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Anti-angiogenesis effect of trichosanthin and the underlying mechanism

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ABSTRACT

The growth and metastasis of tumors depend on angiogenesis. Tumor angiogenesis is initiated by the secretion of growth factors from tumor cells; downstream signals are then triggered in pre-existing blood vessels to sprout a new vascular network. Trichosanthin (TCS) is a type I ribosome-inactivating protein that has anti-tumor activity, but the underlying mechanism remains unclear. In this study, we found that a non-toxic dose of TCS decreased the wound-healing and the migration of H5V mouse heart capillary endothelial cells (ECs) induced by human choriocarcinoma (JAR) cells, as well as the JAR-induced angiogenesis of rat third-order mesenteric arteries. TCS was effective on both tumor cells and ECs/arteries. First, TCS decreased vascular endothelial growth factor transcription and secretion by JAR cells. Second, TCS consequently inhibited the tumor cell-induced, extracellular signal-regulated kinase-mediated angiogenic signal in ECs and blood vessels. In conclusion, the ability of TCS to inhibit tumor angiogenesis contributes to its anti-tumor activity.

1. Introduction

Angiogenesis is the formation of a new vascular network from pre-existing vessels [1]. It is a vital process in the growth of blood vessels and wound healing. Furthermore, angiogenesis plays a key role in tumor development. Tumor cells are able to activate a process very similar to normal angiogenesis. Tumor cells can secrete a large number of growth factors, such as interleukin (IL)-8 and vascular endothelial growth factor (VEGF), which stimulate new blood vessel formation by nearby endothelial cells (ECs) from pre-existing vessels. Angiogenesis is a crucial step in cancer growth, providing nutrients to tumor cells, thus enabling the progressive enlargement and metastatic dissemination of tumors.

A series of positive and negative factors [2] control the process of angiogenesis. VEGF is the most well-studied angiogenic factor. In humans, VEGF mRNA is alternatively spliced and the proteins are classified as $VEGF_{121}$, $VEGF_{121b}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ [3]. VEGF stimulates angiogenesis by binding to VEGF receptors (VEGFRs) on the plasma membrane of ECs. Upon activation, VEGFRs elicit a proliferative signal in the ECs and promote endothelial cell–cell interactions and capillary formation.

Trichosanthin (TCS) is a 27-kDa protein isolated from the root tuber of the Chinese medicinal herb *Trichosanthes kirilowii* Maximowicz. TCS is a type I ribosome-inactivating protein (RIP) and has been used to treat trophoblastic tumors, but the underlying mechanism remains unclear. Nevertheless, evidence shows that TCS at a low dose can interfere with humoral immunity and immune-related processes [4,5]. Because tumor growth, the immune response, and angiogenesis are co-dependent and share key regulatory growth factors and cytokines [6], in this study, the effect of TCS at non-toxic levels on choriocarcinoma cell line-induced angiogenesis was explored. Our results demonstrated that TCS profoundly decreased the angiogenic response of ECs and angiogenesis induced by tumor cells, and the effect of TCS is likely to be mediated through its functional interference with VEGF production in tumor cells and the extracellular regulation of protein kinase activation in ECs.

2. Materials and methods

2.1. Cells, viruses and reagents

The H5V mouse heart capillary endothelial cells and human choriocarcinoma JAR cells were obtained from the ATCC (Rockville, MD, USA). H5V cells were grown in DMEM and JAR cells in RPMI 1640. TCS was from Shanghai Jinshan Pharmaceutical Co., Ltd. (Shanghai, PRC).

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2.2. Cell viability assessment by MTT assay

MTT was used to assess the viability of cells in 96-well plates following the kit protocol (Roche Applied Science). Briefly, H5V and JAR cells were treated with TCS (0.16–5 μg/mL in twofold serial dilutions) for 48 h. After the treatment, to measure the number of viable cells, 10 μL MTT was added to form formazan crystals, which were later dissolved in solubilization solution. Absorbance was read at 570 nm.

2.3. Wound-healing assay

Cell motility was investigated by measuring cell migration into a wound made in a confluent monolayer. Briefly, H5V and JAR cells were separately cultured in 12-well plates and transwell inserts (0.4 μm pore size; Falcon, USA) in complete media with 10% FBS. After overnight growth and attachment, both media were replaced with serum-free ones for another 24 h. A wound was then made by scraping with a P10 pipette tip on the monolayer of H5V cells; floating cells were washed off with PBS. The transwells with JAR cells were then placed into the culture plate wells to co-culture with H5V cells for 48 h. During certain treatments, 1.5 μg/mL TCS and 80 nM of the extracellular signal-regulated kinase (ERK) inhibitor U0126 (Cell Signaling, USA) or 0.1 μg/mL of the ERK activator epidermal growth factor (EGF, Cell Signaling, USA) were added into both the wells and transwell inserts.

The ability of cells to migrate into the wound gap was observed and recorded photographically. Healing was analyzed by measuring the percentage of wound reduction with ImageJ software (NIH, USA).

2.4. Migration assay

H5V migration was assessed with transwell inserts (5.0 μm pore size; Corning, USA). Briefly, JAR cells were cultured in 24-well plates and allowed to grow and attach. Culture medium was then replaced with serum-free medium. The transwell inserts were placed into the wells of the cell culture plates. H5V cells (10^6 cells/ml) were then harvested, washed twice with PBS and resuspended in serum-free DMEM with 0.2% BSA. The cell suspension (200 μL) was then seeded into each upper chamber of the inserts and co-cultured with JAR cells. During certain treatments, 1.5 μg/mL TCS or 5 μg/mL of the pan-VEGF inhibitor GW654652 (Glaxo-SmithKline, Collegeville, PA) was added. FBS (10%) without JAR

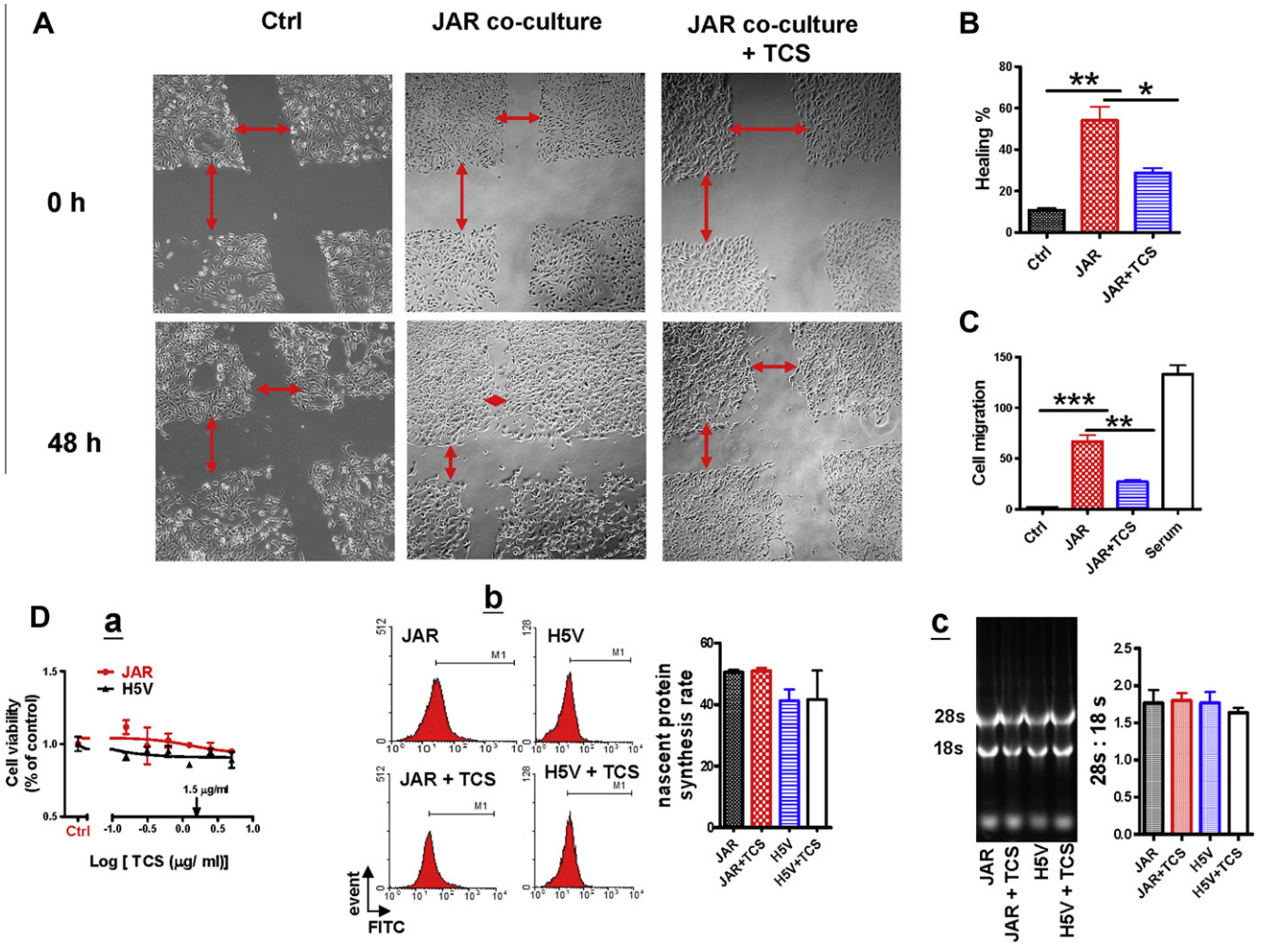

Fig. 1. TCS suppresses JAR cell-derived endothelial migration. (A and B) Representative images and statistics of the effect of TCS on the JAR cell-induced wound-healing response in H5V cells. A wound was generated by scraping the monolayer of H5V cells, and JAR cells were co-cultured with H5V cells in transwell inserts. Untreated or with 1.5 μg/mL TCS for 48 h, the ability of cells to migrate into the wound was observed and analyzed. (C) Statistics summarizing the effect of TCS on JAR-induced migration in H5V cells. H5V cell suspensions were seeded in transwell inserts and co-cultured with pre-seeded JAR cells with or without 1.5 μg/mL TCS for 18 h. H5V cells that migrated through the filter were stained with crystal violet and counted under a microscope. (D) Cytotoxicity in H5V and JAR cells assessed by MTT assay (a). RIP activity of TCS was analyzed by status of nascent protein synthesis (b) or ribosome RNA agarose gel (c). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

cells in the lower well was used as a chemoattractant for positive control. After 18 h stimulation, H5V cells that had not migrated through the filter in the transwell inserts (on the upper surface of the filter) were removed with a cotton swab. Cells that had migrated to the lower surface of the filter were stained with crystal violet and counted under a microscope.

2.5. Nascent protein synthesis rate assay

The Click-iT® AHA kit (invitrogen, USA) was employed to detect nascent protein synthesis rate. H5V or JAR cells were untreated or given 1.5 μg/mL TCS for 72 h. Then the total proteins of cells in each treatment were labeled with L-azidohomoalaine (AHA) and detected by alkyne-containing molecule with a FACSCalibur flow cytometer on FITC channel.

2.6. Ribosome RNA integrity assay

H5V or JAR cells were untreated or given 1.5 μg/mL TCS for 72 h. Total RNA was extracted using the Trizol protocols (Qiagen, USA). Products were resolved on 1% agarose gels. Band intensity was analyzed by G:BOX Chemi system (Syngene, USA).

2.7. Real-time PCR

JAR cells were untreated or given 1.5 μg/mL TCS. cDNA was made with an iScript™ cDNA synthesis kit (Bio-Rad Laboratories, CA). Real-time PCR was performed using IQ™ SYBR Green Supermix (Bio-Rad) for 40 cycles of 95 °C for 15 s and 58 °C for 40 s. The data were analyzed with iCycler Multicolor Real-time PCR Detection System Software (Bio-Rad). The primer pair for VEGF was: forward 5′-GCAGAATCATCACGAAGTGG-3′, reverse 5′-GCATGGTGATGTTGGACTCC-3′ [7].

2.8. Immunoblotting analysis

Lysates of H5V cells and arteries with indicated treatments were prepared using the CytoBuster™ protein extraction reagent (Novagen, USA) at 4 °C and separated by 12% SDS–PAGE. The antibody binding was detected with an Odyssey imaging system (LI-COR Biosciences, USA). The anti-human phospho-ERK was purchased from Cell Signaling (USA).

2.9. Aortic ring model for angiogenesis

Angiogenesis was assessed with the aortic ring model [8] with modifications. Briefly, male Sprague–Dawley rats at 28 weeks were sacrificed by CO_2 asphyxiation in accordance with the guidelines for the Care and Use of Laboratory Animals. Segments of third-order mesenteric arteries were separated and dissected into ~5-mm rings. The rings were then embedded in rat tail collagen (Gibco, USA) and maintained in endothelial basal medium (EBM; Clonetics, USA). With specified treatment, the rings were co-cultured with JAR cells, or treated with 1.5 μg/mL TCS, 5 μg/mL GW654652, 80 nM U0126 or 1 ng/ml EGF for 96 h. The angiogenic response was quantitated by counting the endothelial sprouts from the edges of the rings under a microscope.

2.10. VEGF enzyme-linked immunosorbent (ELISA) assay

VEGF concentrations were determined using ELISA kit (Invitrogen, USA). Briefly, JAR cells were untreated or given 1.5 μg/mL TCS for 48 h. The supernatants of the cells were collected and pipetted into ELISA plates coated with polyclonal antibody specific for human VEGF. The antibody binding was then detected by a biotinylated monoclonal antibody against human VEGF. Bound anti-VEGF was quantified at 450 nm after addition of streptavidin peroxidase and substrate.

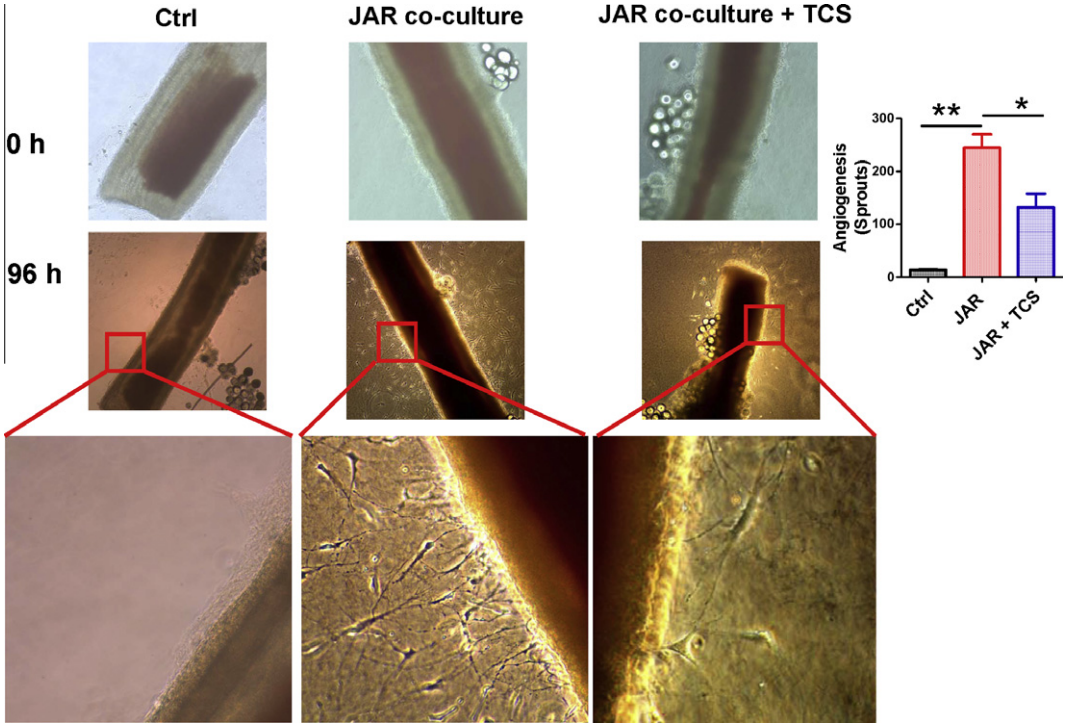

Fig. 2. TCS suppresses JAR cell-derived endothelial migration. Segments of third-order mesenteric arteries embedded in rat tail collagen EBM were co-cultured with JAR cells, with or without 1.5 μg/mL TCS for 96 h. The angiogenic response was quantitated by counting endothelial sprouts from the edge of arteries under a microscope. $^{*}p < 0.05$, $^{**}p < 0.01$.

2.11. Data analysis

Data were analyzed using one-way ANOVA and presented as the mean ± SEM ($n = 3$).

3. Results and discussion

Tumor progression is tightly associated with the immune response and angiogenesis. Many cancers derive from sites of inflammation, where immune-related factors stimulate angiogenesis and potentiate tumor growth [6,9]. TCS has both anti-tumor and immune-modulation activity; although its anti-tumor action is believed to be due to its cytotoxicity [10], it is possible that TCS may also interfere with immune and angiogenic signals during carcinogenesis to inhibit tumor growth. Therefore, we explored the effect of TCS on choriocarcinoma cell-induced angiogenesis.

3.1. TCS suppresses JAR cell-derived endothelial migration

The migration of ECs is an important early step in the angiogenic cascade. This migration can be stimulated by a large number of growth factors secreted by tumor cells, after which the migrated ECs finally differentiate into new blood vessels [1]. We found that choriocarcinoma JAR cells activated marked H5V migration, as evaluated by both wound-healing and transwell-migration assays (Fig. 1 A–C), suggesting that in the co-culture system, JAR cells create an environment to initiate this early step in angiogenesis. However, when both JAR and H5V cells were treated with 1.5 μg/mL TCS, the migration significantly decreased, indicating TCS interferes with this critical JAR-induced endothelial event. During the treatments, the 1.5 μg/ml of TCS did not show cytotoxicity to JAR or H5V (Fig. 1D a). Additionally, because TCS is a RIP, the possible changes in nascent protein synthesis and ribosome integrity were tested in TCS treated cells. There were not significant changes between control and treated samples in the total amount of the nascent synthesized proteins during 72 h period of treatment (Fig. 1D b), suggesting the non-toxic level of TCS does not interfere with protein synthesis. The result was supported by detecting ratio of 28S to 18S in total RNA samples (Fig. 1D c). Usually, degradation of RNA shows a ratio significantly lower than 2:1. However, in H5V or JAR cells treated with TCS, the ratios were all around 2:1, indicating the intact of ribosome RNA. These data together could rule out the RIP effect of TCS on the angiogenesis-related proteins discussed in our following studies.

3.2. TCS suppresses JAR cell-induced endothelial migration

To further elucidate the effect of TCS on angiogenesis, we used an *ex vivo* aortic ring model. Consistent with the results of H5V migration, when the rings from third-order rat mesenteric arteries

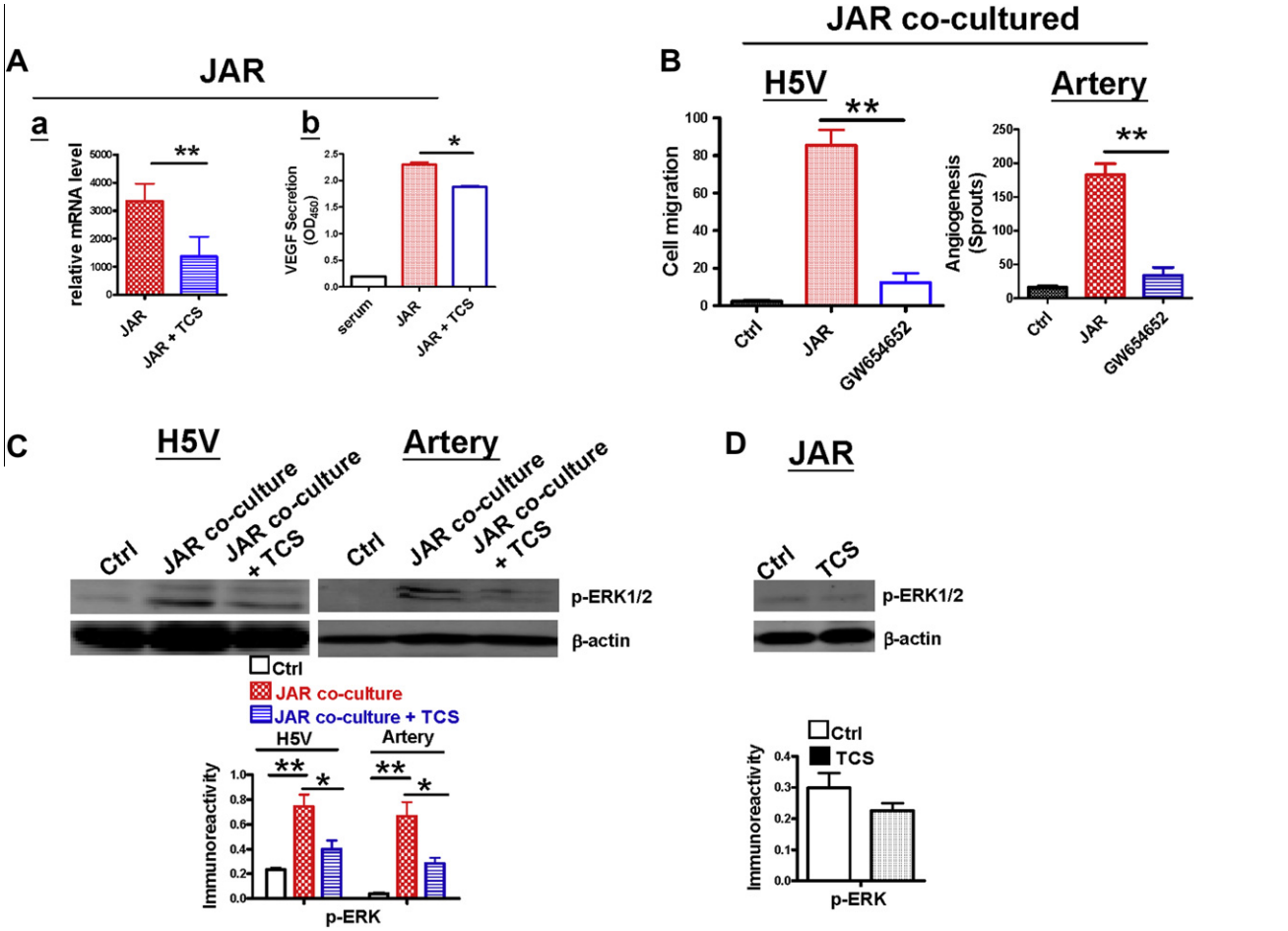

Fig. 3. Involvement of VEGF and ERK pathway in the anti-angiogenic activity of TCS. (A a and b) Effect of TCS on mRNA transcription and protein secretion of VEGF by JAR cells with or without 1.5 μg/mL TCS for 48 h. Levels of VEGF mRNA and VEGF protein were analyzed by real-time PCR and ELISA, respectively. (B) The VEGF inhibitor GW654652 (5 μg/mL) decreased JAR cell-induced H5V migration and angiogenesis. (C and D) Representative images and statistics of Western blot showing the effects of TCS on H5V cells and rat mesenteric arteries (C) and JAR cells (D). H5V cells and arteries were co-cultured with JAR cells and untreated or given 1.5 μg/mL TCS for 48 h, or JAR cells alone were treated with TCS for 48 h. Proteins were extracted and separated by SDS–PAGE ($^{*}p < 0.05$, $^{**}p < 0.01$).

were co-cultured with JAR cells, the number of endothelial sprouts significantly increased (Fig. 2). When treated with TCS, the number of sprouts significantly decreased. Therefore, these data demonstrate the inhibitory effect of TCS on tumor-derived angiogenesis.

3.3. Roles of VEGF and the ERK pathway in the anti-angiogenic activity of TCS

At a non-toxic concentration, TCS modulates the activity of some growth factors, such as IL-2 in activated splenocytes [4], IL-4 in mesenteric lymph node cells and ovalbumin cells, [11], as well as IL-10 and monocyte chemoattractant protein-1 in peritoneal macrophages. To unravel the molecular mechanisms involved in the anti-angiogenic activity of TCS, we evaluated the activation of one angiogenesis-related growth factor, VEGF. The expression and secretion of VEGF is strictly controlled in human and animal tissues, but in many human tumors, abnormally high levels of VEGF are produced and angiogenesis is triggered [3,7]. Indeed, we found that a high level of VEGF mRNA was transcribed in JAR cells (Fig. 3A a), and large amounts of VEGF protein were found in the supernatant from JAR cells (Fig. 3A b). However, when treated with TCS, the VEGF expression and secretion were significantly decreased. VEGF is reported to directly induce the growth of sprouts from preexisting blood vessels [12], so our data strongly suggest that the anti-angiogenic effect of TCS is at least partly due to its ability to inhibit VEGF activation.

Previous studies have shown that after binding to the VEGFR, VEGF activates several signaling cascades in ECs, such as the extracellular signal-regulated kinase (ERK), and tyrosine phospholipase C pathways [13]. It is widely accepted that ERK activation is one of the most important downstream events in VEGF signaling [3].

Therefore, following VEGF activation in JAR cells, we next evaluated the downstream ERK signal in endothelial H5V cells. Previous studies suggested that inappropriate activation of ERK is frequently found in ECs that are near human cancers [3]. Indeed, we found that the phosphorylation of ERK significantly increased in H5V cells when they were co-cultured with JAR cells (Fig. 3B), indicating that JAR co-culture initiated activation of the ERK signaling pathway. The ERK activation in H5V cells depended on growth factors secreted by JAR cells, especially VEGF as we demonstrated before. Removing the JAR cells significantly reduced the level of ERK phosphorylation. Furthermore, consistent with the decreased VEGF activation in TCS-treated JAR cells, the tumor cell-induced ERK activation in H5V cells was also significantly decreased by TCS. In the downstream of the VEGF signaling pathway, ERK triggers deregulated mitogenesis and cell survival signaling, then promotes ECs proliferation, migration, and neovascularization [14]. Therefore, it is reasonable to speculate that when ERK activation is inhibited by TCS, the migration of ECs and the original process of angiogenesis could be also affected.

In addition, we found that untreated JAR cells themselves showed modest ERK phosphorylation, and TCS treatment had no significant effect on this. We previously found that TCS at higher concentrations (>10 μg/ml) interferes with the ERK pathway in JAR cells (unpublished data), and other studies showed that VEGF expression can be modulated by the ERK pathway [15]. However, in this study we found that although the activation of ERK in JAR cells was not affected by TCS, the latter still inhibited VEGF

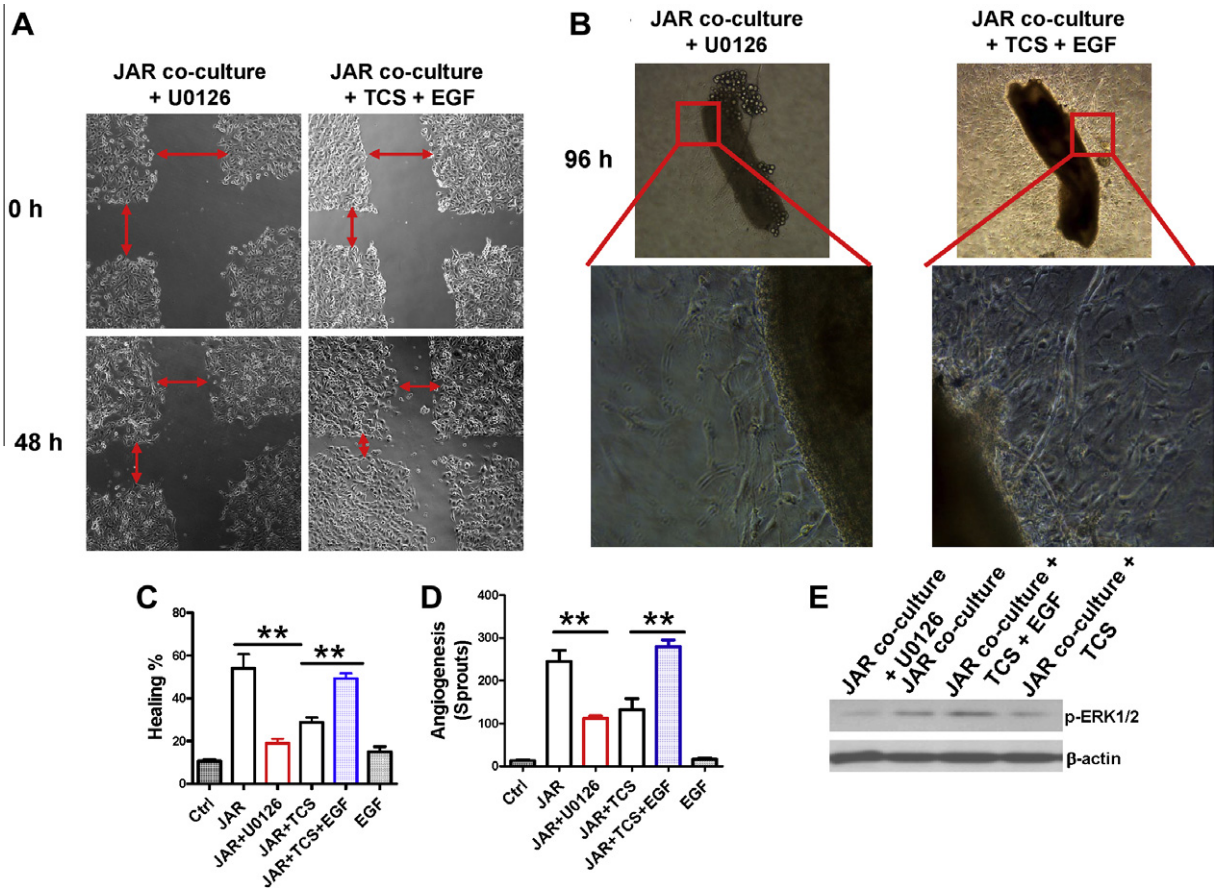

Fig. 4. The effect of ERK inhibitor and activator on the anti-angiogenic activity of TCS. H5V cells or arteries were pre-treated with 80 nM U0126 or 1 ng/ml EGF for 6 or 24 h, then the U0126 or EGF were washed off and cells or arteries were co-cultured with JAR cells, with or without 1.5 μg/mL TCS for 48 h or 96 h. The wound-healing response (A and C), angiogenesis (B and D) and ERK activation (E) were analyzed (**$p < 0.01$).

expression and secretion. This result was probably due to the concentration of TCS, this concentration was too low to show an effect on ERK activation that can be detected by Western blot. Furthermore, VEGF expression could be also controlled by other factors, such as non-receptor tyrosine kinase receptors and signal transducer and activation of transcription 3 [16,17], which are widely involved in VEGF activation in tumor cells and whose activity could be affected by TCS.

3.4. Effects of ERK inhibitor and activator on the anti-angiogenic activity of TCS

By applying an ERK inhibitor and an activator to the co-culture system, we further confirmed the role of ERK in the anti-angiogenic activity of TCS. To avoid errors caused by the detectable ERK activation in JAR cells, after incubation with ERK inhibitor U0126 or activator EGF (Fig. 4E) in H5V cells for 6 h or in arterial rings for 24 h, the activator or inhibitor were washed off. JAR cells were then co-cultured with H5V cells or arterial rings. The ERK inhibitor U0126 significantly decreased the JAR-induced wound-healing response of H5V cells, as well as the JAR-induced angiogenesis in arterial rings (Fig. 4). These data are consistent with previous studies [13] and our earlier observations (see Fig. 3), suggesting that JAR cells and their secreted VEGF depend on ERK activation in H5V cells or arteries to initiate an angiogenesis cascade.

Furthermore, TCS inhibited ERK activation during JAR cell-induced angiogenesis by decreasing the VEGF secretion (Fig. 3B); when JAR cells were treated with TCS to maintain an unchanged level of VEGF secretion, the ERK activator EGF significantly increased the TCS-inhibited wound-healing and angiogenesis responses. However, without JAR cell stimulation, EGF alone did not increase the wound-healing and angiogenesis responses (Fig. 4C and D). This result demonstrated that TCS interferes with the tumor cell-induced angiogenesis cascade through the VEGF–ERK pathway in ECs and arteries.

However, based on our results, it is worth noting that TCS at a non-toxic concentration was found to be more potent on ECs and arteries than tumor cells in its ability to interfere with ERK pathways. This may because ERK plays different roles in response to TCS in tumor cells, ECs and arteries. TCS is an anti-tumor agent because it kills tumor cells to inhibit carcinogenesis. The apoptosis induced by TCS in tumor cells usually involves mitogen-activated protein kinase signal transduction pathways [18], which include the ERK pathway. However, at a non-toxic concentration, the ERK-related apoptotic response to TCS may be greatly reduced. When TCS decreases the VEGF, the effects of TCS and ERK tend to be more directed to the angiogenesis signaling cascade.

Taken together, our study demonstrated that a low concentration of TCS at a non-toxic level inhibits tumor cell-induced angiogenesis. TCS inhibits the secretion of VEGF by tumor cells and activation of the ERK pathway in ECs and arteries.

Application of anti-angiogenic drugs in clinical practice can inhibit tumor angiogenesis and increase the curability of cancers. To date, although TCS shows anti-tumor activity because it kills tumor cells, its clinical application is limited because of undesirable complications such as cytotoxicity and causing an allergic response at high doses. However, in this study, we found that TCS can inhibit angiogenesis at a low dose with less cytotoxicity, making it an attractive option as an anti-tumor agent *via* inhibiting the vascular network during tumor growth. However, great caution is advisable because the pleiotropic activities of low-dose TCS in normal tissues and under other pathological conditions remain unknown.

Acknowledgments

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