Tanshinone IIA induces apoptosis in human lung cancer A549 cells through the induction of reactive oxygen species and decreasing the mitochondrial membrane potential

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Abstract. Tanshinone IIA (Tan-IIA) is extracted from Danshen and known to inhibit proliferation and induce apoptosis in many cancer cells. We aimed to elucidate its anticancer activity and molecular mechanism in human lung cancer A549 cells. The cytotoxicity of Tan-IIA in A549 cells were measured by the MTT assay. The effects of Tan-IIA on the cell cycle, mitochondrial membrane potential (MMP), calcium and reactive oxygen species (ROS) released in A549 cells were detected by flow cytometry. The protein expressions of p53, Bax, Bcl-2 and ß-actin in A549 cells were tested by Western blotting. The proliferative rates of A549 cells were obviously inhibited by Tan-IIA in a dose- and time-dependent manner. The results of FACS showed that the sub-G1 phase was increased when A549 cells were cultured with various concentrations of Tan-IIA (control, 2.5, 5 and 10 μg/ml) for 48 h. Tan-IIA induced the production of ROS, Ca^{+2} and decreased MMP. The outcome of Western blotting showed that protein expressions of p53 and bax were increased, but proto-oncogene bcl-2 was notably decreased, after culturing with Tan-IIA (5 μg/ml) for 6, 12 and 24 h. Tan-IIA inhibited the proliferation of non-small cell lung cancer A549 cells, possibly by decreasing the MMP and inducing apoptosis due to the induction of a higher ratio of Bax/Bcl-2.

Introduction

Lung cancer remains deadly, prevalent and costly to patients and society. According to a 2004 report from the 'People's Health Bureau of Taiwan', lung cancer is the leading cause of cancer related deaths in Taiwan. Lung cancer is also the leading cause of cancer-related morbidity and mortality in Western countries (1). Consequently, it has become a significant public health problem. However, chemotherapeutic treatment for lung cancer is still unsatisfactory. There is clearly an ongoing need for more effective treatments. Alternative medicines offer likely therapeutic agents to treat a large variety of malignant cancers. Tanshinone IIA (Tan-IIA; $C_{19}H_{18}O_3$) was extracted from Danshen, *Salviae Miltiorrhizae* Radix (2,3), which is widely adopted and prescribed in traditional Chinese medicine to treat cardiovascular diseases (4-6). It was well documented that Tan-IIA possesses anti-inflammatory activities (7,8) and anti-oxidant properties (9,10). In addition, previous studies have also demonstrated that Tan-IIA causes significant growth inhibition and apoptosis induction in human leukemia cells (11), breast cancer MDA-MB-231cells (12), colon cancer colo205 cells (13) and hepatocellular carcinoma cells (14). However, it is still unclear whether Tan-IIA has the ability to induce cell growth reduction and apoptosis in human lung cancer cells. In the present study, we investigated the roles of Tan-IIA in inducing proliferation inhibition and apoptosis-associated molecular protein expression in human non-small cell lung cancer A549 cells.

Materials and methods

Chemicals and reagents. Tan-IIA (molecular formula, $C_{19}H_{18}O_3$; >96% HPLC) was purchased from Herbasin Co. (Shenyang, China). Aprotinin, antipain, sodium deoxycholate, leupeptin, propidium iodide (PI), sodium orthovanadate, Triton X-100, Tris-HCl, ribonuclease-A and MTT [3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazolium bromide] were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer were purchased from Merck Co. (Darmstadt, Germany). RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). 10X SDS-PAGE running buffer, Tris, Tween-20, SDS, 5X TBE buffer were obtained from Amresco (St. Louis, MO, USA). BioMax Flim was obtained from Kodak. DioC6 (84715) and $H_2DCFDA_{(C6827)}$ were obtained from Biocompare, USA). Anti-ß-actin (MAB1501; Chemicon), anti-p21 (MS-891-PO; NeoMarkers)

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anti-p53 (MS-256-PO; NeoMarkers), anti-Cytochrome c (cat#pc323; Calbiochem), anti-Bcl-2 (N-19-sc-492; Santa Cruz Biotechnology, Inc), anti-Bax (N-19-sc-492; Santa Cruz Biotechnology, Inc). Goat anti-mouse IgG (HRP) horseradish peroxidase conjugated antibody (AP124P), was obtained from Chemicon.

Human lung cancer cell line (A549 cells). The human lung cancer A549 cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The A549 cells were placed into 25-cm^2 tissue culture flasks and maintained in RPMI-1640 contained with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin and grown at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. All data presented in this report are from at least three independent experiments.

The effects of Tan-IIA on viability of A549 cells. The A549 cells were plated in a density of $1x10^5$ cells per well and grown for 24 h. Various concentrations of Tan-IIA were added and cells were grown for 24, 48 and 72 h, while only adding DMSO 0.2% (solvent) for the control regimen. For determining cell viability, after 24, 48 and 72 h of culture, viability was evaluated by MTT assay (15) in triplicate. Briefly, A549 cells were seeded in a 6-well plate at a density of $1x10^5$ cells/well and allowed to adhere overnight. After removing the medium, 2,000 μl of fresh medium per well, containing 10 mM HEPES was then added and 200 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to the wells and the plate was incubated for 30 min at 37˚C in the dark. Medium was removed and 1,000 μl DMSO was added to the wells. Absorbance was measured using an ELISA plate reader at 590 nm.

Cell cycle analysis for A549 cells treated with Tan-IIA by flow cytometry assay. The percentage of cells in sub-G1, G0/G1, S and G2/M phases were determined by flow cytometry as described previously (16). Briefly, A549 cells ($1x10^6$/10 cm dishes) were incubated with various concentrations of Tan-IIA (0, 2.5, 5 and 10 μg/ml) for 48 h before cells were harvested by centrifugation. After being harvested, cells were washed with PBS, then fixed gently (drop by drop) in 70% ethanol (in PBS) in ice overnight and resuspended in PBS containing 40 μg/ml PI, 0.1 mg/ml RNase (Sigma) and 0.1% Triton X-100. After 30 min at 37˚C in the dark, cells were analyzed with flow cytometry (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm. Then, cell cycle and apoptosis were determined and analyzed using the ModFit software. The average of the percentage of each phase in the cell cycle was representative of three independent experiments.

Flow cytomertry detection of ROS in A549 cells after treatment with Tan-IIA. The levels of ROS in A549 cells were examined and determined by flow cytometry (Becton Dickinson FACS Calibur) as described previously (17). Briefly, 2, 7-dichlorodi-hydrofluorescein diacetate (DCFH-DA, Biocompare, USA) was used for staining. Cells were treated with or without Tan-IIA for different durations (0, 6, 12, and 24 h) to detect changes of ROS. Cells were harvested and washed twice, resuspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate solution (10 μM), incubated at 37˚C for 30 min and analyzed by flow cytometry.

Flow cytometry detection of Ca^{2+} concentrations in A549 cells after treatment with Tan-IIA. The Ca^{2+} levels in A549 cells were determined by flow cytometry as described previously (17). Briefly, using Indo 1/AM (Calbiochem; La Jolla, CA, USA) for staining. Cells were pre-treated with or without Tan-IIA for different durations (0, 6, 12, and 24 h), harvested and washed twice, then resuspended in Indo 1/AM (3 μg/ml), incubated at 37˚C for 30 min, and the changes of Ca^{2+} concentrations were analyzed by flow cytometry.

Flow cytometry detection of MMP in A549 cells after treatment with Tan-IIA. The MMP levels in A549 cells were determined by flow cytometry as described previously (17). Briefly, DiOC6 (4 mol/l, Biocompare) was used for staining. Cells were treated with or without Tan-IIA for different durations (0, 6, 12, and 24 h), harvested and washed twice, resuspended in 500 μl of DiOC6 (4 mol/l), incubated at 37˚C for 30 min and the changes of MMP were analyzed by flow cytometry.

Western blotting for examination of the effect of Tan-IIA on p53, Bcl-2, Bax, and Cytochrome c levels in A549 cells. Total proteins were collected from A549 cells after treatment with or without various concentrations of Tan-IIA for 0, 6, 12, and 24 h before p53, Bcl-2, Bax, and Cytochrome c (Cyto-c) levels were examined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, as described previously (12,18).

DNA fragmentation electrophoresis analysis from A549 cells co-treated with different concentrations of Tan-IIA. A549 cells were plated in 6-well plates at a density of $5x10^6$ cells/well and grown for 24 h. Then different concentrations of Tan-IIA (0, 2.5, 5 and 10 μg/ml) were added, while only adding DMSO (solvent) for the control regimen and grown at 37˚C, in humidified 5% CO_2 and 95% air for 24 h. The DNA was prepared using Genome DNA isolation kit protocol (BIO 101, La Jolla, CA, USA). DNA fragmentation electrophoresis assay was used as described previously (19). We ran DNA on a 1.2% agarose gel containing EtBr on the power supply to 75 volts. It was visualized on a UV Trans illuminator.

Statistical analysis. Values are presented as percentage ±SD of control. The Student's t-test was used to analyze the statistical significance between the Tan-IIA treated and control groups. A p-value <0.05 was considered significant for all tests.

Results

Effects of Tan-IIA on cell viability. To investigate the inhibitory effect of Tan-IIA on cell growth of human lung cancer A549 cells, A549 cells were cultured and exposed to Tan-IIA at concentrations of 1, 2, 3, 4 and 5 μg/ml for 24, 48 and 72 h. The cytotoxicity of Tan-IIA in A549 cells was determined using the MTT assay. The percentages of viable cells relative to control were 85.94±0.35, 46.96±0.4, 21.27±0.12, 20.4±0.1, 17.97±0.15%, respectively, when cultured with various concentrations of Tan-IIA (1, 2, 3, 4 and 5 μg/ml) for 24 h. The

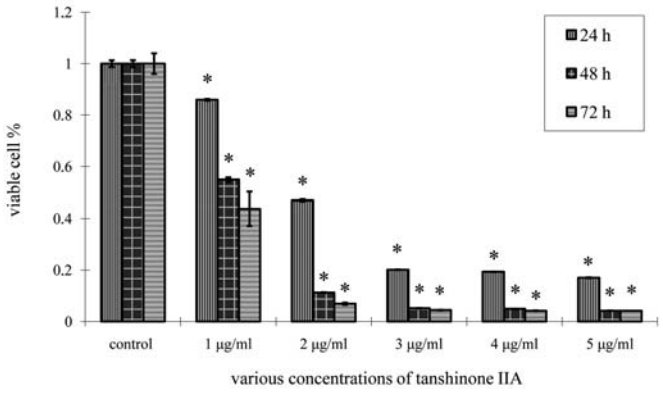

Figure 1. A549 cells were treated with increased Tanshinone IIA concentrations of 0, 1, 2, 3, 4 and 5 μg/ml for 24, 48 and 72 h. The cytotoxicity of Tanshinone IIA in A549 cells was determined using the MTT assay as described in Materials and methods. Tanshinone IIA significantly inhibited A549 cell growth. Each point is the mean ±SD of three experiments. *P<0.05.

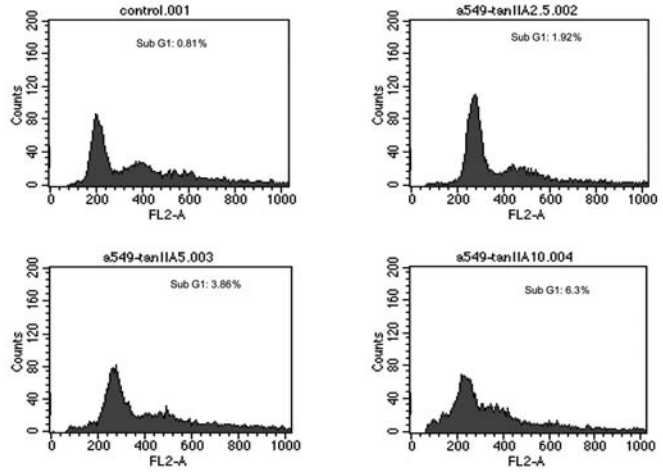

Figure 2. A549 cells were exposed to Tanshinone IIA concentrations of control, 2.5, 5 and 10 μg/ml for 48 h. Cells were harvested and cell cycle analysis was performed by flow cytometry as described in Materials and methods. The percentages of cells in the sub-G1 phase exhibited a significant increase after treatment with 2.5, 5 and 10 μg/ml of Tanshinone IIA for 48 h.

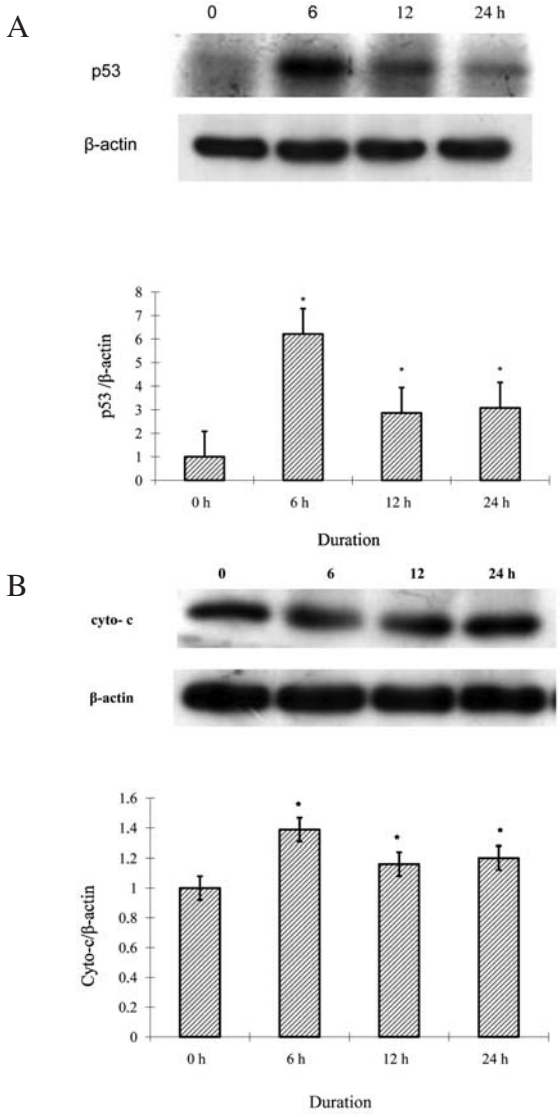

Figure 3. Protein expression of p53 and Cytochrome c (Cyto-c) in A549 cells after exposure to Tan-IIA. A549 cells (5x10^6/ml) were treated with Tan-IIA (2 μg/ml) for different durations (0, 6, 12 and 24 h) before being harvested by centrifugation. Total protein was prepared and determined as described in Materials and methods. Levels of (A) p53 and (B) Cyto-c expression were estimated by Western blotting as described in Materials and methods. Protein expression of p53 (A) and Cyto-c (B) was significantly increased after treatment with Tan-IIA for 6 h. Each point is the mean ±SD of three experiments. *P<0.05.

percentages of viable cells relative to control were 54.95±0.86, 11.23±0.14, 5.14±0.12, 4.91±0.06 and 4.18±0.06%, respectively, when cultured with various concentrations of Tan-IIA (1, 2, 3, 4 and 5 μg/ml) for 48 h. The percentages of viable cells relative to control were 43.7±6.59, 6.9±0.4, 4.44±0.12, 4.18±0.12 and 4.13±0.00% respectively, when cultured with various concentrations of Tan-IIA (1, 2, 3, 4 and 5 μg/ml) for 72 h. The IC_{50} concentrations for Tan-IIA to treat A549 cells for different durations (24, 48 and 72 h) were 2, 1.5 and 1 μg/ml. Results showed that Tan-IIA exhibited remarkable inhibition against the growth of A549 cells in a time- and dose-dependent manner (Fig. 1). BALB 3T3 fibroblast cells were used for positive control. The IC_{50} concentrations for Tan-IIA to treat BALB3T3 cells for different durations (24, 48 and 72 h) were 5.58, 6.08 and 9.77 μg/ml in our previous study (13).

Effects of Tan-IIA on cell cycle. In order to determine the effect of Tan-IIA on cell growth and apoptosis in A549 cells, cell cycle distribution was analyzed by flow cytometry. When A549 cells were treated with Tan-IIA (0, 2.5, 5 and 10 μg/m) for 48 h, the percentage of cells in the sub-G1 phase increased from 0.81 to 1.92, 3.86 and 6.3%, respectively (Fig. 2). Results showed that apoptosis increased after A549 cells were treated with various concentrations of Tan-IIA (0, 2.5, 5 and 10 μg/m) for 48 h.

Western blot investigation of the effect of Tan-IIA on p53, Bax, Bcl-2, and Cytochrome c levels. Protein expression of p53 and Cyto-c in A549 cells after exposure to Tan-IIA. A549 cells (5x10^6/ml) were treated with Tan-IIA (2 μg/ml) for

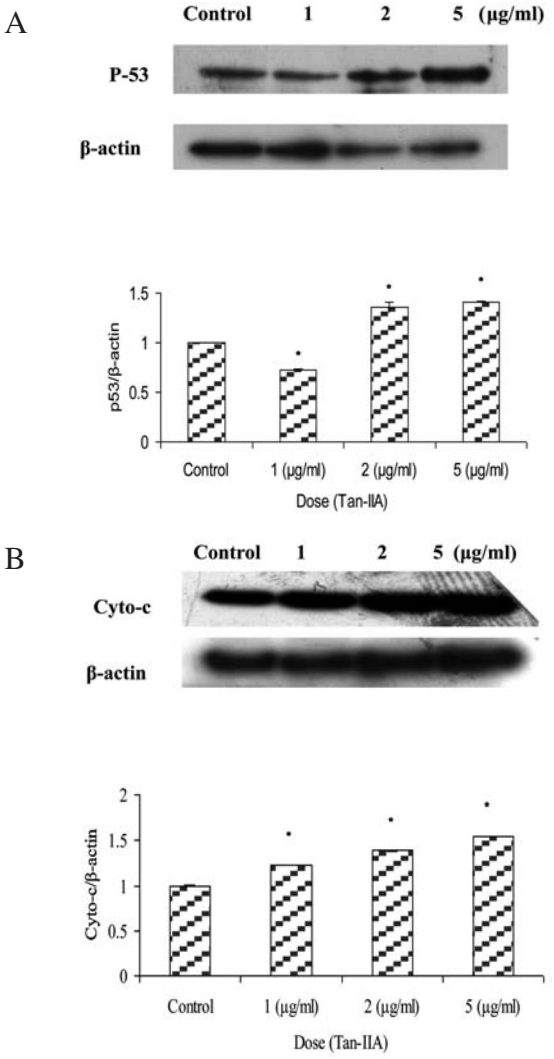

Figure 4. Representative Western blot showing changes on the levels of p53 (A) and Cyto-c (B) in A549 cells after exposure to Tan-IIA. The A549 cells ($5x10^6$/ml) were treated with various concentrations (1, 2 and 5 μg/ml) of Tan-IIA for 24 h, then cytosolic fraction and total protein were prepared and determined as described in Materials and methods. This was followed by evaluation of the levels of p53 (A) and Cyto-c (B) expressions which were estimated by Western blotting as described in Materials and methods. Tan-IIA increased the protein expression of p53 (A) and Cyto-c (B) at the concentrations of 2 and 5 μg/ml for 24 h. Each point is the mean ±SD of three experiments. *P<0.05.

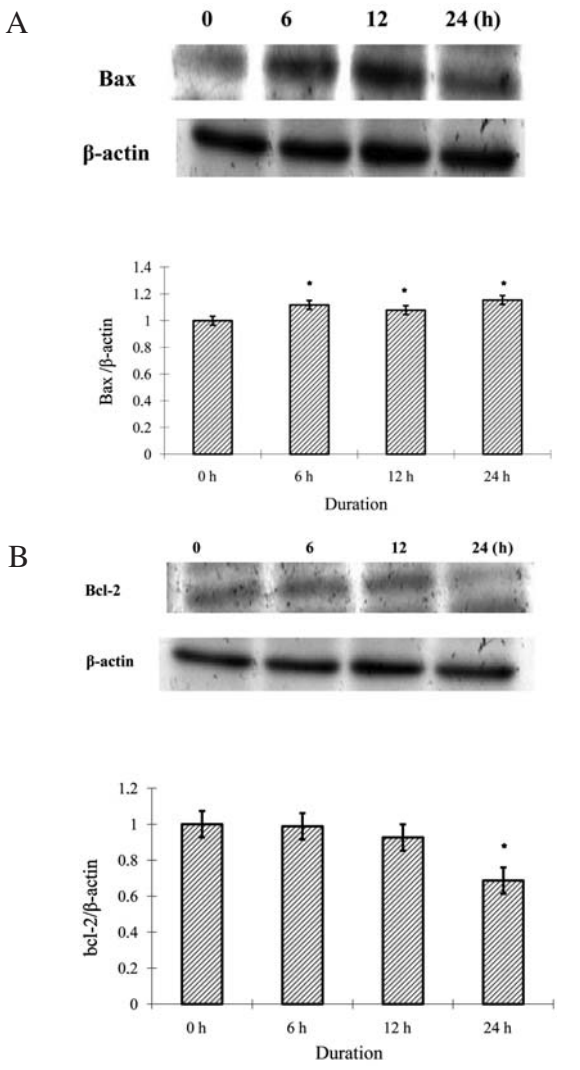

Figure 5. Protein expression of Bax and Bcl-2 in A549 cells after exposure to Tan-IIA. A549 cells ($5x10^6$/ml) were treated with Tan-IIA (2 μg/ml) for different durations (0, 6, 12 and 24 h) before being harvested by centrifugation. Total protein was prepared and determined as described in Materials and methods. Levels of (A) Bax and (B) Bcl-2 expression were estimated by Western blotting as described in Materials and methods. Protein expression of Bax (A) was significantly increased after treatment with Tan-IIA for 6, 12 and 24 h. The protein expression of Bcl-2 (B) was significantly decreased after treatment with Tan-IIA for 24 h. Each point is the mean ±SD of three experiments. *P<0.05.

different durations (0, 6, 12 and 24 h) before being harvested by centrifugation. Total protein was prepared and determined as described in Materials and methods. Levels of p53 and Cyto-c expression were estimated by Western blotting as described in Materials and methods. Protein expression of p53 (Fig. 3A) and Cyto-c (Fig. 3B) was significantly increased after treatment with Tan-IIA for 6 h. Representative Western blotting showed changes on the levels of p53 and Cyto-c in A549 cells after exposure to Tan-IIA. The A549 cells ($5x10^6$/ml) were treated with various concentrations of Tan-IIA (1, 2 and 5 μg/ml) for 24 h, then total protein was prepared and determined as described in Materials and methods. The levels of p53 and Cyto-c expressions were estimated by Western blotting. Tan-IIA increased the protein expression of p53 (Fig. 4A) and Cyto-c (Fig. 4B) at the concentrations of 2 and 5 μg/ml for 24 h. The protein expressions of Bax and Bcl-2 in A549 cells were exposed to Tan-IIA. A549 cells ($5x10^6$/ml) were treated with Tan-IIA (2 μg/ml) for different durations (0, 6, 12 and 24 h) before being harvested by centrifugation. Total protein was prepared and determined as described in Materials and methods. Levels of Bax and Bcl-2 expression were estimated by Western blotting. The protein expression of Bax (Fig. 5A) was significantly increased after treatment with Tan-IIA for 6, 12 and 24 h. The protein expression of Bcl-2 (Fig. 5B) was significantly decreased after treatment with Tan-IIA for 24 h.

Effects of Tan-IIA on the production of ROS of A549 cells. When A549 cells were treated with Tan-IIA (2 μg/m) for different durations (0, 6, 12 and 24 h), the ROS increasing percentages were 1.20±0.58, 12.35±2.12, 67.62±5.67 and 83.17±3.21%, respectively. Increasing time of incubation led

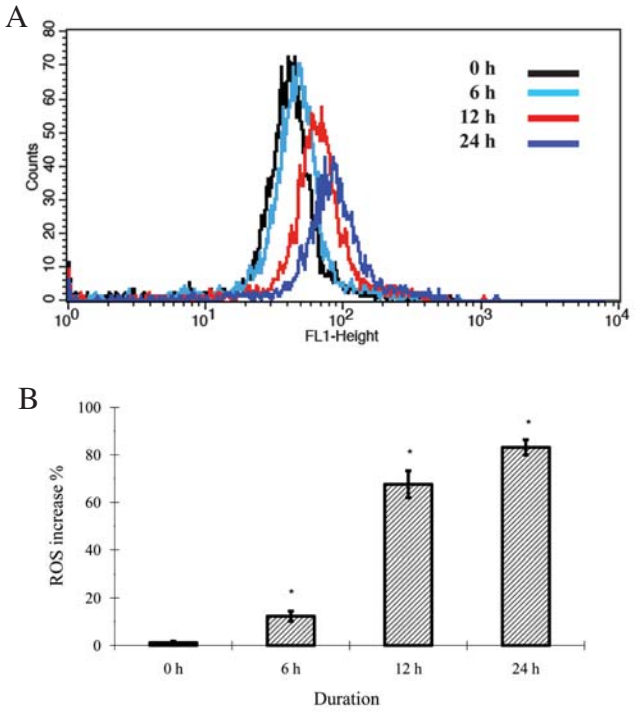

Figure 6. Effects of Tan-IIA on the production of ROS of A549 cells. A549 cells were treated with Tan-IIA (2 μg/ml) for different durations (0, 6, 12 and 24 h). ROS was evaluated as described in Materials and methods. Increasing ROS percentages were 1.20±0.58, 12.35±2.12, 67.62± 5.67 and 83.17±3.21%, respectively. Increasing the time of incubation led to an increase in the level of ROS in the examined A549 cells. Each point is the mean ±SD of three experiments. *P<0.05.

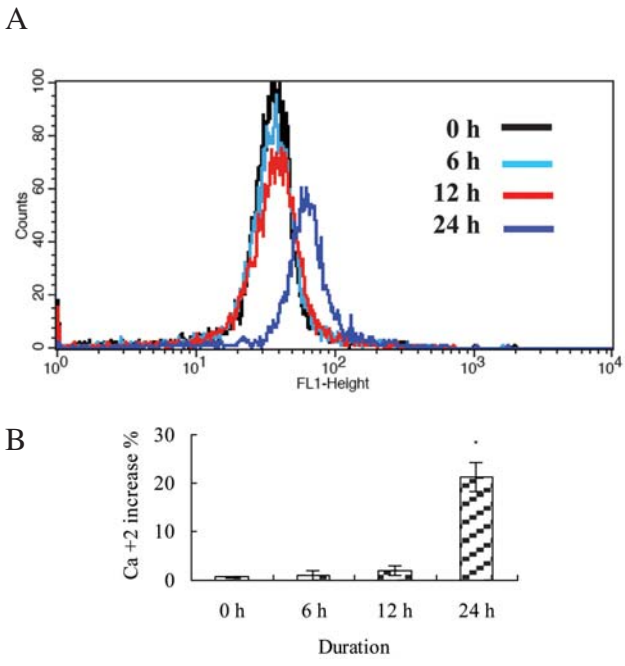

Figure 7. Effects of Tan-IIA on the production of Ca^{2+} of A549 cells. When A549 cells were treated with Tan-IIA (2 μg/ml) for different durations (0, 6,12 and 24 h). The Ca^{2+} was evaluated as described in Materials and methods. The increasing Ca^{2+} concentration percentages were 0.65±0.11, 1.02±1.00, 2.01±1.09 and 21.21±3.01%, respectively. The Ca^{2+} concentrations were significantly different between the Tan-IIA treated group and the control group. Increasing the time of incubation led to an increase in the concentrations of Ca^{2+} in the examined A549 cells. Each point is the mean ±SD of three experiments. *P<0.05.

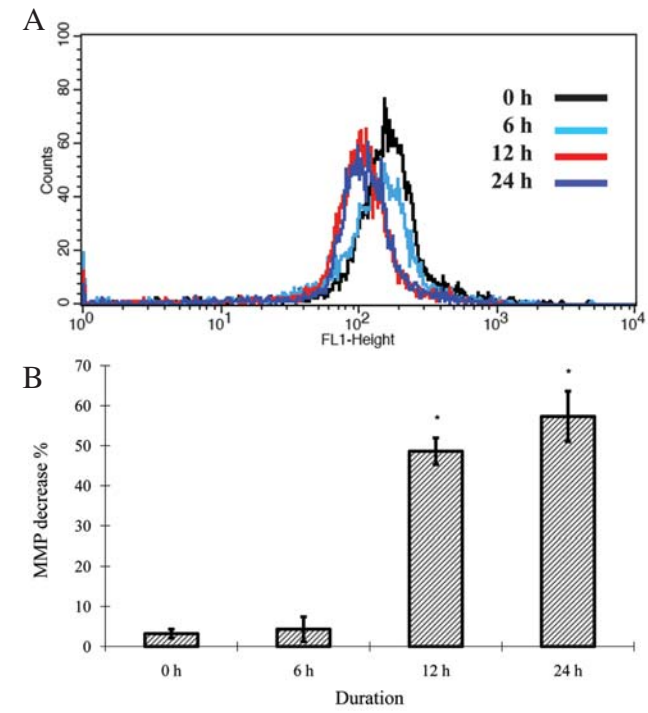

Figure 8. The effects of Tan-IIA on the MMP of A549 cells. When A549 cells were treated with Tan-IIA (2 μg/ml) for different durations (0, 6, 12 and 24 h), The MMP was evaluated as described in Materials and methods. The decreasing MMP percentages were 3.21±1.11, 4.32±3.10, 48.62±3.32 and 57.33±6.30%, respectively. Increasing the time of incubation led the MMP decreasing in the examined A549 cells. Each point is the mean ±SD of three experiments. *P<0.05.

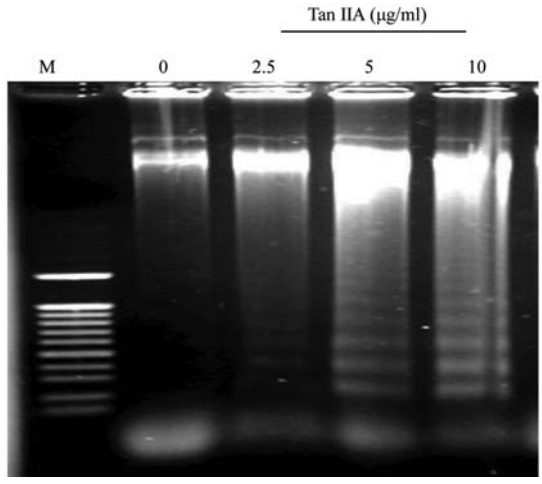

Figure 9. DNA fragmentation electrophoresis analysis was evaluated as described in Materials and methods. A549 cells were treated with Tan-IIA at the concentration of 2.5, 5 and 10 μg/ml for 24 h. The multiples of fragments were significantly increased after A549 cells were treated with Tan-IIA.

to an increase of the level of ROS in the examined A549 cells (Fig. 6A and B).

Effects of Tan-IIA on the production of Ca^{2+} of A549 cells. When A549 cells were treated with Tan-IIA (2 μg/m) for different durations (0, 6, 12 and 24 h), the Ca^{2+} increasing percentage concentrations were 0.65± 0.11, 1.02±1.00, 2.01±1.09 and 21.21±3.01%, respectively. The Ca^{2+}

concentrations were significantly different between the Tan-IIA treated group and the control group. Increasing the time of incubation led to an increase in the concentrations of Ca^{2+} in the examined A549 cells (Fig. 7A and B).

The effects of Tan-IIA on MMP in A549 cells. When A549 cells were treated with Tan-IIA (2 μg/m) for different durations (0, 6,12 and 24 h), the MMP decreasing percentages were 3.21±1.11, 4.32±3.10, 48.62±3.32 and 57.33±6.30%, respectively. Increasing the time of incubation led to the deacrease of MMP in the examined A549 cells (Fig. 8A and B).

DNA fragmentation electrophoresis analysis. The cleavage of the genomic DNA into multiples of oligonucleosomal fragments (180-200 bp) is one of the classic features of apoptosis. DNA fragmentation electrophoresis analysis was performed to investigate Tan-IIA-mediated A549 cells apoptosis. The results showed that the presentation of multiples of fragments were significantly increased after A549 cells were treated with Tan-IIA at the concentrations of 2.5, 5 and 10 μg/ml for 24 h (Fig. 9). That Tan-IIA induces apoptosis in A549 cells was confirmed.

Discussion

Our results showed that Tan-IIA induces apoptosis and inhibits proliferation in non-small cell lung cancer A549 cells in a dose- and time-dependent manner. Our Western blotting data indicated that Tan-IIA decreased expression of Bcl-2 and increased expression of p53, Bax and Cyto-c in A549 cells. This is in agreement with other reports which have demonstrated that Tan-IIA induces apoptosis in human lung cancer SPC-A-1 cells through upregulating protein expression of p53 and Bax and downregulating protein expression of Bcl-2 (20). Apoptosis is a programmed mechanism of cell death. It is well documented that mitochondrial structure and function have a close relationship with apoptosis (21). MMP decreasing induces Cyto-c release and apoptosis (22,23). Over-expression of the protein Bcl-2 prevents the decrease of MMP, but over-expression of the Bax protein decreases MMP and then induces apoptosis (24,25). Our results also showed that Tan-IIA increases the induction of ROS and decreases MMP. Therefore, the pathways for Tan-IIA-induced apoptosis in human A549 cells are mitochondria-dependent. Since Tan-IIA may be through the induction of ROS and a higher ratio of Bax/Bcl-2, then decreasing MMP leads to Cytochrome c release and causes apoptosis.

Acknowledgements

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