The anti-atherosclerotic effect of tanshinone IIA is associated with the inhibition of TNF-α-induced VCAM-1, ICAM-1 and CX3CL1 expression

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ABSTRACT

Tanshinone IIA is one of the major diterpenes in Salvia miltiorrhiza. The inhibitory effect of Tanshinone IIA on atherosclerosis has been reported, but the underlying mechanism is not fully understood. The present study aimed to study the anti-atherosclerosis effect of Tanshinone IIA on the adhesion of monocytes to vascular endothelial cells and related mechanism. Results showed that Tanshinone IIA, at the concentrations without cytotoxic effect, dose-dependently inhibited the adhesion of THP-1 monocytes to the TNF-α-stimulated human vascular endothelial cells. The expressions of cell adhesion molecules including VCAM-1, ICAM-1 and E-selectin were induced by TNF-α in HUVECs at both the mRNA and protein levels. The mRNA and protein expressions of VCAM-1 and ICAM-1, but not E-selectin, were both significantly suppressed by Tanshinone IIA in a dose dependent manner. In addition, the TNF-α-induced mRNA expression of fractalkine/CX3CL1 and the level of soluble fractalkine were both reduced by Tanshinone IIA. We also found that Tanshinone IIA significantly inhibited TNF-α-induced nuclear translocation of NF-κB which was resulted from the inhibitory effect of Tanshinone IIA on the TNF-α-activated phosphorylation of IκKα, IκKβ, IκB and NF-κB. As one of the major components of Salvia miltiorrhiza, Tanshinone IIA alone exerted more potent effect on inhibiting the adhesion of monocytes to vascular endothelial cells when compared with Salvia miltiorrhiza. All together, these results demonstrate a novel underlying mechanism for the anti-inflammatory effect of Tanshinone IIA by modulating TNF-α-induced expression of VCAM-1, ICAM-1 and fractalkine through inhibition of TNF-α-induced activation of IκK/NF-κB signaling pathway in human vascular endothelial cells.

Introduction

Salvia miltiorrhiza (S. miltiorrhiza), referred to “Danshen” in traditional Chinese Medicine, had been commonly used for the treatment of various diseases including cardiovascular diseases (Xia et al. 2003), cerebrovascular diseases, hepatitis, cirrhosis and even cancer (Wang et al. 2007). Danshen had also been shown to exhibit strong antioxidant activity by scavenging reactive oxygen species (ROS) (Fu et al. 2007), modulate the endothelial cell permeability, inhibit platelet aggregation (Liu et al. 2011), and protect human umbilical vein endothelial cells (HUVECs) against homocysteine-induced endothelial dysfunction (Chan et al. 2004).

Tanshinone IIA (Tan IIA) is one of the major diterpenes identified from S. miltiorrhiza. It possesses anti-oxidant (Fu et al. 2007), anti-inflammatory (Fan et al. 2009), anti-angiogenesis (Tsai et al. 2011) and anti-cancer properties (Wang et al. 2005) in recent studies. It had also been demonstrated that Tan IIA can inhibit atherosclerosis in the rabbits fed with high fatty diet not only due to the anti-oxidation effect but also to the anti-inflammation effect by decreasing the expression of CD40 and MMP-2 activity (Fang et al. 2008). Moreover, the suppression of inflammatory process in the atherosclerosis by Tan IIA can decrease the monocyte/macrophage infiltration (Kim et al. 2009) that protects endothelial cells from the damage caused by H2O2.

As we know, the atherosclerosis is a process of inflammation (Libby 2002). The adhesion molecules such as VCAM-1, ICAM-1 and Fractalkine/CX3CL1 play a critical role in the atherosclerosis. VCAM-1 is involved primarily in the early adhesion of monocyte...
to the endothelium of atherosclerosis (Libby 2002). ICAM-1 is involved in both monocyte and lymphocyte adhesion to the endothelium (Weber and Noels 2011). The Fractalkine/CX3CL1 is closely related to the monocytes and T lymphocyte homeostasis in the advanced stage of atherosclerosis (Stolla et al. 2012; Weber and Noels 2011). The gene expression of these molecules is modulated by cytokines such as TNF-α and IL-1β. Shortly after the stimulation, the mRNA level of these adhesion molecules is known to be up-regulated by the activation and nuclear translocation of transcription factor NF-κB (Ye et al. 2008).

The present study aimed to investigate the anti-inflammatory effect of Tan IIA on the expression of VCAM-1, ICAM-1 and fractalkine in TNF-α-stimulated human vascular endothelial cells. The effect of Tan IIA on the nuclear translocation and the activation of IKK/NF-κB pathway in human vascular endothelial cells were also investigated.

**Materials and methods**

**Cell culture**

HUVECs were isolated from the vein of human umbilical cords and grown in EGM provided by Clonetics (MD, USA). The experimental use of vascular endothelial cells isolated from human umbilical cord was approved by the Institutional Review Board of Chang-Gung Memorial Hospital, and informed consent was obtained from each donor before labor. Cells were maintained in a humidified atmosphere with 5% CO₂/95% air at 37 °C and passaged 3–5 times prior to use in experiments. Cells at 80–90% confluency were treated with 1–20 μM Tan IIA and 0.5–5 mg/ml S. miltiorrhiza for indicated time period. The human monocyte leukemia cell line, THP-1, was obtained from ATCC and grown in suspension culture in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and antibiotics at 37 °C in a humidified atmosphere with 5% CO₂/95% air.

**Cytotoxicity assay**

Lactate dehydrogenase (LDH) activity was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Mannheim, Germany). Disruption of plasma membrane integrity leads to the release of LDH into the supernatant and results in the conversion of a tetrazolium salt into a red formazan product. HUVECs were treated with various concentrations of Tan IIA and S. miltiorrhiza for 24 h and 50 μl of the supernatant was collected and the LDH activity was measured and read at 490 nm using a spectrophotometer.

**Monocyte adhesion assay**

Monolayers of HUVECs were pretreated with or without Tan IIA and S. miltiorrhiza for 18 h, followed by induction with TNF-α for 6 h (Fu et al. 2007; Ren et al. 2010; Tang et al. 2011; Tsai et al. 2011). HUVECs were then incubated with 2 × 10⁴ THP-1 cells for 30 min in a humidified atmosphere with 5% CO₂/95% air at 37 °C. After incubation, non-adherent cells were removed by washing with PBS twice. Total six random high-power microscopic fields (HPF) (100 ×) were photographed and the numbers of adhesion cells were directly counted.

**RNA isolation and real-time polymerase chain reaction**

Total cellular RNA was isolated by lysis of cells in a guanidinium isothiocyanate buffer, followed by single step phenol-chloroform-isoamyl alcohol extraction procedure modified from that previously described (Chomczynski and Sacchi 1987). Briefly, untreated or treated cells with Tan IIA, S. miltiorrhiza or 6-Amino-4-(4-phenoxynaphthylamino)quinazoline (NF-κB transcriptional activation inhibitor) were harvested and lysed in 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium sarcosine and 0.1 M β-mercaptoethanol. Sequentially, 1/10 volume of 2 M sodium acetate (pH 4.0), one volume of phenol and 1/5 volume of chloroform-isoamyl alcohol (49:1, v:v) were added to the homogenate. After vigorous vortexing for 30 s, the solution was centrifuged at 10,000 × g for 15 min at 4 °C. After removal of the aqueous phase, RNA was precipitated by the addition of 0.5 ml isopropanol. For real-time PCR analysis, reverse transcription was performed using 1 μg of total RNA and oligo (dT) primers in a 20 μl reaction according to the manufacturer’s protocol (PE Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using the Mx3000 QPCR system (Strategene, La Jolla, CA, USA) with SYBR green (Applied Biosystems) as a dsDNA-specific binding dye. The PCR was cycled 40 times after initial denaturation (95 °C, 2 min) with the following parameters: denaturation, 95 °C, 15 s; and annealing and extension, 60 °C, 1 min. The threshold cycle was recorded for each sample to reflect the mRNA expression level. Sequences for the specific primers used in the PCR are VCAM forward primer (5′-CATGACCTGTTCACGCG-GAG-3′) and reverse primer (5′-CATTACGAGGCACCACTC-3′); ICAM forward primer (5′-GCAAGAAGATGCAACCCA-3′) and reverse primer (5′-TTGCTTTACCACCGTC-3′); E-selectin forward primer (5′-GTTTGAGTGTGATGCTGTG-3′) and reverse primer (5′-GAAGTGAATGCTTCAACCCGTTC-3′); Fractalkine forward primer (5′-ATACACGAAACGCGGAC-3′) and reverse primer (5′-CGGGCATTCTCGAGGCTTAG-3′); GAPDH forward primer (5′-GACCTGACCTGGCGTCTA-3′) and reverse primer (5′-AGGACTGGGTGTCGGTG-3′).

**Isolation of nuclear proteins**

Nuclear proteins were isolated from treated and control HUVEC cells and were subjected to Western blotting to assess NF-κB p65 subunit. Briefly, cells treated with different concentration of Tan IIA for 18 h, followed by induction with TNF-α for 6 h were harvested and then nuclear extracts were prepared. Cells were harvested and washed with PBS containing 5 mM NaF, 1 mM Na₃VO₄ and lysed with hypotonic buffer containing 20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM Na₃VO₄, 1 mM Na₂PO₄, 1 mM EGTA, 0.5 mM PMSF, 1 mM EDTA and 1 μg/ml leupeptin. Cell nuclei were resuspended in high salt buffer (hypotonic buffer containing 420 mM NaCl and 20% glycerol) for 30 min at 4 °C, then, centrifuged to obtain the nuclear extracts in the supernatant. Nuclear extracts were dialyzed for 5 h at 4 °C in buffer containing 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT and 20% glycerol and ready for Western blotting.

**Western blotting of cell lysates**

Protein concentrations were determined by the Bradford method (Bio-Rad, CA). Samples with equal amount of proteins were subjected to 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) (Millipore, Bedford, MA, USA) membrane. The membrane was incubated at room temperature in blocking solution (1% BSA (bovine serum albumin)), 1% goat serum in PBS for 1 h, followed by 2 h incubation in blocking solution containing an appropriate dilution (1:1000) of primary antibody, e.g. anti-VCAM, anti-ICAM, anti-E-selectin, anti-fractalkine (CX3CL1) and anti-tubulin antibody (NeoMarkers, Fremont, CA, USA), anti-phosphorylated IKK α/β, anti-total IKK-α, anti-total IKK-β, anti-phosphorylated p65 NF-κB, anti-total p65 NF-κB, anti-phosphorylated IkB-α, anti-total IkB-α (Cell Signaling, Boston, MA, USA) and anti-lamin B (Santa Cruz Biotechnology, Inc.).
washed, the membrane was incubated in PBS containing goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO, USA) for 1 h. The membrane was washed and the positive signals were developed with chemiluminescence reagent (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, England). Then the membrane was exposed to Fuji medical X-ray film (Fuji Ltd., Tokyo, Japan) for 5 min.

Fractalkine ELISA

A human fractalkine-specific ELISA kit (R&D Systems) was used to determine the levels of fractalkine in conditioned media collected from HUVECs. The experimental steps were carried out as described in the protocol provided by manufacturer. This study was performed three times and each time was analyzed in duplicate.

Immunocytochemical staining of NF-κB in HUVECs

Cells grown on coverslips were fixed in 4% formaldehyde (pH 7.5) for 15 min at room temperature and immersed in blocking solution containing 1% BSA and 1% goat serum in PBS for 30 min followed by the incubation with 50 × dilution of monoclonal antibody against NF-κB p65 (Santa Cruz Biotechnology, Inc.) in blocking solution for 60 min. After washing, cells was incubated in PBS containing biotinylated goat anti-rabbit IgG for 15 min followed by washing and incubated with streptavidin conjugated with horseradish peroxidase (Thermo, Scientific) for 10 min. Cells were washed and the positive signals were developed with ImmPACTTM 5G Peroxidase Substrate for 15 min. After counter-staining with nuclear fast red (Vector Labs) and washing, cells were mounted and analyzed by light microscope.

Preparation of Salvia miltiorrhiza extract

Salvia miltiorrhiza (S. miltiorrhiza) used in this study was identified and authenticated by Shu-Tuan Chiang R&D Manager of the Chuang Song Zong Pharmaceutical Company. The extract was prepared according to Taiwanese good manufacturing practice (GMP) methodologies and guidelines. Briefly, the whole plant (200 g) was minced, extracted with 95% ethanol 1:10 (v/w) and boiled for 1 h and collected the extract. Two more cycles of the same extraction were performed and the resulting crude extract was filtered and lyophilized down to a dry powder. The extract used in the experiments was prepared by dissolving the powder in ethanol at the desired concentration and storing at −20°C. The extraction rate was 9.37%, i.e. 1 g of lyophilized powder is equal to 20.55 g original herb.

High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) was performed on Waters 2690 Separations Module (Waters Corporation Taiwan). Waters 2996 Photodiode Array Detector was used at λ = 270 nm. The chromatographic separation was carried out on an Lichrospher RP-18e column (4 mm × 250 mm i.d.; 5 μm particle size) eluted with the mixture of 0.03% phosphoric acid (A) and acetonitrile (B). The linear gradient program was set from 80% of 0.03% phosphoric acid and 20% of acetonitrile to 20% of 0.03% phosphoric acid and 80% of acetonitrile for 90 min. Flow rate was 0.8 ml/min. Sample injecting volume was 20 μl each. Tanshinone IIA, Cryptotanshinone (Delta Corporation, Wuhu, China) and Salvianolic acid B (Beta Corporation, Nanchang, China) were used as reference compounds and dissolved separately in methanol (MeOH).

Statistical analysis

All statistical analyses were performed using SigmaStat statistical software (version 2.0, Jandel Scientific, CA, USA). Results were represented as means ± standard deviation (SD). ANOVA was carried out when multiple comparisons were evaluated. Values were considered to be significant at p < 0.05. All experiments were repeated at least three times independently.

Results

No cytotoxic effect of Tan IIA on HUVECs

To rule out the possible cytotoxic effect of Tan IIA on HUVECs, cells were treated with Tan IIA at concentration up to 20 μM for 24 h and conditioned medium were processed for the determination of LDH activity. As shown in Figs. 1A and 7B, there was no cytotoxicity detected under the experimental conditions. The phase-contrast microscopic examination (100 ×) on the cell morphology of HUVECs after Tan IIA treatment did not reveal any obvious change and there was no sign of apoptosis or necrosis (data not shown).

Tan IIA inhibited the adhesion of monocytes to TNF-α-stimulated HUVECs

To assess the effect of Tan IIA on the monocyte-endothelium adhesion, HUVECs were treated with or without the indicated concentration of Tan IIA (1, 5, 10 and 20 μM) for 18 h and then with TNF-α (10 ng/ml) for 6 h. THP-1 monocytes were added to the HUVECs culture to study the adhesion. Without TNF-α stimulation, very few THP-1 monocytes could adhere to HUVECs, however, TNF-α greatly increased the adhesion of THP-1 monocytes to HUVECs. Tan IIA pretreatment dose-dependently inhibited the TNF-α-induced adhesion of THP-1 monocytes to HUVECs as shown in Fig. 1B. Tan IIA at the concentration of 10 μM reduced more than half of the adhesion compared to TNF-α control without pretreatment. Similar dose-dependent inhibitory effect of Tan IIA was also observed when Jurkat T lymphocytes were used in the adhesion assay (data not shown).

Effects of Tan IIA on the expressions of VCAM-1, ICAM-1, and E-selectin in TNF-α-stimulated HUVECs

To explore the mechanism responsible for the inhibitory effect of Tan IIA on monocyte-endothelial cell interaction, we investigated the expressions of cell adhesion molecules in HUVECs. It has been reported that TNF-α markedly increases the gene expression of VCAM-1 in HUVEC time-dependently starting at 4 h and with a peak expression at 6 h, followed by gradual decrease (Choi et al. 2004). In the present study, the expression of cell adhesion molecules was therefore analyzed at 6 h after TNF-α stimulation. As shown in Figs. 2 and 3, the mRNA and protein expression levels of VCAM-1, ICAM-1, and E-selectin were markedly increased by TNF-α stimulation for 6 h in HUVECs. The inhibitory effect of Tan IIA treatment (1, 5, 10 and 20 μM) for 18 h was demonstrated on the TNF-α-induced expressions of VCAM-1 and ICAM-1 at both the mRNA and protein levels. The TNF-α-induced mRNA expression of E-selectin was slightly inhibited by Tan IIA treatment, but not influenced the expression of protein in E-selectin.

Effects of Tan IIA on the expression of fractalkine in TNF-α-stimulated HUVECs

The expression of fractalkine at both the mRNA and protein levels in HUVECs was also investigated. Stimulation of HUVECs with TNF-α (10 ng/ml) for 6 h resulted in a significant increase
Fig. 1. Tan IIA inhibited the adhesion of leukemic monocytes (THP-1) to TNF-α-stimulated HUVECs. (A) The cytotoxic effect of Tan IIA was evaluated by the LDH assay using the conditioned medium collected from HUVECs treated with different concentrations of Tan IIA for 24 h. (B) HUVECs were stimulated with TNF-α (10 ng/ml) for 6 h with/without Tan IIA (1, 5, 10 and 20 μM) pretreatment for 18 h. The adhesion of THP-1 cells to HUVECs was carried out for 30 min at 37 °C. Microphotographs (100×) showing the adhesion of THP-1 to TNF-α-stimulated HUVECs under conditions as indicated. (C) The number of THP-1 cells adhered to TNF-α-stimulated HUVECs was counted and compared. Data were mean ± SEM calculated from three individual experiments. (Significance compared with positive control of TNF-α treatment only, *p < 0.05.) Values were mean ± SEM of three independent tests.

Tan IIA inhibited TNFα-induced nuclear translocation of NF-κB and activation of IKK/NF-κB pathway in HUVECs

NF-κB acts as a transcription factor and the nuclear translocation of NF-κB after inflammatory stimulation is critical to trigger subsequent immune response. Therefore, the nuclear translocation
of NF-κB was used in the present study to evaluate its activity. As shown in Fig. 5A, the TNF-α-induced translocation of NF-κB with color developed by the reaction of peroxidase with ImmPACTTM SG substrate (VECTOR) and counterstained with nuclear fast red (VECTOR) was demonstrated by the dark blue colored nuclei which was then significantly inhibited by treatment with Tan IIA. These results demonstrated that Tan IIA inhibited TNF-α-induced translocation of NF-κB in a dose-dependent manner (Fig. 5B). As shown in Fig. 5C and D, the amount of NF-κB p65 subunit in the nucleus increased by TNF-α stimulation and dose-dependently inhibited by Tan IIA. This result further confirmed that the nuclear translocation of NF-κB in HUVECs was indeed inhibited by Tan IIA.

In order to further understand how the nuclear translocation of NF-κB was inhibited by Tan IIA, we analyzed the upstream activation pathway of NF-κB including IKKα, IKKβ, IκB-α and NF-κB itself. As shown in Fig. 6A and B, TNF-α significantly activated the phosphorylation of these four proteins. Pretreating HUVECs with different concentrations of Tan IIA for 18 h dose-dependently suppressed the phosphorylation of these TNF-α-activated proteins in the IKK/NF-κB pathway. Although it is known that the phosphorylation of IκB-α could lead to the degradation of this protein, the suppressed phosphorylation of IκB-α by Tan IIA did not result in the increase or less degradation of IκB-α in TNF-α-stimulated HUVECs. Recent reports (Gupta et al. 2010; Kim et al. 2008; Tang et al. 2011) have showed that the expressions of ICAM-1, V-CAM-1 and E-selectin could be inhibited by suppressing NF-κB signaling pathway.

6-Amino-4-(4-phenoxphenylethylamino)quinazoline, a more specific inhibitor of NF-κB activation was used, in order to better demonstrate the causal role of NF-κB in our study (Li et al. 2009; Souslova et al. 2010), to further elicit the inhibitory effect of NF-κB on the down-regulation of ICAM-1, V-CAM-1, E-selectin and fractalkine by qRT-PCR. As shown in Fig. 6C, the TNF-α-induced mRNA expressions of ICAM-1, V-CAM-1 and E-selectin in vascular endothelial cells were suppressed by 6-Amino-4-(4-phenoxphenylethylamino)quinazoline in a dose-dependent manner. However, the TNF-α-induced mRNA expression of E-selectin in vascular endothelial cells was not affected under the same condition. These results indicated that the TNF-α-induced mRNA expressions of ICAM-1, V-CAM-1 and fractalkine were mainly contributed by NF-κB activation. Since similar inhibitory
effect was observed by treating cells with Tan IIA, it is reasonably to suggest that the inhibition of NF-κB activation by Tan IIA is responsible for the observed inhibition of ICAM-1, VCAM-1 and fractalkine.

The effect of the Salvia miltiorrhiza extract on inhibiting the adhesion of monocytes to TNF-α-stimulated HUVECS

Since tanshinone IIA is one of the major components in Salvia miltiorrhiza, we prepared the whole extract of Salvia miltiorrhiza and studied its activity on human vascular endothelial cells. The extract of Salvia miltiorrhiza was analyzed by HPLC and nine peaks were revealed as shown in Fig. 7A. The identities of three major peaks including peak 2 (Rt at 14.8 min), peak 5 (Rt at 62.4 min) and peak 8 (Rt at 75.1 min) were further confirmed to be salvianolic acid B, cryptotanshinone and tanshinone IIA, respectively.

Result of LDH assay demonstrated that the viability of human vascular endothelial cells was not affected by Salvia miltiorrhiza extract at concentration up to 5 mg/ml in which tanshinone IIA was calculated to be 40 μM based on the result of HPLC analysis (Fig. 7B). The whole extract of Salvia miltiorrhiza dose-dependently reduced the adhesion of monocytes to vascular endothelial cells with IC50 determined to be 1.3 mg/ml in which tanshinone IIA was 10.53 μM (Fig. 7C). Interestingly in Fig. 1C, tanshinone IIA alone actually exerted higher potency in inhibiting the monocyte adhesion since the IC50 was determined to be 7.3 μM. As shown in Fig. 8, Salvia miltiorrhiza extract exerted the similar effect on inhibiting the mRNA expression levels of ICAM-1, VCAM-1 and fractalkine as tanshinone IIA did. However, Salvia miltiorrhiza extract could reduce the mRNA expression level of E-selectin more significantly when compared with that of Tanshinone IIA.

Fig. 5. Effect of Tan IIA on the nuclear translocation of NF-κB in TNF-α-treated HUVECs. HUVECs were stimulated with TNF-α (10 ng/ml) for 1 h with/without Tan IIA (1, 5, 10 and 20 μM) pretreatment for 18 h. NF-κB localization was visualized by immunocytochemical method. (A) Representative microphotographs (×100) were taken by using light microscope. (B) The number of positive signal (dark blue) in the cell nuclei was determined and compared. (C) NF-κB (p65) translocation to the nucleus was examined in nuclear fractions and lamin B was used as an loading control. (D) Results from densitometric analysis of protein levels in nucleus were shown below the representative data, respectively. Data were quantified and presented as mean values ± SD of three independent experiments. (Significance compared with positive control of TNF-α treatment only, *p<0.05.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Discussion

The initial process of inflammation is achieved by the increased movement of leukocytes from the circulating blood to the foci of inflammation. First is the leukocyte migration, rolling and adhesion in the lumen. Second is the diapedesis to transmigration across the endothelium. Final is the migration of leukocytes in the extracellular matrix (ECM) toward the stimuli (Ley et al. 2007). Atherosclerosis actually involved an ongoing inflammatory response activated by lipid and oxLDL accumulation in the artery wall (Hansson 2005). Tan IIA, a major lipid-soluble active compound of Danshen (S. miltiorrhiza), has been reported to have anti-inflammatory and anti-atherosclerosis properties in recent years (Fan et al. 2009). In the present study, we demonstrated that Tan IIA, a major compound in S. miltiorrhiza, reduced the adhesion of THP-1 cells, which is a human monocyteic cell line derived from an acute monocyteic leukemia to the vascular endothelial cells, suggesting an inhibitory effect on the initial step of inflammatory process to activate atherosclerosis.

Endothelial cells can respond to various stimuli such as TNF-α, IL-1β and LPS in the inflammation process and up-regulate the expression of cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and chemokine (fractalkine/CX3CL1). In addition to the inhibitory effect of Tan IIA on the protein expression levels of VCAM-1 and ICAM-1 that has been reported in brain microvascular endothelial cells (Tang et al. 2011), our results also demonstrated that Tan IIA-mediated inhibition of TNF-α-induced VCAM-1 and ICAM-1 protein expression was correlated well with the decrease at the mRNA levels in HUVECs. Thus, it implies that Tan IIA can down-regulate the TNF-α-activated gene transcription of VCAM-1 and ICAM-1 in HUVECs. Selectins are the main receptors that mediate the initial capture of circulating leukocytes to vascular endothelial surfaces (McEver 2002). However, our result indicated that Tan IIA affected TNF-α-induced gene expression of E-selectin in HUVECs, but not protein level. It may imply that the expression of E-selectin alone, without the expression of appropriate amounts of VCAM-1 and ICAM-1, is not enough to support the adhesion of T lymphocytes to vessel wall. The differential effect of Tan IIA on
the expression of VCAM-1, ICAM-1 and E-selectin in HUVECs may also suggest a different pathway involved in the up-regulation of E-selectin by TNF-α which is not affected by Tan II A.

Fractalkine is an atypical chemokine that exits in two forms: either the membrane-anchored form or the soluble CX3C chemokine (Bazan et al. 1997). The membrane-anchored CX3CL1, which is induced in activated endothelial cells, enables the integrin-independent capture and firm adhesion of leukocytes via the G1 coupled receptor CX3CR1. The soluble CX3C chemokine, which binds CX3CR1 on nearby cells will induce cell chemotaxis and survival (White and Greaves 2009). Therefore, the soluble CX3C chemokine play a critical role for nearby T cells and monocytes homeostasis in the advanced stage of atherosclerosis (Stolla et al. 2012). The association between CX3CR1 and integrins through the coexpression of fractalkine/CX3CL1 and integrin ligands, such as ICAM-1 and VCAM-1, greatly enhances cell adhesion function more than each system alone.

This study demonstrated that the TNF-α-induced expressions of CX3CL1 at mRNA and protein levels were also significantly inhibited by Tan II A in HUVECs. Therefore, not only the typical
adhesion molecules but also the novel chemokine which enable the integrin-independent capture and firm adhesion of leukocyte (White and Greaves 2009) were affected by Tan IIA.

NF-κB is considered as an inflammatory marker that is associated with various inflammatory diseases. After activation, the NF-κB p65 subunit translocates into the nucleus and activates the transcription of various adhesion molecules, acute phase proteins, cytokines and chemokines (Li and Verma 2002). In the resting state, the NF-κB dimmers are retained in the cytoplasm by associating with the inhibitory proteins, IκBα (IκB, and IκBe) (Ockinghaus and Ghosh 2009). Upon stimulation with TNF-α, IκB proteins are phosphorylated and degraded. NF-κB is then activated and translocate to the nucleus where it can activate certain genes through binding to transcription-regulatory elements (Ockinghaus and Ghosh 2009). This study demonstrated that the phosphorylations of NF-κB and IκBα were suppressed by Tan IIA in a dose-dependent manner, although the degradation of IκBα in TNF-α-stimulated HUVECs was not prevented by Tan IIA. It may imply that the inhibitory effect of Tan IIA on the phosphorylation of NF-κB contributes mostly to the inhibited nuclear translocation of NF-κB. Furthermore, the upstream IKK (IκBα kinases) complex plays a central role on triggering the NF-κB cascade. The IKK complex phosphorylate the IκK and the subsequent degradation of IκK results in the activation of NF-κB (Israel 2010). We also demonstrated that the phosphorylation of IKKα/β was increased in TNF-α-stimulated HUVECs which was inhibited by Tan IIA pretreatment in a dose dependent manner. All together, it indicates that Tan IIA may inhibit TNF-α-induced NF-κB activation by the suppression of the IKK pathway in HUVECs.

Tanshinone IIA was confirmed to be one of the three major components in Salvia miltiorrhiza extract by HPLC analysis. Tanshinone IIA alone exerted higher potency on inhibiting the monocyte adhesion when compared with Salvia miltiorrhiza extract even though Salvia miltiorrhiza extract could reduce the E-selectin mRNA expression level in vascular endothelial cells more significantly when compared with tanshinone IIA. These results suggest that certain component(s) in Salvia miltiorrhiza extract could particularly suppress the E-selectin gene expression and some other component(s) in the Salvia miltiorrhiza extract might modulate the overall activity. Further studies are required to investigate the synergistic effect of these components in Salvia miltiorrhiza.

In conclusion, the present study demonstrated a mechanism underlying for the anti-inflammatory activity of Tan IIA which involves down-regulation of cell adhesion molecules and chemokine expression through inhibition of TNF-α-induced NF-κB activation and IκBα phosphorylation by the suppression of the IKK pathway. Cell adhesion molecules and chemokine play an essential role in the process of inflammation as they are necessary for adhesion of leukocytes to the endothelium and for migration of leukocytes from the blood to inflammatory sites. Therefore, Tan IIA might be a potential candidate for the development of drug for the prevention of atherosclerosis.

Conflict of interest

The authors have declared no conflict of interests existed.

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