Original Paper

Glycyrrhizin Suppresses Lung Adenocarcinoma Cell Growth Through Inhibition of Thromboxane Synthase

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Key Words

Lung adenocarcinoma • Glycyrrhizin • Thromboxane synthase • Cell growth

Abstract

Background/Aims: The effects of glycyrrhizin treatment in lung cancer remain undetermined, despite extensive studies of the anti-tumor activities of glycyrrhizin. **Methods:** Lung adenocarcinoma A549 and NCI-H23 cell lines were used in this study. Cell growth was examined by MTS assays, while apoptosis and cell cycle were determined by flow cytometric analysis. Both real-time PCR and western blotting were used to examine the expression levels of thromboxane synthase (TxAS), and TxAS activity was measured using EIA detection of the biosynthesis of TxA₂. TxAS was overexpressed in NCI-H23 cells by transfection with TxAS cDNA, while TxAS was inhibited by transfection with TxAS siRNA in A549 cells. For the mouse model of lung adenocarcinoma, the effects of glycyrrhizin on tumor growth were analyzed by western blot evaluation of TxAS, PTEN and survivin. TxAS activity was determined by EIA assay. **Results:** Glycyrrhizin suppressed cell growth in A549 cells, but not in NCI-H23 cells, by induction of apoptosis. TxAS was overexpressed in A549 cells, but the TxAS levels in NCI-H23 cells were minimal. Moreover, TxAS expression and activity were suppressed by glycyrrhizin. Glycyrrhizin had no additive effects with TxAS siRNA knockdown in suppressing A549 cell growth, whereas it completely suppressed cell growth of NCI-H23 cells transfected with TxAS cDNA. These results were further confirmed by the *in vivo* study. **Conclusion:** Our study suggests that the anti-tumor effect of glycyrrhizin in lung adenocarcinoma is, at least in part, TxAS-dependent. Therefore, glycyrrhizin is a promising anti-cancer agent for the treatment of lung adenocarcinoma.

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Introduction

Lung cancer leads to over one million deaths worldwide each year, making it the leading cause of cancer-related death. Adenocarcinoma is the major subtype of lung cancer, with an average 5-year survival rate of 15% [1]. The poor survival for lung adenocarcinoma patients is mainly due to the lack of an effective therapy [1]. The drug cisplatin is currently employed as a first-line treatment for lung cancer patients. However a major problem in using cisplatin as a lung cancer treatment is the high incidence of chemoresistance in patients. Moreover, the cytotoxicity of cisplatin affects multiple organs, such as the kidneys, peripheral nerves and inner ear [2]. Although a great effort has been made to design more powerful drugs for the treatment of lung adenocarcinoma, the results are still unsatisfactory. In fact, some herbal drugs in both traditional and alternative medicine have been suggested to be a better choice to improve the current therapeutic strategy.

Glycyrrhizin, also known as glycyrrhizic acid, belongs to a class of triterpenes naturally extracted from the roots of licorice plants. Since the discovery of the anti-viral properties of glycyrrhizin in 1979 [3], extensive studies have been done to investigate the pharmacological effects of glycyrrhizin. Recently, glycyrrhizin has been demonstrated to have both anti-inflammatory and anti-tumor effects in many types of cancer, such as glioma [4], hepatocarcinoma [5] and prostate cancer [6]. However, the effects of using glycyrrhizin in the treatment of lung cancer remain undetermined. Glycyrrhizin has been shown to be able to suppress cyclooxygenase (COX)-2 and its downstream products [7, 8] by directly binding to high mobility group protein B1 (HMGB1) [9, 10]. Recent studies to determine the mechanisms underlying the pharmacological effects of glycyrrhizin have documented that glycyrrhizin is capable of preventing induction of COX-2, thereby exerting anti-inflammatory and anti-cancer effects [10-12].

COX-2 is a well-known oncogene that plays an important role in non-small cell lung cancer (NSCLC) [13]. It is an inducible enzyme, becoming abundant in sites of inflammation and tumor tissues [14]. COX-2 catalyzes the conversion of arachidonic acid into prostaglandin H₂, an unstable intermediate from which all other prostanoids, prostaglandin (PG)-E₂, prostacyclin (PGI) and thromboxane (Tx)-A₂, in principle, are finally derived by a variety of terminal synthases [14, 15]. These synthases are prostaglandin E synthase (PGES), prostacyclin synthase (PGIS), and TxAS, and thus, the activity of TxAS can be reflected by the biosynthesis of TxA₂ [14, 15]. It was reported that an increased expression of COX-2 with a concomitant increase in TxAS and PGES, but not PGIS, was detected in lung adenocarcinoma specimens compared to normal lung tissues [16]. While PGES has been documented to promote the progression of lung adenocarcinomas [17], its roles in cancer progression are paradoxical [18, 19]. To date, it is clear that both TxAS and PGIS play important roles in lung cancer [15], but particularly TxAS as it has also been recently established to be a tumor promoter [15, 20-25].

Although it is clear that COX-2 is a main target of glycyrrhizin, the effects of glycyrrhizin on the downstream targets of COX-2 are still unknown. In light of the observations above, this study was designed to determine the effects of glycyrrhizin in lung adenocarcinoma cell growth and its molecular mechanisms based on the COX-2/TxAS pathway.

Materials and Methods

Cell culture and chemicals

The human lung adenocarcinoma NCI-H23 and A549 cell lines and immortalized normal lung fibroblast CCL-75.1 cells were purchased from the American Type Culture Collection (Rockville, MD). Both NCI-H23 and CCL-75.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), and A549 cells were cultured in RPMI 1640 medium. All cells were maintained in culture medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂.

Glycyrrhizin (>98% purification) and cisplatin were supplied by ALADDIN Chemical Co., Ltd. (Shanghai, China).

Animal treatments

All the procedures involving animals abided by the guidelines of Guangzhou University of Chinese Medicine for the care and use of laboratory animals and were approved by the institutional animal research ethical committee.

Mouse embryonic fibroblast (MEF) cells were generated according to the following protocol: embryonic day 15.5-17.5, embryos were dissected from pregnant female CF-1 mice, and they were minced following removal of the heads and internal organs. The tissue masses were subsequently plated and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

For tumor formation in nude mice, a total of 1×10^7 monodispersed A549 cells were suspended in 200 µl of serum-free RPMI 1640 medium and injected into the tail vein of 6-week old female nude mice. For histopathological examination, mice were killed at 6 weeks post-inoculation, and then, lung tissue was trimmed and fixed with 10% formalin. Paraffin sections of 6 µm were obtained and stained by standard Hematoxylin and Eosin (H&E) methods.

Transient transfections

Transfection of small interference RNA (siRNA) was used to obtain gene knockdown, and transfection of cDNA was used to obtain protein overexpression in this study. Cells were seeded at a uniform density into 6-well culture plates and then incubated overnight. For siRNA transfections, 2 µg of control siRNA or siRNA against TxAS was used for A549 cells because A549 overexpresses TxAS. Because NCI-H23 expresses limited TxAS, for overexpression experiments, 2 µg of vacant pCMV6 (control) or pCMV6-TxAS plasmid was transfected into NCI-H23 cells. The transfection experiments were performed according to the manufacturer's instruction (OriGene technologies, Rockville, MD). After 18h of incubation, the medium was changed to normal growth medium for an additional 72h before analysis in the absence or presence of glycyrrhizin. The extent of the specific gene knockdown or overexpression was determined by western blot analysis.

Cell growth detection

To measure the rate of cell growth, cells were seeded into 96-well plates at a density of 1×10^3 cells and incubated overnight. CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was performed according to the manufacturer's instruction (Promega, Madison, WI). Viable cells were determined using a microplate reader at an absorbance of 490 nm.

Flow cytometric analysis

The cells were plated at a density of 1×10^5 cells/10 ml of medium and incubated overnight. Live cells were collected and washed twice with ice-cold PBS. For the detection of cell cycle progression, cells were stained with 5 µl of propidium iodide (PI, 1 mg/ml) and subsequently incubated at room temperature for 30 min in the dark. For apoptosis analysis, cells were stained with Annexin V fluorescein dye and PI at room temperature in the dark for 15 min and then resuspended in 400 µl of Annexin-binding buffer (Beckman Coulter, Inc., Brea, CA). The stained cells were analyzed using Beckman Flow Cytometers.

Enzyme immunoassay (EIA)

TxA2 is chemically unstable *in vivo*, therefore TxA2 production is monitored by assaying the level of thromboxane B2 (TxB2), a stable product of the non-enzymatic hydration of TxA2 [14]. A549 cells were seeded at a uniform density into 6-well culture plates and incubated overnight. Following treatment, the culture supernatant was collected, centrifuged and analyzed for the secretion of TxB2 with a TxB2 EIA kit (Cayman Chemical, Ann Arbor, MI). The level of serum TxA2 in the animal model was also determined by this method.

Real-time quantitative PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA). cDNA for templates was synthesized from 1 µg of total RNA using a high capacity cDNA reverse transcription kit (Promega). cDNA was used as a template for PCR by co-amplifying the genes using β-actin as a reference gene using the corresponding gene-specific primer sets. SYBR Green qPCR SuperMix (Invitrogen) was used in real-time PCR reactions. The gene-specific primer sequences used were as follows: 5'-TTCCTCCTGTGCCTGATG-3' (sense)

and 5'-CTGATGCGTGAAGTGCTG-3' (antisense) for human COX-2; 5'-TGACCGCCTCCTCCTT-3' (sense) and 5'-CCGTTTCCCATCCTTGTGTA-3' (antisense) for human PGIS; 5'-AATAAGAACCGAGACGAACT-3' (sense) and 5'-GGCTTGACCCAGTAGAG-3' (antisense) for human TxAS; 5'-GGAACGACATGGAGACCATCTAC-3' (sense) and 5'-TCCAGGCGACAAAAGGGTTA-3' (antisense) for human PGES; and 5'-GGAAATCGTGCGTGACATT-3' (sense) and 5'-CAGGCAGCTCGTAGCTCTT-3' (antisense) for human β -actin. Real-time PCR was performed using the CFX96 Touch Deep Well™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc. Berkeley, CA) and the following steps were performed: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles for 15 s at 95°C and 1 min at 60°C. The fold change in the expression of each target gene was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Lung organs removed from the mice were snap frozen in liquid nitrogen within 15 min after extirpation. Following homogenization of tissues in liquid nitrogen, pulverized tissue powders were lysed in RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 8.0, 1% Triton X-100, 0.1% SDS, and 1% deoxycholate) with a complete protease inhibitor cocktail (Roche Ltd., Basel, Switzerland) for 15 min on ice. Total proteins were extracted from cancer cell lines using RIPA buffer containing protease inhibitors. Equal amounts of total protein (30 μ g) were resuspended in loading buffer [100 mmol/L Tris-HCl (pH 8.8), 0.01% bromophenol blue, 36% glycerol, 4% SDS, and 1 mmol/L DTT], boiled for 5 minutes, separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The following antibodies were used: rabbit polyclonal antibodies against GAPDH (1:1000), phosphatase and tensin homolog (PTEN) (1:500), poly ADP ribose polymerase (PARP) (1:1000) and survivin (1:1000), which were bought from Cell Signaling Technology (Beverly, MA). The mouse monoclonal antibody against TxAS (1:1000) was purchased from OriGene technologies. The mouse monoclonal antibody raised against recombinant β -tubulin of human origin and the goat polyclonal antibody against β -actin (1:1000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse IgG-HRP and anti-rabbit IgG-HRP antibodies were purchased from Cell Signaling Technology. Anti-goat IgG-HRP antibody was bought from Santa Cruz Biotechnology. To ensure equal protein loading, membranes were stripped and then probed with anti-GAPDH or anti- β -actin antibodies.

Statistical analyses

Data are expressed as the means $\pm$ SD of at least three independent experiments done in triplicate. Student's *t* test was employed for the comparisons between groups. One-way ANOVA followed by Dunnett's test was used to compare the differences among three or more groups. A two-side *p* value of <0.05 was considered statistically significant for all experiments. The statistical analyses were performed using SPSS 11.6 statistical software (SPSS, Chicago, IL).

Results

The differential effects of glycyrrhizin on A549 and NCI-H23 cell growth

To determine the effects of glycyrrhizin on lung adenocarcinoma cell growth, both A549 and NCI-H23 cells were treated with graded concentrations of glycyrrhizin for 72 h. The selection of concentrations was based on a report showing that the ID50 of glycyrrhizin in HepG2 cells was more than 1.2 mM [26]. As shown in Fig. 1A, glycyrrhizin inhibited A549 cell growth in a dose-dependent manner. The inhibitory rate of growth in cells treated with glycyrrhizin at 1.0 mM was nearly 50% that of the control cells (*p*=0.009). However, glycyrrhizin failed to suppress NCI-H23 cell growth significantly (Fig. 1B).

Cisplatin has been used as a first-line chemotherapeutic treatment for lung cancer patients [27]. The suppressing effects of glycyrrhizin on A549 cell growth raised the possibility that glycyrrhizin may have an additive or supra-additive effect with cisplatin treatment on tumor growth. Both A549 and NCI-H23 cells were grown for 72h either in the presence of cisplatin alone or of cisplatin in combination with increasing concentrations of glycyrrhizin. MTS assays showed that the cell growth rate of A549 was suppressed by cisplatin up to 39.3 $\pm$ 6.3% at concentrations of 2.5 μ g/ml and up to 42.5 $\pm$ 8.5% at 4 μ g/ml

Fig. 1. The effects of glycyrrhizin on lung adenocarcinoma cell growth. A. Glycyrrhizin inhibits A549 lung adenocarcinoma cell growth in a dose-dependent manner. Figure was drawn using the raw data; percentage to control is shown in the lower panel. B. Glycyrrhizin moderately suppresses NCI-H23 lung adenocarcinoma cell growth (no statistical significance observed). Figure was drawn using the raw data; percentage to control is shown in the lower panel. C. Glycyrrhizin has a synergistic effect with cisplatin (DDP) in suppressing A549 cell growth. D. There is no significantly additive effect of glycyrrhizin with DDP in suppressing NCI-H23 cell growth. E. Glycyrrhizin does not significantly affect MEFs cell growth.

Figure was drawn using the raw data; percentage to control is shown in the right panel. All of these experiments were performed by MTS and data are expressed as the mean±SD of three independent experiments done in triplicate. * $p < 0.05$, ** $p < 0.01$.

($p < 0.01$, Fig. 1C). Importantly, treatment of A549 cells with 2.5 µg/ml cisplatin abrogated cell growth up to $61.1 \pm 3.5\%$ of the growth of the control by additional administration of 1.0 mM glycyrrhizin ($p < 0.001$) and up to $84.7 \pm 2.4\%$ of the growth of the control by the addition of 2.0 mM glycyrrhizin ($p < 0.001$). These results suggest that glycyrrhizin is able to sensitize A549 cells to cisplatin. However, as shown in Fig. 1D, there were no additional effects observed in NCI-H23 cells treated with cisplatin (2.5 µg/ml) and 0.5 mM or 1.0 mM glycyrrhizin compared to cells treated with cisplatin (2.5 µg/ml) alone. Additionally, the addition of 2.0 mM glycyrrhizin did not have an additive effect with cisplatin in suppressing NCI-H23 cell growth. Cisplatin can induce apoptosis at 2 µg/ml in several cancer cell lines [24, 28], and the IC₅₀ of cisplatin for A549 is approximately 12 µM, i.e., 3.6 µg/ml [29]. Therefore, 2.5 µg/ml and 4 µg/ml of cisplatin were used in this study.

To test if the inhibitory effect of glycyrrhizin on A549 cell growth had low toxicity and was tumor specific, MEF cells were treated with different concentrations of glycyrrhizin for 72h. Fig. 1E shows that glycyrrhizin did not have a significant inhibitory effect on MEF cell growth.

Glycyrrhizin induces apoptosis but not cell cycle arrest in A549 cells

Both cell cycle progression and prevention of apoptosis are required for tumor growth. We therefore determined whether apoptosis or cell cycle arrest was induced by glycyrrhizin

Fig. 2. The effects of glycyrrhizin on apoptosis and cell cycle in A549 cells. A. Flow cytometric analysis of cell apoptosis induced by glycyrrhizin (1.0 mM). Percentage of cells in early or late apoptosis is provided in the lower right and upper right quadrants, respectively. B. The effect of glycyrrhizin (1.0 mM) on PARP activation. Levels of full length (116 kDa) and cleaved (85 kDa) PARP were determined by western blot analysis. Actin served as the loading control. C. The effect of glycyrrhizin (1.0 mM) on cell cycle progression. The sub-G1 phase in the glycyrrhizin treatment group suggests that glycyrrhizin induces cell apoptosis, while the other profiles (G1, S and G2/M) are similar between the control and glycyrrhizin treated groups. The figure shown is representative of three independent experiments.

in lung adenocarcinoma A549 cells. Following a 72h treatment of glycyrrhizin (1.0 mM), cells were stained with annexin-V-fluorescein isothiocyanate and PI. Flow cytometric analysis was performed to measure the fraction of apoptotic cells. Glycyrrhizin treatment increased the percentage of PI-positive cells, which indicates the fraction of cells in early apoptosis, and double positive cells, which indicates the fraction of cells in late apoptosis [24], by approximately 5.6-fold and 8.4-fold, respectively ($p < 0.001$) compared to control cells (Fig. 2A). The induction of apoptosis by glycyrrhizin was also confirmed by western blot analysis using a PARP antibody detecting both the full length and cleaved forms of PARP. Fig. 2B shows the appearance of a 116 kDa native form (full length) and an 85 kDa cleaved form in A549 cells treated with glycyrrhizin (1.0 mM) for 72h.

In addition, after treating A549 cells with 1.0 mM glycyrrhizin for 72h, cell cycle profiles were examined by flow cytometric analysis. Unexpectedly, there were no significant changes in the G1, S or G2/M phases between control and treated groups (Fig. 2C). The occurrence of a sub-G1 phase in treated cells confirmed the apoptotic effects of glycyrrhizin observed in A549 cells.

Glycyrrhizin suppresses the expression and activity of TxAS

COX-2 is one of main targets of glycyrrhizin [11, 12]. To gain some insight into the molecular mechanisms behind the effects of glycyrrhizin in suppressing cell growth of A549 and NCI-H23 cells, the expression of COX-2 and its downstream enzymes, PGIS, PGES and TxAS, were examined. The expression of these genes in CCL-75.1 served as a control as CCL-75.1 is an immortalized lung fibroblast cell (normal lung cell line). Lung fibroblasts are a likely source of pulmonary TXA₂, as shown by early *in vivo* studies [30, 31]. Real-time PCR experiments illustrated that compared to normal lung cells, there was lower expression of PGIS in cancer cell lines, which is in line with the anti-tumor role of PGIS in lung cancer [15] (Fig. 3A). Relative to normal lung cells, PGES has been shown to be highly expressed in both A549 and NCI-H23 cells, supporting its role in the progression of lung

Fig. 3. The effects of glycyrrhizin on the expression of COX-2-related genes, TxAS protein expression and TxAS activity. **A.** mRNA levels of COX-2 and its downstream enzymes in CCL-75.1, NCI-H23 and A549 cells were detected by real-time PCR. A549 cells express higher levels of both COX-2 and TxAS than NCI-H23 cells, and NCI-H23 cells have low levels of TxAS. The dotted line of 1 represents the controls (gene expression in the normal CCL-75.1 cell line). Data are expressed as the mean $\pm$ SD of three independent experiments done in triplicate. * p < 0.05 and ** p < 0.01. **B.** Expression of TxAS-related genes in A549 cells were determined by real-time PCR. mRNA levels of COX-2 and TxAS are significantly down-regulated by 1.0 mM glycyrrhizin. mRNA levels of PGIS are mildly increased, while those of PGES are mildly decreased. The mRNA levels of those enzymes in cells without treatment were used as controls, which is represented by the dotted line of 1. Data are expressed as the mean $\pm$ SD of three independent experiments done in triplicate. ** P < 0.01 versus control. **C.** Western blot evaluation of the effects of glycyrrhizin on TxAS protein expression. Actin was used as a loading control. Figures are a representative result selected from three independent experiments, and densitometry for blots are shown in the right panel. ** P < 0.01 compared to control. **D.** EIA detection of TxAS2 biosynthesis in response to glycyrrhizin (1.0 mM) treatment. The TxAS2 biosynthesis reflects TxAS activity [14, 15]. The results are presented as the percentage of the control. Data are expressed as the mean $\pm$ SD of three independent experiments done in triplicate. ** p < 0.01 compared to control.

adenocarcinoma [17]. Importantly, levels of COX-2 and TxAS were, respectively, 2.0-fold and 3.0-fold higher in A549 cells than in NCI-H23 cells (p < 0.01). In particular, the levels of TxAS in NCI-H23 cells were even below those in normal CCL-75.1 cells. Thus, it is possible that the extraordinary difference in the expression of TxAS in A549 and NCI-H23 contributes to the differential effects of glycyrrhizin in these two cell lines. To verify this possibility, the effects of glycyrrhizin on the transcription of COX-2, PGIS, TxAS and PGES were determined. As expected, real-time PCR experiments showed that a 72h treatment of A549 cells with glycyrrhizin (1.0 mM) dramatically inhibited mRNA expression of both COX-2 and TxAS (Fig. 3B). COX-2 was utilized as a positive control for high expression because it is one of the main targets of glycyrrhizin. Expression of PGIS was moderately increased, which indicates

Fig. 4. The effects of TxAS cDNA or siRNA transfection on lung adenocarcinoma cell growth. **A.** Western blot evaluation of the efficacy of TxAS cDNA transfection. Actin served as a loading control, and the figure shown was selected from three independent experiments. Densitometry for blots is shown in the right panel. ****** $p < 0.01$ versus vacant plasmid transfection. **B.** MTS assay detection for NCI-H23 cell growth after transfection of pCMV6 vacant or pCMV6-TxAS plasmids. pCMV6-TxAS transfection promotes NCI-H23 cell growth but this effect is reversed by glycyrrhizin (1.0 mM). Data are presented as percentages of the control and expressed as the mean $\pm$ SD of three independent experiments done in triplicate. ****** $p < 0.01$. **C.** Western blot evaluation of the efficacy of TxAS-siRNA transfection. Actin served as a loading control, and the figure shown was selected from three independent experiments. Densitometry for blots is shown in the right panel. ****** $p < 0.01$ versus non-target transfection. **D.** MTS assays for A549 cell growth after transfection of control-siRNA or TxAS-siRNA. A549 cell growth is reduced by TxAS-siRNA, and glycyrrhizin treatment has no significant additional effects. Data are presented as percentages of the control and expressed as the mean $\pm$ SD of three independent experiments done in triplicate. ****** $p < 0.01$ versus control.

the low toxicity of glycyrrhizin. Importantly, PGES was weakly decreased, which strongly suggests that TxAS is the main target of glycyrrhizin downstream of COX-2.

The effects of glycyrrhizin on the expression level and activity of TxAS were further evaluated. A549 cells were treated with 1.0 mM or 2.0 mM glycyrrhizin for 72h, and total protein was extracted and subjected to western blot analysis. As demonstrated in Fig. 3C, glycyrrhizin suppressed the expression of TxAS in a dose-dependent manner. The densitometric analysis for three independent blots showed that glycyrrhizin suppressed TxAS expression by approximately 60.3% at a concentration of 1.0 mM and by almost 86.4% at 2.0 mM. Glycyrrhizin was subsequently used at 1.0 mM to suppress the biosynthesis of TxA2, the direct product of TxAS [20]. A549 cells were treated with 1.0 mM glycyrrhizin for 72h, and the culture medium was collected to perform EIA experiments. Fig. 3D shows that glycyrrhizin significantly inhibited TxA2 production by $45.2 \pm 4.3\%$ ($p < 0.01$). Collectively, these findings imply that the pharmacological effects of glycyrrhizin in lung adenocarcinoma may be dependent on TxAS.

Fig. 5. *In vivo* studies of the effects of glycyrrhizin in lung adenocarcinoma. A. H&E staining of the establishment of lung adenocarcinoma in nude mice. The left panel shows low power ($\times 40$) and the right panel shows high power ($\times 400$). The irregular arrangement of cancer cells and the irregular shape of the nucleus are shown, and the phase of cell division is visible. Blue, nuclear staining; red, erythrocyte in blood vessels. B. The coefficient of lung weight to body weight was calculated as the ratio of tissue wet weight (g) to body weight (g). ** $p < 0.01$. C. Western blot analysis for PTEN (47 kDa), survivin (17 kDa), TxAS (60 kDa) and human origin β -tubulin (55 kDa). Figures shown were selected from three independent experiments, and GAPDH (37 kDa) served as a loading control. D. EIA detection of serum TxAS levels among normal and xenograft model with and without glycyrrhizin treatment (50 mg/kg) groups. ** $p < 0.01$.

The anti-tumor effect of glycyrrhizin in lung adenocarcinoma cells is dependent on TxAS

To determine whether the anti-tumor effect of glycyrrhizin in lung adenocarcinoma cells is TxAS-dependent, knockdown and overexpression of TxAS was performed using transfection of siRNA and cDNA, respectively. Because TxAS expression is low in NCI-H23 cells, pCMV6-TxAS or the control plasmid (pCMV6-vacant) were transiently transfected into these cells in the presence or absence of 1.0 mM glycyrrhizin for 72h. Western blotting was performed to test the efficacy of TxAS cDNA transfection. Fig. 4A shows that transfection of pCMV6-TxAS greatly increased TxAS protein levels. MTS experiments demonstrated that the overexpression of TxAS in NCI-H23 cells resulted in an enhancement of cell growth (approximately $158.8 \pm 4.8\%$ that of the control, $p < 0.01$), and such an effect was totally inhibited to the level below the control by the additional administration of glycyrrhizin (Fig. 4B).

siRNA against TxAS or non-target siRNA was transfected into A549 cells to reduce TxAS expression as TxAS is overexpressed in these cells. As shown in Fig. 4C, transfection of TxAS-siRNA could efficiently reduce the expression of TxAS. The densitometry for three independent blots demonstrated that siRNA against TxAS reduced TxAS protein levels in A549 cells by approximately 85.2%. Moreover, MTS experiments showed that TxAS-siRNA significantly reduced cell growth by $55.9 \pm 7.2\%$ compared to control ($p < 0.01$), while the administration of glycyrrhizin had no additional effects (Fig. 4D). The result that glycyrrhizin dramatically suppressed cell growth in control-siRNA transfection is consistent with the data observed above (Fig. 1A). These findings strongly suggest that the suppressing effect of glycyrrhizin on lung adenocarcinoma cell growth is TxAS-dependent.

The effects of glycyrrhizin on lung adenocarcinoma cell growth in vivo

Based on these *in vitro* results, we proceeded to examine the effect of glycyrrhizin on tumor growth *in vivo*. Eight nude mice were used in each of the two experimental groups (with or without glycyrrhizin treatment), and six were used in the control group. The establishment of a xenograft mouse model was described in the Materials and Methods and confirmed by the pathological evaluation at six weeks after tail injection (Fig. 5A). The nude mice with xenografts were divided into groups with or without glycyrrhizin treatment. The model xenograft group had no treatment, while mice in the glycyrrhizin treatment group were given 50 mg/kg glycyrrhizin every two days [11]. Another six mice without xenografts kept in the same condition served as controls. Eight weeks after injection, animals were sacrificed by an excess dose of anesthetic ether and cervical dislocation, and the body weight of all animals was recorded. Whole blood was collected from the orbital sinus and then centrifuged to yield serum. The lung organ was excised and weighed. The coefficient of lung weight to body weight was calculated as the ratio of tissue wet weight (g) to body weight (g). As shown in Fig. 5B, the organ coefficient of the lungs in the model xenograft group was markedly higher than that in the normal group ($p=0.036$), confirming the successful establishment of a lung adenocarcinoma model. In addition, the organ coefficient of lung in the glycyrrhizin-treated group was significantly lower than in the model group ($p=0.038$), suggesting inhibitory effects of glycyrrhizin on tumor growth *in vivo*.

The above results were further confirmed by western blot analysis. As demonstrated in Fig. 5C, when using a human-specific β -tubulin antibody, β -tubulin was only detected in tumor-bearing mice (model and glycyrrhizin-treated group), but not in normal mice. Compared to the model xenograft group, the level of β -tubulin (human origin) in the glycyrrhizin-treated group was much lower, suggesting that lung adenocarcinoma cell death could be induced by glycyrrhizin *in vivo*. This finding was further supported by the detection of PTEN and anti-apoptotic protein survivin. Importantly, expression of both TxAS and its product TxA2 were increased in the model xenograft group, while their expression was dramatically inhibited by glycyrrhizin treatment (Fig. 5D), suggesting a role of TxAS in the anti-tumor effects of glycyrrhizin.

Discussion

The present study provides the first evidence of anti-tumor effects of glycyrrhizin administration in lung adenocarcinoma *in vitro* and *in vivo*. A549 and NCI-H23 are lung adenocarcinoma cell lines widely used in cancer research, yet only A549 cell growth was significantly suppressed by glycyrrhizin in a dose-dependent manner. Moreover, the suppression of A549 cell growth by glycyrrhizin was due to an induction of apoptosis but not cell cycle arrest. The synergistic effect of glycyrrhizin and cisplatin was also observed in A549, but not in NCI-H23 cells. In addition, the study in MEF cells revealed low toxicity and tumor-specific properties of glycyrrhizin (to some extent). MEF cells are used as an ideal tool for research into human disease because there are similarities between some types of MEF cells and human embryonic stem cells [32, 33]. Furthermore, the synergistic effect from the treatment with both glycyrrhizin and cisplatin on tumor cell growth suggests that glycyrrhizin could be used as an adjunctive agent not only to enhance the chemotherapeutic effects of cisplatin but also to reduce the negative side effects associated with cisplatin.

We further examined why there was a difference in the effects of glycyrrhizin on the cell growth of A549 and NCI-H23 cells. COX-2 is a main target for the pharmacological effects of glycyrrhizin [11, 12], as stated in the Introduction. PGE2, PGI and TxA2 are three main downstream products of the COX-2 pathway, and PGES, PGIS and TxAS are responsible for their respective biosynthesis [14]. The role of PGES in tumor pathogenesis is controversial [18, 19] as PGES has recently been reported to be unrelated to COX-2 in lung cancer [34]. PGIS has been demonstrated to have anti-cancer activity [15], and TxAS is a well-established survival factor in many tumor types [14]. Collectively, these observations suggest that TxAS

Fig. 6. Graphical abstract. Glycyrrhizin suppresses cell growth and induces apoptosis by suppressing TxAS expression and activity in lung adenocarcinoma cells.

relays COX-2 signaling in lung adenocarcinoma, which is also supported by the fact that the expression of TxAS correlates with COX-2 expression in lung adenocarcinoma [16, 20, 35]. Thus, it is reasonable to hypothesize that the differential effects of glycyrrhizin observed in A549 and NCI-H23 cells may be due to different expression levels of COX-2 or TxAS. The mRNA expression of COX-2 and its downstream enzymes were therefore screened in various lung cell lines, including an immortalized normal cell line CCL-75.1. Strikingly, the expression of TxAS in A549 and NCI-H23 cells was the most different. The expression level of TxAS in normal CCL-75.1 cells was low but was even lower in NCI-H23 cells. A549 cells can best reflect human lung adenocarcinoma pathology as TxAS is overexpressed in lung adenocarcinoma specimens [16]. Moreover, glycyrrhizin may significantly suppress the expression of TxAS mRNA and protein as well as its activity. Because the biosynthesis of TxA2 is directly related to TxAS activity, TxAS activity is monitored by TxA2 production [21, 24, 36].

In light of these results, it is possible that the anti-tumor effect of glycyrrhizin is TxAS-dependent, which is supported by the data showing that the increased cell growth in NCI-H23 cells overexpressing TxAS was blocked by administration of glycyrrhizin. Furthermore, knocking down TxAS in A549 cells decreased cell growth, and the addition of glycyrrhizin did not have any additional effects. In support of these results, there is considerable evidence showing that suppression of TxAS could reduce cell growth in many types of cancer, including lung cancer [20-24, 37-44].

Last, we verified the anti-tumor effects of glycyrrhizin in lung adenocarcinomas by performing *in vivo* studies. The xenograft model was established by intravenous injection of A549 cells into the tail vein of nude mice [45]. The successful establishment of this animal model was validated by H&E staining, increased organ coefficient of the lung and the presence of human origin β -tubulin in mice inoculated with A549 cells. Compared to mice without treatment, lower levels of organ coefficient of the lung and anti-apoptotic protein survivin, as well as higher expression of tumor suppressor PTEN in the xenograft model mice treated with glycyrrhizin, suggest the induction of *in vivo* tumor cell death by glycyrrhizin, which is consistent with the *in vitro* data that glycyrrhizin induced apoptosis in A549 cells. More importantly, glycyrrhizin treatment suppressed TxAS protein expression and activity (reflected by the biosynthesis of TxA2) in animal xenografts, which corresponded to changes in the organ coefficient of the lung and expression of the anti-apoptotic protein survivin. Coupled with previous studies demonstrating that suppression of TxAS reduced tumor growth *in vivo* [46, 47], these results confirmed the critical role of TxAS in the anti-tumor effects of glycyrrhizin.

Overall, our study has revealed anti-tumor effects of glycyrrhizin on lung adenocarcinoma, and these effect are, to some extent, TxAS-dependent (Fig. 6). TxAS has been shown to be a therapeutic target in lung cancer [20, 21, 35]. Our study therefore makes glycyrrhizin a promising anti-cancer agent for the chemoprevention and treatment of lung adenocarcinoma.

Abbreviations

COX (cyclooxygenase); NSCLC (non-small cell lung cancer); PGE2 (prostaglandin E2); PGI (prostacyclin); TxA2 (thromboxane A2); PGES (prostaglandin E synthase); PGIS

(prostacyclin synthase); TxAS (thromboxane synthase); MEF (mouse embryonic fibroblast); PTEN (phosphatase and tensin homolog); PARP (poly ADP ribose polymerase).

Conflicts of Interest

No potential conflicts of interest were disclosed.

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