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Geldanamycin inhibits trichostatin A-induced cell death and histone H4 hyperacetylation in COS-7 cells

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Abstract

As widely believed treating cells with trichostatin A (TSA), an inhibitor of histone deacetylase, results in histone H4 hyperacetylation and cell cycle arrest. This compound is often compared with other potential anticancer drugs in cell cycle, proliferation and differentiation research. Furthermore, geldanamycin (GA), a 90-kDa heat shock protein (HSP90) specific inhibitor, is a well-known potential anticancer agent. This study examines whether GA can affect the cellular functions induced by TSA. When using TSA treatment, although caused COS-7 cell death, pretreatment of 0.5 μg/ml GA for 30 min and an addition of 50 ng/ml TSA (GA + TSA) apparently averted cell death. Our results indicated that the cell survival rate was only approximately 20% when prolonged treatment was undertaken with 50 ng/ml TSA (TSA) alone for 24 h. In contrast, the cell survival rate was enhanced by two folds when treating with GA + TSA. Furthermore, DNA fragmentation assay revealed that fragmented DNA was produced 8 h after prolonged treatment with TSA alone. Within 16 h, the apoptotic percentages of TSA-treated cells were between 15-25%. In contrast, the other treatments did not exceed 6%. Furthermore, GA inhibited TSA-induced histone H4 hyperacetylation. Western blotting analysis further demonstrated that the HSP70 levels did not significantly increase in TSA-treated cells. However, the accumulated 70-kDa heat shock protein (HSP70) markedly increased up to 2 to 3 folds at 8 h in GA- and GA + TSA-treated cells, and the maximum amount up to 5 to 7 folds at 20 h. Conversely, HSP90 did not markedly increase in all treatments. Based on the results in this study,

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we suggest that apoptosis induced by TSA can be prevented by GA-induced increment of heat shock proteins, particularly HSP70.

Keywords: Geldanamycin; Trichostatin A; DNA fragmentation; Cell viability; 70-kDa heat shock protein; 90-kDa heat shock protein

Introduction

Tsuji et al. [1] first isolated trichostatin A (TSA) from *Streptomyces hygroscopicus*, as antifungal antibiotics active against *Trichophyton*. According to their results, TSA inhibits the proliferation of various cell lines. Yoshida et al. [2] identified TSA as a potent and specific inhibitor of the histone deacetylase, thus inducing hyperacetylation of core histones at extremely low concentrations and increasing the level of gene transcription. Synchronous cultures of normal rat fibroblasts revealed that TSA causes a specific arrest of the cell cycle in both G1 and G2 phases [3]. Recent investigations have demonstrated that TSA causes rat thymocytes, tumor cells and human immune cell apoptosis [4–6].

Other investigators have confirmed that geldanamycin (GA), a benzoquinone ansamycin antibiotic, binds specifically to HSP90 [7,8]. In addition, GA also disrupts signaling pathways mediated by steroids [8] and epidermal growth factors [9]. Administration of GA to cells can rapidly and selectively inhibit the activities to HSP90 and of its substrates [8,9], thereby destabilizing client proteins. Its ability to simultaneously stimulate depletion of multiple oncogenic proteins suggests that GA may contribute to cancer therapy [10]. In addition to inducing the activation of heat shock factor 1 (HSF1), GA increases the synthesis and cellular levels of heat shock proteins [11,12], particularly 70-kDa heat shock protein (HSP70). Conde et al. [13] demonstrated that exposed both rat neonatal cardiomyocytes and H9c2 cells to GA induce heat shock proteins, thus providing protection ischemic stress. Above results suggest that GA may offer a pharmacological means of increasing the level of heat shock proteins in cardiac tissue and, in doing so, protecting the heart against ischemic/reperfusion injury.

Nevertheless, the protective role of HSPs in cell death remains contentious [14,15]. First, HSPs do not have the same protective ability in all apoptosis models. Second, some studies reveal no protective effects of HSPs in apoptosis at all, although these molecules are induced during apoptosis. Third, in some models, HSPs have been considered as apoptosis enhancers: overexpression of HSP70 enhances T-cell receptor/CD3- and Fas/Apo-1/CD95-mediated apoptosis in Jurkat T cells, and overexpression of HSP90 increases the tumor necrosis factor (TNF)-α- -and cycloheximide-induced apoptosis of the human U-937 cell line [16,17]. Such conflicting data on HSP functions in apoptosis have been tentatively explained by the differential mechanisms used by distinct cells to respond to different apoptosis-inducing stimuli.

GA may counteract with TSA in cell death because GA increases induction of HSPs [11,12], a group of potential factors to protect cells from damage, while TSA induces cell death. To our knowledge, this study addresses for the first time the interactions between TSA

and GA, as well as in the roles of mediations, HSPs. In this study, used COS-7 cells as an experimental system to determine whether GA affects the cellular functions induced by TSA. Also examined herein is the expression of HSPs to explore whether HSPs are involved in the rescue of TSA-induced DNA fragmentation, an index of apoptosis.

Materials and methods

Reagents

Trichostatin A was obtained from Sigma (St. Louis, MO, USA), while geldanamycin was purchased from Calbiochem (La Jolla, CA, USA). Both reagents were dissolved in DMSO. PK buffer: 100 mM Tris-Cl pH 7.5; 12.5 mM EDTA; 150 mM NaCl; and 200 μg/ml proteinase K. Sample buffer: 62.5 mM Tris-HCl, pH 6.8; 2% SDS; 5% β-mercaptoethanol; 10% glycerol; and 0.002% bromophenol blue. TTBS: 20 mM Tris-HCL, pH 7.4; 500 mM NaCl; 0.05% Tween 20. Developing buffer: 15 mg of nitro blue tetrazolium; 0.7% N,N-dimethylformamide; 30 mg of 5-bromo-4-chloro-3-indolyl phosphate per 100 ml; 1 mM $MgCl_2$; and 100 mM $NaHCO_3$, pH 9.8. All other reagents used herein were of analytical grade.

Cell line

COS-7 cells [18] were maintained in Dulbecco's modified eagle's medium supplemented with 10% FBS at a 5% CO_2 and 37°C incubator. In this study, the cells were cultured in 75 cm^2 flasks or 6-well plates with or without coverslips.

Analysis of cell viability

The cells were seeded on 6-well plates at 1.0×10^5 cells/well. After culturing for 24 h, the cells were treated with 0.2% DMSO (control), 0.5 μg/ml GA (GA), 50 ng/ml TSA (TSA), or pretreatment with 0.5 μg/ml GA for 30 min and then adding 50 ng/ml TSA for 24 h (GA + TSA). The cells were harvest by washed twice with phosphate buffered saline (PBS) and incubated with trypsin-EDTA, then suspended in isotonic buffer and counted by Coulter Counter (Coulter Electronic Inc., Luton, England).

Analysis of DNA fragmentation

The method was modified according to the method of Compton [19]. The cells were collected after treatment with GA, TSA, or GA + TSA at 8 h. The cell pellets were suspended in 400 μl of PK buffer at 50°C for 3 h and extracted by phenol/chloroform. RNA was then degraded with 5 μl RNase at 37°C for 30 min and extracted by phenol/chloroform again. Finally, the samples were precipitated with alcohol at -70°C for 30 min, centrifuged, air dried and resuspended in water. The DNAs were performed on 1% gel electrophoresis in 1x TBE buffer.

Identification of apoptotic cells

The cells were double-staining with Annexin V-FITC (PharMigen) [20] and DNA-specific fluorochrome Hoechst 33258 dye (8μg/ml) [21]. The Annexin V was used to detect the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the cytoplasmic membrane, an early event in the apoptotic process. In addition, Hoechst 33258 was used to exhibit condensed chromatin, a feature of the apoptotic cells. In the experiment, the cells (1.0×10^5 cells) were cultured in 6-well plates with coverslips. After treatment with vehicle, GA, TSA, or GA + TSA for 8, 12, and 16 h, the cells were washed with PBS for twice and fixed with 95% alcohol. Following PBS washed, stained with Annexin V-FITC for 15 min, and washed with PBS for three times, then stained with Hoechst 33258 for 5 min and washed with PBS again, finally mounting the slides with 70% glycerol. Apoptotic cells exhibiting characteristic chromatin condensation and FITC positive were counted by fluorescence microscopy (Zesis, Axiovert 135). In each sample, a minimum of 500 cells was counted and apoptotic nuclei were expressed as a percentage of total nuclei.

Western blotting analysis

Equal amounts of cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [22] and transferred onto a nitrocellulose membrane (Hybond-C extra, Amersham) by a semi-dry method (OWL Scientific Plastics, Cambridge, UK). Protein concentration was determined by adopting the Lowry method [23] in which bovine serum albumin was used as the standard. For Western blotting analysis, the membrane was incubated for 1 hr with 3% gelatin in TTBS and then rinsed with TTBS. Subsequently, a polyclonal antibody against acetylated histone H4 (Serotec; diluted 1:500), porcine HSP90 [24] (diluted 1:500), human HSP70 (Sigma; diluted 1:500) or actin (Sigma; diluted 1:2000) was added and incubated for 1 hr at room temperature. After three washes with TTBS, the membrane was incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma; diluted 1:2000 in TTBS containing 1% gelatin) at room temperature for 30 min. The membrane was then rinsed three times with TTBS and developed at room temperature in a developing buffer. Next, bands of HSPs and actin (internal control) were quantified by densitometric scanning (PD 486-010, Molecular Dynamics, CA, USA), and the relative synthesis rate of HSPs were presented as sum of pixel values of each band divided by that of actin in the same lane.

Results

Effect of TSA and GA on cell viability

The growth response of the cells to TSA was studied by growing cells for 24 h in medium containing 50 ng/ml TSA. Rounded cells were barely detected at 16 h but were

abundantly found at 24 h after TSA treatment. As the result, the cell viability was reduced to 20% of the control (Fig. 1). However, this viability could be increased by up to 2 folds if cells pretreated with GA for 30 min before TSA treatment. Treatment of GA alone did not cause as much cell death as TSA, resulting about 60% viable cells if GA concentration was in the range of 0.25-2.0 μg/ml (data not shown). In the preliminary experiment, pretreatment of higher doses of GA could not more effectively inhibit the cell death induced by TSA. In addition, treatment of 50 ng/ml and 500 ng/ml TSA to cells had no significantly different viability at 24 h (data not shown). The above results indicated that GA prevented the cell death induced by TSA.

Characterization of cell death

To evaluate whether the cell death was caused by apoptosis, DNA fragmentation assay was performed with the DNA samples extracted from the GA-, TSA-, or GA + TSA-treated cells at 8 h. Exposure to 50 ng/ml TSA resulted in DNA ladder formation within 8 h, whereas fragmentation in GA and GA + TSA were absent (Fig. 2).

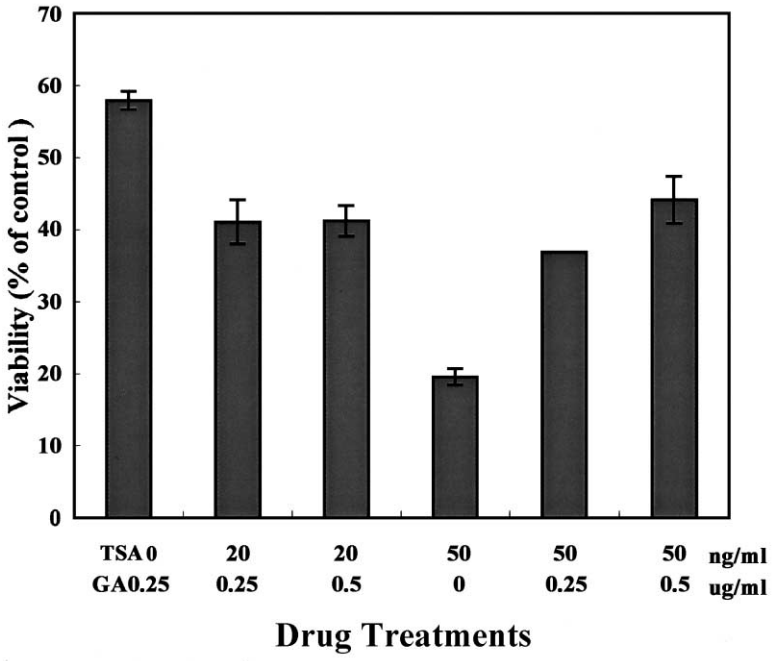

Fig. 1. Survival rate of GA-, TSA-, and GA + TSA-treated cells. The COS-7 cells were treated with 0.2% DMSO (control), 50 ng/ml TSA or pretreated 0.25 μg/ml or 0.5 μg/ml GA for 30 min, and then added 50 ng/ml TSA. After treatment for 24 h, the cell numbers were counted by Coulter Counter (Coulter Electronic Corp., England). The data are the mean ± S.E.M. from three independent experiments.

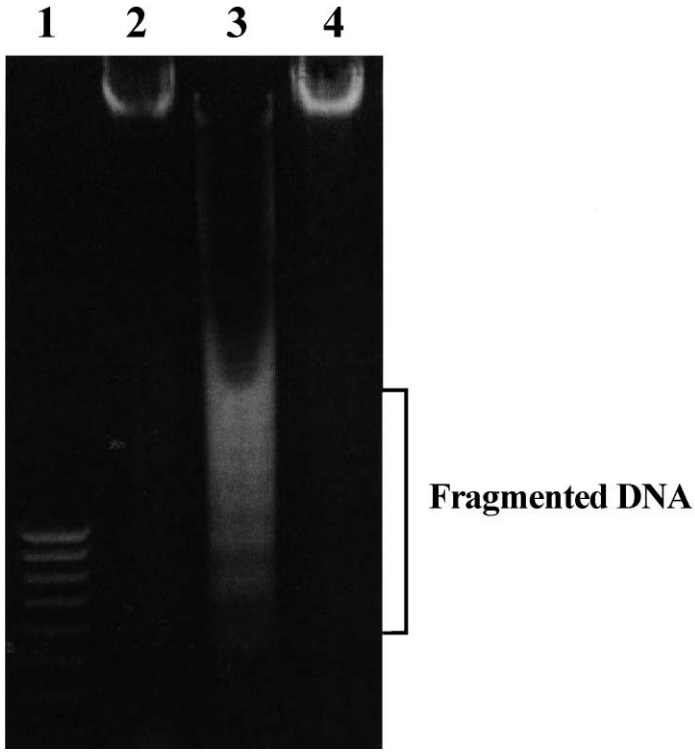

Fig. 2. DNA fragmentation assay of GA-, TSA-, and GA + TSA- treated cells. The DNAs were extracted at 8 h after the treatments, and subjected to electrophoretical separation on 1% agarose gel. Lane 1: 100 bp marker; Lanes 2: the cells were treated with 0.5 μg/ml GA; Lanes 3: the cells were treated with only 50 ng/ml TSA; Lanes 4: the cells were treated with GA + TSA.

Further identification of apoptotic cells was performed by staining cells with Hoechst 33258 and Annexin V-FITC. The Hoechst 33258 staining would reveal the condensation of chromatin of apoptotic cells. Meanwhile, the Annexin V-FITC would stain the PS, which translocated from the inner to the outer leaflet of the plasma membrane in the apoptotic cells. Condense blue lights and Annexin V-positive green lights were presented in the cells with chromatin condensation (Fig. 3). Cells showing the two signals were counted as apoptotic cells. Within 16 h, the apoptotic percentages of TSA-treated cells were between 15-25% (Fig. 4). In contrast, the other treatments did not exceed 6%.

Effects of TSA or GA+TSA on histone H4 acetylation

The effect of GA on histone H4 modification was assessed by monitoring histone H4 acetylation. TSA treatment caused a significant amount of hyperacetylation of histon H4 after 2 h (Fig. 5, lane 2). The maximum levels of acetylation occurred at 8 to 16 h and then deacetylated. However, comparing the cells treated with only TSA revealed that GA + TSA inhibited the acetylation levels of histone H4 in cells from 8 to 16 h.

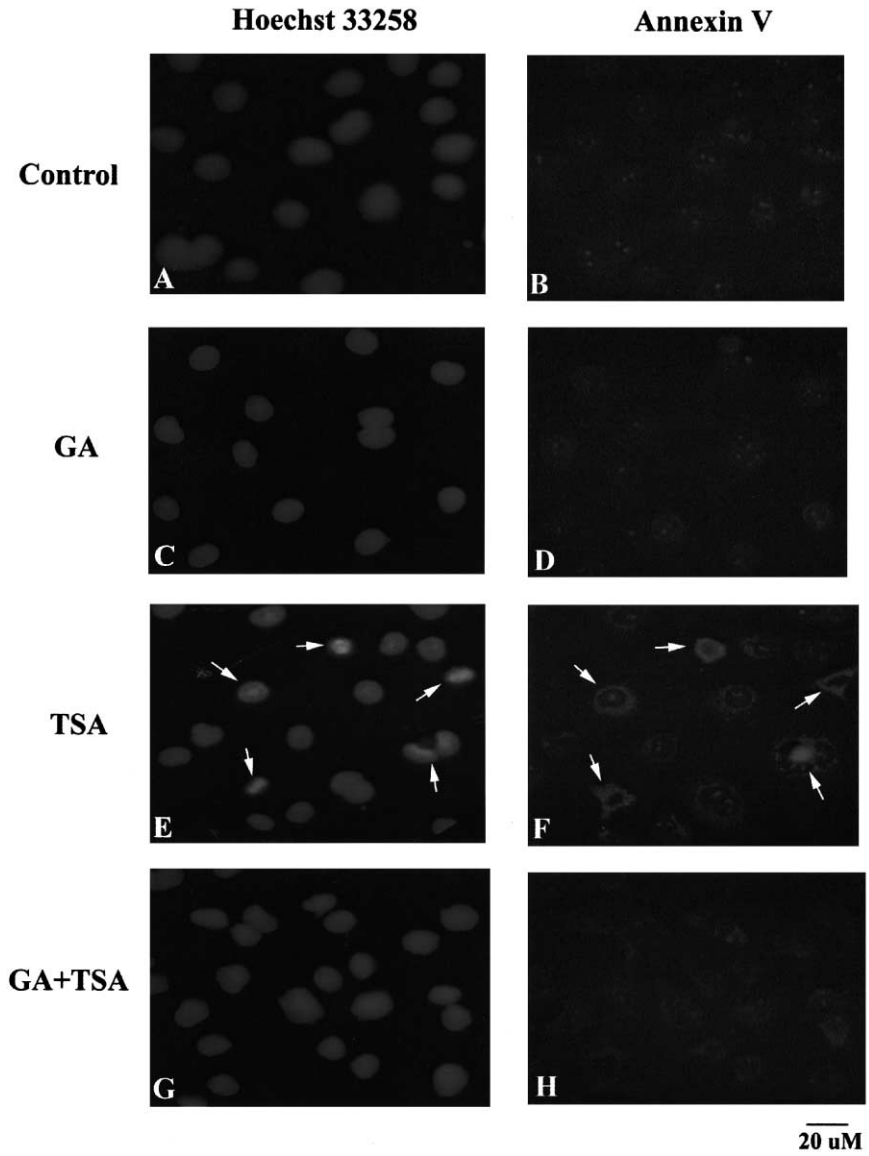

Fig. 3. Morphological study of apoptotic cells. After treatment for 8 h, the DMSO-, GA-, TSA-, and GA + TSA-treated cells were fixed with 95% alcohol and stained with Hoechst 33258 (A, C, E, and G) and Annexin V-FITC (B, D, F, and H). The condense chromatin of the apoptotic cells were stained bright blue (Fig. 3E indicated by arrow) and only the membranes of the apoptotic cells were stained green light (Fig. 3F indicated by arrow).

Induction of heat shock proteins by GA

The effect of GA treatment on the cellular protein induction was examined by analyzing the synthesis of HSPs by Western blotting analysis. Analysis results indicated that HSP70

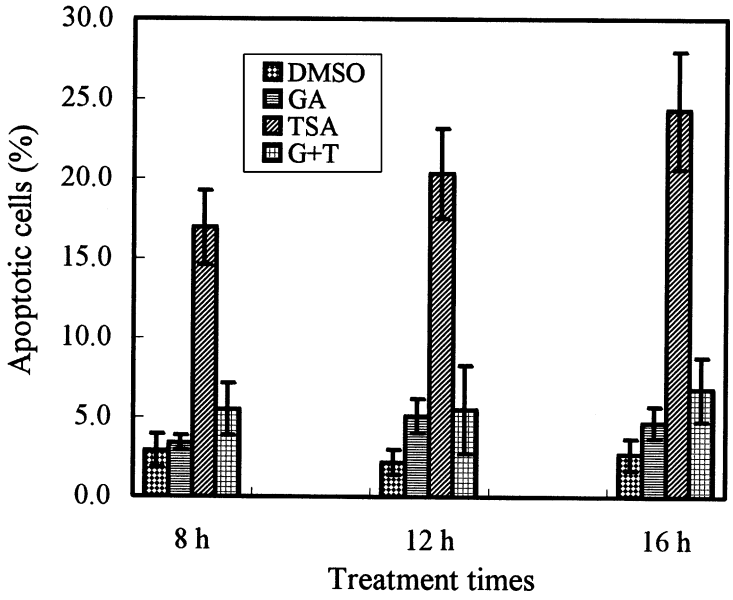

Fig. 4. Percentage of apoptotic cells at various times. After treatment with vehicle, GA, TSA, or GA + TSA for 8, 12, and 16 h, the cells were fixed with 95% alcohol, followed by staining with Hoechst 33258 and Annexin V-FITC. Apoptotic cells exhibiting characteristic chromatin condensation and FITC positive were counted by fluorescence microscopy (Zesis, Axiovert 135). In each sample, a minimum of 500 cells was counted and apoptotic nuclei were expressed as a percentage of total nuclei. The data are the mean ± S.E.M. from three independent experiments.

was significantly induced approximately 2.6 folds more the control after treatment with GA or GA + TSA for 8 h (Figs. 6a and 6b). The maximum amount was up to 5 to 7 folds for 20 h of treatment. In contrast, the cells treatment with only TSA did not significantly induce the

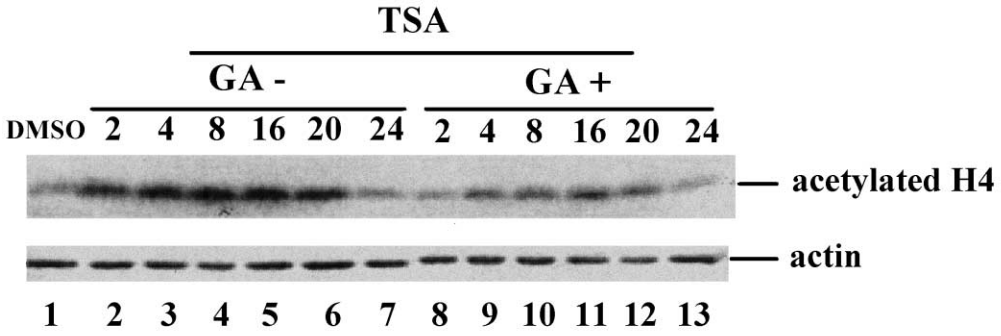

Fig. 5. Western blot analysis of acetylated histone H4. Cells were treated with 0.2 % DMSO (lane 1), 50 ng/ml TSA (lanes 2–7) or pretreated 0.5 μg/ml GA and then adding 50 ng/ml TSA for 2, 4, 8, 16, 20, and 24 h (lane 8–13). The proteins were performed on 14.5 % SDS-PAGE. Actin was an internal control. The position of the acetylated histone H4 and actin are indicated. The results shown are representative of three separate experiments, which yielded similar results.

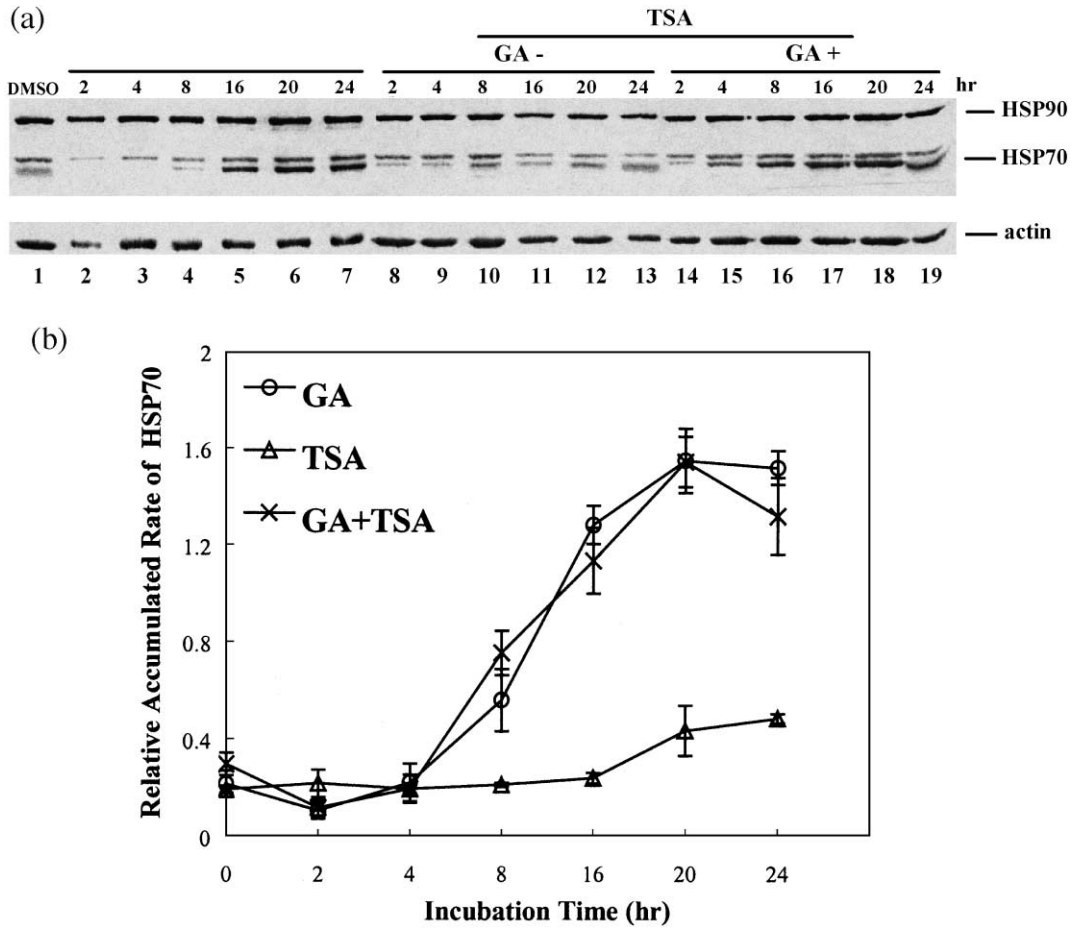

Fig. 6. Western blot analysis of HSPs expression. Cells were treated with 0.2% DMSO (lane 1), 0.5 μg/ml GA (lanes 2–7), 50 ng/ml TSA (lanes 8–13), and pretreated with 0.5 μg/ml GA and then adding 50 ng/ml TSA for 2, 4, 8, 16, 20, and 24 h (lane 14–19). At the time interval indicated, the cell lysates were collected and the proteins were performed on 9% SDS-PAGE. The accumulated HSPs were analyzed by Western blotting analysis (a). The position of the HSP90, HSP70, and actin are indicated. Bands of HSP70 and actin as shown in A were quantified by densitometric scanning, and the relative synthesis rate of HSP70 was presented as a sum of pixel values for each band divided by that of actin in the same lane (internal control) (b). Data represent the means ± S.E.M. of three independent experiments.

synthesis of HSP70. Furthermore, various treatments did not markedly enhance the accumulated levels (Fig. 6a) and *de novo* synthesis of HSP90 (data not shown).

Discussion

This study has demonstrated that TSA induced histone H4 hyperacetylation and DNA fragmentation. However, treatment with GA + TSA inhibited the hyperacetylation of histone

H4 and DNA fragmentation as well. A previous investigation indicated that TSA is a specific inhibitor of histone deacetylase [2]. Herein, acceleration of histone H4 acetylation was observed 2 h after the cells received TSA treatment. This event was found a few hours earlier than the DNA fragmentation. The finding corresponds to that found in other cell system [4].

Apoptosis is a form of cell death, which characteristic changes, including PS externalization, cell shrinkage, plasma membrane blebbing, chromatin condensation and DNA fragmentation [19–21,25]. According to our results, DNA fragmentation, chromatin condensation, and PS externalization occurred after treatment with TSA alone. We believe that the cell death was due to apoptosis and correlated with hyperacetylation of histones. Histones mediate the folding of DNA into chromatin [26]. The acetylation of histones is regulated by histone acetyltransferase and histone deacetylase. Since exposure of cells to TSA leads to histones hyperacetylation and chromatin decondensation which increase DNase I sensitivity [27]. Therefore, hyperacetylation of nucleosomal histone with TSA appears to increase the accessibility of endonucleases to the chromatin DNA, thus causing DNA fragmentation. Conversely GA + TSA-treated cells inhibited DNA fragmentation due to a reduction of the hyperacetylation level of histone H4 and a decrease of the accessibility of endonucleases to the chromatin DNA.

In Fig. 5, the histone H4 of TSA-treated cells were hyperacetylation during 4 to 20 h. However, the acetylation level of histone H4 at 24 h was dramatically decreased to normal level. This phenomenon may be due to histone deacetylase (HDAC) inhibitor trigger an unknown regulatory loop to de novo synthesis HDAC. The evidences revealed that TSA induce HDAC gene expression in several cell types [28,29].

In our study, TSA-treated cells were also enhanced the acetylation levels of histone H2A, H2B, and H3, but GA-pretreated cells did not influence the acetylation levels, which were caused by TSA (data not shown). These results suggest that histone H4 may directly or indirectly interact with GA. Previous investigations have indicated that GA specific binds to HSP90 and disrupts the interaction of HSP90 and target proteins [8,30], implying that HSP90 interacts with histone H4 and regulates the conformation of histone H4. Several investigations have conferred that HSP90 binds histones and induces a condensation of the chromatin structure [31,32]. Once GA inhibits the function of HSP90, the lysines of histone H4 cannot be modified properly by TSA.

In addition to inhibiting DNA fragmentation, GA increased the synthesis of HSP70, implying that HSP70 is an effective protector of cells. Jaattela et al. [33] indicated that HSP70 inhibited late caspase-dependent events such as activation of cytosolic phospholipase A2 and changes in nuclear morphology. HSP70 also conferred significant protection against cell death induced by enforcing the expression of caspase-3. Thus, HSP70 rescues cells from apoptosis later in the death-signaling pathway than any known anti-apoptotic protein, making it a tempting target for therapeutic interventions. Furthermore, Creagh et al. [34] also demonstrated that the inhibition of caspase-dependent and –independent apoptosis by HSP70 is an important anti-apoptotic regulator, functioning at a very early stage in the apoptotic pathway. Recent data have demonstrated that HSP70 is mediated through its direct association with the caspase-recruitment domain (CARD) of Apaf-1 and through inhibition of apoptosome formation [35, 36].

Pandey et al. [37] have confirmed that HSP90 forms a cytosolic complex with Apaf-1 and thereby inhibits the formation of the active complex of apoptosis. However, HSP90 exerts its protective function possibly by modulating the conformation of Apaf-1 or by inhibiting the affinity of Apaf-1 for binding to cytochrome c. In our results, GA did not significantly increase HSP90 synthesis. Our future study will further explore the protective role of HSP90 for cell death.

Acknowledgments

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