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Cytotoxic Action of *Ganoderma lucidum* on Interleukin-3 Dependent Lymphoma DA-1 Cells: Involvement of Apoptosis Proteins

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Aqueous extracts and a semipurified fraction obtained by methanol extraction and column chromatography were isolated from *Ganoderma lucidum* [*Ganoderma lucidum* (Curtis) P. Karst.; *Ganodermataceae* Donk] and their effects on interleukin 3-dependent lymphoma cells (DA-1) were studied. Cell viability was reduced by the action of unboiled aqueous extract and by the methanol-extracted column-chromatography semipurified fraction, producing DNA fragmentation in DA-1 cells. Treatments with aqueous extracts showed increments of Bax after 13 h, increments of p53 and Mdm2 after 19 h and a reduction of these three proteins after 24 h. The methanol-extracted semipurified fraction also induced increments of p53 and Mdm2 factors at 19 h with a reduction after 24 h. The methanol-extracted column-chromatography semipurified fraction from *Ganoderma lucidum* produced minor changes in the level of Akt after treatments for 19 h in DA-1 cells with a slight reduction in the levels of NFkB-p65 factor. Both the unboiled aqueous extract and the methanol-extracted column-chromatography semipurified fraction produced cleavage of inactive caspase 3, as a clear indication of induction of apoptosis by compounds present in *Ganoderma lucidum*.

Keywords: Bax; Bcl-2; *Ganoderma lucidum* (Curtis) P. Karst., Ganodermataceae Donk; lymphoma; p53.

INTRODUCTION

In recent times, the search for new more effective therapeutic agents with more selective action on tumor cells has been conducted. The fungus *Ganoderma lucidum* (common names: Reishi, Lingzhi) is a well known mushroom in traditional Chinese medicine. Extracts from different parts (mycelia, spores and fruiting bodies) from *Ganoderma lucidum* have been used as a source of active compounds against tumor processes (Hu *et al.*, 2002; Liu *et al.*, 2002; Hong *et al.*, 2004; Jiang *et al.*, 2004a, 2004b; Lu *et al.*, 2004; Sliva, 2004). Among the different compounds present in *Ganoderma lucidum*, nucleosides, proteins, polysaccharides, fatty acids, sterols and triterpenes have been described as molecules which could be responsible for the antitumor activity (Yeung *et al.*, 2004). *Ganoderma lucidum* extracts inhibit the growth of human prostate and bladder cancer cell lines (Jiang *et al.*, 2004b; Lu *et al.*, 2004) and cell proliferation inducing apoptosis in human colon carcinoma and breast cancer cell lines (Hu *et al.*, 2002; Hong *et al.*, 2004; Jiang *et al.*, 2004a). Polysaccharides present in *Ganoderma lucidum* may be responsible for the growth delay of sarcoma cells (Cao and Lin, 2004). In part, their antitumor activity may be a consequence of their immunomodulatory properties (Wilasrusmee *et al.*, 2002; Williamson, 2001; Lin, 2005). Also, triterpenes present in *Ganoderma lucidum* inhibit the growth of hepatoma cells by altering intracellular phosphorylation pathways (Lin *et al.*, 2003).

We have previously studied the apoptotic action of two antitumor compounds, etoposide and hydroxyurea, on DA-1 lymphoma cells (Olmos *et al.*, 2005a, 2005b) as a model for cytotoxicity by inducing apoptosis. These cells, derived from a murine lymphoma, could be sensitive to the action of antitumor and toxic compounds of therapeutic interest. Additionally, this model shows the advantage of being modulated by interleukin 3 (Gottlieb *et al.*, 1994, 1996; Gottlieb and Oren, 1998). Thus, antiproliferative and apoptotic effects of antitumor compounds can be studied in this cell model. We have demonstrated the possible differential involvement of Bax and Bcl-2 factors in the apoptotic death of these cells after treatment with etoposide or hydroxyurea (Olmos *et al.*, 2005a, 2005b). Thus, the present work studied the cytotoxicity of different *Ganoderma lucidum* extracts, prepared as described by other authors (Sliva *et al.*, 2002; Stanley *et al.*, 2005) on these interleukin 3 dependent lymphoma cells. Aqueous extracts and a methanol-extracted semipurified fraction from *Ganoderma lucidum* demonstrated the ability to reduce cell viability in DA-1 cells and also induced DNA fragmentation. Changes in protein expression of apoptosis factors were also observed after treatments with these extracts showing activation of apoptosis cascades.

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Some authors have previously shown the activation of intracellular kinase cascades by *Ganoderma* extracts in induced neuronal differentiation in rat PC12 cells (Cheung *et al*., 2000). Jiang *et al*. (2004a) also showed inhibition of Akt/NF-kB signalling by *Ganoderma lucidum* in breast cancer cells. Thus, interest was focused on activation and phosphorylation of intracellular kinases which are altered in other cellular model systems (Jiang *et al*., 2004a; Cheung *et al*., 2000). Changes in expression and phosphorylation of Akt have been studied after treatment of DA-1 cells with aqueous extracts and the methanol-extracted semipurified fraction from *Ganoderma lucidum*.

MATERIALS AND METHODS

Cell lines. Two cell lines were used: Mouse interleukin 3 dependent lymphoma DA-1 cells were kept at 37°C and 5% CO_2, in Iscove's MDM medium containing 10% fetal calf serum, 2 mM L-glutamine and 2.5×10^{-5} M β-mercaptoethanol.

The WEHI-3B cell line was employed and applied as a source of conditioned medium containing interleukin 3, which is required for the survival of DA-1 cells. The supernatant containing the medium with secreted IL-3 was obtained by centrifugation at $1200 \times \mathbf{g}$ for 5 min and subsequent filtration. The DA-1 cells were grown in the presence of 5% of this conditioned medium.

Source of *Ganoderma lucidum*. The *Ganoderma lucidum* [*Ganoderma lucidum* (Curtis) P. Karst.; *Ganodermataceae* Donk] strain was isolated from the fruit body tissue (context) of a wood-inhabiting filamentous parasitic fungi growing in a natural habitat on *Quercus ilex* roots in Cáceres (Spain). A small part of the tissue context developed *Ganoderma lucidum* mycelium that was cultured and subcultured in a malt extract solid medium (MA). In order to produce fruiting bodies it was necessary to cultivate the fungus on lignocellulosic solid medium substrate (Chang and Miles, 2004), adequately hydrated and autoclaved. Once the lignocellulosic medium was inoculated with *G. lucidum* mycelia, the substrate was incubated until a proper mycelial colonization. Subsequently, the bulk mycelium developed fruit bodies that reached maturity in 1–2 months.

Extracts of *Ganoderma lucidum*. Fruiting bodies of *G. lucidum* were resuspended in sterile water to a concentration of 50 mg/mL (Sliva *et al*., 2002; Stanley *et al*., 2005), homogenized and then centrifuged at 14000 rpm (in a Beckman J2.21 centrifuge using JA-20.1 rotor) for 5 min, at room temperature, to obtain a supernatant called extract 1 (E1). The pellet was resuspended in the same volume of sterile water, boiled for 5 min, sonicated for 20 s at 50% intensity, centrifuged at 14000 rpm for 5 min and the supernatant obtained was called extract 2 (E2). Afterwards, these extracts were lyophyllized and disolved in 1/10 the initial volume, giving ten-fold concentrated extracts.

Methanol extracts of *G. lucidum* were also obtained. Fruiting bodies (2 g) were disrupted with liquid nitrogen and the resulting material was resuspended at 50 mg/mL concentration in 10% methanol and extracted three times with 10% methanol for 24 h with agitation. The combined extracts were evaporated to dryness in a rotary evaporator, and dissolved in absolute methanol. To purify this extract, it was adsorbed on 0.2 g of silica gel to be applied onto a silica gel column (1×15.5 cm) and subsequently eluted with 3:1:1 butanol: acetic acid: water. Column fractions (0.2 mL) with an R_f near 0.74 were combined, the solvent was evaporated and the final sample (1.6 mg) was resuspended in 0.01 mL DMSO to obtain the extract 3 (E3).

Aqueous extracts E1 and E2 prepared as described by other authors were used. It is highly probable that sugars are present in these extracts. Several polysaccharides present in *G. lucidum* have been shown to have immunomodulatory and antitumor activities (Cao and Lin, 2004; Sliva *et al*., 2002; Stanley *et al*., 2005). With respect to the E3 fraction, isolated on column chromatography from a methanol extract of *G. lucidum*, based on the solvents used it is possible that compounds similar to terpenoids could be present in this fraction (Li *et al*., 2005; Lin *et al*., 2003; Min *et al*., 2000).

Cell treatments. Cultured DA-1 cells were incubated at concentrations of 0.5×10^6 cells/mL at 37°C in an atmosphere with 5% CO_2 in the presence of IL-3 conditioned medium. DA-1 cells were grown in the presence of IL-3 conditioned medium alone or supplemented with either *Ganoderma lucidum* extracts or 100 μM etoposide as a positive control for toxicity and apoptosis induction.

The E1 and E2 extracts and E3 fraction were added separately to 1 mL aliquots of cell culture (0.5×10^6 cell/mL) and allowed to act for 13, 19 and 24 h.

The lyophyllized E1 extract (14.9 mg dried weight) was dissolved in 0.9 mL of sterile distilled water and 60 μL was added per mL of cell culture.

The lyophyllized E2 extract (3 mg dried weight) was dissolved in 0.9 mL of sterile distilled water and 60 μL was added per mL of cell culture.

Fraction 3 (E3) (2.08 mg) dissolved in 13 μL of DMSO was added per mL of cell culture (0.5×10^6 cell/mL).

DMSO (13 μL) was also added to the cells as a negative control and no toxic effect was observed on cell culture (i.e. no reduction of cell viability, no DNA fragmentation, etc.).

Cell viability, permeability of treated cells to propidium iodide and flow cytometry analyses. The cell viability of DA-1 cells incubated in the presence of IL-3 and treated with *Ganoderma lucidum* extracts was determined by flow cytometry by measuring the impermeability to propidium iodide. 2.5×10^5 treated cells were collected, washed in PBS, pelleted at 1200 rpm for 5 min, resuspended in 500 μL of PBS and stained with propidium iodide at 5 μg/mL final concentration and, analysed by flow cytometry either by FACScan or FACScalibur (Becton Dickinson, San José, CA, USA). For each sample the acquisition was finished at 10000 counts. Data analysis was done using the program Win-MDI (Windows Multiple Document Interface for Flow Cytometry, version 2.8, available from Scripps Research Institute, FACS Core Facility, http://facs.scripps.edu/software.html) (Trotter, 2004). Cell fragments were discriminated from the non viable cells on FSC/FL-2-H dot plots, where FL-2-H corresponds to propidium iodide fluorescence.

Cytometric analysis of cell populations with subdiploid DNA and cell cycle. Apoptotic cells were counted on the basis of DNA content per cell after permeabilization of cells with NP40. After treatments with 100 μM etoposide or *G. lucidum* extracts, 2.5×10^5 cells were collected and washed with PBS. The pellet was resuspended in 475 μL of a solution containing 0.5 mg/mL RNase, 0.1% of NP-40 in PBS and incubated for 30 min in order to extract low molecular weight DNA from cell nuclei. The remnant DNA in cells was stained with 0.05 mg/mL propidium iodide, immediately before measuring the fluorescence in the cytometer.

Cells with hypodiploid DNA (apoptotic cells) were distinguished from those containing diploid DNA (non apoptotic cells) on the basis of a different fluorescence intensity of propidium iodide in the flow cytometer. Cell cycle progress was studied in the same samples. Histograms of the untreated cells were used to define the positions of the different peaks for the G1 and G2/M phases in the cell cycle.

Western blot. 5×10^6 cells treated with the extracts for 13, 19 or 24 h were collected by centrifugation at 1200 rpm for 5 min, resuspended in 200 μL of lysis buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF) and incubated for 20 min at 4°C. The cells were sonicated for 20 s (duty cycle 100%, output control 50%) in a Branson Cell Disruptor B15 Sonifier and then centrifuged (14000 rpm, 5 min, 4°C) and the supernatants were analysed by electrophoresis and blotting. Proteins (20 μg/well) were loaded on a 10% polyacrylamide gel with SDS, electrophoretically separated and transferred to nitrocellulose membranes as described (Towbin *et al.*, 1979). Membranes were blocked with 5% powder milk in TTBS (50 mM Tris pH 7.2, 140 mM NaCl, 0.06% Tween 20). Afterwards, they were washed with TTBS and incubated with antibodies specific against several apoptosis factors diluted in TTBS containing milk.

All the following antibodies were obtained from Santa Cruz Biotechnology, CA, USA, and used at the dilution indicated: Anti-Bax mouse monoclonal IgG_{2b} (B-9; sc-7480; 200 μg/mL) 1:100; anti-Bcl-2 mouse monoclonal IgG_1 antibody (C-2; sc7382; 200 μg/mL) 1:200; anti-MDM2 (SMP14) 1:100; anti-Akt1/2/3 (H-136) sc-8312 antibodies, 1:400; anti-pAkt1/2/3 (Ser 437) sc-7985-R antibodies 1:400; anti Erk1/2 sc-154 antibodies 1:3000; anti p-Erk1/2 (Tyr^{204}) sc-7383 antibodies 1:200; anti-NFkB p50 and p105 (E-10) sc-8414 antibodies 1:200; anti-NFkB p65 (F-6) sc-8008 antibodies 1:200.

Anti-p53 mouse monoclonal IgG antibody (Ab-1, OP03; 100 μg/mL) was purchased from Calbiochem (Oncogene Research Products, MA, USA) and diluted 1:50.

Monoclonal anti-β-actin antibodies were purchased from Sigma A5441 and used at a dilution of 1:5000.

Polyclonal caspase 3 antibodies were purchased from Cell Signalling Technology (Reference number 9662) and used at a dilution of 1:1000.

After incubation of the nitrocellulose membranes with specific antibodies overnight at 4°C the bands were revealed using goat anti-mouse horseradish peroxidase-conjugated polyclonal antibodies (1:2000) from Promega (Madison, WI) in an enhanced chemiluminescence (ECL) detection kit (Amersham). The intensities of the bands were corrected with respect to the intensity of actin band in the blot, as a control for constitutive expression, and quantified relative to the intensity of the band in control cells without any treatment, which were considered as 100%.

All the experiments were repeated three or four times. The mean and SEM were graphically represented. In order to determine the significance of the effect, Student's *t*-test was used. The asterisk * indicates $p < 0.05$.

RESULTS

Cell viability analyses of DA-1 cells

The cell viability of DA-1 lymphoma cells was determined after treatment with *Ganoderma lucidum* extracts obtained as described in Methods. As can be observed, etoposide (Fig. 1) at concentrations of 100 μM induced a decrease in cell viability, with values of 70%, 58% and 51% viability in cells treated with etoposide for 13, 19 and 24 h. Also, different extracts obtained from *Ganoderma lucidum* were used: Both E1 and E3 showed an effective reduction in cell viability with a reduction of up to 70% and 36% at 24 h of treatment, respectively (Fig. 1). On the contrary, the E2 extract showed not so clear action on viability. In the case of E1 the effects were clearly visible after 19 h. E3 showed an even more effective action than E1 extract (Fig. 1). Thus, the analyses of cell viability clearly demonstrate the action of these extracts (E1 and E3) on cell membrane permeability which allows the entrance of propidium iodide.

Figure 2 shows some representative diagrams where it is possible to observe the increment in the peak corresponding to cells permeant to propidium iodide due to the alteration of the membrane (see the increased peak of higher fluorescence in FL2-H in samples treated with *Ganoderma lucidum* extracts). The toxic action of E3 is more clearly visible than that of E1 both at 13 and 24 h.

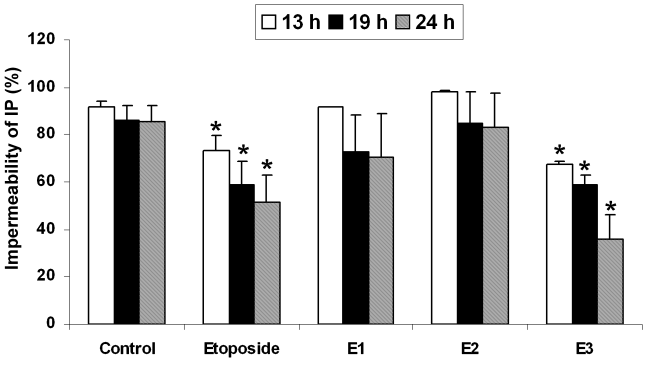

Figure 1. Cell viability of DA-1 cells grown in different culture conditions. DA-1 cells were grown in the conditions indicated in Experimental section in the presence of conditioned medium containing interleukin 3, E1, E2 or E3 represent treatments with *Ganoderma lucidum* extracts. Etoposide 100 μM treatment was used as a control. Different times of treatment are shown by open, black and grey bars. All the experiments were repeated three or four times and the mean and SEM are plotted. Significance was evaluated using Student's *t*-test. *$p < 0.05$.

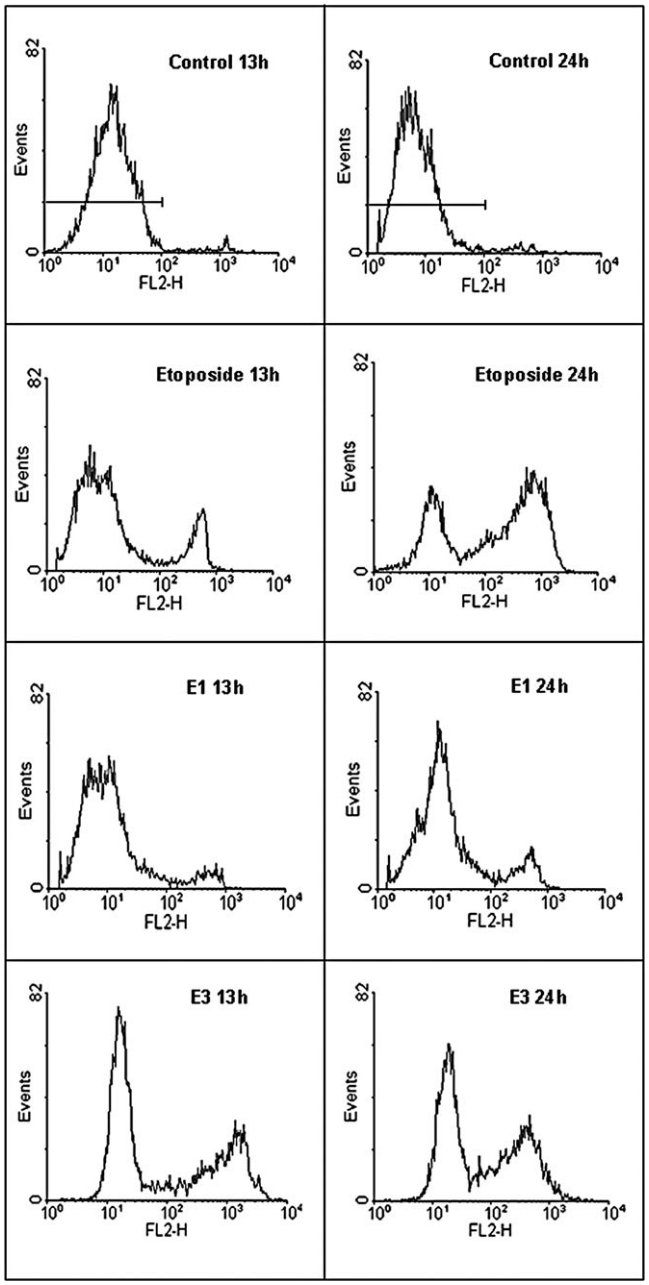

Figure 2. Propidium iodide permeability of DA-1 cells grown in different culture conditions. Cellular viability was measured through propidium iodide permeability. Representative histograms of propidium iodide fluorescence are shown for control cells and cells treated either with 100 μM etoposide, E1 extract or E3 fraction from *Ganoderma lucidum*.

Cell death induction in DA-1 cells

Previously, it was shown that either of two antitumor compounds (hydroxyurea or etoposide) induces apoptosis in DA-1 cells (Olmos *et al.*, 2005a, 2005b), as tested by permeability alteration analyses, DNA fragmentation and annexin-FITC studies. The apoptotic action of these compounds can be related to changes in cell cycle. Thus, we studied the effects on cell cycle as well as on the presence of subdiploid DNA. Figure 3 shows the results obtained for treatments of DA-1 cells in the presence of the extracts obtained from *Ganoderma lucidum*. As can be observed, E1 extract showed a level of cell death induction (as tested by production of

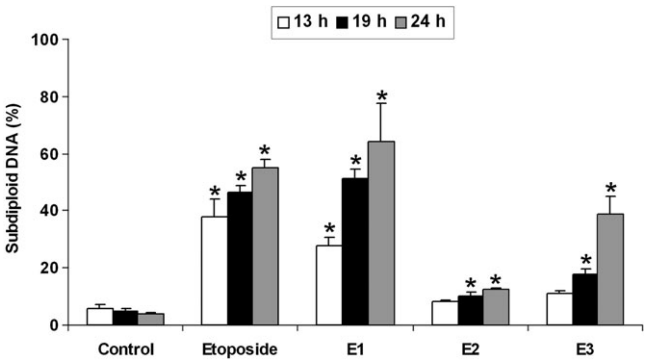

Figure 3. Apoptosis of DA-1 cells induced by extracts from *Ganoderma lucidum*. The apoptosis induced by treatment with extracts from *Ganoderma lucidum* was measured by quantifying subdiploid DNA. Different times (13, 19 and 24 h) are shown by open, black and grey bars. Untreated cells and cells treated with 100 μM etoposide are included as negative and positive controls for apoptosis. All the experiments were repeated three or four times and the mean and SEM are plotted. Significance was evaluated using Student's *t*-test. $*p < 0.05$.

subdiploid DNA) similar to that of 100 μM etoposide. At 24 h, the induction of subdiploid DNA was even higher in the case of E1 than etoposide. E3 fraction also showed an important induction of subdiploid DNA in DA-1 cells, an effect that was more clearly visible at 24 h. On the contrary, E2 extract showed a very limited induction of cell death in DA-1 cells even at 24 h. Figure 4 shows representative diagrams of DNA content of control and E1 and E3 treated DA-1 cells. The effects of E1 extract are clearly visible at 24 h (with an important peak corresponding to subdiploid DNA) although they were already apparent at 13 h. The methanol-extracted semipurified E3 fraction also induced DNA fragmentation (see the panels on the right side of Fig. 4). In this case, it is possible to observe a prominent peak with high fluorescence in FL2-H at 13 and 24 h, that might indicate an arrest in S phase of the cell cycle as a consequence of the treatment.

Analyses of expression of apoptosis related factors

Since some factors such as p53, Bax, Bcl-2 and Mdm-2 are involved in apoptosis processes, the levels of these proteins (Figs 5–8) were analysed by western blot using specific antibodies.

Figure 5 shows representative plots corresponding to the western blot analyses of DA-1 cells treated with E1 extract. p53 expression was reduced with respect to control in cells after 13 h of treatment with E1, an effect which later reverted reaching 160% at 19 h of treatment (Figs 5 and 8). Bax protein increases earlier, doubling the levels of control at 13 h and 19 h of treatment with E1. Analysis of Bcl-2 expression revealed a decrease at 13 h. Longer treatments (19 and 24 h) recovered the level to values similar to untreated cells. Mdm2 showed also a clear reduction at 13 h and an increase at 19 h with restoration of the level at 24 h to those found in control cells (Fig. 5).

The effect of the E2 extract is shown in Fig. 6. At 13 h, the treated DA-1 cells showed increments in Bax and Mdm2, while Bcl-2 showed a reduction. In the case of Mdm2, a high level of this protein was observed with treatments for 19 h.

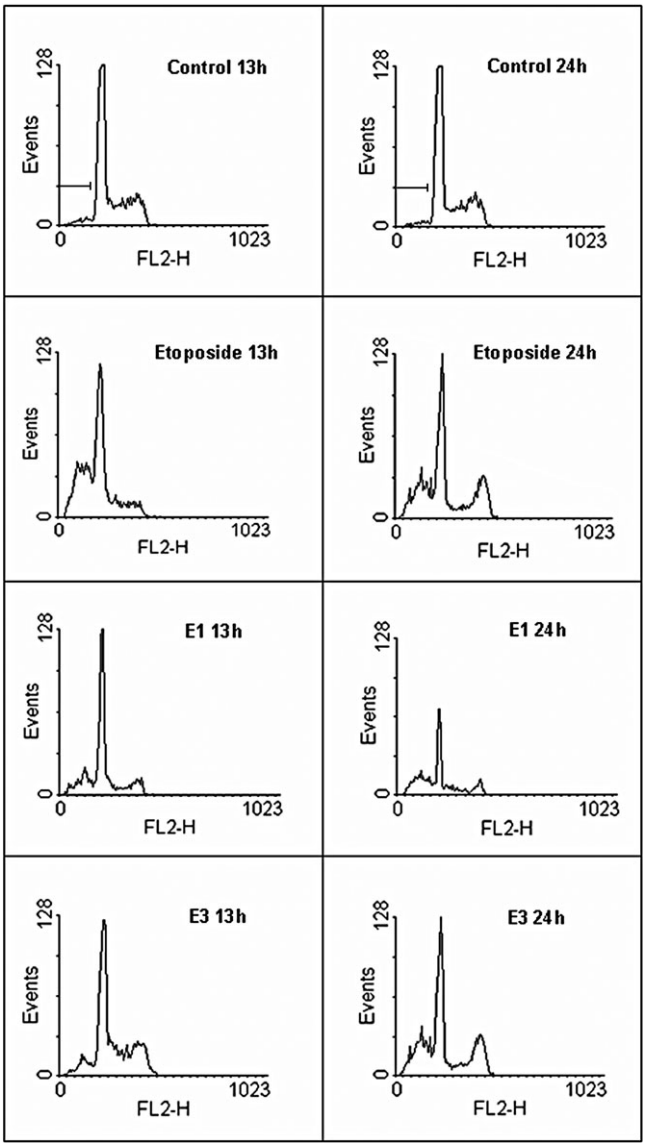

Figure 4. DNA fragmentation on DA-1 cells induced by treatments with different extracts from *Ganoderma lucidum*. The apoptosis induced by treatment with extracts from *Ganoderma lucidum* was measured by quantitation of subdiploid DNA. This figure shows representative histograms of the DNA content, measured with propidium iodide, present in control cells and cells treated with 100 μM etoposide, E1 extract or E3 fraction from *Ganoderma lucidum* after 13 or 24 h. The number of events versus the intensity of fluorescence of propidium iodide is shown.

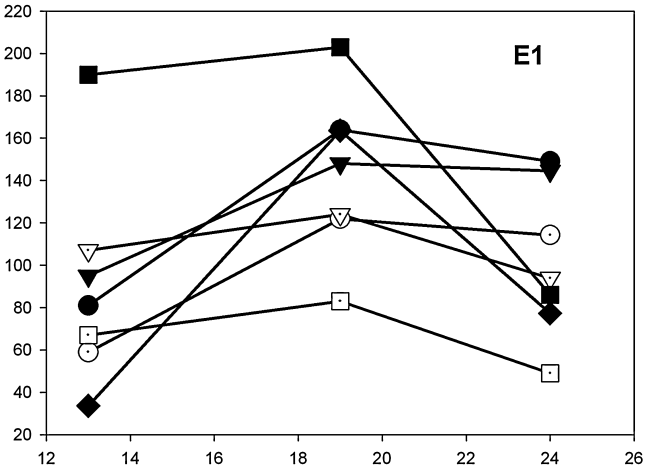

Figure 5. Expression of apoptotic factors in DA-1 cells treated with E1 aqueous extract. DA-1 cells were incubated with E1 and western blots of proapoptotic and antiapoptotic proteins (p53 -●-, Bcl2 -○-, Bax -■-, Akt -▼- , pAkt -▽-, Mdm-2 -◆- and NFkB p65 -□-) were carried out. Control represents untreated cells. Actin was used as a control of constitutive expression. Treatments were applied for 13, 19 and 24 h. The represented values correspond to the mean of three different experiments. All values were corrected to the staining intensity of actin in control cells.

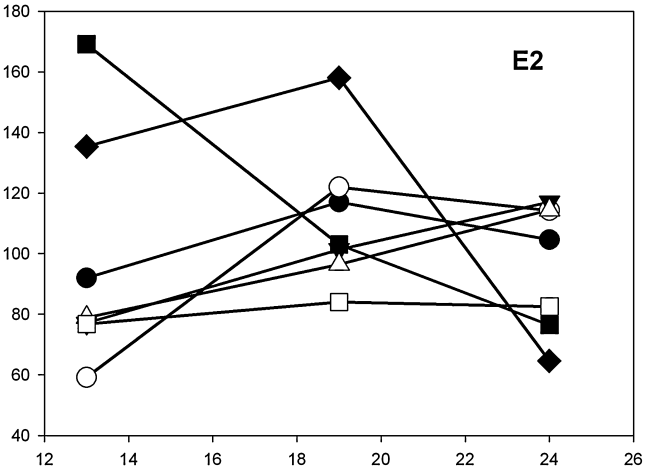

Figure 6. Expression analyses of DA-1 cells levels of apoptotic factors in DA-1 cells treated with E2 aqueous extract. DA-1 cells were incubated with E2 and western blots of proapoptotic and antiapoptotic proteins (p53 -●-, Bcl2 -○-, Bax -■-, Akt -▼- , pAkt -▽-, Mdm-2 -◆- and NFkB p65 -□-) were carried out. Control represents untreated cells. Actin was used as a protein control of constitutive expression. Treatments were applied for 13, 19 and 24 h. The represented values correspond to the mean of three different experiments. All values were corrected to the staining intensity of actin in control cells.

Figure 7 shows the response to the E3 fraction. Increments in p53, Mdm and Bcl-2 were observed after treatment for 19 h (see also Fig. 8).

Variations in the expression of some other factors such as the kinases Akt and NFkB were also checked (Figs 5–8). Akt was increased after 19 or 24 h treatment with E1 extract (Figs 5 and 8) while NFkB was reduced at 13 h of E1 treatment (Fig. 5).

In the case of E2 treatments, minor variations in Akt and slight increments of pAkt were observed as time passed (Figs 6 and 8) and NFkB showed values of 80% of control at all time points (Fig. 6).

After treatment of DA-1 cells with the E3 fraction, the levels of Akt remained close to controls after 13 h, with a slight increase at 19 h and then a decrease. Its phosphorylated form p-Akt remained nearly constant through the treatment, although lower than in untreated cells (61%, 60% and 65% at 13, 19 and 24 h, respectively) (Figs 7 and 8). NFkB was reduced by E3 treatment (Fig. 7).

To further confirm an apoptosis effect induced by either of these extracts uncleaved caspase 3 was quantitated in DA-1 treated cells in comparison with untreated cells (Fig. 9). As can be observed, both E1 extract and semipurified E3 fraction produced a clear reduction of inactive caspase 3 in western blot analyses (Fig. 9), an effect clearly visible at 24 h. To the contrary, no effect was manifested by treatment with E2.

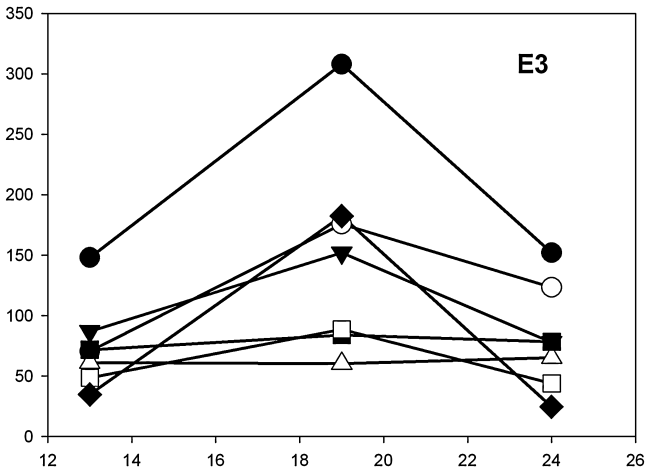

Figure 7. Expression analyses of DA-1 cells levels of apoptotic factors in DA-1 cells treated with methanol-extracted semipurified E3 fraction. DA-1 cells were incubated with E3 and western blots of proapoptotic and antiapoptotic proteins (p53 -●-, Bcl2 -○-, Bax -■-, Akt -▼-, pAkt -▽-, Mdm-2 -◆- and NFkB p65 -□-) were carried out. Control represents untreated cells. Actin was used as a protein control of constitutive expression. Treatments were applied for 13, 19 and 24 h. The represented values correspond to the mean of three different experiments. All values were corrected to the staining intensity of actin in control cells.

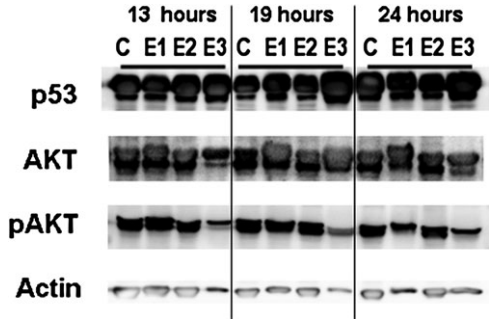

Figure 8. Western blot analyses of p53, Akt and p-Akt in DA-1 cells treated independently with E1 extract, E2 extract or methanol-extracted semipurified E3 fraction. DA-1 cells were incubated as described in the Methods section and western blots of p53, Akt and pAkt were carried out. Treatments were done for different times (13, 19 and 24 h). The figure shows a representative experiment.

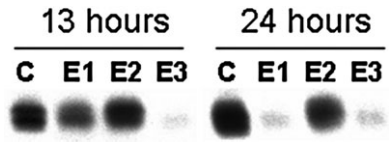

Figure 9. Western blot analyses of procaspase 3 levels in DA-1 cells treated independently with E1 extract, E2 extract or methanol-extracted semipurified E3 fraction. DA-1 cells were incubated as described in the Methods section and western blot of caspase 3 was carried out. The blot shows the region corresponding to the uncleaved procaspase 3 band. Treatments were done for different times (13 and 24 h). The figure shows a representative experiment.

DISCUSSION

Antitumor compounds can exert toxic effects on different kinds of cells and induce apoptosis in tumor cells. *Ganoderma lucidum* is a mushroom with components that have been claimed to show antitumor properties. Some authors have shown inhibition of the growth of solid tumors of sarcoma by using aqueous extracts from *G. lucidum* (Sone *et al*., 1985). For instance, Xie *et al*. (2006) observed significant changes in the induction of cell death in breast cancer and lymphoma cells alter treatment with a polysaccharide extract from *G. lucidum*. In contrast, Sliva *et al*. (2002) or Jiang *et al*. (2004a) in breast and prostate cancer cells, Cao and Lin (2004) in endothelial cells and Müller *et al*. (2006) in different lymphoma cell lines did not observe significant changes in cell viability alter treatment with different extracts from *G. lucidum*.

On the basis of previous studies (Gottlieb *et al*., 1994, 1996; Gottlieb and Oren, 1998; Olmos *et al*., 2005a, 2005b), the activity of aqueous and alcohol *Ganoderma lucidum* extracts were studied on DA-1 lymphoma cells. A reduction in viability was observed after treatment with an unboiled aqueous extract (E1) that was obtained similar to that described by others (Sliva *et al*., 2002; Stanley *et al*., 2005) (Figs 1 and 2). Thus, aqueous extraction from *Ganoderma lucidum* renders some compounds with toxicity on DA-1 lymphoma cells (Figs 1 and 2). The E3 fraction also showed toxic effects on lymphoma cells reducing cell viability up to 36% (Figs 1 and 2). These results are in accordance with those results described by other authors (Lin, 2005) that showed inhibition of cell growth in liver cancer cells (Huh-7) after treatment with alcohol extracts from *G. lucidum*.

The results shown in this work (Figs 3 and 4) indicate that the E1 extract and E3 fraction induced fragmentation of DNA, measured as subdiploid DNA. These data are similar to those described by Jiang *et al*. (2004b) who showed programmed cell death induced by *G. lucidum* in prostate cancer PC-3 cells and by Cao and Lin (2004) who described the apoptosis induction in endothelial cells from umbilical cord by a polysaccharidic extract from *G. lucidum*. Müller *et al*. (2006) also described apoptosis in lymphoma cells induced by a methanol extract from *G. lucidum* containing C2 ganoderic acid. The data are also in accordance to that described by Hu *et al*. (2002) who showed inhibition of the proliferation of breast cancer MCF-7 in a time-dependent way and induction of apoptosis after treatment with an alcohol extract from *G. lucidum*. The results may also be compared with those described by Tang *et al*. (2006) who studied the action of ganoderic acid T in lung cancer 95-D cells finding induction of apoptosis in a time-dependent way. Li *et al*. (2005) also observed induction of apoptosis in human cancer HuH-7 cells by triterpenes from *Ganoderma amboinense*, in particular by ganoderic acid X with inhibition of topoisomerases, which can be correlated to the presence of cytotoxic compounds in *Ganoderma* sp.

The present work checked whether the observed cell death may be mediated by changes in apoptosis factors. p53 is known to play an essential role in apoptosis induced by some antitumor compounds (Lowe *et al*., 1994; Newcomb, 1995). In some cell types, p53 is actually required for an apoptotic response (Messmer and Brune, 1997; Palacios *et al*., 2000). p53 has been identified in many cases as an initiator of apoptotic signals (Merino and Cordero-Campaña, 1998) being a regulator of cell cycle progression and a mediator of apoptosis in response to DNA damage. Tang *et al*. (2006) observed an increase in p53 levels in lung cancer 95-D cells after

treatment with ganoderic acid T assuming that this activation of p53 could be stimulated by DNA damage induced by this compound (Li *et al*., 2005). Cerebrosides isolated from *G. lucidum* inhibited DNA polymerases and induced DNA damage (Mizushina *et al*., 1998). In the present study, treatment of DA-1 cells with E1 or E3 fraction produced increments in p53 levels after 19 and 24 h (Figs 5–7), particularly for E3. Thus, this protein might be involved in cell death induced in DA-1 lymphoma cells by *G. lucidum* (Figs 5–7). In contrast, the modulation of p53 by E2 extract was quite modest.

p53 regulates the expression of Mdm2 and Bcl-2 (Barak *et al*., 1993) both of which inhibit the apoptosis mediated by p53 (Chiu *et al*., 1994; Chen *et al*., 1996). In the case of Mdm2, the pattern of expression showed a reduction for E1 and E3 treatments at 13 h. One common factor between E1 and E3 appears to be the up/down-regulation of p53/Mdm2, whereas this was not the case for the E2 extract.

p53 could also regulate the expression of *bax* (Miyashita and Reed, 1995), which promotes cell death (Allen *et al*., 1998). A reduction of Bcl-2 levels was observed in DA-1 cells after treatment for 13 h with E1 or E2 extracts or E3 fraction from *Ganoderma lucidum* (Figs 5 and 7). Cao and Lin (2004) also observed a reduction of the levels of Bcl-2 and an increment of the levels of Bax, in HUVEC cells treated with polysaccharides from *G. lucidum*. Tang *et al*. (2006) found that Bax expression increased in lung cancer 95-D cells after treatment with ganoderic acid T maintaining the levels of Bcl-2. These authors suggested apoptosis induction by ganoderic acid T via alteration of the Bax/Bcl-2 ratio. Jiang *et al*. (2004b) found that an aqueous extract containing polysaccharides and triterpenes from *G. lucidum* reduced the expression of Bcl-2 in prostate cancer PC-3 cells and increased the levels of Bax, correlating changes in Bax/Bcl-2 ratio with apoptosis induction (Raisova *et al*., 2001). The increase of Bax in cells treated for 13 h with *Ganoderma lucidum* E1 or E2 extract could suggest a role of Bax in the apoptosis induced by these extracts (Figs 5 and 6). In the case of the E3 fraction (Fig. 7), Bax disminished slightly with respect to untreated cells while Bcl-2 increased, showing low values for the Bax/Bcl-2 ratio which could be characteristic of resistant cells.

NF-kB is a factor related to cell growth, survival, angiogenesis, adhesion and migration (Mayo and Baldwin, 2000; Shibata *et al*., 2002). Jiang *et al*. (2004a) studied the effect of an aqueous extract containing polysaccharides and triterpenes from *G. lucidum* in MDA-MB-231 breast cancer cells showing inhibition of NF-kB. A similar result has been described in PC-3 prostate cancer cells (Jiang *et al*., 2004b). In cultures of rat cortical neurons exposed to hypoxia, Zhao *et al*. (2004) also observed a blockage in the activation of NF-kB as a consequence of a polysaccharidic extract from *G. lucidum*. From the results an inhibition of NF-κB can be inferred, since E1, E2 or E3 reduced the levels of NF-kB in DA-1 cells (Figs 5–7).

Jiang *et al*. (2004a) studied the implication of Akt on the inhibitory effect of an aqueous extract containing polysaccharides and triterpenes from *G. lucidum* on NF-kB in MDA-MB-231 cells finding an inhibition of Akt expression in a dose- and time-dependent way. Although they did not find a reduction in phosphorylation of Akt (pAkt) in Thr^{308}, they observed a reduction in the levels of pAkt Ser^{473}. Stanley *et al*. (2005) carried out a similar study in PC-3 cells, finding no changes either in Akt expression or in its phosphorylation in Thr^{308}. However, the levels of pAkt in Ser^{473} disminished in a dose-dependent response. The results show that independent treatments with any of the extracts or E3 fraction from *G. lucidum* slightly increased the levels of Akt at 19 h. In the case of the E1 extract and E3 fraction (Figs 5, 7 and 8) the levels of Akt decreased at 24 h in accordance with Jiang *et al*. (2004a, 2004b) who carried out longer treatments (up to 96 h). The E1 extract treatment also produced a very slight reduction of pAkt with treatment for 24 h which could correlate with that shown by Jiang *et al*. (2004a, 2004b). Stanley *et al*. (2005) also showed a reduction of the level of p-Akt-Ser^{473} with treatments for 24 h with different doses of an aqueous extract from *Ganoderma lucidum* containing polysaccharides and triterpenes. The E2 extract induced a reduction of Akt and pAkt Ser^{473} at 13 h (Figs 6 and 8) with an increase of Akt at longer times. The correlation of the effects of E1, E2 extracts or E3 fraction on cell viability and DNA fragmentation in DA-1 cells (Figs 1 and 3) with the involvement of Akt expression and its phosphorylation on the cytotoxic action of *Ganoderma lucidum* in DA-1 cells can not yet be clearly established.

Finally, apoptosis induction by either the E1 extract or E3 fraction on DA-1 cells was clearly confirmed since caspase 3 activation was stimulated (Fig. 9). Cleavage of inactive caspase 3 demonstrates the role of the apoptosis mechanism in the cytotoxicity induced in DA-1 cells by the E1 extract or E3 fraction from *Ganoderma lucidum*.

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Ethics

This work accomplishes all the ethical institutional or national requirements.

Conflict of Interest

The authors declare no conflict of interest with this work.

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