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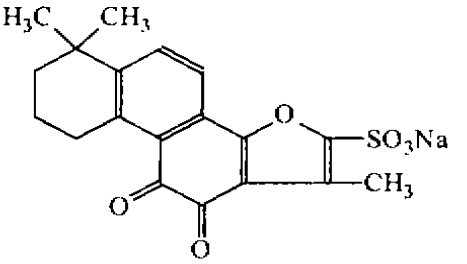
Effects of tanshinone Ⅱ-A sulfonate on adhesion molecule expression of endothelial cells and platelets *in vitro*[1]

JIANG Kai-Yu[2,3], RUAN Chang-Geng[3], GU Zhen-Lun[2,4] ([2]*Department of Pharmacology*, [3]*Jiangsu Institute of Hematology, Thrombosis and Haemostasis Research Unit, Suzhou Medical College, Suzhou 215007, China*); ZHOU Wen-Xuan, GUO Ci-Yi (*The Hong Kong Association for Health Care, Hong Kong, China*)

KEY WORDS vascular endothelium; tanshinone Ⅱ-A; blood platelets; cell adhesion molecules; cell adhesion; umbilical veins; HL-60 cells; flow cytometry; thrombin; P-selectin; tumor necrosis factor

AIM: To study the action of tanshinone Ⅱ-A sulfonate (Tan) on adhesion molecule expression by cultured endothelial cells and platelets. **METHODS**: Tumor necrosis factor α (TNF-α)-induced ICAM-1 expression on the cell surface and endothelial adhesivity toward HL-60 cells were studied using human umbilical vein endothelial cells (HUVEC). Thrombin-induced expression of platelet P-selectin was studied using human blood platelets. Adhesion molecule expression on the cell surface was measured by flow cytometry. The number of HL-60 cells adhering to the HUVEC monolayer was determined by liquid scintillation spectroscopy. **RESULTS**: Pretreatment of HUVEC with TNF-α significantly enhanced ICAM-1 expression and increased HL-60 cells adhesion to HUVEC from 4.6 % ± 0.7 % to 30 % ± 6 %. Tan (25 - 200 μmol · L^{-1}) inhibited the effects of TNF-α in a concentration-dependent manner. Tan also inhibited the increase of P-selectin expression of thrombin-activated platelets in a concentration-dependent manner. **CONCLUSION**: Tan inhibited expression of adhesion molecules (ICAM-1, P-selectin) in HUVEC and in human blood platelets.

Tanshinone Ⅱ-A was isolated from the root of *Salvia milliorrhiza*. Its sulfonate is water-soluble and can inhibit platelet adherence, aggregation and has an antithrombotic activity[1,2]. It suppresses the neutrophil functions including acid-phosphatase release, adhesiveness, phagocytosis, oxygen free radical generation, and chemokinesis[3-6]. It also reduces myocardial necrosis concomitantly[1,3]. To further understand the antithrombotic mechanism of tanshinone Ⅱ-A sulfonate (Tan), the present study was to investigate the effects of Tan on adhesion molecule expression in cultured human umbilical vein endothelial cells (HUVEC) and in platelets.



Tanshinone Ⅱ-A sulfonate

MATERIALS AND METHODS

Reagents TNF-α was from Sigma. Tanshinone Ⅱ-A sulfonate (mp 194 - 196 ℃, purity > 96 %) was supplied by Shanghai Institute of Materia Medica, Chinese Academy of Sciences. ICAM-1 mAb 84H10 was purchased from Immunotech SA (France). The monoclonal antibody against P-selectin was prepared in our laboratory[7,8]. Other reagents were of AR.

Cell cultures HUVEC were isolated[9] and cultured in RPMI-1640 (Gibco), containing 20 % (vol/vol) heat-inactivated fetal calf serum (FCS), benzylpenicillin 100 kU · L^{-1} and streptomycin 100 mg · L^{-1} at 37 ℃ in humidified 95 % air + 5 % CO_2. Confluent HUVEC were obtained after 5 - 7 d. HL-60 cells were cultured in RPMI-1640 medium with 10 % (vol/vol) FCS.

Preparation of platelet Blood was collected from healthy volunteers in 2 % edetic acid (EDTA)-0.7 % NaCl. Platelets were washed and resuspended in Tyrode's buffer (KCl 2.6, $MgCl_2 \cdot 6H_2O$ 4.0, NaCl 135, $NaHCO_3$ 12.0, NaH_2PO_4 0.42, glucose 5.0 mmol · L^{-1}, and 0.25 % FCS, pH 7.4). Adjust the platelet concentration to 1×10^9 · L^{-1}.

Flow cytometry Confluent HUVEC treated with Tan or TNF-α as described below were detached from the plate (6-well) by PBS/EDTA 5 mmol · L^{-1}. Cells were washed with RPMI-1640 plus 10 % FCS, stained with the ICAM-1 mAb 84H10

[1] Project supported by The Hong Kong Association for Health Care.
[4] Correspondence to Prof GU Zhen-Lun.
Phn 86-512-519-5696, ext 4170. Fax 86-512-519-2662.
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diluted in PBS containing 2 % BSA and 0.2 % sodium azide. Platelets were stained with anti-P-selectin mAb followed by fluorescein-conjugated goat anti-mouse IgG (Sino-American Biotechnology Co. Shanghai. China). Cells immunofluorescence analysis was performed with an EPICS XL cytofluorimeter (Coulter, USA) Five thousand cells were analyzed for each experiment.

Cell adhesion assays HUVEC growth to confluence in 24-well plates were treated with Tan and TNF-α as described below. HL-60 cells were labeled with [^{3}H]thymidine 37 MBq·L^{-1} at 37 ℃ for 2 h, washed thrice with RPMI-1640 medium, and incubated at 37 ℃ in RPMI-1640 containing 10 % FCS for 1 h. Labeled HL-60 cells (1×10^6 cells/well) were incubated with the HUVEC monolayer at 37 ℃ for 45 min. Nonadherent cells were removed by washing twice with RPMI-1640 medium prewarmed to 37 ℃. The HL-60 cells adhering to the HUVEC monolayer was detached in the presence of PBS containing EDTA 10 mmol·L^{-1} prechilled to 4 ℃ and its radioactivity was counted by liquid scintillation spectroscopy (Beckman LS600, USA). HL-60 adherence to HUVEC was expressed as % of total cell added.

Experimental protocol Cultured confluent HUVEC were washed twice with RPMI-1640 containing 5 % FCS. Tan was dissolved in redistilled water. The cells were incubated with the indicated concentration of sterilized Tan at 37 ℃ for 2 h, then the TNF-α 5 μg·L^{-1} was added for 16 h. Human blood platelet suspension was incubated with Tan for 30 min (each sample was 1×10^9 platelets), followed by activation with thrombin 1 kU·L^{-1} for 20 min. In the cell adhesion assay, HUVEC were stimulated with TNF-α 1 μg·L^{-1} for 20 h. Tan was added as described above.

Statistics Data were expressed as $x \pm s$ and compared with t test.

RESULTS

ICAM-1 expression and cell adhesion

Treatment of HUVEC with TNF-α 5 μg·L^{-1} for 16 h resulted in an increase in ICAM-1 expression. Pretreatment of the HUVEC with Tan (25 – 200 μmol·L^{-1}) for 2 h before adding TNF-α, reduced TNF-α-induced ICAM-1 expression on the cell surface of HUVEC in a concentration-dependent manner (Tab 1).

Basal HUVEC minimally bound HL-60 cells (4.6 % ± 0.7 %), whereas treatment of HUVEC with TNF-α 1 μg·L^{-1} for 20 h, resulted in a 7-fold increase in the number of HL-60 cells bound. Pretreatment of the HUVEC monolayer with Tan (25 – 200 μmol·L^{-1}), reduced adherence of HL-60 cells to TNF-α-treated HUVEC (Tab 2).

Tab 1. Effect of Tan on ICAM-1 expression in HUVEC. $n = 4$, $x \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* **TNF-α 5 μg·L^{-1}.**

Treatment	Fluorescence intensity (lg)
Control	4.2 ± 0.4^c
TNF-α 5 μg·L^{-1}	18.4 ± 1.3
TNF-α + Tan 25 μmol·L^{-1}	14.5 ± 2.0^b
TNF-α + Tan 50 μmol·L^{-1}	14.2 ± 0.8^c
TNF-α + Tan 100 μmol·L^{-1}	12.8 ± 1.1^c
TNF-α + Tan 200 μmol·L^{-1}	11.0 ± 1.0^c

Tab 2. Effect of Tan on HL-60 cells adhesion to HUVEC. $n = 8$, $x \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* **TNF-α 1 μg·L^{-1}.**

Treatment	Adhesion rate %
Control	4.6 ± 0.7^c
TNF-α 1 μg·L^{-1}	30 ± 6
TNF-α + Tan 25 μmol·L^{-1}	24 ± 5^b
TNF-α + Tan 50 μmol·L^{-1}	20 ± 3^c
TNF-α + Tan 100 μmol·L^{-1}	20 ± 4^c
TNF-α + Tan 200 μmol·L^{-1}	17 ± 3^c

P-selectin expression in human blood platelet The fluorescence associated with unstimulated platelets plus FITC-P-selectin mAb was not different from the background fluorescence of washed platelets alone (1.8 ± 0.1). Treatment of platelet with thrombin 1 kU·L^{-1} for 20 min, resulted in a marked increase of P-selectin expression and reached 9.32 ± 0.15 in the mean fluorescence intensity of the total platelet population. Preincubation of platelet with Tan (25 – 200 μmol·L^{-1}) for 30 min, reduced thrombin-induced P-selectin expression in a concentration-dependent manner (Tab 3). Tan 500 μmol·L^{-1} abolished the effect of thrombin.

DISCUSSION

The ability of HL-60 cells to adhere to cytokine-treated HUVEC monolayers was used to assess functional ICAM-1 expression, because HL-60 cells were found to express the ligands for ICAM-1[10]. The present results showed that pretreatment of HUVEC with Tan inhibited TNF-α-induced ICAM-1 expression, and Tan may significantly decrease adherence of leukocyte-like cell line (HL-60) to

Tab 3. Effect of Tan on thrombin-induced P-selectin expression in platelet. $n = 4$, $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* **thrombin 1 kU·L^{-1}.**

Treatment	Fluorescence intensity (lg)
Control	1.77 ± 0.09^c
Thrombin 1 kU·L^{-1}	9.32 ± 0.15
Thrombin + Tan 10 μg·L^{-1}	9.05 ± 0.29^a
Thrombin + Tan 25 μmol·L^{-1}	7.73 ± 0.11^c
Thrombin + Tan 50 μmol·L^{-1}	7.39 ± 0.58^c
Thrombin + Tan 100 μmol·L^{-1}	7.13 ± 0.36^c
Thrombin + Tan 200 μmol·L^{-1}	6.66 ± 0.64^c

TNF-α-treated HUVEC, demonstrating that Tan may be used to selectively inhibit the expression of endothelial cell-adhesion molecule, inhibited adhesion of HL-60 cells to TNF-α-activated endothelial cells, further demonstrating pharmacological activity of Tan in HUVEC.

Our studies suggest that Tan alters the capacity of HUVEC to respond to TNF-α. Impairment of this capacity would not only inhibit induction of adhesion of ICAM-1, but also could inhibit induction of other adhesion molecules involved in leukocyte/HUVEC interaction. At present, the exact mechanism of action of Tan in inhibiting cytokine-induced adhesion has not yet known, but our findings that Tan inhibited the TNF-α induced adhesiveness of HUVEC for HL-60 as well as ICAM-1 production in HUVEC suggest that these events may contribute to the anti-thrombosis activity of Tan *in vivo*.

P-selectin is an important adhesion molecule on platelets, mediating platelet-leukocyte binding *in vitro*[11]. In inflamation and thrombosis, P-selectin may mediate the interaction of leukocyte with platelets bound in the region of tissue injury and stimulated endothelium[12]. In the present study, we demonstrated that Tan significantly inhibited thrombin-induced P-selectin expression in platelets. This inhibitory effect is probably one of the Tan anti-thrombosis mechanisms..

In conclusion, the results showed that the anti-thrombosis of Tan was related to inhibited cell adhesion molecule expression in both HUVEC and platelets.

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丹参酮Ⅱ-A磺酸钠对内皮细胞和血小板体外表达粘附分子的作用[1]

姜开余[2,3], 阮长耿[3], 顾振纶[2,4]

([2]苏州医学院药理教研室, [3]江苏省血液研究所 苏州医学院血栓与止血研究室, 苏州 215007, 中国);

周文轩, 郭次仪 (香港保健协会, 香港, 中国)

关键词 血管内皮; 丹参酮Ⅱ-A; 血小板; 细胞粘附分子类; 细胞粘附; 脐静脉; HL-60 细胞; 流动血细胞计数; 凝血酶; P-选择素; 肿瘤坏死因子

目的: 探讨丹参酮Ⅱ-A 磺酸钠(Tan)对培养人脐静脉内皮细胞(HUVEC)和人血小板表达粘附分子

的影响. **方法**: 用流动血细胞计数仪测定肿瘤坏死因子(TNF-α)诱导人脐静脉内皮细胞 ICAM-1 和凝血酶诱导人血小板 P-选择素的表达. **结果**: HUVEC 经 TNF-α 处理后, 明显增加细胞表面 ICAM-1 的表达, 增加 HL-60 细胞粘附到内皮细胞表面达加入细胞总数的 30 % ± 6 % (对照组为 4.6 % ± 0.7 %). 在 TNF-α 处理前, 用 Tan (25 – 200 μmol·L^{-1}) 与 HUVEC 共孵育, 则 Tan 剂量依赖性地抑制 TNF-α 的作用. Tan (25 – 200 μmol·L^{-1}) 与人血小板孵育后, 可剂量依赖性地抑制凝血酶诱导人血小板表面 P-selectin 的表达. **结论**: Tan 可抑制内皮细胞和血小板表达粘附分子.

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Pharmacokinetics of recombinant human granulocyte macrophage colony-stimulating factor in *Macaca mulatta*

WANG Ren-Gang, ZHU Xing-Zu[1], SONG Wei (*Department of Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China*)

KEY WORDS recombinant granulocyte macrophage colony-stimulating factor; enzyme-linked immunosorbent assay; pharmacokinetics; *Macaca mulatta*

AIM: To examine the pharmacokinetics of iv and sc recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) in *Macaca mulatta*. **METHODS**: Plasma levels of rhGM-CSF were detected with sandwich enzyme-linked immunosorbent assay. **RESULTS**: Plasma concentration-time curves after iv rhGM-CSF in monkeys were best fitted with 3-compartment model. The 1st, 2nd, and 3rd phase $T_{\frac{1}{2}}$ were 0.05 – 0.07, 0.14 – 0.58, and 1.4 – 4.1 h. Cl and K_{10} were similar between different doses, respectively. C_{max} was 0.93 ± 0.16 μg·L^{-1}, T_{max} was 2.65 ± 0.14 h, and elimination $T_{\frac{1}{2}}$ was 2.5 ± 0.3 h after sc rhGM-CSF. The bioavailability after sc rhGM-CSF was 0.61. **CONCLUSION**: Pharmacokinetics of rhGM-CSF in *Macaca mulatta* provided a useful index for clinical trial.

Human granulocyte macrophage colony-stimulating factor (hGM-CSF) is one of the hematopoietic growth factors which control the proliferation and survival of myeloid cells [1]. Recombinant hGM-CSF (rhGM-CSF) has been developed for treatment of several hematopoietic disorders, and has shown promise in the treatment of myelodysplastic syndromes, as an adjunct to autologous bone marrow transplant, and the treatment of bone marrow suppression induced by high dose chemotherapy[2-4]. In China, rhGM-CSF has been developed into therapeutic use. The present study, as part of the preclinical studies of rhGM-CSF, was initiated to investigate the pharmacokinetics of iv and sc injections of rhGM-CSF.

[1] Correspondence to Prof ZHU Xing-Zu.
Phn 86-21-6431-1833, ext 309. Fax 86-21-6437-0269.
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MATERIALS AND METHODS

Chemicals Bacterial-derived rhGM-CSF (Lot No 95011, purity > 98 %, 300 μg/ampole, 6.67 × 10^{8} IU·μg^{-1}) was provided by Shanghai Huaxin Biological High Techniques Co Ltd, Shanghai, China. Standard GM-CSF was purchased from Schering-Plough. Monoclonal antibody against GM-CSF, biotinylated antibody against GM-CSF, and streptavidin conjugated horseradish peroxidase complex were purchased from GIF, Müster, Germany. 3,3,5,5-Tetramethylbenzidine (TMB), gelatin, and bovine serum albumin (BSA) were from Sigma. Ketamine hydrochloride (50 g·L^{-1}) was purchased from Shanghai Zhongxi Pharmaceutical Co Ltd, Shanghai, China.

Macaca mulatta *Macaca mulatta* (n = 9, ♂) weighing 4 – 5 kg were provided from Shanghai Institute of Physiology, Shanghai, China. Only monkeys that had not received rhGM-CSF previously and that did not demonstrate antibodies to rhGM-CSF were used for this study. Monkeys were randomly assigned to study groups

Pharmacokinetics of rhGM-CSF Monkeys were