Tanshinone IIA down-regulates the protein expression of ErbB-2 and up-regulates TNF-α in colon cancer cells *in vitro* and *in vivo*

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Abstract. Tanshinone IIA (Tan-IIA) was isolated from *Salviae Miltiorrhizae Radix*. Our previous studies showed that Tan-IIA induced apoptosis in human colon cancer colo 205 cells, but the molecular mechanisms of the effect of Tan-IIA on human colon cancer were not clearly elucidated. The protein expression of ErbB-2 was up-regulated and activated in human and experimental colon cancers. In the present study, the effects of Tan-IIA on the protein expression of ErbB-2 in colo 205 cells were investigated. *In vitro*, colo 205 cells were treated with various concentrations of Tan-IIA (1, 2 and 5 μg/ml) for 24 h, and the protein expression of TNF-α, ErbB-2 and caspase-3 was assayed by Western blotting. For the *in vivo* studies, male SCID mice were xenografted with colo 205 cells, and from day 10, Tan IIA (20 mg/kg/day, dissolved in corn oil) was administered by oral feeding for 30 days. As a control, mice with xenografted tumors were separately treated with corn oil (0.1 ml/10 g body weight). Expression of TNF-α, ErbB-2 and caspase-3 proteins was measured by Western blot analysis. Our results showed that Tan-IIA down-regulated the protein expression of ErbB-2 and up-regulated TNF-α and caspase-3 in colo 205 cells *in vitro*. In a colo 205 xenograft model, treatment with Tan-IIA caused up-regulation of TNF-α, caspase-3 and down-regulation of ErbB-2 protein expression as compared to the controls. Based on these observations, one possible molecular mechanisms by which Tan-IIA inhibits the proliferation of colo 205 cells is through the down-regulation of ErbB-2 protein expression and the up-regulation of the protein expression of TNF-α and caspase-3.

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Introduction

Danshen (*Salviae miltiorrhizae Radix*) is widely prescribed in traditional Chinese medicine to treat cardiovascular diseases and dysmenorrhea (1,2). Tanshinone IIA (Tan-IIA), an extract from Danshen (3,4), possesses anti-inflammatory activities (5,6), anti-oxidant properties (7-9) and anti-cancer activity in multiple types of human cancer cells (10-12). Our previous study showed that Tan-IIA has the ability to induce cell growth reduction and apoptosis in human colon cancer colo 205 cells, but the molecular mechanisms were not clearly elucidated. Tumor necrosis factor α (TNF-α), a cytotoxic factor, is produced by macrophages (13). TNF receptor type 1, a cytokine receptor, is expressed in most tissues, and can be activated by binding to TNF-α, forming the death inducing signaling complex. This results in the recruitment of caspases and the induction of its autoproteolytic activation and subsequent cleaving of effector caspase-3 leading to cell apoptosis (14). It is well documented that ErbB-2 (erythroblastosis oncogene B; HER-2/neu) is one of the transmembrane tyrosine kinase receptor that is overexpressed in human colorectal adenocarcinomas and serves as an independent prognostic factor (15-17). This knowledge can be applied to develop new chemotherapeutic medication which blocks the expression of ErbB-2 in colorectal cancer patients. The present study was designed to identify the effects of Tan-IIA on the protein expression of ErbB-2 in colon cancer colo 205 cells *in vivo* and *in vitro*.

Materials and methods

Chemicals and reagents. Tan-IIA (molecular formula $C_{19}H_{18}O_3$, purity >96% by HPLC) was purchased from Herbasin Co. (Shenyang, P.R. China). Aprotinin, antipain, sodium deoxycholate, leupeptin, propidium iodide (PI), sodium orthovanadate, Triton X-100, Tris-HCl, ribonuclease-A and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphate 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).

Cell cultures. The human colon cancer cell line (colo 205, human colon adenocarcinoma) was obtained from the Food Industry Research and Development Institute (Hsin-chu, Taiwan, R.O.C.). The cells were placed into 75-cm^3 tissue culture flasks and grown at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI-1640 medium, containing 10% heat-inactivated fetal bovine serum (FBS), 2% penicillin-streptomycin (10,000 U/ml penicillin; 10 mg/ml streptomycin), 1% HEPES, 1% sodium pyruvate and 1% glutamine.

The effects of Tan-IIA on the protein expression of TNF-α, ErbB-2, caspase-3 and ß-actin in colo 205 cells in vitro

Protein preparation. For protein extraction, colo 205 cells ($2x10^6$) were initially seeded on 10-cm cultured dishes and treated with Tan-IIA at the concentrations of 0, 1, 2 and 5 μg/ml for 24 h before cells were harvested by centrifugation. Proteins were extracted as previously described (18). Briefly, cell pellets were resuspended in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 μg/ml antipain) for 30 min at 4˚C. Lysates were immediately centrifuged at 13,000 x g for 20 min at 4˚C, and the supernatant was collected, aliquated (50 g/tube) and stored at -80˚C until assay. The extracted protein concentrations were measured using the Bradford method (19).

Western blotting. All protein samples were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Life Science Products, Hercules, CA, USA) as previously described (18). The SDS-separated proteins were followed by equilibration in transfer buffer (50 mM Tris, pH 9.0, 40 mM glycine, 0.375% SDS and 20% methanol) and were transferred onto an Immobilon-P membrane (Millipore Corp., Bedford, MA, USA). The membranes were incubated with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 for 1 h. The membranes were then washed and incubated with appropriate dilutions of specific antibodies such as TNF-α, ErbB-2, caspase-3 and ß-actin (Upstate, Lake Placid, NY, USA) at 4˚C overnight. After incubation with anti-mouse peroxidase-conjugated antibody (Santa Cruz, CA, USA), the immunoreactive bands were visualized with an enhanced chemiluminescence (ECL, Amerham Pharmacia Biotech) detection kit. The detection of ß-actin was used as an internal control in all of the data for Western blotting.

The effects of Tan-IIA on the protein expression of TNF-α, ErbB-2, caspase-3 and ß-actin in colo 205 cell xenograft tumors. Three-week-old male nude SCID mice were xenografted with colon cancer colo 205 cells ($2x10^6$/0.2 ml) and maintained in a pathogen-free environment (Laboratory Animal Center of Tzu Chi University, Hualien, Taiwan, R.O.C.). From day 10, Tan IIA (20 mg/kg/day, 0.2 mg Tan IIA dissolved in 0.1 ml corn oil) was administered by oral feeding for 30 days (n=5 mice). The mice were subsequently sacrificed by CO_2 inhalation. As a control, mice (n=4) bearing xenografted tumors were separately treated with corn oil (0.1 ml/10 g body weight). The xenograft tumors were dissected, and protein was extracted for Western blot analysis. The protein expression of TNF-α, ErbB-2 and caspase-3 was

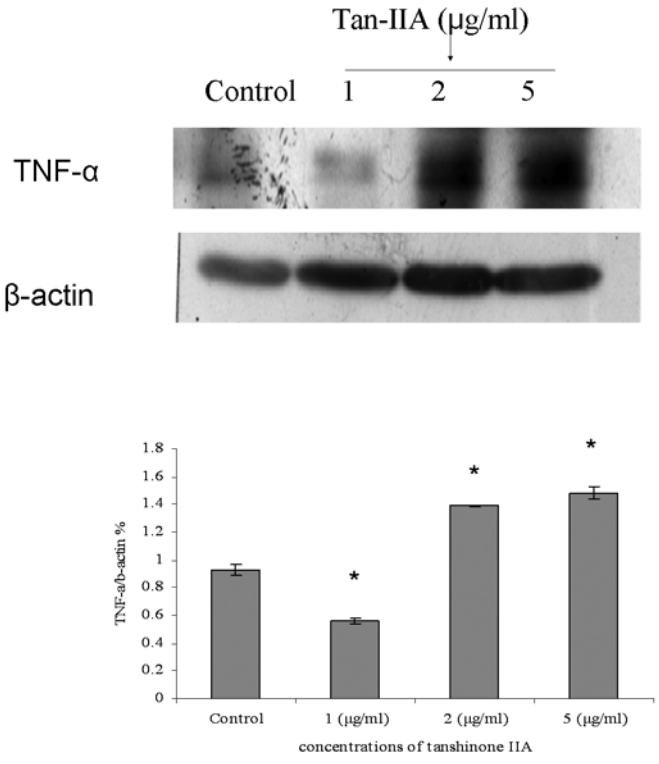


Figure 1. The protein expression of TNF-α in colo 205 cells treated with Tan-IIA (1, 2 and 5 μg/ml) for 24 h. Tan-IIA increased the protein expression of TNF-α at the concentrations of 2 and 5 μg/ml for 24 h. Values are expressed as the means ± SD (n=3). *Significant difference at $p<0.05$ vs the respective groups without Tan-IIA treatment.

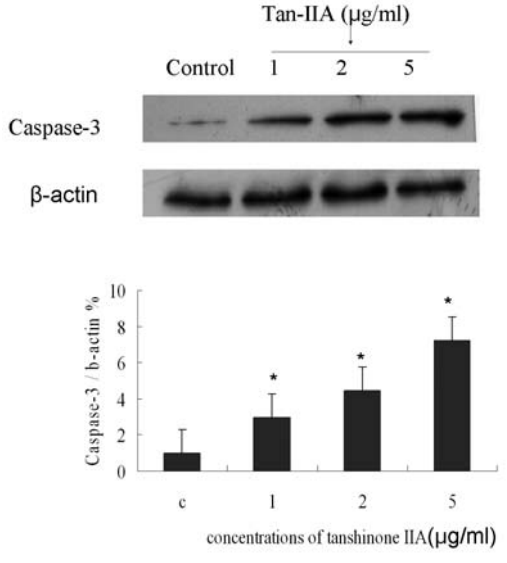


Figure 2. The protein expression of caspase-3 in colo 205 cells treated with Tan-IIA (1, 2 and 5 μg/ml) for 24 h. Tan-IIA increased the protein expression of caspase-3 at the concentrations of 1, 2 and 5 μg/ml for 24 h. Values are expressed as the means ± SD (n=3). *Significant difference at $p<0.05$ vs the respective groups without Tan-IIA treatment.

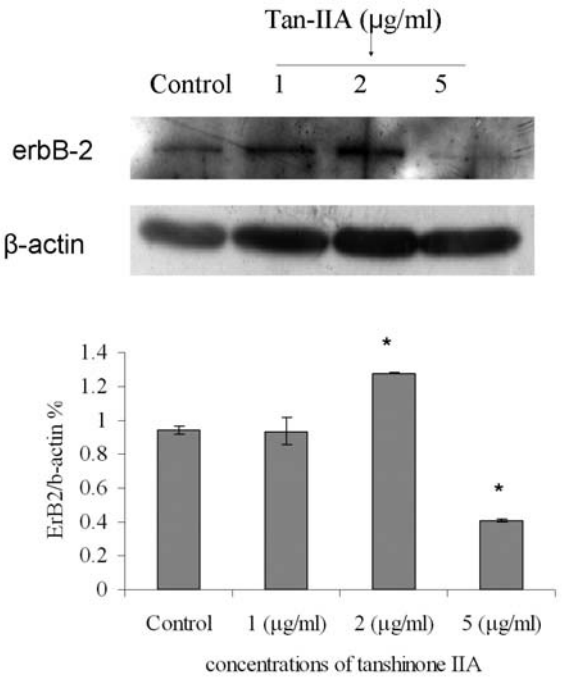


Figure 3. The protein expression of ErbB-2 in colo 205 cells treated with Tan-IIA (1, 2 and 5 μg/ml) for 24 h. The protein expression of ErbB-2 in colo 205 cells was significantly decreased when treated with Tan-IIA at the concentration of 5 μg/ml for 24 h. Values are expressed as the means ± SD (n=3). *Significant difference at p<0.05 vs the respective groups without Tan-IIA treatment.

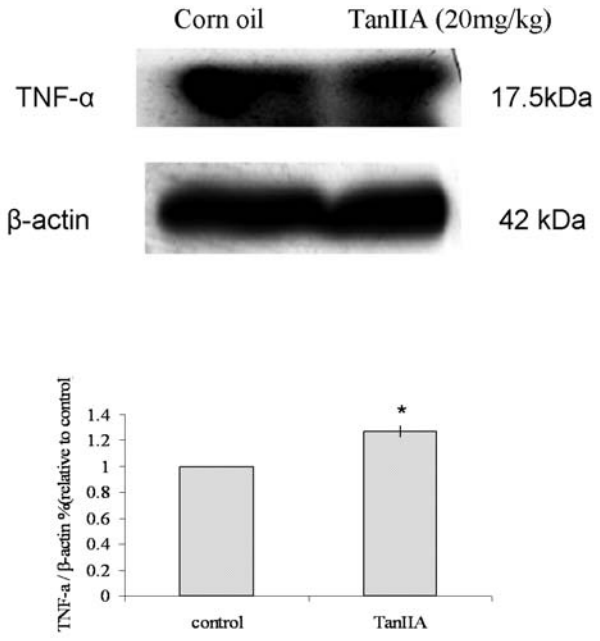


Figure 4. The effects of Tan-IIA on the protein expression of TNF-α in colo 205 cell xenograft tumors. Colo 205 cell xenograft tumors of SCID mice treated with Tan-IIA (20 mg/kg, p.o. x 30 days) exhibited up-regulation of the protein expression of TNF-α when compared to the controls. Values are expressed as the means ± SD (n=3). *Significant difference at p<0.05 vs the respective groups without Tan-IIA treatment.

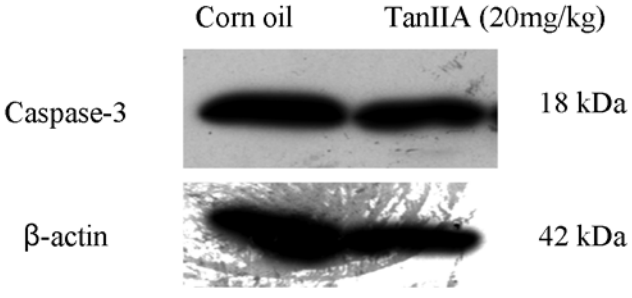
measured by Western blotting. Immunoreactive bands were scanned and analyzed using a digital scanning densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis. Values are presented as the means ± SD. The Student's t-test was used to analyze statistical significance. A p-value <0.05 was considered statistically significant for all tests.

Results

The effects of Tan-IIA on the protein expression of TNF-α, ErbB-2, caspase-3 and β-actin in colo 205 cells in vitro. The colo 205 cells ($2x10^6$/ml) were treated with various concentrations (0, 1, 2 and 5 μg/ml) of Tan-IIA for 24 h, and total protein was prepared and determined as described in Materials and methods. Colo 205 cells were harvested in the presence of Tan-IIA for Western blotting. The results indicated that Tan-IIA increased the expression of TNF-α at the concentrations of 2 and 5 μg/ml for 24 h (Fig. 1) and the expression of caspase-3 at the concentrations of 1, 2 and 5 μg/ml for 24 h (Fig. 2). The protein expression of ErbB-2 in colo 205 cells was significantly decreased when treated with Tan-IIA at the concentration of 5 μg/ml for 24 h (Fig. 3).

The effect of Tan-IIA on the protein expression of TNF-α, ErbB-2, caspase-3 and β-actin in colo 205 xenograft tumors. SCID mice with colo 205 cell xenograft tumors were treated with Tan-IIA (20 mg/kg). The control group was treated with corn oil only. The xenograft tumors were dissected, and the proteins were extracted for Western blot analysis. Colo 205 cell xenograft tumors of SCID mice treated with Tan-IIA showed up-regulation of the protein expression of TNF-α (Fig. 4) and caspase-3 (Fig. 5) and the down-regulation of



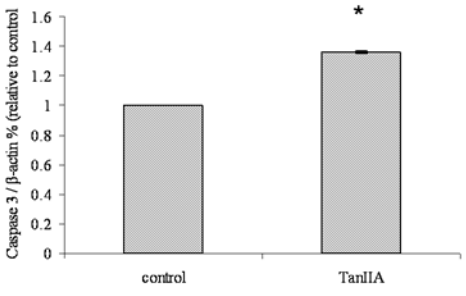


Figure 5. The effects of Tan-IIA on the protein expression of caspase-3 in colo 205 cell xenograft tumors. Colo 205 cell xenograft tumors of SCID mice treated with Tan-IIA (20 mg/kg, p.o. x 30 days) exhibited up-regulation of the protein expression of caspase-3 when compared to the controls. Values are expressed as the means ± SD (n=3). *Significant difference at p<0.05 vs the respective groups without Tan-IIA treatment.

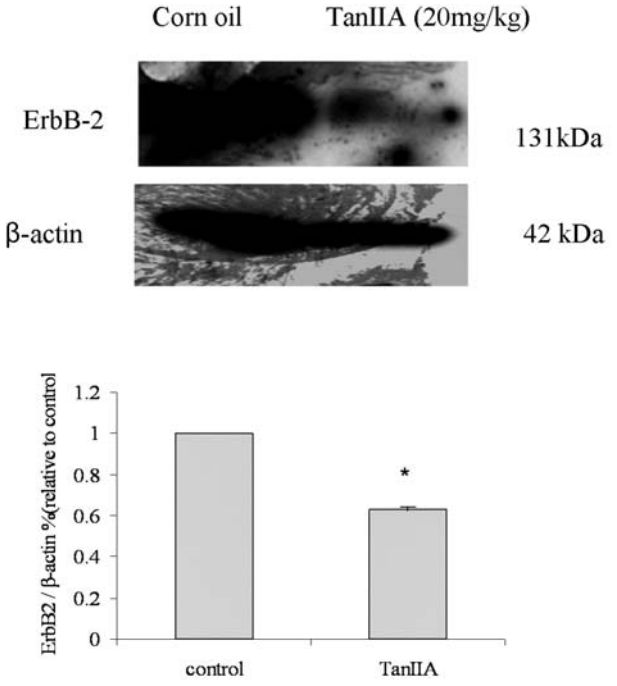


Figure 6. The effects of Tan-IIA on the protein expression of ErbB-2 in colo 205 cell xenograft tumors. Colo 205 cell xenograft tumors of SCID mice treated with Tan-IIA (20 mg/kg, p.o. x 30 days) exhibited down-regulation of ErbB-2 protein expression when compared to the controls. Values are expressed as the means ± SD (n=3). *Significant difference at $p<0.05$ vs the respective groups without Tan-IIA treatment.

ErbB-2 protein expression (Fig. 6) when compared to the controls.

Discussion

Our previous studies demonstrated that Tan-IIA inhibited proliferation and induced apoptosis in human colon cancer colo 205 cells in a dose-dependent manner (20). In the present study, the results showed that Tan-IIA treatment increased the TNF-α and caspase-3 levels in colo 205 cells *in vivo* and *in vitro*. It is well documented that once the TNF-α receptor is ligated, the receptor forms the death-inducing signaling complex, which activates the downstream caspase-3 cascade and consequent apoptosis (14). This process is provoked by external stimuli that bind membrane receptors and ultimately activate caspases resulting in cell death (21). Therefore, Tan-IIA induces apoptosis in colon cancer cells by activating the death receptor-mediated pathways. It is well documented that inhibition of the ErbB-2 activity results in an increased propensity for apoptosis and reduced malignancy *in vivo* (22,23). Our results demonstrate that Tan-IIA down-regulates ErbB-2 protein expression in colo 205 cells *in vivo* and *in vitro*. This is the first study to demonstrate that Tan-IIA down-regulates ErbB-2 and up-regulates TNF-α protein expression in human colon cancer cells suggesting that Tan-IIA may have therapeutic potential for human colon cancer.

Acknowledgements

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