3,4-oxo-isopropylidene-shikimic acid inhibits adhesion of polymorphonuclear leukocyte to TNF-α-induced endothelial cells *in vitro*[1]

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KEY WORDS 3,4-oxo-isopropylidene-shikimic acid; adhesions; vascular endothelium; neutrophils; intercellular adhesion molecule-1

ABSTRACT

AIM: To examine the effect of 3,4-oxo-isopropylidene-shikimic acid (ISA) on human polymorphonuclear leukocyte (PMN) adhesion to human umbilical vein endothelial cells (HUVEC) and explore its mechanism. **METHODS**: Adhesion of PMN to HUVEC was measured by rose bengal staining assay. Cell-ELISA and RT-PCR methods were used to examine the expression of adhesion molecules ICAM-1. Cell viability was detected with MTT assay. **RESULTS:** ISA (1-100 μmol/L) effectively reduced PMN adhesion to TNF-α-induced HUVEC with the inhibitory rate from 17.2 % to 53.5 %, and exerted no effect on PMN adhesion to normal HUVEC. Adhesion molecule ICAM-1 surface protein and mRNA expression induced by TNF-α (400 kU/L) were significantly inhibited by ISA. In addition, the cell viability of HUVEC was unchanged 48 h after treatment with ISA. **CONCLUSION**: ISA inhibited TNF-α-stimulated PMN-HUVEC adhesion and expression of ICAM-1.

INTRODUCTION

Leukocyte infiltration to endothelium plays a major role in the inflammatory response related to thrombosis, arteriosclerosis, and reperfusion injury, *etc*. Leukocyte infiltration requires a chain of reactions between endothelial cells (EC) and leukocytes that initially retards intravascular leukocyte flow and finally leads to leukocyte transmigration through the endothelial monolayer. These events are mediated by sequential interaction of different endothelial adhesion molecules with their receptors on leukocyte surface. A crucial step between the initial contact and final transmigration of leukocytes is their tight adhesion to EC, which is mainly mediated by the endothelial transmembrane receptors intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular adhesion molecule-1 (VCAM-1, CD106). The up-regulation of ICAM-1 on the surface of the endothelium is required for the firm adhesion of rolling polymorphonuclear leukocyte (PMN). Lipopolysaccharide (LPS) and inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1(IL-1), and interferon-γ can stimulate ICAM-1 expression[1,2]. In this study, TNF-α was used to stimulate human umbilical vein endothelial cells (HUVEC) expression adhesion molecule and induced PMN-endothelial

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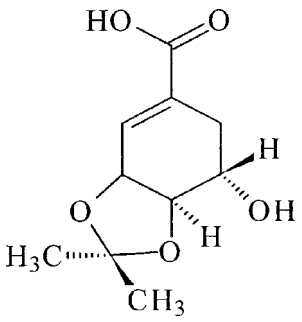
cell adhesion. Based on this model, the effect of 3,4-oxo-isopropylidene-shikimic acid (ISA) on adhesion was observed.

ISA was one of the derivatives of shikimic acid (SA), which was extracted from *Illicium verum* Hookfil. Our previous study suggested that ISA could suppress various experimental thrombosis induced by injuring vascular endothelium[3] and attenuate cerebral ischemic damage[4,5]. To further clarify the above action of ISA, the effect of ISA on PMN-endothelial cell adhesion was observed and its mechanism was explored.



Structural formula of 3,4-oxo-isopropylidene-shikimic acid (ISA)

MATERIALS AND METHODS

Chemicals ISA (purity >98 %), provided by Department of Phytochemistry, Beijing University of Traditional Chinese Medicine, was dissolved in pure water and kept as a stock solution. RT-PCR primer was synthesized by Shanghai Sangon Co. 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide was purchased from Sigma. Freshly discarded human umbilical cords were obtained from China-Japan Friendship Hospital.

Cell culture Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords by collagenase treatment as described by Jaffe *et al*[6]. The cells were grown to confluence in medium 199 (Gibco) supplemented with 20 % fetal bovine serum (Hyclone), benzyl penicillin (100 kU/L), streptomycin (100 mg/L), glutamine (2 mmol/L) and endothelial cell growth factor (20 mg/L, Roche) in culture flasks and dishes coated with 1 % gelatin. To confirm the endothelial origin of the harvested cells, we analyzed them under microscope for the typical cobblestone appearance and tested immunohistochemically for the occurrence of von Willebrand factor. For experiments, the second or third passage of cells was used.

PMN isolation Human peripheral blood PMN were obtained from healthy adult volunteers and separated on a discontinuous gradient consisting of Ficoll-Hypaque solution with the density of 1.077 kg/L and 1.119 kg/L[7,8]. Isolated PMN were resuspended in M199 at a final concentration of 3×10^9 cells/L. PMN purity exceeded 98 % as confirmed by Wright's stain. Cell viability exceeded 95 % by trypan blue exclusion.

Adhesion assays PMN were added to HUVEC monolayers at a PMN-to-HUVEC ratio of 10:1. After coincubation for 40 min, the wells were gently rinsed twice with PBS to remove nonadherent cells. PMN adhesion to endothelial cells was evaluated by rose bengel staining[9]. Color development was measured with a microtiter plate spectrophotometer (Multiskan MK3, Thermo Co) at 570 nm and subtracted A_{570nm} of wells containing EC alone to indicate the adhesion of PMN. Each adhesion assay was performed 12 h after treating monolayers with TNF-α. The effect of ISA on PMN adhesion was assessed by pre-incubation of ISA with monolayers for 6 h before adding TNF-α.

Cell ELISA for ICAM-1[10] HUVEC were seeded in 96-well tissue culture dishes with 3×10^4cells/well. Treatment of HUVEC monolayers with ISA or TNF-α was the same as described in adhesion assays. After stimulation with TNF-α for 12 h, the cell culture medium was removed, and the cells were immediately fixed with methanol. Nonspecific protein binding was blocked subsequently by adding 1 % BSA in PBS for 40 min at 37 ºC. Primary mouse monoclonal antibodies for ICAM-1 (NeoMarkers) were added to each well (1:1000) and incubated at 37 ºC for 1 h. The cells were washed in PBS-0.05 % Tween, and incubated at 37 ºC for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Zhongshan Co, 1:10 000). The cells were washed again with PBS-0.05 % Tween three times, and the solution of substrate (citrate-phosphate buffer containing 0.04 % OPD, Sigma) was added for 15 min. The reaction was stopped with sulfuric acid 2 mol/L (50 μL), and the color development was read on a microtiter plate spectrophotometer (Multiskan MK3, Thermo Co) at 492 nm after subtracting the background values in cells stained only with the second-step antibody.

ICAM-1 mRNA expression with RT-PCR Semiquantitative PCR was used to assess ICAM-1 expression. After incubated with ISA, the cells were treated for 6 h with TNF-α (400 kU/L). Total RNA of HUVEC was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instruction. Quantifi-

cation and purity of RNA were assessed by A_{260}/A_{280} absorption. The RNA samples with A_{260}/A_{280} ratios (above 1.9) were used for further analysis. First-strand cDNA was synthesized from the total RNA by Omniscript RT (Qiagen) following the manufacturer's instructions. cDNA was amplified by PCR in a total volume of 50 μL using 2.5 U *Taq* DNA polymerase (Promega) and 10 pmol each of upstream and downstream primers. After predenaturation at 94 ºC for 3 min, 35 cycles were allowed to run for 30 s at 94 ºC, followed by 30 s at 55 ºC and 30 s at 72 ºC, and a final extension at 72 ºC for 10 min. Primers for ICAM-1 were sense 5'-AAT GCC CAG ACA TCT GTG TCC C-3', antisense 5'-GGC AGC GTA GGG TAA GGT TCT T-3', and for GAPDH, sense 5'-TGG TAT CGT GGA AGG ACT CAT G-3, antisense 5'-TCC TTG GAG GCC ATG TGG GCC AT-3'. The predicted amplification products were 330 bp and 501 bp, respectively. A 15-μL aliquot of the amplified DNA reaction mixture was fractionated by 1.5 % agarose gel electrophoresis, and the amplified product was then visualized by ultraviolet fluorescence after being stained with ethidium bromide. ICAM-1 mRNA expression was normalized to the housekeeping gene GAPDH mRNA expression via densitometric analysis by autogel analysis system. The results were expressed as the relative level of mRNA expression (ratio of ICAM-1/GAPDH).

Cell viability assays Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was used to determine cell viability[12] 48 h after ISA treatment .

Statistical analysis Each experiment was performed in triplicate and repeated at least three times. Data were presented as mean±SD. Differences were analyzed by ANOVA .

RESULTS

Effect of TNF-α on adhesion of PMN to HUVEC PMN adhesion to HUVEC was increased by preincubation of HUVEC with TNF-α (100-800 kU/L) for 12 h, in a concentration-dependent manner (Fig 1).

Effects of ISA on the adhesion of PMN to TNF-α-stimulated and normal HUVEC PMN adhesion to HUVEC was increased by incubation of HUVEC with TNF-α (400 kU/L) for 12 h (P<0.01 *vs* control) . Pretreatment with ISA 1-100 μmol/L inhibited PMN adhension to TNF-α-induced HUVEC in a concentration-dependent manner, with the inhibitory rate from 17.2 % to 53.5 %. Treatment of EC only with ISA for 18 h exerted no effect on PMN adhension to HUVEC compared with control (Tab 1).

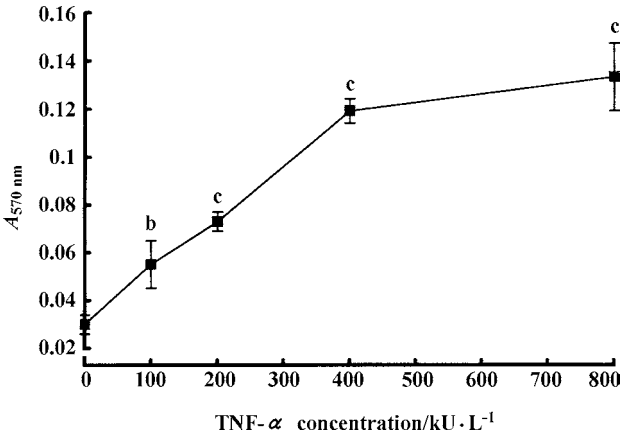


Fig 1. Effect of TNF-α on adhesion of PMN to HUVEC. n=3. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* control.

Tab 1. Effect of ISA on PMN adhesion to TNF-α-induced HUVEC and normal HUVEC. n=3. Mean±SD. ^aP>0.05, ^bP<0.05, ^cP<0.01 *vs* control. ^eP< 0.05, ^fP< 0.01 *vs* TNF-α group.

Group	Concentration/ μmol·L^{-1}	$A_{570\ nm}$	Inhibition rate/%
Control		0.025±0.008^f	
TNF-α	400 kU/L	0.099±0.009^e	
ISA+TNF-α	0.1	0.096±0.008^e	3.0
	1.0	0.082±0.004^{ce}	17.2
	10.0	0.055±0.010^{ef}	44.4
	100.0	0.046±0.004^{bf}	53.5
ISA	0.1	0.018±0.006^a	
	1.0	0.023±0.003^a	
	10.0	0.023±0.010^a	
	100.0	0.021±0.010^a	

ISA: 3,4-oxo-isopropylidene-shikimic acid; HUVEC: human umbilical vein endothelial cells.

Effect of ISA on ICAM-1 surface protein expression in HUVEC ICAM-1 was expressed at low levels on untreated HUVEC and up-regulated by stimulation with TNF-α (400 kU/L) for 12 h. Pretreatment with ISA (10 and 100 μmol/L) attenuated TNF-α-induced ICAM-1 expression in HUVEC, the inhibitory rate was 19.7 % and 40.4 %, respectively (Tab 2).

Effect of ISA on ICAM-1 mRNA expression in HUVEC The products of RT-PCR for ICAM-1 mRNA and GAPDH mRNA were 330 bp and 501 bp respectively,

Tab 2. Inhibitory effects of ISA on expression of ICAM-1 surface protein of HUVEC induced by TNF-α (400 kU/L) by cell-ELISA method. *n*=3. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* control. [e]*P*<0.05, [f]*P*<0.01 *vs* TNF-α group.

Group	Concentration/ μmol·L^{-1}	$A_{492\ nm}$	Inhibitory rate/%
Control		0.20±0.01[f]	
TNF-α	400 kU/L	0.54±0.04[c]	
ISA+TNF-α	1	0.48±0.05[e]	11.8
	10	0.44±0.04[ce]	19.7
	100	0.32±0.03[cf]	40.4

ISA: 3,4-oxo-isopropylidene-shikimic acid; HUVEC: human umbilical vein endothelial cells.

corresponding to the predicted length (Fig 2). In control group, a basic expression of ICAM-1 in HUVEC was found. TNF-α (400 kU/L) enhanced expression of ICAM-1 mRNA in HUVEC significantly. Pre-treatment with ISA (10 μmol/L) obviously decreased the expression level of ICAM-1 mRNA induced by TNF-α (P<0.05).

Effect of ISA on cell viability of HUVEC There was no difference in cell viability between HUVEC incubated with ISA for 48 h and untreated HUVEC (Tab 3).

Tab 3. Effect of ISA on cell viability of HUVEC. *n*=3. Mean±SD. [a]*P*>0.05 *vs* control.

Group	Concentration/μmol·L^{-1}	$A_{570\ nm}$
Control	0	0.119±0.012
ISA	0.1	0.122±0.008[a]
	1	0.125±0.007[a]
	10	0.122±0.009[a]
	100	0.121±0.010[a]

ISA: 3,4-oxo-isopropylidene-shikimic acid; HUVEC: human umbilical vein endothelial cells.

DISCUSSION

Adhesion of circulating PMN to the vascular endothelium is a critical step in the inflammatory response related to thrombosis, arteriosclerosis, and reperfusion injury, *etc.* Our previous study showed that ISA could suppress various experimental thrombosis and attenuate ischemic injury. Whether this effect is mediated by

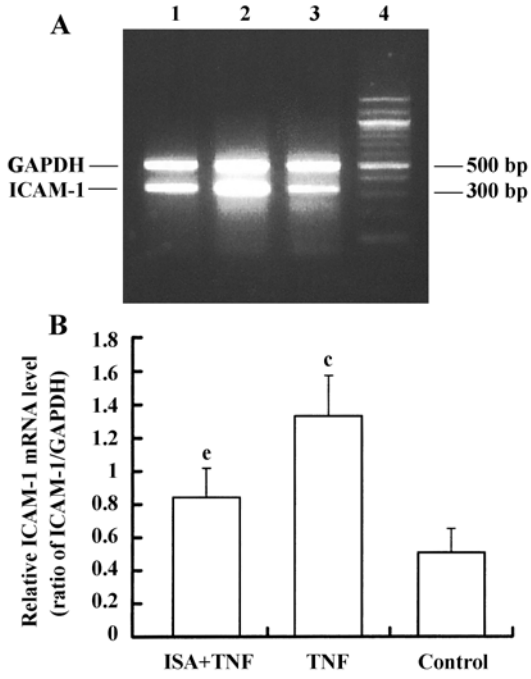


Fig 2. The inhibitory effects of ISA on ICAM-1 mRNA expression in cultured HUVEC stimulated with TNF-α (400 kU/L) for 6 h. A) ICAM-1 (330 bp) and GAPDH (501 bp) mRNA levels in cultured HUVEC were detected by RT-PCR. The samples were loaded on 1.5 % agarose gel. Lane 1: TNF-α+ISA 10 μmol/L; Lane 2: TNF-α; Lane 3: control; Lane 4: 100-bp DNA ladder. B) ICAM-1 mRNA level was expressed as the ratio of ICAM-1 mRNA relative to the GAPDH mRNA levels. *n*=4. Mean±SD. [c]*P*<0.01 *vs* control group. [e]*P*< 0.05 *vs* TNF-α group.

inhibition of adhesion of PMN to vascular endothelium is not clear. The present results showed that ISA effectively reduced PMN adhesion to TNF-α-induced HUVEC in a concentration-dependent manner and exerted no effect on PMN adhesion to normal HUVEC. To exclude the decrease of PMN adhesion to HUVEC for the cytotoxicity induced by ISA, cell viability of HUVEC after treatment with ISA for 48 h was observed. The result showed the cell viability unchanged. So we concluded that ISA inhibited PMN adhesion to TNF-α-induced HUVEC not for its cytotoxicity to HUVEC.

To explore the inhibitory mechanism of ISA on PMN adhesion to HUVEC, the surface protein and messenger RNA expression of ICAM-1 were examined. The up-regulation of ICAM-1 on the surface of the endothelium is required for the firm adhesion of rolling PMN[12]. ICAM-1, a member of the immunoglobulin supergene family, serves as the receptor for leukocyte function-associated antigen-1 (LFA-1; CD11a/CD18), α2-

integrin, which is expressed on PMN, monocytes, lymphocytes, and natural killer cells[1]. ICAM-1 is constitutively expressed at low levels on the cell surface of endothelial and epithelial cells, and up-regulated fast after stimulation with proinflammayory cytokine[13], which is accordance with our study. In our study, TNF-α increased PMN-endothelial cell adhesion in a concentration-dependent manner and upregulated ICAM-1 expression and ICAM-1 mRNA levels in HUVEC. ISA was found to attenuate TNF-α-induced ICAM-1 expression at the dose of 10 μmol/L, but ISA 1 μmol/L could inhibit PMN adhesion to TNF-α -induced HUVEC. These all suggested that the inhibitory effect of ISA on PMN-EC adhesion was not only associated with inhibitory expression of ICAM-1, but also with other effects, which needs further study. ISA also inhibited TNF-α-induced ICAM-1 mRNA expression in HUVEC, which indicated the mechanism of ISA anti-adhesion at least in part related to down-regulation of the gene transcription of ICAM-1.

Taken together, the results suggested that suppressing the surface protein and mRNA expression of ICAM-1 was one of the pathways of ISA inhibiting adhesion of PMN to TNF-α-stimulated HUVEC.

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