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ORIGINAL ARTICLE

Effect of TGF-β1 on apoptosis of colon cancer cells via the ERK signaling pathway

Yunshan Zhao[1]*, Shaoyou Xia[1]*, Chen Cao[2], Xiaohui Du[1]

[1]Department of General Surgery and [2]Department of Pathology, Chinese PLA General Hospital, Beijing 100853, China.

*These authors contributed equally to this study.

Summary

Purpose: *To study the effect of transforming growth factor (TGF)-β1 on apoptosis of colon cancer cells via the ERK signaling pathway.*

Methods: *Human chemosensitive colon cancer cell line HT-29 was used in this study. VEGF mRNA and protein level were detected using PCR and western blot. Enzyme-linked immunosorbent assay (ELISA) was used for prostaglandin (PG) detection. Cell proliferation was determined via MTT assay.*

Results: *TGF-β1 had a significant effect on blocking the cancer cell growth ($p<0.05$). TGF-β1 significantly reduced the VEGF mRNA level ($p<0.05$) and eliminated the COX-2 expression in a dose-dependent manner, while p53 expression was increased ($p<0.05$). TGF-β1-induced inhibitory effect on COX-2 was significantly eliminated by the ERK inhibitor Compound C ($p<0.05$). The basal PGE2 production was eliminated in cells treated with TGF-β1 ($p<0.05$). N-acetylcysteine (NAC) treatment almost completely eliminated the reactive oxygen species (ROS) produced by TGF-β1 and ERK activation. Compared with administration of 5-FU or etoposide alone, TGF-β1 combined with 5-FU or etoposide significantly administration the viability of colon cancer HT-29 cells.*

Conclusion: *ERK is a newly-identified cancer target molecule, which can significantly control COX-2 in colon cancer cells treated with TGF-β1.*

Key words: *apoptosis, colon cancer cells, COX-2, ERK, TGF-β1*

Introduction

Transforming growth factor (TGF)-β1 is a cytokine regulating a variety of basic cell functions, including growth, differentiation, migration and apoptosis. In the early-stage adenoma, TGF-β1 plays a role as a tumor inhibitor in normal epithelial cells through inhibiting cell growth, and is also one of the key cytokines promoting epithelial-mesenchymal transition (EMT) during embryonic development and progression of cancer in the advanced stage, leading to invasion and metastasis of tumor cells [1]. TGF-β1 protein is a key regulatory factor for development and tissue differentiation [2], which sends signals through the complex of TGF-β type II receptor (TβRII) and type I receptor (TβRI) with activity of serine-threonine kinase [3]. However, the kinase domains of TβRI and TβRII are homologous to tyrosine kinase [4], and TβRII phosphorylates itself spontaneously on tyrosine, serine and threonine [5]. The receptor complex is involved in the activation of TβRI, which phosphorylates and activates Smad2 and Smad3 [6]. Then, these proteins combine with Smad4, transfer to the nucleus and bind to DNA-binding complex to regulate gene transcription.

The activation of extracellular signal-regulated kinase (ERK) is important for TGF-β signal transduction. Firstly, both ERK activation and Smad signal transduction are necessary for TGF-β-induced

Correspondence to: Xiaohui Du, MM. Department of General Surgery, Chinese PLA General Hospital, No. 28, Fuxing Rd, Beijing 100853 China.
Tel: +86 010 66 887329, E-mail: zuoy362@126.com
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EMT, a critical event in tumor invasion and metastasis [7]. Secondly, ERK phosphorylation receptor activates Smads to regulate nuclear translocation [8]. Lastly, the ERK substrate interacts with Smads to regulate the gene expression [9].

Mitogen-activated protein kinase (MAPK), also known as ERK, is a metabolism sensor protein kinase, which, as an energy sensor, mainly plays an important role in the absence of adenosine triphosphate (ATP) [10]. Therefore, ERK plays a major protective role under metabolic stress. In the activated state, ERK downregulates several anabolic enzymes, thereby shutting down the metabolic pathways of ATP consumption [11]. According to some reports, ERK possesses great pro-apoptotic potential under activated conditions, such as 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (ERK activator)-treated cells or constitutively activated ERK mutant [12]. The purpose of the study was to explore the possibility that TGF-β1 combined with chemotherapy could improve the therapeutic efficacy.

Methods

Cell culture and reagents

This study was approved by the Ethics Committee of the Chinese PLA General Hospital. Human colon cancer HT-29 cell line was purchased from ATCC (Gaithersburg, MD). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) under normoxia (20% O_2, 5% CO_2 and 75% N_2). Etoposide, 5-fluorouracil (5-FU), methyl thiazolyl tetrazolium (MTT), Hoechst 33342, DCFH, NAC and AICARG were purchased from Sigma (Waltham, MA, USA). Compound C was purchased from Pharmacia, Korea. The prostaglandin assay kit was purchased from Amersham Pharmacia Biotech (New York, USA). The anti-phospho-specific antibody identifying phosphorylated ACC-Ser79 was purchased from Cell Signaling Technology (Danvers, MA, USA). COX-2, p53 and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ELX800 reader (Bio-Tec Instruments, Inc., Winooski, Vermont), fluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan); 2',7'-dichlorofluorescein diacetate (Sigma), TRIzol reagent (Life Technologies, UK), and complementary deoxyribonucleic acid (cDNA) synthesis kit (Amersham Pharmacia Biotech, Piscataway, New Jersey) were also used.

Experimental methods

Protein extraction and Western blotting

Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed using lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_2CO_3 orthovanadate, 1 mM NaF, 1 μg/mL aprotinin, 1 μg/mL leupeptin and 1 μg/mL pepstatin), followed by Western blotting analysis.

Enzyme-linked immunosorbent assay (ELISA) of prostaglandin

The supernatant of control and treated cell culture was collected via centrifugation at 2500 g for 5 min, the sample was added in 6-well plates and the prostaglandin E2 (PGE2) was transferred in all 6-well plates except the blank well. Finally, the anti-PGE2 monoclonal antibody was added into all 6-well plates except the blank well for blank and non-specific binding test, followed by incubation at 4°C for 18 hrs. After the 6-well plates were washed 4 times, the tetramethyl benzidine substrate was added for color development. After incubation at room temperature for 30 min, the reaction was quenched via the addition of 1 M sulfuric acid. The optical density was measured at 450 nm using the ELX800 reader.

Hoechst 33342 chromatin staining

As previously mentioned, apoptosis was observed using Hoechst 33342 chromatin staining. Cells were incubated and the supernatant was discarded. Then, cells were fixed in PBS containing 3.5% formaldehyde at room temperature for 30 min, washed with PBS for 4 times and exposed to 10 μM Hoechst 33342 at room temperature for 30 min. Finally, cells were examined using fluorescence microscope under ultraviolet radiation.

Determination of cell proliferation via MTT assay

Cells inoculated in a 96-well plate (4×10^3 cells/well) were incubated with the test compound for a specified time. Then the medium was taken and incubated with 100 μL MTT solution (PBS containing 2 mg/mL MTT) for 4 hrs. MTT solution turned blue. The optical density was measured at 540 nμ using BioRaD (Hercules, CA, USA) reader.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using the TRIzol reagent according to instructions of the manufacturer, and reversely transcribed into cDNA using the cDNA synthesis kit. The cDNA fragments were amplified via PCR using the following specific primers: VEGF: sense 5'-AGGAGGGCAGAATCATCACG-3', antisense 5'-CAAGGCCCACAGGGATTTTCT-3'. Glucose transporter-1 (Glut-1): sense 5'-CGGGCCAAGAGTGTGAA-3', antisense 5'-TGACGATACCGGAGCCAATG-3'. β-actin: sense 5'-GTGGGGGCGCCCAGGCACCA-3', antisense 5'-CTCCTTAATGTCACGCACCATTTC-3'. PCR was initiated in thermal cycle at 95°C for 5 min, 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, followed by amplification for 25 cycles. The amplified product could be seen on the 1% agarose gel.

ROS measurement

Cells were inoculated into a 12-well microplate covered with the glass. After stimulation for a specified time, cells were incubated with 10 μM 2',7'-dichloro-

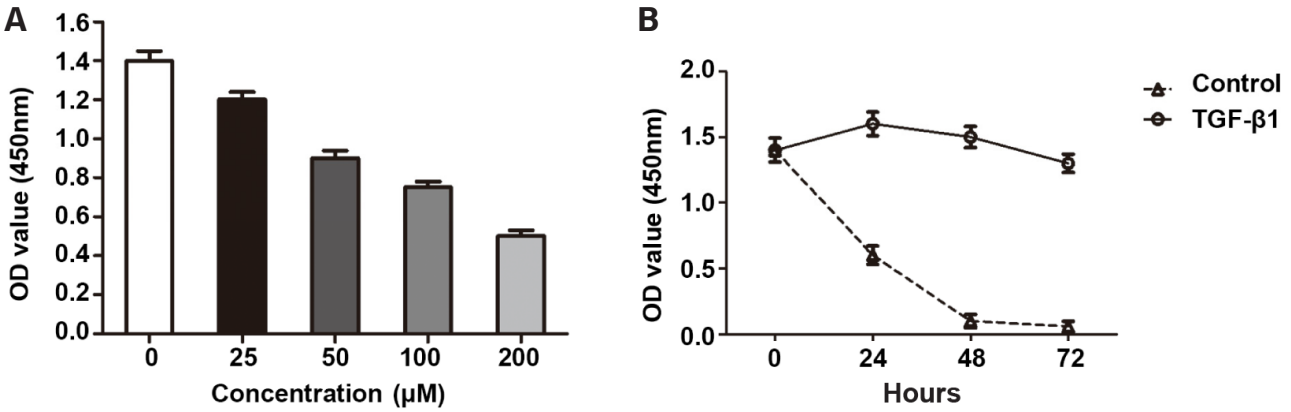

Figure 1. Effect of TGF-β1 treatment on death of colon cancer HT-29 cells. **(A):** Cells are treated with TGF-β1 in different concentrations for 48 h. **(B):** Cells are exposed to TGF-β1 (50 μM) within a specified period of time, and then the cell viability is detected via MTT assay. Data are presented as mean ± standard deviation ($p<0.05$).

fluorescein diacetate (Sigma) for 30 min and washed with PBS, followed by observation under fluorescence microscope.

Results

TGF-β1 inhibited the proliferation of colon cancer HT-29 cells

TGF-β1 inhibited the growth of HT-29 cells in a dose-dependent manner, and 10 μM TGF-β1 inhibited the tumor cell growth within a specified period of time (Figure 1A). TGF-β1 had a significant effect on blocking the cancer cell growth (Figure 1B).

Effect of TGF-β1 on VEGF gene expression

TGF-β1 significantly reduced the VEGF mRNA level (Figure 2).

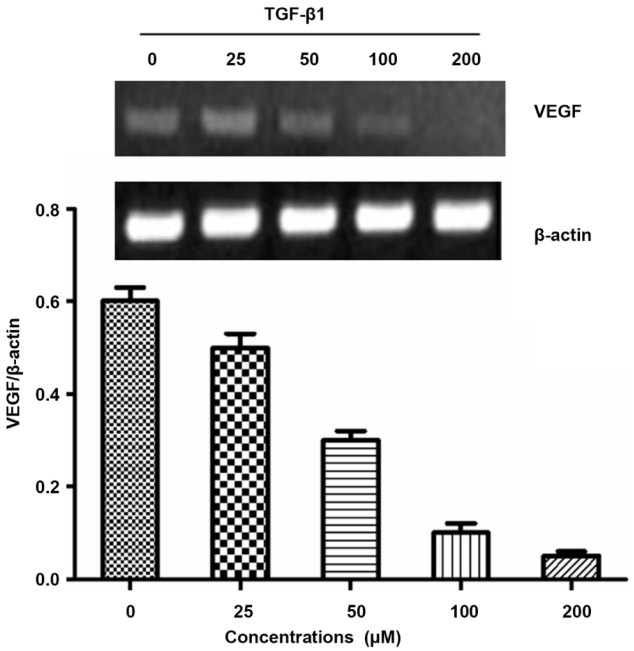

Figure 2. Effect of TGF-β1 on VEGF mRNA level. HT-29 cells were exposed to TGF-β1 in different concentrations for 12 h (Western blotting), and mRNA levels of VEGF and β-actin genes were detected via RT-PCR using specific primers. TGF-β1 reduced significantly the VEGF mRNA level ($p<0.05$).

TGF-β1 eliminated the COX-2 expression and promoted the apoptotic protein expression via ERK phosphorylation

In this study it was observed that TGF-β1 eliminated the COX-2 expression in a dose-dependent manner, while the p53 expression was increased (Figure 3A). The correlation between ERK and COX-2 expression was studied through TGF-β1 treatment, and it was found that TGF-β1 activated ERK in a dose-dependent manner.

The TGF-β1-induced inhibitory effect on COX-2 was significantly eliminated by the ERK inhibitor Compound C (Figure 3B). The basal PGE2 production was eliminated in cells treated with TGF-β1, which was significantly increased by Compound C treatment (Figure 3C). These results suggest that ERK may play an important role in regulating COX-2 in TGF-β1-treated cancer cells. Besides, the regulatory effect of ERK on COX-2 was further detected using the permeable ERK activator AICAR (Figure 3D). The results showed that ERK inhibited COX-2 in TGF-β1-treated cancer cells.

ROS was an upstream signal of ERK activation after TGF-β1 treatment

The detection of H_2O_2 production in TGF-β1-treated cells using DCFH-DA revealed that TGF-β1 significantly increased ROS (Figure 4). To determine whether the ERK activation observed involved the ROS, cells were incubated with the antioxidant NAC, an extensive ROS scavenger. NAC treatment almost completely eliminated the ROS

produced by TGF-β1 and ERK activation (Figure 4). The above results suggest that TGF-β1 is able to produce ROS, and ROS is an upstream signal of ERK activation.

New role of TGF-β1 in combined chemotherapy

The efficacy of TGF-β1 combined with anti-cancer drugs, such as 5-FU or etoposide, was studied. Compared with administration of 5-FU or etoposide alone, TGF-β1 combined with 5-FU or etoposide significantly reduced the viability of colon cancer HT-29 cells (Figure 5).

Discussion

Cancer cells usually display high potential of proliferation, migration and matrix invasion through regulating signal molecules [13]. In this study, whether TGF-β1 inhibited the HT-29 cell proliferation via regulating important signal mol-

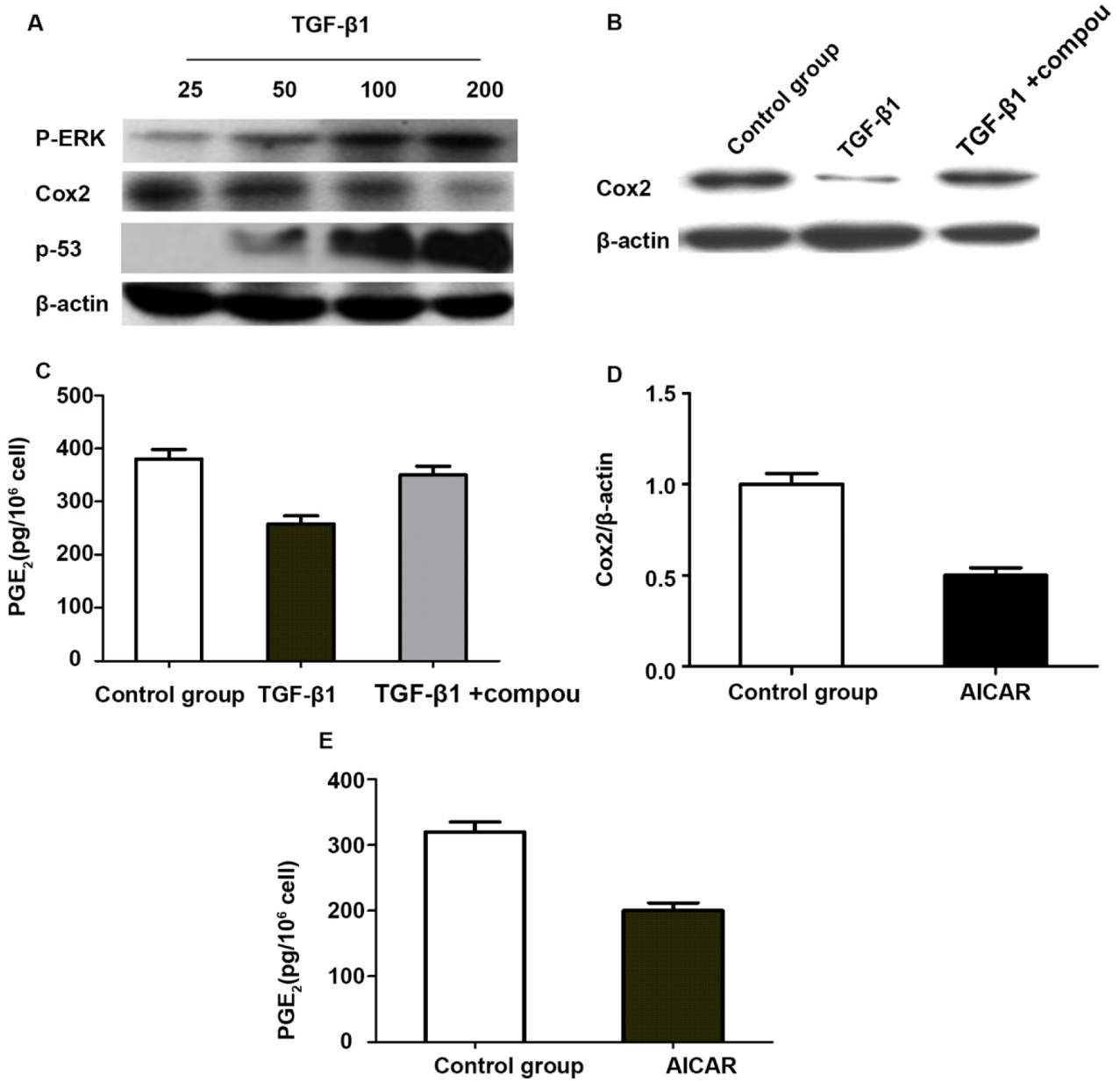

Figure 3. Effects of ERK activator or inhibitor on COX-2 and p53 levels or PGE2 production in TGF-β1-treated cells. **(A):** HT-29 cells were treated with TGF-β1 in different concentrations for 6 h, and COX-2, p53 and ERK levels were analyzed via Western blotting. **(B)**: Cells were pretreated with Compound C (10 μM) for 45 min and exposed to TGF-β1 (100 μM) for 6 h, and COX-2 was analyzed via Western blotting. **(C):** HT-29 cells were pretreated with Compound C (10 μM) for 30 min and then exposed to TGF-β1 (100 μM) for 12 h, and the PGE-2 content in the culture supernatant was detected via ELISA. **(D & E):** Cells were exposed to AICAR (1 mM) for 6 h, and PGE2 and COX-2 production was detected via Western blotting ($p<0.05$).

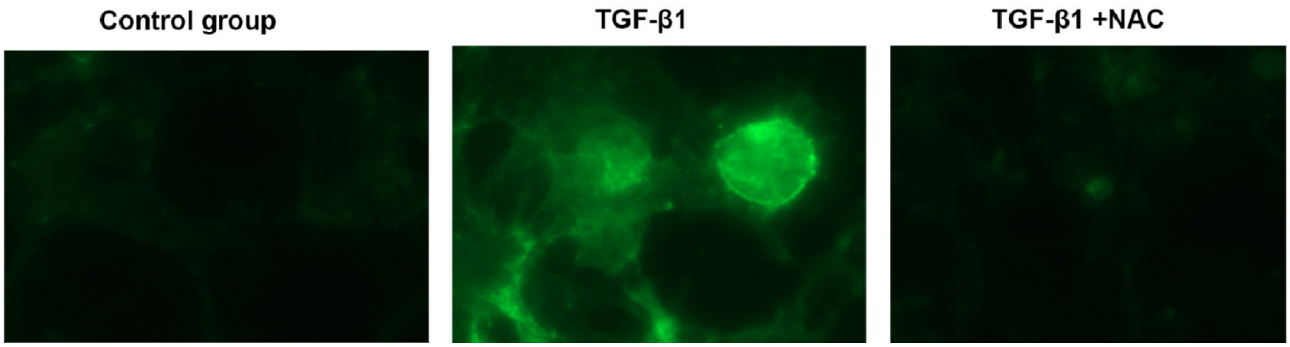

Figure 4. TGF-β1 produces ROS in colon cancer HT-29 cells. Cells were exposed to TGF-β1 for 6 h in the presence or absence of NAC (5 mM). After incubation for another 30 min in the presence of 10 μM DCFH-DA, changes in the fluorescence intensity were measured via the fluorescence cell scan analysis. TGF-β1 significantly increased ROS production. NAC treatment almost completely eliminated the ROS produced by TGF-β1 and ERC activation.

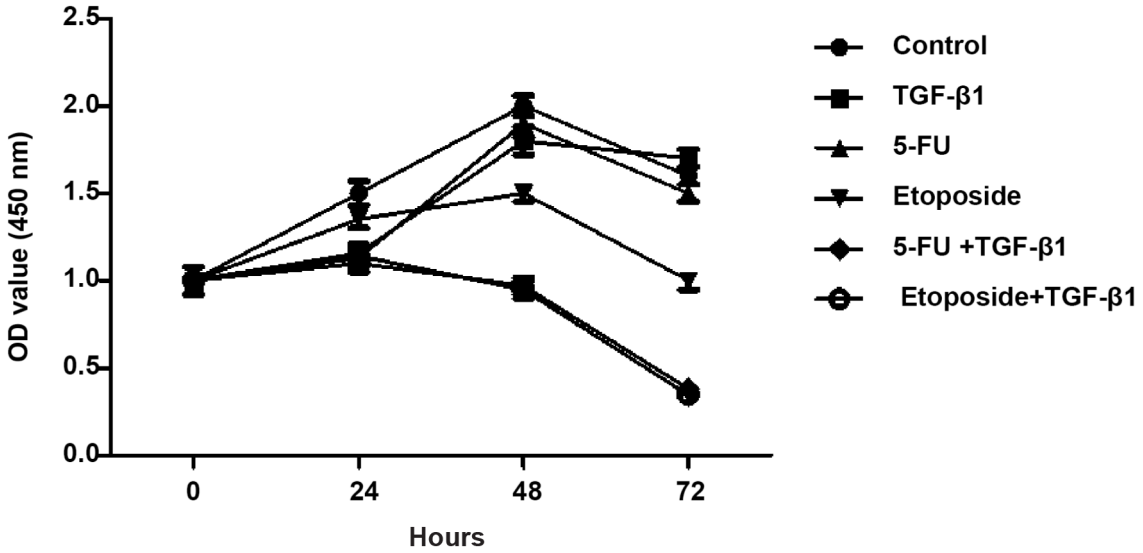

Figure 5. Synergistic apoptotic effect of TGF-β1 combined with different chemotherapeutic drugs in colon cancer HT-29 cells. HT-29 cells were pretreated with TGF-β1 (100 μM) and exposed to 5-FU (50 μM) or etoposide (50 μM) within a specified period of time, and the cell viability was determined via MTT assay ($p<0.05$).

ecules involved in cell survival or apoptosis was evaluated. According to extensive research, the regulation of various signaling pathways involved in tumor survival, growth, invasion or metastasis is an important goal in the development of specific cancer therapeutic method using TGF-β1 [14].

In this study, it was proved that the treatment of chemotherapy-resistant colon cancer HT-29 cells with TGF-β1 weakened the cell proliferation comparable to selective COX-2 inhibitor. Some reports have demonstrated that the COX-2 inhibitor is sensitive to apoptosis of chemotherapy-resistant cancer cells [15], and a variety of COX-2 inhibitors, such as celecoxib, indomethacin, aspirin and NSAIDs, are major drug candidates in the prevention and intervention tests of colon cancer [16]. Currently, the exact cellular and molecular events of COX-2 inhibitor-induced cancer cell sensitization remain unknown. However, many researchers have paid attention to the efficacy of natural COX-2 regulator in cancer prevention [17]. In this study, the efficacy of TGF-β1 combined with 5-FU or etoposide in inhibiting the growth of chemotherapy-resistant cell lines was proved. HT-29 cells displayed high proliferation and resistance to chemotherapeutic drugs [18], and the basal levels of proliferative proteins, such as COX-2, were increased [19]. The antitumor activity of TGF-β1 was related to the inhibition of COX-2 through activating ERK. TGF-β1 treatment eliminated the increase in the basal level of COX-2, and it was observed that TGF-β1 widely phosphorylated ERK. ERK is involved in the homeostasis of cells, and it has been confirmed recently that ERK is a pivot point between cell survival and apoptosis [20]. In addition, LKB1 is a tumor-inhibiting factor, which has been determined as the upstream kinase of ERK. Obviously, the regulatory effect of TGF-β1 on ERK is an important antitumor molec-

ular mechanism [21]. Another ERK activator in a synthetic form, AICAR, has been proved to be an inhibitor of anabolism in tumor cells [18]. It was observed in this study that TGF-β1 could activate ERK, thereby eliminating the production of COX-2 and PGE2 and further increasing the apoptosis of cancer cell lines. The above results indicate that ERK is a cancer target molecule, which can significantly control COX-2 in colon cancer cells treated with TGF-β1.

It has been demonstrated that several kinds of natural compounds used in cancer treatment will lead to an increase in ROS production in cells [22], resulting in damage to mitochondrial membrane permeability. ROS is also one of the upstream signals of ERK activation, which is involved in the inhibition of COX-2. The role of ERK in the ROS-induced inhibition of COX-2 in colon cancer cells was studied, and it was found that TGF-β1 significantly produced ROS, which could induce ERK. The ROS scavenger NAC completely eliminated the ROS production and ERK activation and apoptosis of TGF-β1-treated cells.

In conclusion, this study indicates that TGF-β1 is involved in a variety of molecular events in colon cancer HT29 cells, including inhibition of cell growth, induction of apoptosis and ROS production, decline in COX-2 expression and inactivation of ERK. ROS has been determined as one of the upstream regulators of ERK, and the ERK activation is a key factor in regulating the COX expression. Further investigating and clarifying TGF-β1 in inhibiting ROS and COX-2 through activating ERK is helpful to expounding the molecular regulatory mechanism of tumor cells.

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Authors' contributions

YZ and SX helped with western blot. CC performed ELISA. YZ and XD were responsible for Hoechst 33342 chromatin staining. All authors read and approved the final manuscript.

Conflict of interests

The authors declare no conflict of interests.

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