Original Paper

Tanshinone II A Attenuates TNF- α -Induced Expression of VCAM-1 and ICAM-1 in Endothelial Progenitor Cells by Blocking Activation of NF- κ B

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Key Words

Adhesion molecule • Endothelial progenitor cell • Nuclear factor κ B • Tanshinone IIA • Tumor necrosis factor- α

Abstract

Background/Aims: Tanshinone IIA (Tan IIA) is effective in the treatment of inflammation and atherosclerosis. The adhesion of inflammatory cells to vascular endothelium plays important role in atherogenic processes. This study examined the effects of Tan IIA on expression of adhesion molecules in tumor necrosis factor- α (TNF- α)-induced endothelial progenitor cells (EPCs). **Methods:** EPCs were pretreated with Tan IIA and stimulated with TNF- α . Mononuclear cell (MNC) adhesion assay was performed to assess the effects of Tan IIA on TNF- α -induced MNC adhesion. Expression of vascular cell adhesion molecule-1 (VCAM-1)/intracellular adhesion molecule-1 (ICAM-1) and activation of Nuclear factor κ B (NF- κ B) signaling pathway were measured. **Results:** The results showed that the adhesion of MNCs to TNF- α -induced EPCs and expression of VCAM-1/ICAM-1 in EPCs were promoted by TNF- α , which were reduced by Tan IIA. TNF- α increased the amount of phosphorylation of NF- κ B, I κ B- α and IKK α / β in cytosolic fractions and NF- κ B p65 in nucleus, while Tan IIA reduced its amount. **Conclusion:** This study demonstrated a novel mechanism for the anti-inflammatory/anti-atherosclerotic activity of Tan IIA, which may involve down-regulation of VCAM-1 and ICAM-1 through partial blockage of TNF- α -induced NF- κ B activation and I κ B- α phosphorylation by the inhibition of IKK α / β pathway in EPCs.

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Introduction

Danshen is a popular traditional Chinese medicine and has been widely used in China, Japan, the United States, and also in many European countries for the treatment of several diseases including cardiovascular diseases and cerebrovascular diseases [1]. It is known that danshen contains lipophilic quinones (tanshinone I, tanshinone IIA) and hydrophilic phenolics (salvianolic acid B and danshensu) and displays various pharmacological properties [2]. Among these, tanshinone IIA (Tan IIA) is present in the great amount, served as a marker component and has been used as a reference for some medicine [3].

In recent years, Tan IIA has been reported to have anti-inflammatory and anti-atherosclerosis properties [4]. Atherosclerosis is a process of inflammation and recruitment of circulating mononuclear cells (MNCs) and formation of endothelial cell MNC adhesion play a pivotal role in the development of atherosclerosis [5]. Cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) are crucial in the interaction between endothelial cells and MNCs [5]. It was found that VCAM-1 and ICAM-1 are involved in the adhesion of monocyte and lymphocyte to the endothelium [6-9].

Endothelial progenitor cells (EPCs) comprise a cell population that has the capacity to circulate, proliferate and differentiate into mature endothelial cells (ECs) but that has not yet acquired characteristic mature endothelial markers nor formed a lumen. VCAM-1 and ICAM-1 were showed to be expressed by EPCs and play a critical role in the atherosclerosis [10-12]. The circulating EPCs may stick MNCs through VCAM-1 and ICAM-1 and format EPC-MNC adhesion. These MNCs were engrafted into injured endothelium when EPCs incorporating into endothelium. Incorporated EPCs and differentiated ECs could also continue sticking the circulating MNCs. These processes may contribute to the development of atherosclerosis.

We hypothesize that Tan IIA may exert anti-inflammatory and anti-atherosclerosis properties through down-regulating adhesion molecule expression of EPCs. Tumor necrosis factor- α (TNF- α), a pro-inflammatory cytokine that is released in pathological condition, is elevated in atherosclerosis and could aggravate the process of atherosclerosis [13]. In this paper, we tried to examine the ability of Tan IIA to modulate the expression of adhesion molecules by TNF- α -induced EPCs. Nuclear factor κ B (NF- κ B) serves as a transcription factor and the nuclear translocation of NF- κ B after inflammatory stimulation is essential to induce subsequent immune response [10]. So, we also attempted to find out whether the modulation is NF- κ B dependent.

To the best of our knowledge, this is the first report of the effects and the mechanisms of Tan IIA on the expression of adhesion molecules in TNF α -induced EPCs.

Materials and Methods

Cell culture

All animal investigations were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by NIH. Male Sprague-Dawley rats of 6 to 7 weeks old (200 g) were fed with conventional diet.

In vitro expansion of rat bone marrow-derived EPCs was performed as we previously described [14-16]. Briefly, EPCs were collected from the femurs of rats. The MNCs fraction was obtained by density gradient centrifugation. Cells were then suspended in EBM-2 medium (Lonza) supplemented with 10% FBS (Gibco) and plated on 6-well plates (Corning). In order to remove rapidly adherent mature ECs and hematopoietic cells, the non-adherent cells were aspirated and transferred to new plates after 24 h and 48 h. The collected fraction was cultured in EBM-2 medium supplemented with EGM-2 MV single aliquots containing 10% FBS, vascular endothelial growth factor, epidermal growth factor, fibroblast growth factor-2, insulin-like growth factor-1 and ascorbic acid. Non-adherent cells were removed by washing after 4 d in culture and new media was applied every 3 days.

EPC fluorescent staining

Fluorescent chemical detection of EPCs was performed on attached MNCs after 14 d in culture. Direct fluorescent staining was used to detect dual binding of 1, 1-dioctadecyl-3, 3, 3-tetramethylindocarbocyanine (DiI)-labeled acetylated low-density lipoprotein (acLDL; Molecular Probe) and fluorescein isothiocyanate (FITC)-conjugated Ulex europaeus agglutinin (UEA)-I (Sigma). The cells were first incubated with acLDL (2.4 µg/ml) at 37°C and later fixed with 2% paraformaldehyde for 10 min. After washing, EPCs were reacted with UEA-I (10 µg/ml) for 1 h. After staining, samples were viewed with fluorescence microscopy (×200). Fluorescence microscopy identified double-positive cells as EPCs.

Measurement of cytotoxicity

The cytotoxicity of Tan IIA was evaluated using a standard 3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. After being cultured for 7 d, cells were digested with 0.25% trypsin and then cultured in EBM-2 medium containing 10% FBS in 96-well culture plate (200 µl/well). After being cultured for 48 h, the supernatant was then discarded by aspiration and serum-free EBM-2 medium was added. Tan IIA (0, 1, 5, 10, and 20 µM) was added (200 µl/well), respectively. After being incubated for 24 h, they were supplemented with 20 µl MTT (5 g/l, Fluka Co. Product) and incubated for another 4 h. The supernatant was aspirated and the EPCs preparation was shaken with 150 µl dimethyl sulfoxide (DMSO) for 10 min, before the OD value was measured at 490 nm.

MNCs adhesion assay

EPC monolayers, grown as described earlier, were established in culture dishes. Then Tan IIA (0, 1, 5, 10, and 20 µM) was added, respectively. After being incubated for 18 h, EPCs of each well were treated with TNF-α (10 ng/ml) and cultured for 6 h. EPCs were then incubated with 2×10^5 MNCs for 30 min in a humidified atmosphere with 5% CO₂ at 37°C. After incubation, non-adherent cells were removed by washing with PBS twice. Total six random high-power microscopic fields (100×) were photographed and the numbers of adhesion cells were directly counted.

Analysis of expression of cellular adhesion molecules

After being cultured for 7 d, EPCs were pretreated with Tan IIA (0, 1, 5, 10, and 20 µM) for 18 h and then stimulated for 6 h at 37°C with 10 ng/ml TNF-α. Expression of cell-surface VCAM-1 and ICAM-1 in EPCs was measured by fluorescence-activated cell sorter (FACS) analysis. Cells were washed with ice-cold PBS twice, harvested with 0.5 mL of 0.1 mol/L EDTA, washed with ice-cold PBS twice, incubated with PE-connected antibody against VCAM-1 and ICAM-1 (BD Biosciences) for 1 h. Then cells were fixed with 4% paraformaldehyde for 10 min. After washing, samples were analysed by using a FACStar flow cytometer (Beckman Coulter).

Isolation of nuclear proteins

Nuclear proteins were isolated from treated and control EPCs 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. EPCs 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 Na₂P₂O₇, 1 mM Na₃VO₄, 1 mM EGTA, 0.5 mM PMSF, 1 mM DTT and 1 µg/ml leupeptin. Cell nuclei were resuspended in high salt buffer (hypotonic buffer containing 20% glycerol and 420 mM NaCl) for 30 min at 4°C, and 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 DTT, 0.5 mM PMSF and 20% glycerol and ready for Western blotting.

Western blotting of cell lysates

The protein contents of the cell lysates were determined using a micro BCA kit (Beyotime). Samples with equal amount of proteins were subjected to 10% sodium dodecylsulfate-polyacrylamide gels. Following transfer onto polyvinylidene Fluoride (PVDF, Millipore) membranes and blocking, membranes were incubated with antibodies against VCAM, ICAM, tubulin (1:1000, Santa Cruz Biotechnology), phosphorylated IκB kinase (IKK) α/β, IKK-α, IKK-β, phosphorylated p65 NF-κB, p65 NF-κB, phosphorylated IκB-α, and IκB-α (1:1000, Cell Signaling). After washing, membranes were subsequently incubated with

horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:3000, MultiSciences) for 1 h. The signals were detected by enhanced chemiluminescence reagents (Thermo) and exposure to X-ray film. The density of the bands was quantified by using Image J software (National Institutes of Health).

Immunocytochemical staining of NF- κ B in EPCs

Cells grown on cover slips were fixed in 4% paraform 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 $\times$ dilution of monoclonal antibody against NF- κ B p65 (Santa Cruz Biotechnology, Inc.) in blocking solution for 1 h. After washing, cells were incubated in PBS containing FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc.) for 30 min followed by three washes in PBS and incubated with 4, 6-diamidino-2-phenylindole (DAPI, Beyotime) for 5 min. Then cells were washed in PBS for three times and analyzed by fluorescence microscopy ($\times$ 400).

Electrophoretic mobility shift assay (EMSA)

First, nuclear extracts were prepared from EPCs. Reactions were performed in a total volume of 24 μ L in a buffer consisting of 10 mM HEPES, 50 mM KCl, 1 mM EDTA, 5 mM MgCl₂, 10% glycerol, 5 mM dithiothreitol, 1 mg/mL bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM Na₃VO₄ with 1 μ g of poly (dI-dC) and 0.3 ng of ³²P-labelled high affinity sis-inducible element (hSIE). Following incubation for 15 min at room temperature, DNA-protein complexes were resolved on 4% native polyacrylamide gels and visualized by autoradiography.

Statistical analysis

All data are presented as mean $\pm$ SD. Differences between group means were assessed by ANOVA for multiple comparisons using SPSS 16.0. Values of $P < 0.05$ were considered significant.

Results

Characterization of EPCs

Total MNCs isolated and cultured for 14 d resulted in a spindle-shaped, ECs-like morphology (Fig. 1A). EPCs were characterized as adherent cells double positive for DiLDL uptake and lectin binding by using fluorescence microscopy (Fig. 1B-D).

No cytotoxic effect of Tan IIA on EPCs

To rule out the possible cytotoxic effect of Tan IIA on EPCs, cell viability was assessed by incubating EPCs with various concentrations of Tan IIA for indicated times. When incubated with 1, 5, 10, and 20 μ M Tan IIA for 24 h, cell viability did not show marked changes compared with control group (Fig. 2A). The result showed that Tan IIA did not exhibit cytotoxic effect on EPCs.

Effects of Tan IIA on TNF- α -induced MNC-EPC adhesion

To assess the effects of Tan IIA on TNF- α -induced MNC adhesion, EPCs were treated with indicated concentration of Tan IIA (0, 1, 5, 10, and 20 μ M) for 18 h and then with TNF- α (10 ng/ml) for 6 h. MNCs were added to the EPCs culture to study the adhesion. Without TNF- α stimulation, very few MNCs could adhere to EPCs, however, TNF- α greatly increased the adhesion of MNCs to EPCs. Tan IIA pretreatment dose-dependently inhibited the TNF- α -induced adhesion of MNCs to EPCs, which became apparent at 5 μ M (TNF- α vs. 5 μ M Tan IIA + TNF- α : 190.2 $\pm$ 24.6 vs. 139.4 $\pm$ 9.8, $P < 0.05$), with a peak at 20 μ M (TNF- α vs. 20 μ M Tan IIA + TNF- α : 190.2 $\pm$ 24.6 vs. 62.0 $\pm$ 9.1, $P < 0.01$), as shown in Fig. 2B, C.

Effect of Tan IIA on expression of adhesion molecules VCAM-1 and ICAM-1 in TNF- α -induced EPCs

The expression of cell surface adhesion molecules VCAM-1 and ICAM-1 was measured by FACS analysis. The cell adhesion molecules VCAM-1 and ICAM-1 were expressed at low

Fig. 1. Immunofluorescence identification and immunophenotype of bone marrow derived-EPCs. The attached cells exhibited a spindle shaped, endothelial cells like morphology (A), and adherent cells DiLDL uptake (B: red, exciting wave-length 543 nm) and lectin binding (C: green, exciting wave-length 477 nm) were assessed under a fluorescence microscopy. Double positive cells appearing yellow in the overlay (D) were identified as differentiating EPCs ($\times 200$). White bar indicates 100 μ m.

Fig. 2. Effects of Tan IIA on TNF- α -induced MNC-EPC adhesion. (A) Effects of Tan IIA on the viability of EPCs analysed by the MTT method. Tan IIA did not exhibit cytotoxic effects on EPCs. (B) EPCs were treated with indicated concentration of Tan IIA (0, 1, 5, 10, and 20 μ M) for 18 h and then with TNF- α (10 ng/ml) for 6 h. Adhesion assay using MNCs was performed. Data are presented as mean $\pm$ SD, n = 5. * P < 0.05 vs. TNF- α group. ** P < 0.01 vs. TNF- α group; (C) Microphotographs showing the adhesion of MNCs to TNF- α -stimulated EPCs treated with indicated concentration of Tan IIA (200 $\times$). White bar indicates 100 μ m.

levels in unstimulated EPCs. TNF- α (10 ng/mL) treatment significantly increased expression of these molecules, with about 50% cells showing positive expression of VCAM-1 and ICAM-

Fig. 3. Effects of Tan II A on expression of VCAM-1 and ICAM-1 in EPCs. EPCs were treated with Tan II A (0, 1, 5, 10, and 20 μ M) for 18 h and then with TNF- α (10 ng/ml) for 6 h. (A) the expression of cell surface adhesion molecules VCAM-1 and ICAM-1 was measured by FACS analysis. (B) Mean fluorescence intensity (MFI) of VCAM-1 and ICAM-1 in (A). Data are presented as mean $\pm$ SD, $n = 3$. * $P < 0.05$ vs. TNF- α group. ** $P < 0.01$ vs. TNF- α group; (C) Cell extracts were subjected to 12% SDS-PAGE for Western blot analysis. GAPDH was used as an internal control. (D) Results from densitometric analysis of protein levels were shown below the representative data, respectively. Data are presented as mean $\pm$ SD, $n = 3$. * $P < 0.05$ vs. TNF- α group. ** $P < 0.01$ vs. TNF- α group.

1. Tan II A significantly attenuated the TNF- α -induced expression of VCAM-1 and ICAM-1 in a dose-dependent manner, which became apparent at 1 μ M ($P < 0.05$), with a peak at 20 μ M ($P < 0.01$), (Fig. 3A, B).

To confirm these findings, Western blotting analysis was performed. As illustrated in Fig. 3C, D, amounts of VCAM-1 and ICAM-1 were low in control untreated group, but their expression were markedly increased by TNF- α stimulation for 6 h in EPCs ($299.0 \pm 28.7\%$ of control for VCAM-1, $P < 0.01$; $240.2 \pm 15.5\%$ of control for ICAM-1, $P < 0.01$). Tan II A pretreatment (1, 5, 10 and 20 μ M) for 18 h dose dependently inhibited expression of VCAM-1 and ICAM-1 in TNF- α -stimulated EPCs, which became apparent at 10 μ M for VCAM-1 (TNF- α vs. 10 μ M Tan II A + TNF- α : $299.0 \pm 28.7\%$ of control vs. $231.3 \pm 34.7\%$ of control, $P < 0.05$) and 1 μ M for ICAM-1 (TNF- α vs. 10 μ M Tan II A + TNF- α : $240.2 \pm 15.5\%$ of control vs. $179.8 \pm 13.5\%$ of control, $P < 0.05$), with a peak at 20 μ M (VCAM-1: TNF- α vs. 20 μ M Tan II A + TNF- α : $299.0 \pm 28.7\%$ of control vs. $190.1 \pm 28.6\%$ of control, $P < 0.01$; ICAM-1: TNF- α vs. 20 μ M Tan II A + TNF- α : $240.2 \pm 15.5\%$ of control vs. $80.3 \pm 6.6\%$ of control, $P < 0.01$).

Fig. 4. Effect of Tan IIA on nuclear translocation of NF- κ B in TNF- α -stimulated EPCs. EPCs were treated with indicated concentration of Tan IIA (0, 1, 5, 10, and 20 μ M) for 18 h and then with TNF- α (10 ng/ml) for 6 h. (A) NF- κ B translocation from cytoplasm to nuclei was examined by immunocytochemical staining of NF- κ B (p65) in EPCs. Representative microphotographs were taken by using fluorescence microscopy ($\times 400$). White bar indicates 100 μ m. (B) NF- κ B (p65) translocation to the nucleus was examined in nuclear fractions by Western blot analysis and lamin B was used as a loading control. (C) Densitometric analysis of the protein levels in (B). Data are presented as mean $\pm$ SD, $n = 3$. ** $P < 0.01$ vs. TNF- α group. (D) Nuclear extracts were prepared and NF- κ B DNA binding activities were measured by EMSA. Competition experiments were performed with 100-fold excesses of unlabeled nucleotides corresponding to the NF- κ B binding sequences. Lane 1, negative control; Lane 2, unstimulated cells; lane 3, TNF- α alone; lane 4, TNF- α + 1 μ M Tan IIA; lane 5, TNF- α + 5 μ M Tan IIA; lane 6, TNF- α + 10 μ M Tan IIA; lane 7, TNF- α + 20 μ M Tan IIA; lane 8, TNF- α + excess unlabeled NF- κ B probe; lane 9, positive control. Experiments were performed in triplicate.

Effect of Tan IIA on translocation of NF- κ B p65 in TNF- α -stimulated EPCs

NF- κ B acts as a transcription factor and the nuclear translocation of NF- κ B heterodimers from cytoplasm after inflammatory stimulation is critical to trigger subsequent immune response. To determine whether NF- κ B activation and nuclear translocation were involved in the regulation of Tan IIA on adhesion molecule expression, we studied the effects of Tan IIA on NF- κ B p65 protein levels in the nuclei of TNF- α -treated EPCs by immunofluorescence and Western blots. The DNA binding activity of NF- κ B in the nuclei was also assessed by EMSA.

TNF- α -treated EPCs showed marked NF- κ B p65 staining in the nuclei, while Tan IIA-pretreated EPCs showed weaker staining in the nuclei, but stronger staining in the cytoplasm (Fig. 4A). The result suggested that TNF- α treatment promoted the translocation of NF- κ B p65 protein from cytoplasm to nuclei, and Tan IIA significantly attenuated TNF- α -induced translocation of the NF- κ B p65 protein. Consistent with the result of immunofluorescence

Fig. 5. Effect of Tan II A on the phosphorylation of NF-κB (p65), IκB-α and IKKα/β in TNF-α-stimulated EPCs by Western blotting assays. EPCs were treated with indicated concentration of Tan II A (0, 1, 5, 10, and 20 μM) for 18 h and then with TNF-α (10 ng/ml) for 6 h. (A) Cell extracts were subjected to 12% SDS-PAGE for Western blot analysis. Tubulin was used as an internal reference for semiquantitative loading in parallel lanes. (B) Results from densitometric analysis of protein levels were shown below the representative data, respectively. Data are presented as mean ± SD, n = 3. * P < 0.05 vs. TNF-α group. ** P < 0.01 vs. TNF-α group.

assay, when analyzed by Western blots, a higher level of NF-κB p65 protein was found in the nuclei of TNF-α-induced EPCs compared with the control group. Furthermore, Tan II A pretreatment obviously reduced NF-κB p65 protein levels (P < 0.01), as shown in Fig. 4B, C.

In addition to the immunocytochemistry assay and Western blotting, we further studied the DNA binding activity of NF-κB by EMSA in TNF-α-treated EPCs. As shown in Fig. 4D, a low level of DNA binding activity was observed for NF-κB in the nuclei of untreated EPCs. TNF-α stimulation led to an increase in NF-κB DNA binding activity, whereas pretreatment of EPCs with Tan II A for 18 h dose-dependently decreased the level of NF-κB DNA binding activity.

Effect of Tan II A on activation of IKK/NF-κB pathway in TNF-α-stimulated EPCs

We subsequently explored how Tan II A inhibited nuclear translocation of NF-κB in TNF-α-stimulated EPCs. The activation of upstream pathway of NF-κB including NF-κB itself, IκB-α, IKKα, and IKKβ was observed in the study. The results showed that TNF-α treatment markedly promoted the phosphorylation of NF-κB, IκB-α, IKKα, and IKKβ. Tan II A pretreatment (1, 5, 10 and 20 μM) for 18 h inhibited the phosphorylation of these four proteins in TNF-α-stimulated EPCs (Fig. 5A-D).

Discussion

Herbal medicine which has been used in the treatment of several diseases for thousands of years, is a current focus of interest for the general public and the medical profession [17]. *Salvia miltiorrhiza*, referred to “Danshen” in traditional Chinese Medicine, is commonly used in traditional oriental herbal medicine and has been widely used in both Asian and Western countries for the treatment of cardio-cerebral vascular diseases [18]. Both aqueous and lipid

soluble fractions of Danshen contain active components responsible for the therapeutic effects. The two active hydrophilic components of Danshen are danshensu and salvianolic acid B, whereas Tan IIA and cryptotanshinone are the two lipophilic components [19]. Tan IIA is served as a marker component among these components. In recent years, many studies in animal models and patients have reported that Tan IIA is effective in the treatment of inflammation and atherosclerosis, the pathological basis for most clinical cardiovascular diseases [4, 20, 21].

Inflammation is involved in all stages of the atherosclerosis, including lesion formation and plaque stability [22]. The adhesion of inflammatory cells to the vascular endothelium plays important role in atherogenic processes [23]. These processes depend on the interaction between cell adhesion molecules expressed on endothelium and their cognate ligands on leukocytes [24]. Increased expression of adhesion molecules such as VCAM-1 and ICAM-1 in arterial endothelium may promote adhesion and recruitment of inflammatory cells, thus contribute to the development of atherosclerosis and plaque instability [25, 26]. *In vitro* experiments indicated that Tan IIA decreases the expression of ICAM-1 in human umbilical vein endothelial cells induced by TNF- α [27]. Tang et al. found that Tan IIA pretreatment inhibits the expression of VCAM-1 and ICAM-1, and decreases TNF- α -induced adhesion of neutrophils to brain microvascular endothelial cells in a dose-dependent manner [28]. Chang et al. also found that Tan IIA can suppress TNF- α -induced expression of VCAM-1 and ICAM-1 in human vascular endothelial cells [10].

It was known that endothelial cell dysfunction is a major promoter for atherosclerosis and cardiovascular events [29]. Endothelial dysfunction eventually represents an imbalance between the magnitude of injury and the ability for repair [30]. Endothelial cells, the most abundant cells in the endothelium, do not have significant replicative capacity; however, EPCs also participate in vascular repair [31]. EPCs proliferate in the bone marrow and other tissues, and are released in response to vascular damage, migrate to the site of injury and further replicate and mature to endothelial cells [31]. This whole process is called endothelial (vascular) repair. EPCs could be used as a marker of vascular function and served as a cellular reservoir that could replace injured endothelium [30]. Recently, EPC transplantation represents a novel approach to treat cardiovascular diseases. TNF- α is a kind of pro-inflammatory cytokine that is released in response to a pathological condition. TNF- α is increased in atherosclerosis and could enhance the process of atherosclerosis [13]. TNF- α was found to reduce proliferation, adhesion, migration and tube formation ability of EPCs [32]. Meanwhile, the elevated level of TNF- α may represent a hostile microenvironment which may induce EPCs differentiation into aberrant cells [33]. So drug regimen before or in combination with cell transplantation may be a promising strategy for the future EPC therapy for cardiovascular diseases [33]. The purpose of this study was to examine the effect of Tan IIA on expression of adhesion molecules in TNF- α -induced EPCs.

The results showed that TNF- α induced the expression of VCAM-1 and ICAM-1 in EPCs and promoted the adhesion of MNCs to TNF- α -induced EPCs. When pretreated with Tan IIA (1, 5, 10 and 20 μ M) for 18 hours, down-regulation of VCAM-1 and ICAM-1 was observed, and the adhesion of MNCs to TNF- α -induced EPCs was significantly reduced in a dose-dependent manner. This is the first study to show that Tan IIA reduces the expression of adhesion molecules and consequently decreases MNCs adhesion to EPCs. The elevated expression of adhesion molecules by EPCs in atherosclerotic lesions may result in further recruitment of monocytes to atherosclerotic sites. The findings suggested that Tan IIA may exert anti-atherosclerotic property through the suppression of adhesion molecules in endothelium. Furthermore, we also explored the underlying mechanism of these effects.

It has been shown that the NF- κ B signaling pathway regulates the transcription of several cell adhesion molecules, including VCAM-1 and ICAM-1 [34]. Accordingly, the role of NF- κ B signal transduction pathway activation in TNF- α -induced expression of cell adhesion molecules in EPCs was explored in the present study. NF- κ B is a major transcription factor that plays an important role in many diseases, such as atherosclerosis, diabetes, cancer, and so on [35]. In resting cells, NF- κ B proteins are kept in the cytoplasm in an inactive form as

a p50/p65 protein heterodimer in association with inhibitory I κ B proteins including I κ B- α , I κ B- β , and I κ B- ϵ among which I κ B- α is the most abundant [36]. It is well known that I κ B- α protein was phosphorylated by IKKs (IKKs contain two major kinase subunits, IKK α and IKK β) upon TNF- α stimulation. IKK α and IKK β phosphorylate I κ B proteins, including I κ B- α , at specific serines within their amino termini, thus leading to site-specific ubiquitination and degradation by the 26S proteasome [37]. Upon stimulation with cytokine such as TNF- α , I κ B- α undergoes phosphorylation, ubiquitination and subsequent degradation, thereby unmasking the nuclear localization signal on p65 and allowing translocation of NF- κ B to the nucleus where it can activate certain genes through binding to initiate transcription of many target genes including VCAM-1 and ICAM-1 [38].

In the present study, it was found that the phosphorylation of IKK α / β was increased in TNF- α -stimulated EPCs which was inhibited by Tan IIA pretreatment. The findings also showed that the phosphorylations of NF- κ B and I κ B- α in the cytoplasm of TNF- α -stimulated EPCs were suppressed by Tan IIA in a dose-dependent manner. TNF- α -treated EPCs were found to contain elevated levels of nuclear NF- κ B p65. Tan IIA reduced the amount of nuclear NF- κ B p65. The results suggested that Tan IIA inhibitory effect on the nuclear translocation of NF- κ B might mainly through suppressing the phosphorylation of NF- κ B. It is worthy to note that although I κ B- α /NF- κ B complexes are localized mainly in cytosol, I κ B- α and NF- κ B as well as I κ B- α /NF- κ B complexes have been reported to shuttle between cytoplasm and nucleus [39]. I κ B- α /NF- κ B complexes have been shown to mask the nuclear localization sequences on p65, resulting in a partial inhibition of nuclear translocation of NF- κ B [38]. To test directly whether NF- κ B was attenuated in EPCs treated with Tan IIA, we analyzed the DNA binding activity of NF- κ B by EMSA. It was found that Tan IIA treatment significantly inhibited TNF- α -induced NF- κ B activation in a dose-dependent manner. These data confirmed that Tan IIA inhibited VCAM-1 and ICAM-1 expression in EPCs at least partially through NF- κ B-dependent signaling pathways.

It should be noted that we cultured EPCs derived from healthy rats to investigate the effect of Tan IIA on expression of adhesion molecules in TNF- α -induced EPCs in this study. The findings suggested that Tan IIA might exert therapeutic effect on inflammatory-related diseases such as atherosclerosis through down-regulation of cell adhesion molecules such as VCAM-1 and ICAM-1. However, if we wanted to study the anti-atherosclerotic effects of Tan IIA, we should culture EPCs derived from atherosclerotic animals. Furthermore, animal experiments should be performed to determine the change of EPCs treated with Tan IIA in mature ECs able to repair injured vessels.

Conclusion

The current study demonstrated a novel mechanism underlying for the anti-inflammatory or anti-atherosclerotic activity of Tan IIA which may involve down-regulation of cell adhesion molecules including VCAM-1 and ICAM-1 through partial blockage of TNF- α -induced NF- κ B activation and I κ B- α phosphorylation by the inhibition of IKK α / β pathway in EPCs.

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Disclosure Statement

None declared.

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