Tanshinone I suppresses growth and invasion of human breast cancer cells, MDA-MB-231, through regulation of adhesion molecules

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The role of cell adhesion molecules has been studied extensively in the process of inflammation, and these molecules are critical components of carcinogenesis and cancer metastasis. This study investigated the effect of tanshinone I derived from the traditional herbal medicine, Salvia miltiorrhiza Bunge, on the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in tumor necrosis factor-α (TNF-α)-stimulated endothelial cells. Furthermore, this study investigated the effect of tanshinone I on cancer growth, invasion and angiogenesis on human breast cancer cells MDA-MB-231, both in vitro and in vivo. Tanshinone I dose dependently inhibited ICAM-1 and VCAM-1 expressions in human umbilical vein endothelial cells (HUVECs) that were stimulated with TNF-α for 6 h. Pretreatment with tanshinone I significantly reduced adhesion of either monocyte U937 or MDA-MB-231 cells to HUVECs. Interestingly, the inhibitory effect of tanshinone I on monocyte and cancer cell adhesion to HUVECs was mimicked with transfection of ICAM-1 and VCAM-1 small interfering RNA. In addition, tanshinone I effectively inhibited TNF-α-induced production of vascular endothelial growth factor (VEGF) and VEGF-mediated tube formation in HUVECs. Tanshinone I also inhibited TNF-α-induced VEGF production in MDA-MB-231 cells and migration of MDA-MB-231 cells through extracellular matrix. Additionally, reduction of tumor mass volume and decrease of metastasis incidents by tanshinone I were observed in vivo. In conclusion, this study provides a potential mechanism for the anticancer effect of tanshinone I on breast cancer cells, suggesting that tanshinone I may serve as an effective drug for the treatment of breast cancer.

Introduction

Danshen (Salvia miltiorrhiza Bunge) is an herb that has been widely used in traditional Chinese medicine for treating coronary heart diseases, such as angina pectoris and myocardial infarction. Along with >20 phenolic acids, ~30 diterpene compounds, including the relatively abundant tanshinones tanshinone I, tanshinone IIA, cryptotanshinone and dihydrotanshinone, have been isolated from Danshen (1). These abundant tanshinones are the major diterpenes isolated from Danshen and show cytotoxic effects on cell lines derived from various human carcinomas of the colon, ovary, lung and mouth (2,3). A recent report has shown that tanshinone IIA inhibits human breast cancer proliferation (4) and induces apoptosis in human leukemia cells through the activation of caspase-3, the downregulation of Bcl-2 and Bcl-XL and the upregulation of Bax (5,6). Other tanshinones, such as tanshinone I, cryptotanshinone and dihydrotanshinone, are structurally similar to tanshinone IIA and may possess similar cytotoxic effects on tumor cells. In preliminary data, tanshinone I possesses the strongest inhibitory effect on tumor necrosis factor-α (TNF-α)-induced adhesion molecules (I.T. Nizamutdinova, K.H. Son, S.S. Kang, Y.S. Kang, Y.S. Kim, K.C. Chang, H.J. Kim, unpublished data).

Advanced breast cancer kills >40 000 American women each year and 10 times that number worldwide (7,8). Western European and North American populations are at highest risk, with a lifetime risk of ~8-10% up to age 74 years. Generally, the lowest risk, ~1%, is seen in Asian populations (9), but has been steadily increasing. In all 70–80% of primary breast cancers are estrogen receptor (ER) and/or progesterone receptor positive (+) and are considered to be hormone responsive (10). Importantly, if the primary tumors are ER+, >80% of lymph node metastases and 65–70% of distant metastases retain their receptors (11,12). The ER+ human breast cancer cell line MCF-7 is most widely used for orthotopic xenograft studies (13). It develops into tumors in mammary glands of nude mice in response to estradiol supplementation (14,15). However, most reports state that such tumors are poorly invasive and rarely, if ever, metastasize (15). Indeed, most metastasis models do not use this cell line, relying instead on ER− cell lines, like MDA-MB-231 (16); these cells express putative aggressiveness markers and do metastasize in nude mice. Generally, ER+ breast cancers have a better prognosis and are often responsive to antiestrogen therapy; however, ER-independent breast cancers are more aggressive, possess high potential to metastasize and are unresponsive to antiestrogens (17). Moreover, there is still no effective cure for the patients with advanced stages of the disease, especially in cases of hormone-independent cancer (18,19). Therefore, the effect of tanshinone I on ER− MDA-MB-231 breast cancer cells was investigated in this study.

Multiple and diverse cell adhesion molecules (CAMs) take part in intercellular and cell–extracellular matrix (ECM) interactions of cancer. Cancer progression is a multistep process in which some adhesion molecules play a pivotal role in the development of recurrent, invasive and distant metastasis. Several CAMs, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), CD106, vascular cell adhesion molecule-1 (VCAM-1, CD106) and endothelial leukocyte adhesion molecule-1 (E-selectin, CD62E), have been implicated in cancer growth and metastasis (20,21). CAMs are expressed on a variety of cells, including vascular endothelial cells (ECs), lymphocytes, fibroblasts, hematopoietic cells and tumor cells (22–26). Some CAMs, such as VCAM-1, are expressed preferentially or at higher levels on breast cancer endothelium compared with normal endothelium (22,27), and E-selectin, VCAM-1 and ICAM-1 are expressed on ECs that have been activated by cytokines such as interleukin-1β, interleukin-6 or TNF-α (28,29), in which proinflammatory cytokines are elevated in the peripheral blood after major surgery (30–32). A major site of TNF-α action for mediating inflammation and immune responses is the vascular endothelium, and it was previously reported that TNF-α increases the expression of ICAM-1 and VCAM-1 in ECs (33–35). Thus, the inflammatory reaction provoked by surgical trauma leads to the

Abbreviations: CAM, cell adhesion molecule; EC, endothelial cell; ECM, extracellular matrix; ER, estrogen receptor; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; sRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

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activation of leukocytes and monocytes with the release of proinflammatory cytokines, such as TNF-α. ICAM-1 and VCAM-1 have been shown to be involved in cell–cell and cell–ECM interactions and are mechanistically important for the extravasation of both monocytes during inflammation (36) and cancer cells during metastasis (37,38). The adhesion of circulating tumor cells to the microvascular endothelium of organs at distant sites—like the liver and the lungs—is an important step in blood-born metastasis.

This study aimed to examine the effect of tanshinone I on the expression of ICAM-1 and VCAM-1 in human umbilical vein endothelial cells (HUVECs) and the role of ICAM-1 and VCAM-1 in the adhesion of cancer cells to ECs. Furthermore, we investigated the effect of tanshinone I on angiogenesis and invasion of MDA-MB-231 breast cancer cells.

Materials and methods

Materials

Tanshinone I was isolated and identified as described in Kim et al. (39) (Figure 1). Tanshinone I purity (≥99%) was validated by high-performance liquid chromatography. Tissue culture medium 199, fetal bovine serum (FBS), antibiotics (penicillin-streptomycin), glutamine and collagenase were supplied by Gibco-BRL (Rockville, MD). VCAM-1 and ICAM-1 small interfering RNA (siRNA), siRNA transfection kit, anti-ICAM-1 and anti-VCAM-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence western blotting detection reagent was purchased from Amersham (Bucks- hamshire, UK). All other chemicals, including EC growth supplements and heparin, were supplied by Sigma–Aldrich (St Louis, MO).

Cell culture

HUVECs were isolated from umbilical cord veins by collagenase treatment, as described previously (35), and grown in medium 199 supplemented with 20% FBS, 2 mM l-glutamine, 5 U/ml heparin, 100 IU/ml penicillin, 10 µg/ml streptomycin and 50 µg/ml EC growth supplements. Cells were cultured in 100 mm dishes and grown in a humidified 5% CO₂ incubator. HUVECs were washed three times with M199 before the dye-loaded cells were added and incubated at 37°C. After 30 min, cell suspensions were withdrawn, and the HUVECs were gently washed with M199. Fluorescent images were obtained using a high-resolution video camera (DXC-960MD; Sony) mounted on a BH-2 Olympus microscope (Melville, NY) and the immunoreactivity of these was measured using SigmaGel 1.0 (Jandel Scientific, Erkrath, Germany). Analyses were repeated three times, over the same region, and the results are the mean values of the three independent experiments.

Quantitative Human Vascular endothelial growth factor immunoassay

The level of vascular endothelial growth factor (VEGF) in the conditioned medium was determined using a VEGF enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacture’s instructions. All assays were performed on triplicate plates, and data are presented as mean values ± SD.

Tube formation assay

Twenty-four-well culture plates (Nalge Nunc International, Naperville, IL) were coated with 50 µl low-growth-factor synthetic matrix (Matrigel, BD Biosciences, San Jose, CA) and incubated for 30 min at 37°C. HUVECs that were serum starved for 4 h were treated with trypsin-ethylenediaminetetra-acetic acid and suspended in a conditioned medium obtained from the VEGF and/or tanshinone I-treated cells (1, 5 and 10 µM) (N = 5) for 22 h at 37°C. HUVECs were seeded at a density of 1 × 10⁴ cells on the synthetic matrix and then incubated for 17 h at 37°C. Micrographs of the 96-well plates were taken by phase contrast microscopy at a magnification of ×100. Images of tube formation were selected randomly in three fields at ×100 magnification.

Matrigel invasion assay

MDA-MB-231 cells were cultured for 3 days. The upper chamber of 24-well cell culture inserts (8 µm pore size, Falcon, Franklinlakes, NJ) were washed with a serum-free medium, coated with 100 µl of Matrigel (1 mg/ml) and dried for 30 min at 37°C. MDA-MB-231 cells treated with tanshinone I were collected; 2 × 10⁶ cells per insert in serum-free media were added to the upper chambers, and 500 µl of RPMI media containing 10% FBS was added to the lower chambers. The invasion chambers were incubated for 24 h in a 37°C cell culture incubator. The non-invasive cells that remained on the upper surface of the insert membranes were removed by scrubbing. The cells on the lower insert membranes were stained with 4’,6-diamidino-2-phenylindole, and cells were counted under the light microscope. Each sample was measured in triplicate, and each experiment was repeated three times.

Animal experiments

MDA-MB-231 cells were grown in serum-containing culture medium until the cell density was ~70–80%. Cells were then trypsinized, and cell pellets were resuspended in serum-free RPMI at 5–6 × 10⁶ cells/100 µl of cell suspension. Nude mice were injected subcutaneously with MDA-MB-231 cells. All experiments were performed in compliance with institutional guidelines set by the Institutional Animal Care and Use Committee of the Gyeongsang National University. Tumors were allowed to grow until they reached 4 mm. At this time, animals were randomized into two groups (seven mice per group): control (without any treatment) and tanshinone I-treated (10 mg/kg) mice. Tanshinone I was intraperitoneally injected every day. Tumor size (longest and shortest diameter) and body weight were measured every third day. Tumor volume was calculated as described previously (40) using the following formula: (smallest diameter² × widest diameter)/2. At the end of the 4 week period, when the tumor in the control mice reached ~0.9–1.0 cm, the mice were killed. Liver and lung tissue were fixed overnight in 4% paraformaldehyde, followed by paraffin infiltration and embedding. Sections of 5 µm were

Fig. 1. Chemical structure of tanshinone I extracted from Danshen (Salvia miltiorrhiza).
mounted onto ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA), stained with hematoxylin and eosin and examined for cellularity by a light microscope.

**Statistical evaluations**

Scanning densitometry was performed using an Image Master® VDS (Pharmacia Biotech, San Francisco, CA). Treatment groups were compared using one-way analysis of variance, and any significant differences identified in the analysis of variance were located with the Newman–Keuls test. P values <0.05 were accepted as significant.

**Results**

**Tanshinone I suppressed TNF-α-induced ICAM-1 and VCAM-1 expression in HUVECs**

First of all, the cell viability of HUVECs in response to tanshinone I was examined by MTT assay. Figure 2A shows that tanshinone I has mild cytotoxicity in HUVEC in the dose of 1–50 μM for 24 h. Treatment of HUVECs with tanshinone I (1, 5, 10 and 50 μM for 24 h) decreased cell viability by ~98, 81, 74 and 67%, respectively. Next, the effect of tanshinone I, which was derived from *S. miltiorrhiza* Bunge, on ICAM-1 and VCAM-1 expression was examined after stimulating HUVECs with TNF-α. The cells were pretreated with tanshinone I in a dose-dependent manner (1, 5, 10 and 50 μM) for 24 h and were then cotreated with TNF-α (10 ng/ml) for 6 h. Results showed that TNF-α increased both ICAM-1 and VCAM-1 expression, which was completely suppressed by tanshinone I at 5–50 μM (Figure 2B). It suggests that tanshinone I exerts inhibitory effect on adhesion molecule expression independent of cell death in HUVECs.

**Tanshinone I inhibited the TNF-α-stimulated adhesion of monocyte and breast cancer cells to ECs**

Monocyte–EC interactions, as well as cancer cells–EC interactions, are regulated in part by the expression of specific adhesion molecules. Among these, ICAM-1 and VCAM-1 play important roles in the adhesion of monocytes or cancer cells to EC. Therefore, the effect of tanshinone I on the adhesion of monocytes or cancer cells to HUVECs was investigated after TNF-α stimulation. Adhesion of human monocyte U937 cells to HUVECs that were stimulated with TNF-α at 10 ng/ml for 6 h was increased by four times compared with unactivated HUVECs. In contrast, HUVECs treated with 1–50 μM tanshinone I for 24 h before TNF-α stimulation showed significant reduction of U937 cells adhering to ECs (Figure 3A). In addition, adhesion of MDA-MB-231 cells to HUVECs increased about six times after TNF-α stimulation, which was also dose dependently significantly inhibited by tanshinone I (Figure 3B).

**ICAM-1 and VCAM-1 play an important role in the adhesion of monocytes and human breast cancer cells to the ECs**

To verify that the reduction of adherent monocytes or cancer cells to ECs by tanshinone I is due to tanshinone I-induced ICAM-1 and VCAM-1 downregulation, we treated HUVECs with siRNA against either ICAM-1 or VCAM-1 alone or a mixture of siRNA against both adhesion molecules; cells were then stimulated with TNF-α. The effectiveness of ICAM-1 and VCAM-1 silencing in HUVECs was determined by western blot analysis (Figure 3C). ICAM-1 siRNA resulted in a marked inhibition (>60%) of TNF-α-increased adhesion of monocytes to ECs. VCAM-1 siRNA also showed significant reduction in the adherent monocytes by TNF-α (>50%). Furthermore, the mixture of siRNA against both adhesion molecules reduced the number of adherent monocytes >70%, as compared with the TNF-α-treated group (Figure 3C). Moreover, similar results were shown in the adhesion of MDA-MB-231 cells to ICAM-1 or VCAM-1 knocked down HUVECs (Figure 3D). Thus, these results indicate that TNF-α-mediated expression of ICAM-1 and VCAM-1 on EC plays an important role in the adhesion of U937 monocyte or MDA-MB-231 breast cancer cells to EC and tanshinone I may inhibit monocyte and cancer cell adhesion to EC by downregulating TNF-α-mediated ICAM-1 and VCAM-1 expression in ECs.
It was previously reported that TNF-α induces tumor cells to secrete VEGF through nuclear factor kappa B-mediated VEGF transcription, implying that inflammatory signaling pathways play important roles in promoting tumor angiogenesis (41,42). Thus in this study, we investigated the effect of tanshinone I on the production of VEGF by both ECs and cancer cells with or without TNF-α. HUVECs were pretreated with varying doses of tanshinone I for 24 h and then co-treated with TNF-α (10 ng/ml) for 24 h. Results showed that HUVECs cultured in media without TNF-α produced low level of VEGF (12 pg/ml), whereas HUVECs incubated with TNF-α showed significant enhancement of VEGF production (120 pg/ml), which was dose dependently suppressed by tanshinone I (Figure 4A). To confirm if tanshinone I plays an important role in the suppression of VEGF production in breast cancer cell lines, MDA-MB-231 cells were pretreated with increasing doses of tanshinone I for 24 h and then stimulated with TNF-α for another 24 h (Figure 4A). Compared with HUVECs (12 pg/ml), MDA-MB-231 in media without TNF-α produced a much higher level of VEGF (2620 pg/ml), which was enhanced to 3100 pg/ml after stimulation with TNF-α. Tanshinone I (10 and 50 μM) significantly inhibited TNF-α-enhanced VEGF production as well as the basal level of VEGF in MDA-MB-231 (Figure 4A).

Interestingly, the mixed siRNA treatment, against both adhesion molecules, showed a stronger decrease than the two individual siRNAs in the invasion of MDA-MB-231 cells (>80%; Figure 4D). Thus, these results suggest that the effect of tanshinone I on invasion of MDA-MB-231 cells could be via downregulating ICAM-1 and VCAM-1.

Tanshinone I attenuated growth and metastasis of breast cancer cells in nude mice

To confirm the effectiveness of tanshinone I in in vivo tumor progression, tanshinone I was injected into tumor-bearing mice for 4 weeks. First of all, we determined whether tanshinone I has a toxic effect in vivo, the mice were received intraperitoneally with a daily injection of 10 or 50 mg/kg tanshinone I for 7 days. A total of 50 mg/kg concentration of tanshinone I caused marked lethality in mice (11 of 21 mice were died), whereas 10 mg/kg dose did not have any toxic
effect (Figure 5A). Thus, we used 10 mg/kg of tanshinone I for further experiment. Control animals developed significant tumor growth during the 4–5 week follow-up period, as shown by the tumor volume in the histogram. In contrast, animals that received 10 mg/kg tanshinone I daily showed that tumor growth was markedly inhibited (Figure 5B and C). Hematoxylin and eosin staining results revealed that there were no differences in the liver between control and tanshinone I-treated mice; however, a high number of cancer cells had invaded into lymph node, bronchi and vessels of control mice lungs but not in tanshinone I-treated mice lungs (Figure 5D). These results suggest that tanshinone I not only inhibits breast cancer invasion in vitro but also has therapeutic activity in vivo.

**Discussion**

Cell adhesion is essential in all aspects of cell growth, migration and differentiation in vertebrate cells. CAMs are important participants in cell–cell interactions and interactions between cells and ECM components (43). These molecules have been implicated in a wide variety of cellular functions, including signal transduction, cellular communication and recognition, embryogenesis, inflammatory and immune responses and apoptosis (43). Evidence suggests that CAMs may be associated with invasion and metastasis in a variety of human malignancies. This study demonstrated that 10 ng/ml TNF-α significantly induced ICAM-1 and VCAM-1 expression in HUVECs, which was dramatically inhibited by tanshinone I. Additionally, tanshinone I significantly reduced TNF-α-mediated adhesion of monocytes or cancer cells to ECs, which may be due to inhibition of ICAM-1 and VCAM-1 expression. These are supported by experiments with siRNA against icam-1 and vcam-1 genes. Knockdown of these adhesion molecules dramatically decreased the adhesion of monocyte U937 cells and breast cancer cells MDA-MB-231 to HUVECs. Thus, these findings suggest that tanshinone I can modulate or prevent breast cancer metastasis by regulating adhesion molecules, such as ICAM-1 and VCAM-1.

Numerous studies have provided suggestive evidence that angiogenesis is of critical importance for the growth and metastasis of many solid tumors, including breast carcinomas. In fact, breast cancer patients whose tumors had high vessel counts had poor clinical prognoses, whereas patients with low vessel counts generally had minimal if any detectable metastatic disease (44–46). Enhanced angiogenesis and increased vascular density are pathological features of cancer, underscoring the importance of antiangiogenic therapies that ameliorate this condition. VEGF is a well-studied proangiogenic cytokine shown to mediate angiogenic activity under various physiological circumstances, including embryogenesis, skeletal growth and ovarian follicular growth. However, high levels of VEGF have also been observed in many disease states that involve pathological angiogenesis (47). The proangiogenic effects of VEGF include increased endothelial permeability, proliferation and chemotaxis and upregulated endothelial adhesion molecule expression, including VCAM-1 and ICAM-1. In this study, tanshinone I attenuated the production of VEGF in HUVECs and MDA-MB-231 cells that were stimulated with TNF-α.
In addition, tanshinone I could decrease VEGF-mediated tube formation in HUVECs. Previous studies have suggested that angiogenesis contributes to the invasive and metastatic spread of tumor cells (44,48). This study also showed that tanshinone I suppressed the invasion of MDA-MB-231 cells through the ECM. This activity may be another important function of tanshinone I in tumor development. It has been known that inflammatory CAMs may modulate EC migration and angiogenesis through unknown mechanisms. However, recent data suggest a novel role of adhesion molecules such as ICAM-1 in modulating VEGF-A-induced angiogenesis (49). Thus, this report provides a new insight into the role of adhesion molecules in the regulation of VEGF angiogenic function, and we suggest that tanshinone I inhibits cancer cell adhesion to ECs and invasion via down-regulation of adhesion molecules, such as ICAM-1 and VCAM-1. Our results also showed that tanshinone I inhibited growth and metastasis to lung tissue of MDA-MB-231 breast cancer cells in vivo.

Fig. 4. The effect of tanshinone I on angiogenesis and metastasis of breast cancer cells. (A) HUVECs or MDA-MB-231 cells were treated in a fresh medium containing 1% calf serum with increasing doses of tanshinone I (1, 5, 10 and 50 μM) for 24 h and then cells were stimulated with or without TNF-α (10 ng/ml) for 24 h. The concentration of VEGF was determined using a quantitative VEGF ELISA kit. (B) HUVECs were treated with VEGF, and the ability to induce endothelial tube formation was examined with/without tanshinone I (1, 5 and 10 μM). HUVECs that were treated with VEGF formed significantly vessel-like structures. Micrographs of HUVECs on 96-well plates subjected to VEGF and/or tanshinone I were taken under a phase contrast light microscope at ×100 magnification. MDA-MB-231 cells were incubated in medium with tanshinone I for 24 h (C) or transfected with ICAM-1 and/or VCAM-1 siRNA for 10 h (D). MDA-MB-231 cells were transfected with siRNA against ICAM-1 and/or VCAM-1, and western blot analysis showed that ICAM-1 and VCAM-1 were effectively knocked down in MDA-MB-231 cells transfected with siRNA against either ICAM-1 or VCAM-1. Cells were stained with 4',6-diamidino-2-phenylindole, and images from three randomly selected fields were presented. The number of invaded cells was counted under a light microscope. Data represent mean ± SD of three separate experiments performed in triplicate. Analysis of variance was used to compare the multiple group means, followed by Newman–Keuls test (significance compared with control, **P < 0.01; significance compared with TNF-α, *P < 0.05, **P < 0.01).
Cancer metastasis is a complex process involving co-ordinated cellular responses of both cancer cells and normal cells. Metastasis involves several steps: (i) invasion of the stroma, (ii) intravasation of the blood vessel, (iii) circulation in the blood, (iv) lodging and adhesion in the target capillaries, (v) extravasation from the blood vessels and (vi) proliferation of secondary tumors. This study showed that tanshinone I attenuated adhesion molecules, such as ICAM-1 and VCAM-1; thereby, it prevented adhesion of monocytes as well as cancer cells to ECs stimulated with TNF-α. It also suppressed endothelial tube formation and invasion of MDA-MB-231 cells through the ECM. Taken together, this research suggests that tanshinone I from *S. miltiorrhiza* Bunge can be used as useful drug to modulate tumor growth and metastases.

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