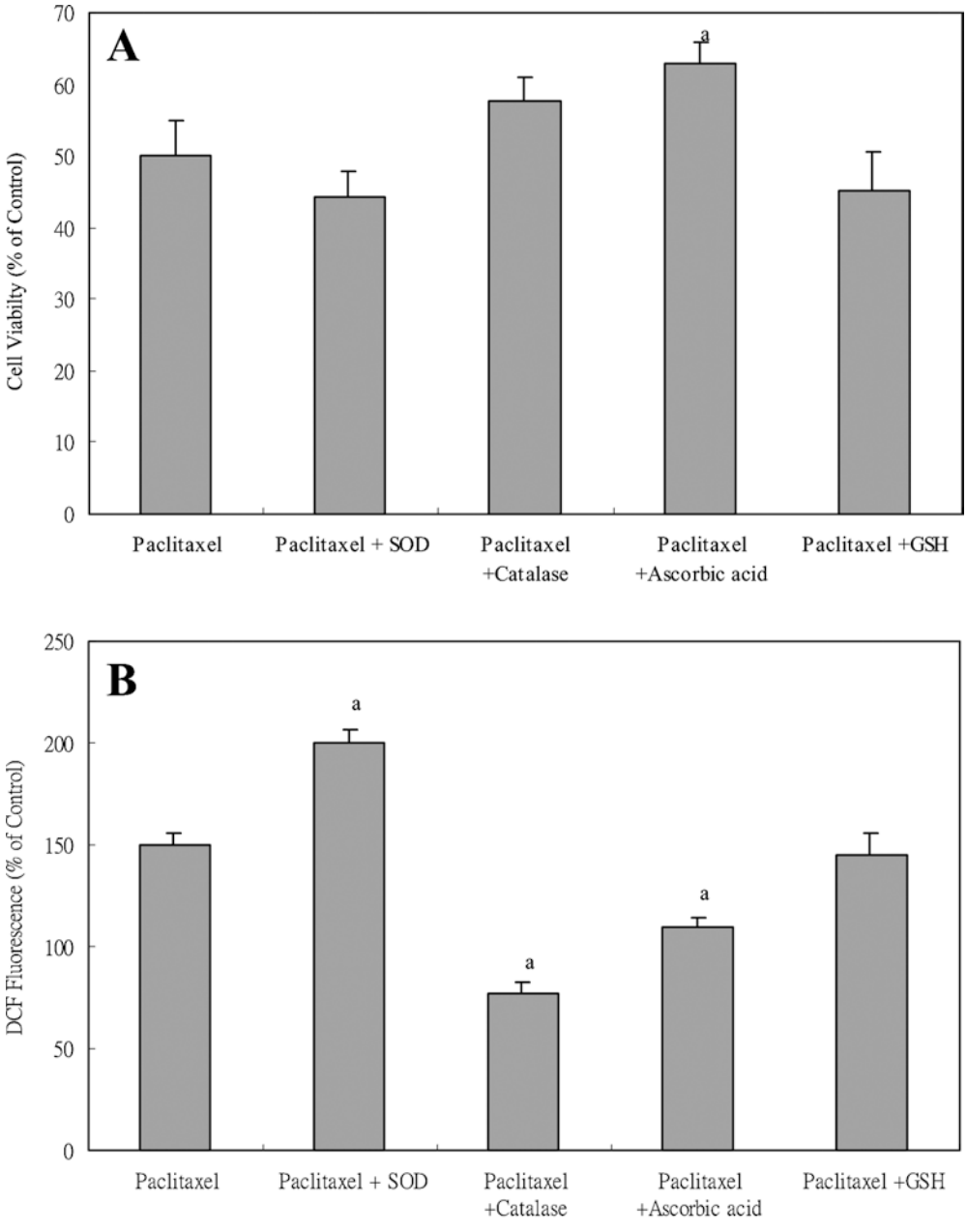

Fig. 3 The effects of antioxidants on the formation of ROS and cell viability in paclitaxel-treated BCBL-1 cells. BCBL-1 cells were treated with 50 n*M* paclitaxel in the absence or presence of various antioxidants (100 U/ml SOD, 50 U/ml catalase, 25 μ*M* ascorbic acid, or 250 μ*M* GSH) for 18 h: **A** cell viability was determined by trypan blue exclusion assay; **B** formation of ROS was detected by staining with DCF-DA. BCBL-1 cells in supplemented medium were used as control. The data shown are the means ± SD from three independent experiments. [a] $P < 0.05$ vs control (untreated BCBL-1 cells)

amounts of cell lysate were separated by SDS-PAGE and electrophoretically transferred onto PVDF membrane (Millipore). The membrane was blocked with 5% nonfat dried milk in TBST buffer (20 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 0.1% Tween 20), and incubated overnight at 4°C with specific primary antibodies including anticaspase-2 (Pharmingen), anticaspase-3 (Transduction Laboratories), anticaspase-8 (Oncogene), antiBax and antiBcl-2 (Transduction Laboratories), and antiactin (Santa Cruz Biotechnology). Subsequently, the membrane was washed with TBST buffer and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Protein bands were visualized by enhanced chemiluminescence kits (ECL Plus, Amersham) according to the manufacturer's instructions.

Data and statistics

All data are presented as means ± SD. Data were analyzed by analysis of variance (ANOVA) and Dunnett's test. *P* values smaller than 0.05 were considered significant.

Results

Paclitaxel affected the cell viability of BCBL-1 cells and induced oxidative stress in BCBL-1 cells

To investigate the cytotoxicity of paclitaxel in terms of the level of ROS, we used the trypan blue exclusion assay to determine the cytotoxicity of paclitaxel, and measured DCF fluorescence to detect the formation of

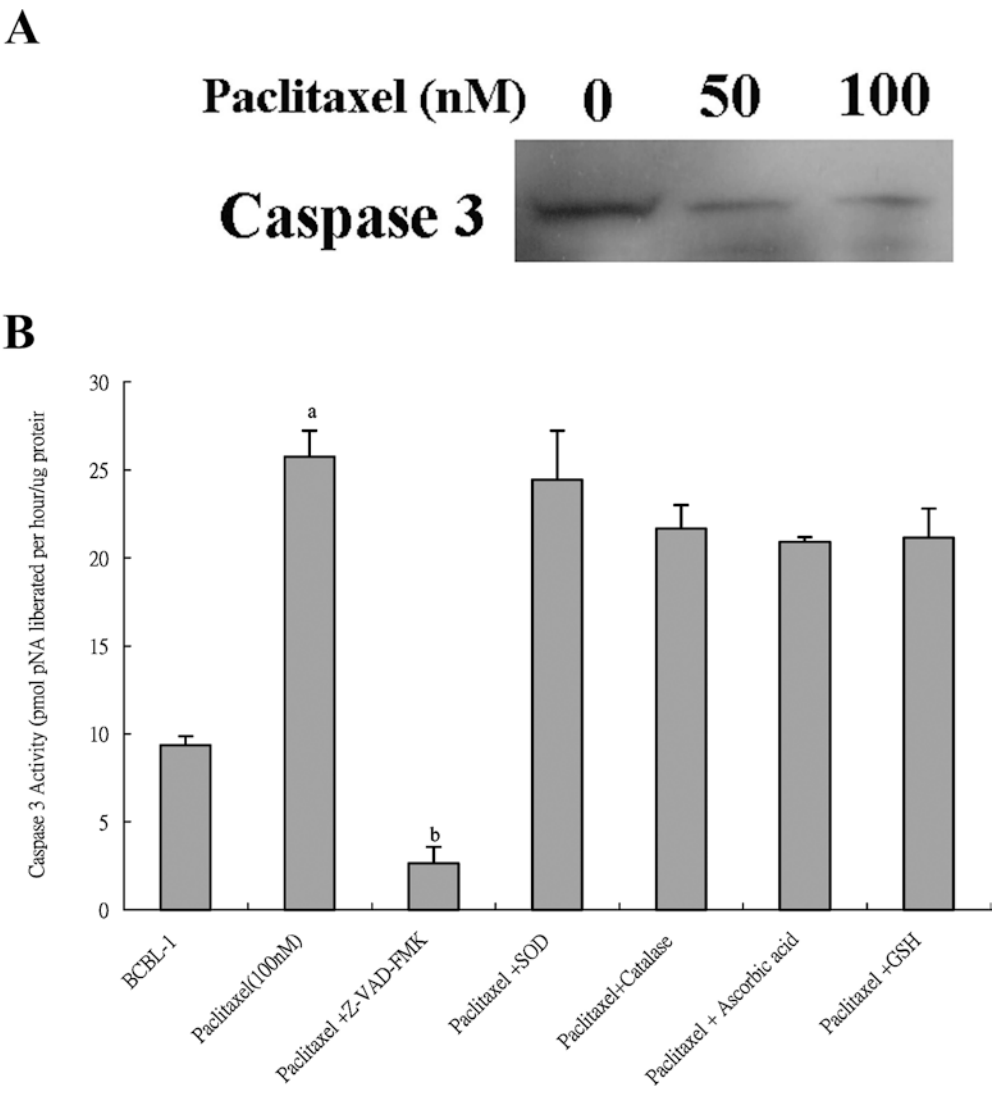

Fig. 4 Caspases-3-like activity in paclitaxel-treated BCBL-1 cells in the absence and presence of antioxidants. **A** Activation of caspase-3 in paclitaxel-treated BCBL-1 cells. BCBL-1 cells were untreated or treated with paclitaxel (50 or 100 n*M*) for 18 h. Protein extract (50 μg) was immunoblotted with specific antibodies as described in Materials and methods. **B** Antioxidants did not affect paclitaxel-induced caspase-3 activation. BCBL-1 cells were untreated, treated with 100 n*M* paclitaxel in the absence or presence of different antioxidants (100 U/ml SOD, 50 U/ml catalase, 25 μ*M* ascorbic acid, or 250 μ*M* GSH) and 100 n *M* paclitaxel in the presence of the caspase-3 inhibitor z-VAD-fmk for 18 h. Cell lysates were then prepared and incubated with the caspase-3 substrate Ac-DEVD-pNA for assay of caspase-3 activity using the CaspACE Assay System, Colorimetric (Promega). The results shown are the means + SD of three separate experiments. [a] $P < 0.05$ vs control (untreated BCBL-1 cells), [b] $P < 0.05$ vs paclitaxel-treated BCBL-1 cells

ROS. Paclitaxel was active at nanomolar concentrations and induced a dose-dependent reduction in cell viability in BCBL-1 cells (Fig. 1A). Paclitaxel also induced the production of ROS in a dose-dependent manner (Fig. 1B). The time-dependence (2, 4, 6, 18, and 30 h) of the effects of paclitaxel on BCBL-1 cells was investigated. Paclitaxel affected cell viability and ROS production in a time-dependent manner, with the effects on BCBL-1 cells apparent after 4 h of treatment (data not shown). In addition, apoptosis of BCBL-1 induced by paclitaxel was monitored by flow cytometric analysis (Fig. 2).

Effects of antioxidants on paclitaxel-induced cytotoxicity and ROS production

To determine whether antioxidants can protect the BCBL-1 cells against paclitaxel-induced oxidative stress, cells were treated with 50 n*M* paclitaxel in the absence or presence of various antioxidants, including SOD, catalase, ascorbic acid, and GSH. The various antioxidants had different effects. Cotreatment with SOD increased the generation of ROS, but had no effect on cytotoxicity. GSH had no effects on the production of ROS or cytotoxicity. Both catalase and ascorbic acid suppressed paclitaxel-induced ROS, but only ascorbic acid increased the viability of paclitaxel-treated BCBL-1 cells (Fig. 3).

Antioxidants had no effects on paclitaxel-induced activation of caspases

To assess whether the cytotoxicity of paclitaxel is mediated by caspase activation, caspase-3-like activity was determined. Caspase-3 p32 was decreased in paclitaxel-treated BCBL-1 cells (Fig. 4A), and the pan-caspase inhibitor z-VAD-fmk blocked paclitaxel-induced caspase-3-like activity (Fig. 4B). Even though ascorbic acid

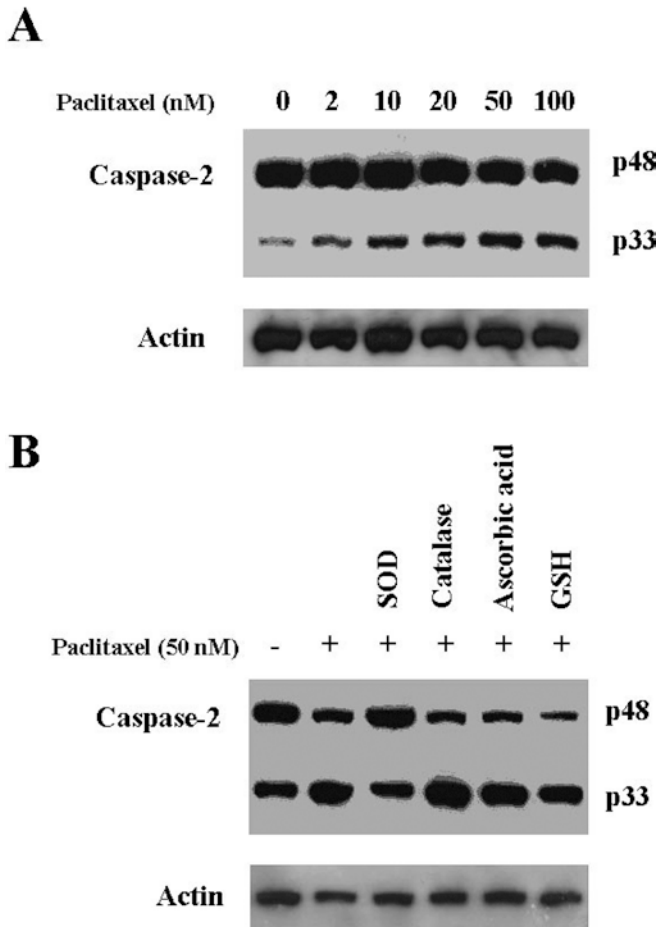

Fig. 5 Caspase-2 activation in paclitaxel-treated BCBL-1 cells in the absence and presence of antioxidants. **A** Activation of caspase-2 in paclitaxel-treated BCBL-1 cells. **B** Antioxidants did not affect the paclitaxel induced caspase-2 activation. BCBL-1 cells were treated with various concentrations of paclitaxel or cotreated with 50 n*M* paclitaxel and different antioxidants (100 U/ml SOD, 50 U/ml catalase, 25 μ*M* ascorbic acid, or 250 μ*M* GSH) for 18 h. Protein extract (50 μg) was immunoblotted with specific antibodies as described in Materials and methods. Protein levels of actin were used to ensure equal amounts of total protein in each lane. Representative results from three independent experiments are shown

showed a small preventive effect against paclitaxel-induced cytotoxicity, it still failed to suppress the caspase-3-like activity induced by paclitaxel (Fig. 4B). SOD, catalase and GSH did not affect paclitaxel-induced caspase-3-like activity (Fig. 4B).

The activation of caspase-2 and caspase-8 was next detected by Western blot analysis. Paclitaxel induced the activation of caspase-2 (Fig. 5A) and caspase-8 (Fig. 6A). Cotreatment with SOD slightly inhibited the activation of caspase-2 by paclitaxel, and catalase increased the caspase-2 activity. Neither ascorbic acid nor GSH affected caspase-2 activity (Fig. 5B). None of the antioxidants affected the activation of caspase-8 (Fig. 6B). In general, none of the antioxidants affected the paclitaxel-induced activation of caspases, even though SOD and catalase showed some effects on caspase-2 activity.

Antioxidants induced Bcl-2 protein expression and decreased paclitaxel-induced expression of Bax protein

To determine whether the Bcl-2 family proteins are involved in paclitaxel-induced apoptosis of the BCBL-1 cells, Western blotting was used to detect the expression of the proapoptotic and antiapoptotic proteins, Bax and Bcl-2. Paclitaxel increased the protein levels of Bax, and slightly decreased the levels of Bcl-2 (Fig. 7A). Cotreatment with antioxidants decreased the expression of Bax protein and increased the levels of Bcl-2 protein (Fig. 7B). Cotreatment with ascorbic acid resulted in the highest Bcl-2/Bax ratio (quantified by densitometric analysis; data not shown).

Discussion

In previous studies, paclitaxel has been demonstrated to be highly toxic against several kinds of human tumor cell lines [17, 19], and it has been utilized for the treatment of KS which is closely related to HHV-8 [3, 20]. However, the mechanism of paclitaxel action in KS remains unclear. In this study, we utilized BCBL-1 HHV-8-harboring cells to study the possible mechanisms of paclitaxel in HHV-8-related tumors.

First, we tried to assess the involvement of oxidative stress in the cytotoxicity of paclitaxel-treated BCBL-1 cells. We found that both the cytotoxicity and the production of ROS in paclitaxel-treated BCBL-1 cells were dose-dependent (Fig. 1). Various antioxidants showed different effects on the paclitaxel-induced cytotoxicity and generation of ROS. Even though catalase decreased the production of ROS, it did not significantly attenuate the cytotoxicity ($P = 0.055$; Fig. 3). Only ascorbic acid was able to decrease both the cytotoxicity of paclitaxel and the generation of ROS (Fig. 3). Previous studies have also shown that ascorbic acid inhibits ROS generation and cell death induced by vincristine, which is also a microtubule-interfering anticancer agent [10]. However, the reduction of ROS by cotreatment with catalase or ascorbic acid did not correlate with the decrease in cytotoxicity, suggesting that oxidative stress is only partially involved in the cytotoxicity of paclitaxel.

Paclitaxel acts on both mitochondria [1] and microtubules [9]. The mitochondrial antiapoptotic protein Bcl-2 is a potential candidate for paclitaxel binding [24]. It is known that Bcl-2 exerts a protective effect against apoptosis. Previous studies have shown that paclitaxel-induced apoptosis in gastric cancer cells is mediated by downregulation of the apoptosis-regulated gene Bcl-2 and by upregulation of the apoptosis-regulated gene Bax [31]. In our experiments, paclitaxel

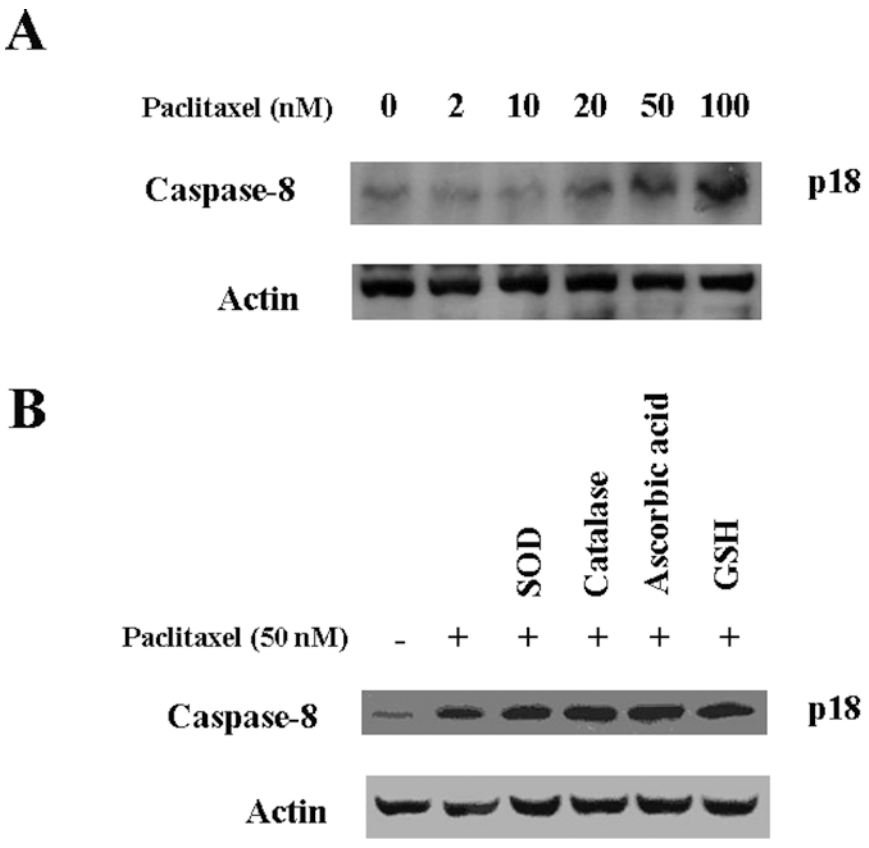

Fig. 6 Activation of caspase-8 (p18) in paclitaxel-treated BCBL-1 cells in the absence and presence of antioxidants. **A** Activation of caspase-8 in paclitaxel-treated BCBL-1 cells. **B** Antioxidants did not affect paclitaxel-induced caspase-8 activation. BCBL-1 cells were treated with various concentrations of paclitaxel or cotreated with 50 n*M* paclitaxel and different antioxidants (100 U/ml SOD, 50 U/ml catalase, 25 μ*M* ascorbic acid, or 250 μ*M* GSH) for 18 h. Protein extract (50 μg) was immunoblotted with specific antibodies as described in Materials and methods. Protein levels of actin were used to ensure equal amounts of total protein in each lane. Representative results from three independent experiments are shown

treatment resulted in a slight decrease in the protein levels of Bcl-2 and an increase the protein levels of Bax in BCBL-1 cells (Fig. 7A). Cotreatment with the antioxidants induced the expression of Bcl-2, and cotreatment with SOD, catalase, and ascorbic acid decreased the expression of Bax protein. Cotreatment with ascorbic acid resulted in the highest Bcl-2/Bax ratio (data not shown). It has been reported that ascorbic acid and vitamin E are able to reduce lipopolysaccharide-induced apoptosis by an increase in Bcl-2 and a decrease in Bax protein levels [11]. This suggests that ascorbic acid could attenuate the cytotoxicity of paclitaxel in BCBL-1 cells by upregulation of Bcl-2 and downregulation of Bax protein.

The activation of caspase-3 and caspase-8 has been reported to play a crucial role in paclitaxel induced apoptosis [22]. In our experiments, caspase-3-like activity was induced by paclitaxel treatment in BCBL-1 cells, and the pan-caspase inhibitor z-VAD-fmk inhibited caspase-3 activation. The presence of z-VAD-fmk inhibited the cytotoxicity of paclitaxel in BCBL-1 cells, but could not completely block the cytotoxicity of paclitaxel (data not shown). Activation of caspase-8 was also observed in paclitaxel treated BCBL-1 cells (Fig. 6A). However, none of the antioxidants, including ascorbic acid, was able to prevent paclitaxel-induced activation of caspase-3 (Fig. 4) and caspase-8 (Fig. 6B). Caspase-2, a regulative caspase, is able to induce mitochondrial dysfunction and activate the apoptosome through direct or indirect Bid processing [23]. In our experiments, caspase-2 was activated in paclitaxel-treated BCBL-1 cells and its activation was not affected by the antioxidants utilized in this study (Fig. 5). However, the cleavage of caspase-2 was also observed in untreated cells. Since BCBL-1 cells are latently infected by HHV-8 and its lytic cycle can be spontaneously turn on in a small proportion of cells to generate virus progenies, cleavage of caspase-2 may occur in untreated BCBL-1 cells. However, we could not explain the increase of active p33 subunit following cotreatment with paclitaxel and catalase. These findings suggest that caspase activation is important in the cytotoxicity of paclitaxel in BCBL-1 cells, and oxidative stress is not involved in paclitaxel-induced caspase activation.

In conclusion, paclitaxel induced oxidative stress in BCBL-1 cells, and this oxidative stress altered the protein levels of Bcl-2 family members that lead to cell death. However, oxidative stress was only partially involved in the paclitaxel-induced cytotoxicity in BCBL-1 cells. Paclitaxel also activated caspase-2, caspase-3, and caspase-8 to cause death of BCBL-1 cells. However, caspase activation was not due to oxidative stress. These results imply that apoptosis of BCBL-1 cells induced by paclitaxel is both caspase-dependent and caspase-independent, and occurs via a caspase-dependent pathway.

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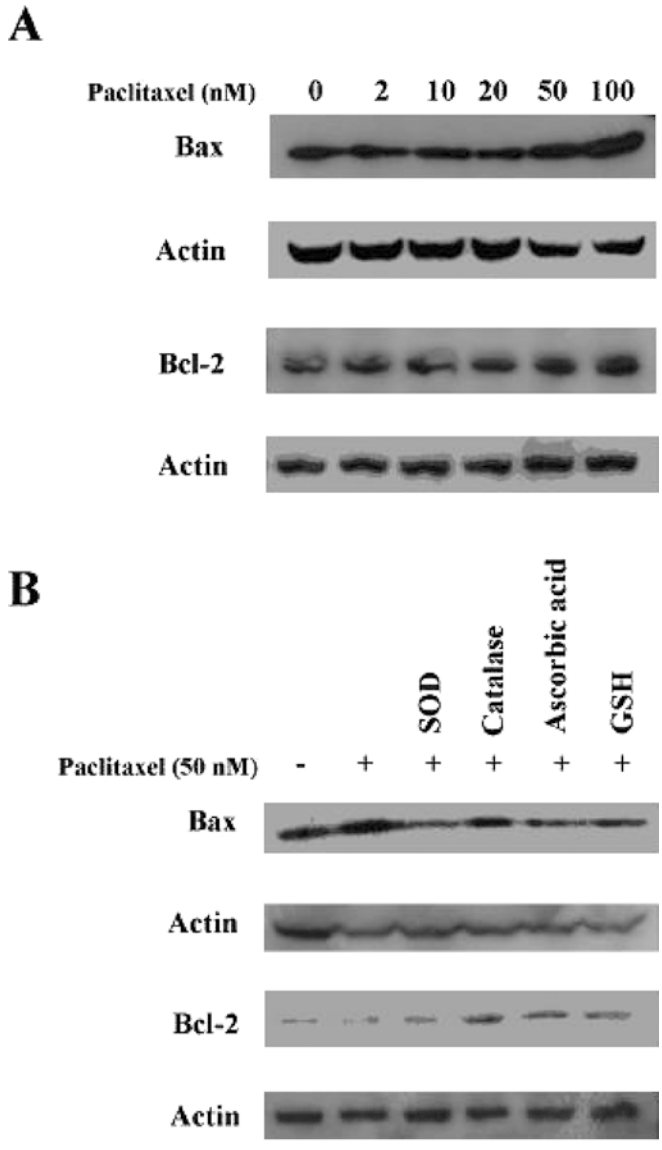

Fig. 7 Western blot analysis of Bax and Bcl-2 proteins in paclitaxel-treated BCBL-1 cells in the absence and presence of antioxidants. **A** Expression of Bax and Bcl-2 proteins in paclitaxel-treated BCBL-1 cells. **B** Expression of Bax and Bcl-2 proteins with cotreatment with antioxidants and paclitaxel in the BCBL-1 cells. BCBL-1 cells were treated with various concentrations of paclitaxel or cotreated with 50 n*M* paclitaxel and various antioxidants (100 U/ml SOD, 50 U/ml catalase, 25 μ*M* ascorbic acid, or 250 μ*M* GSH) for 18 h. Protein extract (50 μg) was immunoblotted with specific antibodies as described in Materials and methods. Protein levels of actin were used to ensure equal amounts of total protein in each lane. Representative results from three independent experiments are shown

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