Tanshinone IIA decreases the migratory ability of AGS cells by decreasing the protein expression of matrix metalloproteinases, nuclear factor κB-p65 and cyclooxygenase-2

CHIN-CHENG SU1-4

1Tumor Research Center of Integrative Medicine; 2Comprehensive Breast Cancer Center; 3Department of Surgery, Changhua Christian Hospital, Changhua, Changhua 50006; 4School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan, R.O.C.

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Abstract. During progression of gastric cancer, degradation of the extracellular matrix by matrix metalloproteinases (MMPs) has been associated with poor prognosis. Tanshinone IIA (Tan-IIA) exerts antitumor activity in a variety of human cancer cells. It is extracted from Danshen (Salviae miltiorrhizae radix), and induces apoptosis and inhibits the proliferation of gastric cancer cells. However, the molecular mechanisms underlying the inhibition of migration in gastric cancer by Tan-IIA have not been fully elucidated. In the present study, AGS cell migration ability was evaluated using a wound-healing assay. The protein expression levels of nuclear factor (NF)-κB-p65, cyclooxygenase (COX)-2, MMP-2, -7, and -9 and β-actin in AGS cells were measured by western blotting. The results demonstrated that AGS cells treated with Tan-IIA exhibit decreased protein expression levels of NF-κB-p65, COX-2, and MMP-2, -7 and -9. The results also indicate that Tan-IIA inhibits migration ability in a dose- and time-dependent manner. These findings demonstrate that Tan-IIA inhibits the migration ability of AGS human gastric cancer cells and that decreasing the protein expression of NF-κB-p65, COX-2, and MMP-2, -7 and -9 may be an underlying molecular mechanism.

Introduction

Tanshinone IIA (Tan-IIA; C19H18O3) is extracted from Danshen (Salviae miltiorrhizae radix) (1,2). Tan-IIA exerts antitumor activity in a variety of human cancer cells, such as lung (3), breast (4,5), hepatic (6), pancreatic (7) and colon (8). These studies (3-8) suggest that Tan-IIA may be administered as a complementary therapeutic agent to treat gastric cancer. Furthermore, our previous studies demonstrated that Tan-IIA inhibits the proliferation of AGS cells in a time- and dose-dependent manner, by decreasing the level of protein expression of binding immunoglobulin (Ig) protein, myeloid cell leukemia 1 protein, B-cell lymphoma-extra-large and translationally-controlled tumor protein, and increasing caspase-12, C/EBP-homologous protein, Bcl-2-associated X protein, and caspase-12, -9 and -3 to induce apoptosis (9). The half-maximal inhibitory concentration was 5.5, 3.7 and 3.5 µg/ml at 24, 48 and 72 h, respectively (9). In addition, Tan-IIA was demonstrated to inhibit AGS human gastric cancer cells by increasing the protein expression level of phosphorylated (p)-p38 and p-Jun-amino-terminal kinase, and decreasing that of p-extracellular-signal-regulated kinases to induce G2/M phase arrest. In addition, Tan-IIA increased the protein expression levels of tumor necrosis factor-α, FAS ligand, and caspase-8 and -3 to induce apoptosis (10). It is well documented that nuclear factor (NF)-κB functions as a tumor promoter in inflammation-associated cancer and is, therefore, a potential target for cancer prevention in chronic inflammatory diseases (11). During malignant tumor metastasis, the degradation of the extracellular matrix (ECM) is mediated by matrix metalloproteinases (MMPs) (12-14). As the underlying mechanisms of Tan-IIA inhibiting the migration ability of gastric cancer cells remain to be elucidated, this was the aim of the present study, as well as evaluating the levels of metastatic-associated protein expression in AGS human gastric cancer cells.

Materials and methods

Materials. The AGS human gastric adenocarcinoma cell line [Bioresource Collection and Research Center (BCRC); BCRC no. 60102] was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Tan-IIA (CAS no. 568-72-9) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Ham’s F-12K (Kaign’s) Medium, fetal bovine serum (FBS), 1% penicillin/streptomycin and
glutamine were obtained from Thermo Fisher Scientific, Inc. (Gibco; Waltham, MA, USA). The polyclonal rabbit anti-human NF-κB-p65 (cat. no. 6956; molecular weight, 65kDa), monoclonal rabbit anti-human cyclooxygenase (COX)-2 (cat. no. 12282; molecular weight, 74kDa) and monoclonal rabbit anti-human MMP-2 (cat. no. 13132; molecular weight, 72kDa) antibodies were all obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Polyclonal goat anti-human MMP-7 (cat. no. NB600-1069; molecular weight, 28kDa) and polyclonal rabbit anti-human MMP-9 (cat. no. NBP1-57940; molecular weight, 78kDa) antibodies were obtained from Novus Biologicals, LLC (Littleton, CO, USA). Sodium deoxycholate, leupeptin, Triton X-100, Tris-HCl, ribonuclease-A, sodium pyruvate, HEPES, dimethyl sulfoxide, Tween-20 and mouse anti-β-actin antibodies were obtained from Sigma-Aldrich. BioMax film was obtained from Kodak (Rochester, NY, USA), and potassium phosphate and 0.2-mm polyvinylidene difluoride (PVDF) membranes were purchased from Merck Millipore (Darmstadt, Germany).

Cell culture was conducted as described in a previous study (9). The AGS cells were placed into 75-cm² tissue culture flasks and maintained in Ham’s F-12K Medium containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown for 48-72 h at 37°C in a humidified atmosphere of 5% CO₂.

Wound-healing assays were performed as described in a previous study (15). Cells were plated at a density of 1x10⁶ cells per 60-mm Petri dish in complete medium for 16-20 h. Different concentrations (0, 2.0, 3.7 and 5.5 µg/ml) of Tan-IIA were administered for 0, 24, and 48 h and, once the cells reached confluency, a plastic pipette tip was drawn across the center of the plate to produce a clean, wide, wound area.

Cell movement into the wound area was examined under an Olympus 1X81 microscope (Olympus Corporation, Tokyo, Japan).

**Western blot analysis.** The western blotting procedures were conducted as described in previous studies (9,10). The cells treated with Tan-IIA were lysed in ice-cold radioimmuno-precipitation assay buffer (Merck Millipore) containing a protease inhibitor cocktail (Gibco; Thermo Fisher Scientific, Inc.). The lysate was agitated for 30 min at 4°C and centrifuged at 12,281 x g for 10 min. The protein concentration was measured using the Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Equal quantities of proteins (10 µg) were subjected to electrophoresis using 12% sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA; stacking gel: 70 V, 400 mA, 30 min; separating gel: 100 V, 400 mA, 90 min). To verify equal protein loading and transfer, the proteins were transferred to PVDF membranes, which were blocked for 1 h at 4°C using blocking buffer [5% dried, skimmed milk in solution containing 50 mM Tris-HCl (pH 8.0), 2 mM CaCl₂, 80 mM sodium chloride, 0.05% Tween-20 and 0.02% sodium azide (Merck Millipore)]. The membranes were subsequently incubated for 2 h at room temperature with the following primary antibodies: Rabbit polyclonal anti-NF-κB-p65, monoclonal rabbit anti-COX-2, monoclonal rabbit anti-MMP-2, polyclonal goat anti-MMP-7, polyclonal rabbit anti-MMP-9 (all diluted to 1:1,000), followed by incubation at room temperature for 1 h with anti-rabbit (cat. no. sc:2004) or anti-mouse (cat. no. sc:2005) IgG-horseradish peroxidase-conjugated secondary antibodies (1:5,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The membranes were washed three times for 10 min with 1X phosphate-buffered saline with 0.05% (v/v) Tween 20.
Figure 2. Protein expression levels of NF-κB-p65, COX-2, MMP-2, -7, and -9 and β-actin in AGS cells. The AGS cells were treated with various concentrations of Tan-IIA (0, 2.0, 3.7 and 5.5 µg/ml) for 24 or 48 h and the protein expression levels were evaluated by western blot analysis. The results indicate that Tan-IIA significantly decreases the protein expression levels of (A) NF-κB-p65, (B) COX-2, (C) MMP-2, (D) MMP-7 and (E) MMP-9 in a dose-dependent manner. Tan-IIA, tanshinone IIA; NF-κB-p65, nuclear factor κB-p65; COX-2, cyclooxygenase-2; MMP, matrix metalloproteinase. *P<0.05, **P<0.01 and ***P<0.001, compared with the control.
The protein bands were visualized on X-ray film using an enhanced chemiluminescence detection system (PerkinElmer, Inc., Waltham, MA, USA).

Statistical analysis. All data presented are from a minimum of three independent experiments. Values are presented as the mean ± standard deviation. Student’s t-test was used to analyze...
statistical significance and P<0.05 was considered to indicate a statistically significant difference.

Results

Wound-healing assays. The wound-healing assay is one of the methods used to investigate cell migration in vitro. A wound was created in the cell monolayer using a plastic pipette tip and AGS cells were treated with various concentrations of Tan-IIA (0, 2.0, 3.7 and 5.5 µg/ml) for 24 or 48 h. Images were captured to compare the cell migration for wound closure. The results indicate that Tan-IIA inhibits the migration of AGS cells in a time- and dose-dependent manner (Fig. 1).

Effects of Tan-IIA concentration on the protein expression levels of NF-κB-p65, COX-2, MMP-2, -7, and -9 and β-actin in AGS cells. The AGS cells were treated with various concentrations of Tan-IIA (0, 2.0, 3.7 and 5.5 µg/ml) for 24 or 48 h and the protein expression levels of NF-κB-p65, COX-2, MMP-2, -7, and -9 and β-actin were evaluated by western blot analysis. The results demonstrate that Tan-IIA significantly decreases the protein expression levels of NF-κB-p65 (Fig. 2A), COX-2 (Fig. 2B), and MMP-2 (Fig. 2C), -7 (Fig. 2D) and -9 (Fig. 2E) in a dose-dependent manner.

Effects of Tan-IIA treatment duration on the protein expression of NF-κB-p65, COX-2, MMP-2, -7, and -9 and β-actin in AGS cells. The AGS cells were treated with Tan-IIA (3.7 µg/ml) for different durations (0, 24 and 48 h) and the protein expression levels were evaluated by western blot analysis. The results demonstrate that Tan-IIA significantly decreases the protein expression levels of NF-κB-p65, COX-2, and MMP-2, -7 and -9 in a time-dependent manner (Fig. 3).

Discussion

Cell migration in vitro is commonly evaluated using a wound-healing assay. A wound is created in the cell monolayer using a plastic pipette tip. Images are captured upon initiation of the assay and at regular time-points during cell migration (that is taking place to close the wound), and these images are compared to measure the cell migration. This method aims to mimic cell migration during wound healing in vivo (16). The present study indicates that Tan-IIA inhibits the migration ability of AGS human gastric cancer cells in a time- and dose-dependent manner in vitro. This finding is consistent with previous studies, which demonstrated that Tan-IIA markedly decreased migratory and invasive abilities in SGC7901 gastric cancer cells (15). It is well known that MMP-2 overexpression is significantly associated with poor overall survival (OS) of gastric cancer patients, and has a negative impact on OS in Asian and European countries (17). A previous meta-analysis indicated that MMP-2 overexpression may be a predictive factor for poor prognosis in gastric cancer (17). de la Peña et al (18) demonstrated that MMP-2 expression may present as a potential molecular marker for advanced human gastric cancer. An additional meta-analysis indicated that abnormal MMP-2 expression may be markedly associated with poor prognosis in gastric cancer patients (19). Yang et al (20) demonstrated that the MMP-7-181 A>G polymorphism may contribute to gastric cancer susceptibility. Furthermore, Huang et al (21) demonstrated that interleukin (1L)-1β-induced p38 activation significantly increased the messenger (m)RNA and protein expression, and activity of MMP-2 and -9. This indicated that IL-1β/p38/activator protein 1 (c-Fos)/MMP-2 and -9 signaling pathway may be important in metastasis in MKN-45 and AGS cells. In our previous study, AGS cells were treated with Tan-IIA and it was demonstrated that treatment increased p-p38 expression levels in a time- and dose-dependent manner (10). In the present study, the results indicate that Tan-IIA decreases MMP-2, -7 and -9 expression levels in a time- and dose-dependent manner. The results indicate that Tan-IIA may inhibit the metastasis of AGS cells by decreasing MMP-2, -7 and -9 expression levels.

Carcinogen exposure and chronic inflammation are significant underlying conditions resulting in tumor development. It is well documented that NF-κB is essential in promotion of inflammation-associated cancer, and is therefore being considered as a potential target for cancer prevention in chronic inflammatory diseases (11). MGC803 gastric cancer cells treated with chloroquine inhibited the mRNA expression levels of COX-2, MMP-2 and -7 and NF-κB-p65. This prevented the migration of MGC803 cells in a dose-dependent manner. Furthermore, the results indicated that the Toll-like receptor 9/NF-κB signaling pathway was involved in gastric cancer cell migration (22). The results from the current study indicate that Tan-IIA decreases NF-κB-p65 and COX-2 expression levels in a time- and dose-dependent manner. In addition, the results from the wound-healing assay indicate that Tan-IIA inhibits the migration of AGS cells for wound closure in a time- and dose-dependent manner. These results suggest that Tan-IIA inhibits the migration ability of AGS cells by decreasing the protein expression of NF-κB-p65, COX-2, and MMP-2, -7 and -9.

In conclusion, to the best of our knowledge, this is the first study to report that Tan-IIA inhibits the migration ability of AGS cells via decreasing the protein expression of NF-κB-p65, COX-2 and MMPs. However, these findings warrant further investigation in the future.

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References

SU: TAN-IIA INHIBITS AGS CELL MIGRATION BY DECREASING MMPs AND NF-κB


