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Toxicarioside A, isolated from tropical *Antiaris toxicaria*, blocks endoglin/TGF-β signaling in a bone marrow stromal cell line

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ABSTRACT

Objective: To investigate possible mechanism of toxicarioside A in HS-5 bone stromal cells. **Methods:** HS-5 bone stromal cells were cultured in media supplemented with various concentrations of toxicarioside A or control DMSO (not treatment). Endoglin and TGF-β were detected by Northern and Western blot analysis and quantified in a standard method. Downstream molecules of endoglin and TGF-β (Smad1, Smad2 and their active phosphorylated counterparts, pSmad1 and pSmad2) were also detected and quantified by Western blot analysis. In addition, cell proliferation assay and small interfering RNA (siRNA) against endoglin were used to certificate the function of endolgin in the HS-5 cells. **Results:** Compared with the not treated (0 μg/mL) or DMSO treated control HS-5 cells, HS-5 cells treated with toxicarioside A were found significant attenuation of endolgin and TGF-β expression. Significant inhibition of cell proliferation was also found in the HS-5 cells treated with toxicarioside A. ALK1-related Smad1 and ALK5-related Smad2 were decreased in HS-5 cells treated with toxicarioside A. In addition, phosphorylated Smad1 (pSmad1) and Smad2 (pSmad2) were also found attenuation in toxicarioside A-treated HS-5 cells. RNA interference showed that blockage of endoglin by siRNA also decreased Smad1 and Smad2 expression in HS-5 cells. **Conclusions:** Our results indicate that toxicarioside A can influence bone marrow stromal HS-5′s function and inhibit HS-5 cell proliferation by alteration of endoglin-related ALK1 (Smad1) and ALK5 (Smad2) signaling.

1. Introduction

Bone marrow is a delicate microenvironment in which many cell types (such as hematopoietic cells and immunocytes) are generated, keeping a very important role in body homostatasis and body health. However, in some disease conditions, such as breast and prostate cancers, bone marrow also offers a suitable microenvironment for tumor metastasis and development[1]. At present, prostate cancer has been confirmed as one of the most common cancer and leading cause of cancer-related deaths in the world[2]. It is characterized by a very predictive progression from an early hormone-sensitive form that can be treated by androgen deprivation therapy to a hormone-refractory form that occurs within the axial skeleton[3]. Once prostate cancer becomes hormone refractory, it is essentially incurable and is more preferent to transfer into the state of the secondary bone metastases, in which their sequelae are lethal to patients[2-4]. Thus, it is very important to uncover the potential mechanisms behind the preferential metastasis and growth of certain cancers (such as breast and prostate cancer) in bone marrow microenvironment if want to cure these cancers in the future. It is generally accepted that cancer is not simply a disease involving a single tumor cell that grows out of control, but is a dynamic interaction between the tumor cells and its surrounding microenvironment[5]. Factors present within the bone microenvironment, whether insoluble components of the extracellular matrix or soluble factors (such as cytokines

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and mitogens) secreted by host cells, and direct cell-to-cell interactions are thought to contribute to the preferential metastasis and growth of prostate cancer cells in bone marrow[5-7].

More and more evidence has demonstrated that bone stromal cells affect prostate cancer cells and can be changed by their interactions with the cancer cells[5,8]. Chung and collaborators indicated that growth of both benign and malignant prostate cancers *in vivo* is heavily dependent on the cells of the host environment, including the stromal cells[9, 10]. O'Connor and collaborators found that bone marrow tromal cells coculture with prostate cancer cells alters endoglin expression and attenuates transforming growth Factor-β signaling in reactive bone marrow stromal cells[11].

Previous studies have demonstrated that endoglin is a homodimeric transmembrane glycoprotein that can bind specifically to transforming growth factor β1 (TGF-β1) and TGF-β3, as well as activin A and several bone morphogenic proteins, after incorporation with one of two transmembrane serine-threonine kinases known as TGF-β receptor I and TGF-β receptor II[12]. Endoglin is constitutively phosphorylated, but it is not an active signaling molecule and only works as a auxiliary regulatory component by formation of a heteromeric complex with the TGF-β receptor, by which it regulates the signaling of distinct TGF-β receptor I isotypes known as activin receptor-like kinase (ALK)-1 and ALK-5[13,14]. After formation of the activated heteromeric complex, endoglin initiates an intracellular signaling cascade by which specific Smad proteins are activated and further signals are transduced into the nucleus where they regulate the transcription of series genes involved in maintaining normal physiologic functions such as cell proliferation, apoptosis, cell motility and adhesion[15].

Antiaris toxicaria is widely growing over tropical areas in Southeast Asia. Early studies of the toxic agents of this plant in Indonesia and Malaysia have resulted in the isolation of several kinds of cardenolides from the latex, seeds, and stem[16,17]. Traditionally, cardenolides are generally accepted in treatment of congestive heart failure and as anti-arrhythmic agents[18-20], but recent studies have demonstrated that certain cardenolides extracted from some plants or animals are involved in complex cell signal transduction mechanisms that may have important consequences in blocking tumor cell proliferation and inducing tumor apoptosis[21-27]. In recent years, our research group has isolated three new cytotoxic cardenolides from the latex of *Antiaris toxicaria*, which were further proved to possess the capabilities of significant cytotoxicity against K562, SGC-7901, SMMC-7721 and HeLa cell lines[28, 29]. In the present study, we found that toxicarioside A, isolated from *Antiaris toxicaria* in Hainan China, had the capability of inhibiting endoglin expression and altering TGF-β signaling in a bone marrow stromal cell line, which results in inhibition of cell proliferation. Our results uncover a possible mechanism of toxicarioside A in action against metastatic bone tumors.

2. Materials and methods

2.1. Isolation and identification of toxicarioside A

Latex of *Antiaris toxicaria* was collected in Lingshui county of Hainan Province, China, in November 2005. The plant was validated by Professor Zhu-nian Wang in the Institute of Crops Genetic Resources, Chinese Academy of Tropical Agricultural Sciences. The voucher specimen was numbered as AN200511 and deposited in the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences. Toxicarioside A was isolated from the fractionation of the 60% ethanol extract of the latex of *Antiaris toxicaria* as we previously reported[28] and its structure was elucidated by comprehensive analysis of 1D and 2D NMR spectra (data not shown). The resultant toxicarioside A was dissolved in DMSO in a stock concentration (1 mg/mL) for subsequent experiments.

2.2. Cell culture and toxicarioside A treatment

Human bone marrow stromal cell lines HS-5 (CRL-11882) was purchased from the American Type Culture Collection (ATCC). The HS-5 cells were cultured in low-glucose DMEM medium (Gibical) and supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 ℃ in a humidified atmosphere with 5% CO_2. For analysis of endoglin expression and Smad signaling in HS-5 cells, recombinant TGF-β1 or BMP-2 (eBioscience) 100 ng/mL was added into the DMEM medium. The logarithmic phase or different time point cells were used for subsequent experiments.

2.3. RNA isolation and Northern blot analysis

Total RNA was isolated directly from HS-5 cells using TRIzol reagent (Gibco-BRL/Invitrogen) as recommended by the manufacturer's instructions. For Northern blot analysis, RNA was transferred to Hybond N^+ membranes and then hybridized with full-length cDNA probes for murine endoglin and β-actin in PerfectHyb Plus hybridization buffer (Sigma-Aldrich) according to the manufacturer's instructions. Digital images were acquired and analyzed with a gel imaging system (Bio-Rad Gel Doc1000, Bio-Rad). The resultant mRNA level were relatively compared to β-actin and expressed as percent of β-actin.

2.4. Western blot analysis

Western blot analysis was performed as described previously[30-32]. Total cellular protein was prepared by solubilizing the HS-5 cells with SEB. The protein concentration of each sample was determined by the Lowry methods. Fifteen micrograms of total cell protein were used to analysis for endoglin, TGF-β, Smad1, Smad2, pAmad1 and pSmad2. All membranes were probed with antibodies to β-actin to correct for loading and transfer differences among samples. Before loading, all cell lysates were added to an equal volume, heated at 100 ℃ for 5 min. Thereafter, the proteins were separated by SDS-PAGE using Bio-Rad Criterion Tris-HCl gels with a 4% (w/v) polyacrylamide stacking gel and a 10% (w/v) polyacrylamide resolving gel in gel running buffer (150 mmol/L glycine, 50 mmol/L Tris base, 0.1% (v/v) SDS, pH 8.8) under constant voltage. The fractionated proteins were transferred to a polyvinylidene Fluoride (PVDF) membrane at 4 ℃ for 5 h at 50 V in transfer buffer (100 mmol/L glycine, 100 mmol/L Tris base, pH 8.3).

The PVDF membranes were blocked overnight at 4 ℃ with gentle rotary agitation in TBS/0.1% (v/v) Tween 20 (TBST). The primary antibodies for β-actin (1:15 000; Abcam), endoglin (1:250; Santa Cruz Biotechnology), Smad1 (1:1 000; Cell Signaling Technology, Inc.), Smad2 and pSmad1 (1:1 000; Cell Signaling Technology) were applied in 5% (v/v) bovine serum albumin. All primary antibodies were incubated overnight at 4 ℃ with gentle rotary agitation. The blots were washed at least three times for 10 min each at room temperature with PBST. The secondary antibodies, horseradish peroxidase-conjugated sheep anti-mouse IgG, were added to blocking solution at 1:10 000 dilutions and incubated for 2 h at room temperature with gentle rotary agitation. After three 10 min washes with PBST at room temperature, detection was carried out using the enhanced chemiluminescence system (Amersham) as previously reported[33]. Digital images were acquired and analyzed with a gel imaging system (Bio-Rad Gel Doc1000, Bio-Rad). The resultant protein level was relatively compared to β-actin and expressed as percent of β-actin.

2.5. Cell proliferation assay

For evaluation of cell proliferation, HS-5 cells were plated into 96-well plates at a concentration of 5 000 per well and cultured as the condition as indicated above. Exposures were done on six wells for each conditioned medium feeder cell line. On test day 3, growing cultures of HS-5 cells were labeled with 100 μmol/L 5-bromo-2-deoxyuridine for 4 h at 37 ℃ in a humidified air/CO_2 (95:5, v/v) atmosphere. After labeling, cell proliferation was measured using a Roche Cell Proliferation ELISA kit (Roche Diagnostics). In brief, the medium from each well was aspirated, HS-5 cells were fixed for 30 min with 200 μL fixative buffers, and the fixed cells were incubated with an immunoreagent containing antibodies specific for 5-bromo-2-deoxyuridine for 90 min at room temperature. The ELISA plates were washed and incubated for about 15 min with a colorimetric substrate, the reaction was stopped by the addition of 1 mol/L sulfuric acid, and cell proliferation was determined by measuring the absorbance at 450 nm. Total cell protein per well was quantitated on at least three wells that were plated concurrently to normalize cell proliferation for cell number. Wells designated for protein quantification were lysed with 25 μL of RIPA buffer and quantified using the Pierce BCA Protein Assay as reported previously[11].

2.6. Preparation of small interference RNA against endoglin and cell infection

Small interference RNA (siRNA) against endoglin was prepared as previously reported[11,34]. Briefly, siRNA duplexes against murine endoglin were designed and synthesized by a commercial biotechnological company (Takara, China). Scrambled siRNA was also obtained from Takara and was used as the control siRNA. HS-5 cells were transfected with the siRNA duplexes by electroporation using a electroporation system (Bio-Rad Gene Pulser II). For transfection, 2×10^6 cells were resuspended in 1 mL electroporation buffer with 200 nmol/L dsRNA. Thereafter, the resuspended cells were transferred to a Gene Pulser cuvette (Bio-Rad) and electroporated (1 pulse, 0.2 kV, 0.3 μF, 73.8 ms). The electroporated cells were then combined, mixed with 4 mL cultured medium, and plated onto four wells of a six-well culture plate at a concentration of 1×10^6 per well. All cultures were incubated at 37 ℃ in a humidified air/CO_2 (95:5, v/v) atmosphere for the duration of the experiment. At 24 h post-transfection, the toxicarioside A and DMSO were added into the cultured medium for the next 24 to 48 h. The cultured cells were collected and used for Western blot analysis as above.

2.7. Statistical analysis

All data are expressed as mean±SEM. For analysis of differences between two groups, a Student's t test was performed. For multiple groups, an ANOVA was carried out followed by a Student-Newman-Keuls test. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Inhibition of endoglin expression by toxicarioside A

Endoglin mRNA expression was analyzed by Northern blot. HS-5 cells were cultured in different concentration of toxicarioside A (0.5 to 9.0 μg/mL) and total mRNA was isolated to perform Northern blot analysis. The results of Northern blot analysis showed that toxicarioside A treatment inhibited endoglin mRNA expression in a dose-dependent pattern. toxicarioside A 0.5 to 9.0 μg/mL induced significant inhibition of endoglin expression when compared with not treated (0 μg/mL) cells and different does (Figure 1A, $P<0.001$). In addition, endoglin protein was isolated by SDS/PAGE and detected by Western blot analysis. Similar results were found as in the Northern blot analysis (Figure 1B). Endoglin protein expression was also shown in a pattern of dose-dependent in HS-5 cells (Figure 1B). These results indicated that toxicarioside A has powerful capabilities of inhibiting endoglin expression in HS-5 cells. Meanwhile, these results also indicated that the optimum effective dose of toxicarioside A was between 1.5 to 4.5 μg/mL. Therefore, the concentration of toxicarioside A for subsequent experiments related to treatment of HS-5 cell treatment was 2.5 μg/mL.

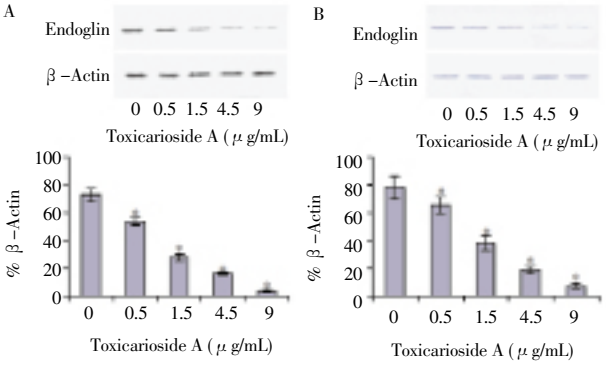

Figure 1. Inhibition of endoglin expression by toxicarioside A.
HS-5 cells were cultured in various dose of toxicarioside A (0-9 μg/mL) for 48 h. Endoglin mRNA and protein were detected by Northern blot (A) and Western blot (B) analysis. *$P < 0.001$ or less, compared to the various dose of toxicarioside A.

3.2. Inhibition of TGF-β expression by toxicarioside A

To understand whether toxicarioside A could attenuate TGF-β expression in HS-5 cells, we still used Western blot analysis to detect the TGF-β protein in the HS-5 cells. HS-5 cells were cultured in DMEM medium contained toxicarioside A 2.5 μg/mL or DMSO for 24 h. Thereafter, HS-5 cells were isolated by SDS-PAGE and blotted by a monoclonal antibody against TGF-β. The Western blot results showed that the TGF-β expression was significantly attenuated in the HS-5 cells treated with toxicarioside A when in comparison with the HS-5 cells treated with DMSO, 11.5±1.6 (percent of β-actin) in toxicarioside A-treated cells versus 77.3±8.8 in DMSO-treated cells, more than 6 folds decrease (Figure 2, $P < 0.001$).

Figure 2. Inhibition of TGF-β expression by toxicarioside A.
HS-5 cells were cultured in DMEM medium supplemented with toxicarioside A 3 μg/mL for 48 h. TGF-β protein was detected by Western blot analysis. *P<0.001 or less, compared to the DMSO-treated HS-5 cells.

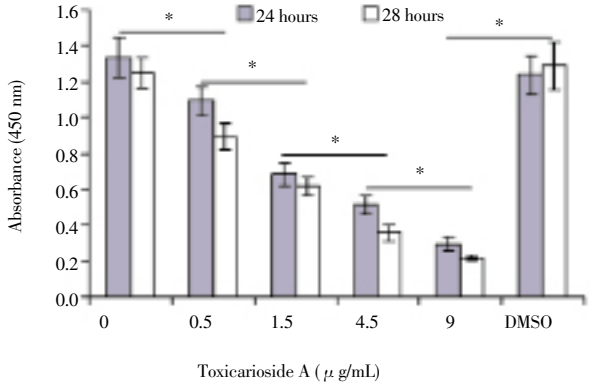

Figure 3. Suppression of cell proliferation by toxicarioside A.
HS-5 cells were cultured in various dose of toxicarioside A (0-9 μg/mL) for 24 or 48 h Cell proliferation was detected by Proliferation ELISA kit (Roche Diagnostics). *$P < 0.001$ or less, compared to the various dose or DMSO-treated HS-5 cells.

3.3. Suppression of cell proliferation by toxicarioside A

To know whether decreased expression of endoglin and TGF-β influence cell functions in HS-5 cells, we thus we detected the cell proliferation induced by toxicarioside A. Cell proliferation was performed by commercial Proliferation ELISA kit. Compared with the HS-5 cells treated with DMSO, the cell proliferation in the HS-5 cells treated with toxicarioside A showed significantly decreased also in a dose-dependent pattern (Figure 3, $P < 0.001$) when compared with not treated (0 μg/mL) cells, DMSO-treated cells and different does (0.5 to 9 μg/mL), which was consistent with the decreased levels of endoglin and TGF-β in the toxicarioside A-treated HS-5 cells, suggesting endoglin and TGF-β signaling is related to the HS-5 cell proliferation.

3.4. Alteration of Smad signaling by toxicarioside A

Previous studies have concluded that endoglin acts as a positive regulator of ALK1 signaling and a negative regulator of ALK5 signaling in endothelial cells. Therefore, we detected Smad1, Smad 2 and their active phosphorylated counterparts, pSmad1 and pSmad2. In HS-5 cells, both Smad1 and Smad2 could be attenuated by toxicarioside A when compared with the HS-5 cells treated with DMSO (Figure 4, $P < 0.000\ 1$). In addition, similar results were found to the active phosphorylated Smad1 and Smad2, pSmad1 and pSmad2. Western blot analysis indicated that pSmad1 and pSmad2 were also attenuated in HS-5 cells treated by toxicarioside A, but keep unaffected in the HS-5 cells treated with DMSO, Smad1, pSmad1, Smad2 and pSmad2 were 12.3±1.6, 15.1±2.1, 9.1±1.3 and 10.5±1.5 in the HS-5 cells treated with toxicarioside A versus 71.7±6.9, 61.2±2.1, 70.3±7.3 and 58.9±4.6 in the HS-5 cells treated with DMSO (Figure 4). Significant differences were found between toxicarioside A-treated and DMSO-treated HS-5 cells ($P < 0.001$). These results also indicate that inhibition of endoglin and TGF-β expression in HS-5 cells lead to attenuation of both ALK1 and ALK5 signaling pathways, which resulted in suppression of HS-5 cells proliferation.

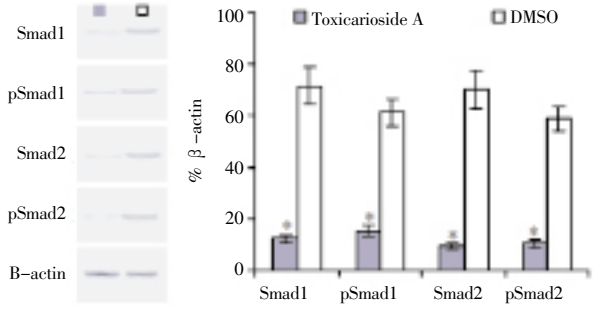

Figure 4. Alteration of Smad signaling by toxicarioside A.
HS-5 cells were treated with toxicarioside A (3 μg/mL) or DMSO, Smad1, Smad2 and active phosphorylated Smad1 (pSmad1), phosphorylated Smad2 (pSmad2) were detected by Western blot analysis. *P<0.001 or less, compared to the DMSO-treated HS-5 cells.

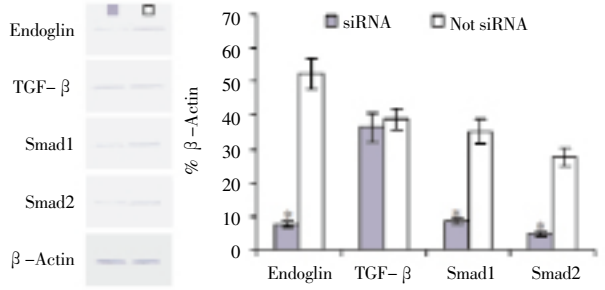

Figure 5. Alteration of Smad signaling by endoglin interference. HS-5 cells were interfered or not interfered with siRNA against endoglin. Endoglin, Smad1 and Smad2 were then detected by Western blot analysis. *P<0.001 or less, compared to the not siRNA HS-5 cells.

3.5. Alteration of Smad signaling by endoglin interference

Due to the role of endoglin in TGF-β signaling and decreased expression of endoglin and TGF-β in HS-5 cells treated with toxicarioside A, we next used RNA interference to uncover the roles played by endoglin or TGF-β in the effects on Smad signaling in HS-5 cells. We thus used a siRNA against endoglin to attenuate endoglin protein level in HS-5 cells. Our results showed that endoglin protein level was decreased by about 7 folds (7.6±1.0 in siRNA HS-5 cells and 52.4±4.6 in not siRNA HS-5 cells, respectively) after transfection with siRNA against endoglin in HS-5 cells (Figure 5, P<0.001), suggesting the siRNA against endoglin effectively blocked the endolgin expression in HS-5 cells.

To know whether attenuation of endoglin expression is the major reason that caused Smad1 and Smad2 signaliing atteration in HS-5 cells, we next detected the TGF-β, Smad1 and Smad2 proteins in HS-5 cells. After blocking the expression of endoglin by siRNA, we found TGF-β expression was not affected by the endoglin interference, 36.4±4.2 versus 38.9±3.2 (Figure 5, P>0.05). However, Smad1 and Smad2 proteins were significantly decreased in endoglin siRNA HS-5 cells when comparison with the not siRNA HS-5 cells, 8.6±0.9 (% to β-actin) and 4.9± 0.7 versus 35.2±3.7 and 27.6±2.8, respectively (Figure 5, P < 0.001). These results indicated that the alteration of the Smad1 and Smad2 singling in HS-5 cells by toxicarioside A treatment was direct results of decreased endolgin expression and not related to other cellular changes, such as self-secreted TGF-β.

4. Discussion

In general, endoglin is considered as angiogenesis-related molecule as vascular endothelial growth factor by many previous studies. Endoglin works as a auxiliary regulatory component by formation of a heteromeric complex with the TGF-β receptor, by which it regulates the signaling of distinct TGF-β receptor I isotypes ALK-1 and ALK-5[13,14]. In addition, many study results also indicate that endoglin mainly expresses in active endothelial cells, in which it help new vessel formation in such conditions as tumor and inflammation[35,36]. However, in our present study, we found that both endoglin and its one of ligands, TGF-β, were expressed in a normal bone marrow stromal cell line, HS-5, and their expressions was attenuated by toxicarioside A, a cardenolide isolated from the latex of a tropical *Antiaris toxicaria*, in Hainan Island, China. Moreover, in the present study, we still found that toxicarioside A treatment also caused inhibition of cell proliferation in HS-5 cells. Therefore, we next used Western blot analysis to detect the ALK1 and ALK5's downstream molecules, Smad1, Smad2 and their active phosphorylated counterparts, pSmad1 and pSmad2. Our results showed that Smad1, Smad2, pSmad1 and pSmad2 were all attenuated in HS-5 cells treated with toxicarioside A, but not in the control HS-5 cells treated with DMSO. Because we found both endoglin and TGF-β in the HS-5 cells treated with toxicarioside A were attenuated, we further used RNA interference (siRNA) to knockdown the endoglin expression in HS-5 cells in order to identify which the major molecules that took the special role in controlling ALK1 and ALK5 signal pathways between endoglin and TGF-β. Our results indicated that both ALK1 and ALK5's downstream molecules, Smad1 and Smad2 were significantly decreased in the siRNA HS-5 cells, but keep unaffected in the not siRNA HS-5 cells. Taking all the results together in the present study, we can conclude that it is endoglin but not TGF-β that causes the normal ALK1 and ALK5 signal pathways and keep HS-5 cell in normal function, and that toxicarioside A treatment can induce inhibiton of endoglin and TGF-β expression in HS-5 cells, which lead to decreased HS-5 cell proliferation at last.

Stromal cell is one of the important cell types in bone marrow microenviroment. Recently, O'Connor and their coworkers used tumor cultured media to culture bone-marrow stromal cells and found that some unknown substances in some prostate cancer cells can inhibit endoglin expression in some stromal cell lines, the inhibition of endolgin expression also decrease stromal cell proliferation and potentially facilitate prostate cancer metastasis in the bone marrow[11]. As we know, endoglin has been considered as one of the hallmark molecules that lead to new vessel formation, and monoclonal antibodies and other strategies to block the funtion of endoglin reach convincible antitumor activities[37,38]. In our present study, we found that toxicarioside A can inhibit endoglin expression and cell proliferation in HS-5 cells, which indicate toxicarioside A is not suitable for treatment of prostate cancer if use toxicarioside A as cytotoxic agent. Reversely, we still found that toxicarioside A can inhibit endoglin expression in some tumor cells and induce effective antitumor activities in vivo through suppression of endoglin-related tumor angiogenesis, which let the tumor lack of blood support and nutrition and cell apotosis at last (unpublished data). From this conflict results, we think that toxicarioside A posses antitumor activities but is not suitable for all the tumor type, such as breast and prostate cancers that are preferential metastasis and growth in bone marrow microenvironment.

Antiaris toxicaria is growth widespread in tropical areas, such as southeastern Asia. At present, there are many fairy tales about the plant in the ancient times, especially its poison for arrows, darts and blow-darts. Modern biomedical studies have isolated many chemical effective ingredients from its latex sap[39], in which the most focus is on the active complex mixture of cardenolide glycosides[28,39-42]. Besides the traditional effect of the cardenolides on the inhibition of the ubiquitous cell surface Na^+, K^+-ATPase, there has been a substantial increase in the number of studies investigating the effects of cardiac glycosides on the growth of human malignant tumor cells and its possible molecular mechanisms in the last decade[23-27]. In our previous works, we have isolated three new cytotoxic cardenolides from the latex of *Antiaris toxicaria* and have proved them possess the capabilities of significant cytotoxicity against several human tumor cell lines *in vitro*[28, 29]. In the present study, we further investigated its other potential medicinal value in HS-5 cells. Our results indicate that toxicarioside A really has other function except for cardiac and antitumor activities. Although its inhibiting function against endoglin expression in HS-5 cells in this study is a potential reason that promote cancer metastasis and growth in bone marrow microenvironment, it really suggests that toxicarioside A must be reconsidered if use it as a cytotoxic antiumor agent for tumor therapy in the future.

In the present study, we found both endoglin and TGF-β were attenuated in the HS-5 cells treated with toxicarioside A. In order to determine if the effects were the result of a primary affect on endoglin or TGF-β attenuation or the result of other substance changes in the HS-5 cells, endoglin expression was selectively blocked in HS-5 cells using siRNA. Similar to what was observed in the HS-5 cells treated with toxicarioside A, endoglin protein levels were decreased and both Smad1 and Smad2 signaling pathways were altered in the HS-5 cells transfected with endoglin siRNA. Smad1 but Smad2 protein levels were significantly decreased, suggesting the attenuation of the Smad1-related ALK1 and Smad2-related ALK5 signaling pathways is induced by endoglin but not by other factors (such as TGF-β).

Many previous studies in endothelial cells have certified that endoglin is a positive regulator of ALK1 signaling and a negative regulator of ALK5 signaling[43,44]. However, in the present study, our results demonstrated that toxicarioside A have the capabilities of inhibiting endoglin expression and attenuating Smad1-related ALK1 and Smad2-related ALK5 signaling in HS-5 bone marrow stromal cells. In addition, the results of endoglin siRNA showed that blocked endoglin by siRNA itself caused significant attenuation of Smad1 and Smad2. These results suggest that endoglin is a positive regulator of both ALK1-induced Smad1/5/8 activation and ALK5-induced Smad2/3 activation. This is in contrast to the role that endoglin plays in endothelial cells.

In summary, the results we found in this study indicate that toxicarioside A can inhibit endoglin expression in HS-5 bone marrow stromal cells. The decreased expression of endoglin induces attenuation of both of both ALK1-induced Smad1/5/8 activation and ALK5-induced Smad2/3 activation, which results in inhibition of HS-5 cell proliferation. Our data disclose a possible mechanism of toxicarioside A in action against bone marrow microenvironment.

Conflict of interest statement

We declare that we have no conflict of interest.

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