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The anti-angiogenic effect of sinomenine

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Abstract

Sinomenine is an alkaloid extracted from the Chinese medicinal plant, *Sinomenium acutum*, which has been utilized to treat rheumatoid arthritis (RA) in China for over 2000 years. Sinomenine has been shown to mediate a wide range of pharmacological actions which includes anti-inflammatory and anti-rheumatic effects. RA has been classified as a chronic immune-mediated disease that exhibits overlapping manifestation of inflammatory, abnormal cellular and hormonal immune responses with synovial hyperplasia. Since, angiogenesis is recognized to play a critical role in the development of RA and anti-angiogenic therapy has been proposed as a new therapeutic strategy for treatment of RA, we would like to see if sinomenine possesses anti-angiogenic property. In this study, sinomenine inhibited bFGF-induced proliferation of human umbilical vein endothelial cells (HUVEC) and arrested its cell cycle in G1 phase. Sinomenine disrupted tube formation of HUVEC on Matrigel and suppressed the chemotaxis of HUVEC. In addition, sinomenine reduced neovascularization in Matrigel plug assay as well as microvascular outgrowth in rat aorta ring sprouting assay. These results suggest that sinomenine inhibited bFGF-induced angiogenesis *in vitro* and *in vivo*. As the leukocytes–endothelial adhesive interactions also play an important role in inflammation, we found that sinomenine reduced the transmigration of granulocytic differentiated HL60 cells across IL-1β activated HUVEC monolayer. Therefore, the inhibition of leukocytes migration across blood vessel walls and the anti-angiogenic effect of sinomenine may contribute towards its therapeutic mechanisms in alleviating the pathogenesis of RA.

Introduction

The Chinese medical plant *Sinomenum acutum* has been used for the treatment of various rheumatoid diseases for over 2000 years. Sinomenine(7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methylmorphinane-6-one) is the active principle of this herb. It has previously been demonstrated that sinomenine exhibited a wide range of pharmacological actions including anti-inflammatory [1], anti-rheumatic [2], immunosuppressive, analgesic [3, 4] and anti-arrhythmic effects. Sinomenine inhibits inflammatory reactions by suppressing the production of inflammatory mediators, such as prostaglandin E_2 (PGE_2), leukotriene C_4 (LTC_4) and nitric oxide (NO) from macrophages [1]. Furthermore, sinomenine has been shown to inhibit *in vitro* lymphocytes proliferation [5] and improve adjuvant arthritis (AA) and antigen-induced arthritis (AIA) in rats [2]. In clinical studies, the therapeutic efficacy of sinomenine was also confirmed in patients with rheumatoid arthritis (RA). Actually, RA is a chronic immune-mediated disease that exhibits overlapping manifestations of inflammation, abnormal cellular and humoral immune responses and synovial hyperplasia [8–12]. However, the primary cause of RA is still not known. About 80% of RA patients showed significant improvement in these disease states after the administration of sinomenine tablets for 3 months [6, 7].

Angiogenesis, the formation of new blood vessels [13], is recognized to play a critical role in RA pathogenesis [14]. Angiogenesis has been proposed to enhance the joint damage by facilitating the growth of synovial cells, the formation and maintenance of invasive synovial pannus, generating proteases from activated endothelial cells (EC) to degrade cartilage and promoting the chondrocytes invasion that leads to ossification and consequence in joint damage [15, 16]. Moreover, while the synovial tissue which is normally relatively poor in microvasculation become inflammatory, the release of huge amounts of inflammatory mediators such as cytokines from the injured tissues and inflammatory cells can trigger the cascade of angiogenic pathway and sequentially facilitate the

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pathogenesis of RA. However, angiogenesis under normal physiological conditions would not cause pathological disorders; conversely, it is essential and critical in many physiological processes such as female reproductive cycle, embryogenesis, growth and wound healing [17]. This multi-step process occurs under tight regulation through the balance of angiogenic inhibitors and stimulators [18–20]. As the importance of angiogenesis has been focused recently, anti-angiogenic therapy is proposed as a new therapeutic strategy for the treatment of 'angiogenic diseases' [21, 22]. In the case of RA, histological studies found that pannus is a highly vascular structure that significantly modulates the pathological processes of RA [23]; By inhibiting neovascularization, the formation of endothelial lining, the primary targets for those RA-mediators, such as cytokines, permeability factors and matrix-degrading enzymes will be eliminated [8]. Thus the development of pannus will be attenuated, and results in a therapeutic benefit.

Endothelial cells play an important role in leukocytes trafficking in the development of chronic inflammation in RA. They adhere and recruit circulating leukocytes from the blood stream. Previous study indicated that inflammatory cytokines, such as IL-1β and TNF-α, stimulated expression of endothelial adhesion molecules E-Selectin, ICAM-1 and VCAM-1 and promoted trans-endothelial migration of leukocytes towards inflammatory sites [24, 25].

In the present study, we investigated the potential anti-angiogenic activities of sinomenine using different bioassays. The effect of sinomenine on trans-endothelial migration of HL60 was also investigated. We demonstrated that sinomenine is a new type of anti-angiogenic agent and that its therapeutic prospects towards RA may be partly contributed by it anti-angiogenic activity.

Materials and methods

Materials

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (TCS Biologicals, Buckingham, UK). Cell culture flasks and tissue culture inserts were purchased from NUNC. RPMI medium, phosphate-buffered saline (PBS), fetal calf serum (FCS), fetal bovine serum (FBS), serum-free endothelial growth medium and trypsin-EDTA were supplied by Invitrogen. Penicillin – streptomycin, EC growth supplement (ECGS), heparin, amphotericin-B, M199 medium, dimethyl sulphoxide (DSMO, TC grade), gelatin, MTT powder, Drakin's reagent, basic fibroblast growth factor (bFGF), RNase, propidium iodide and Triton X-100 were obtained from Sigma. Growth factor reduced-Matrigel was obtained from Becton Dickinson Bioscience and the radioactive [^{3}H]-thymidine was purchased from Amersham Pharmacia and glass fiber filters were obtained from Whatman. IL-1β was obtained from Roche. The Hematoxylin and Eosin stain were purchased from Merck.

Chemicals

Sinomenine was kindly provided by Shanxo Institute of Pharmacological Industry, Xian, China and the purity of the drug was confirmed by reverse phase-HPLC. A stock solution of sinomenine (50 mM) was freshly prepared in 1% DMSO in PBS.

Animals

Female Balb/c mice and Sprague–Dawley (SD) rats (6–8 weeks old) were provided by the Laboratory Animal Service Centre of the Chinese University of Hong Kong. Balb/c mice and SD rats were housed in a temperature and humidity controlled animal unit with 12/12 light-dark cycle, and free access to purified water (National PJ-24 water purifier) and food (Laboratory Rodent Diet No. 5001, PMT Feed, St. Louis, USA). Animals were acclimated to this environment for at least 2 days prior to the start of experiments.

Cell culture

HUVEC were cultured in M199 medium supplemented with 30 μg/ml ECGS, 20% heat-inactivated FCS, 0.1% (v/v) penicillin – streptomycin, 50 ng/ml amphotericin-B and 90 μg/ml heparin on 0.1% gelatin-coated culture flasks. HUVEC were grown at 37 °C in humidified air with 5% CO_2. Cells were cultured on 0.1% gelatin-coated 75 cm^2 culture flasks and passed for a week with change of medium every 2 days. Between each passage, cells were washed twice with PBS and then harvested with 0.05% trypsin containing 0.02% EDTA. Cells were centrifuged at $350 \times g$ for 5 min and the cells were resuspended in medium and for seeding to a new gelatin-coated flask. All experiments were conducted with HUVEC from passages 2–6.

Briefly, HL60 cell is a maturation-arrested promyelocytic cell line. The cells were cultured on 25 cm^2 culture flasks and maintained in RPMI supplemented with 10% heat-inactivated FBS, penicillin (50 U/ml) streptomycin (50 μg/ml) and neomycin (10 μg/ml). The cells were kept at 37°C incubator in a humidified atmosphere containing 5% CO_2. Between each passage, the cells (1:8, v:v) were suspended in fresh medium and passed to a new flask.

Meta-tetrazolium reduction MTT assay

HUVEC (2×10^4 cells/well) were seeded in gelatin-coated 96-well plates and incubated in M199 medium supplemented with 20% FCS for 24 h. Cells were then incubated with different concentrations of

sinomenine (31.25–1000 μM) for 20 h. After treatment, cells were washed twice with PBS and 200 μl of M199 medium containing 20% FCS and 10% (v/v) MTT reagent (5 mg/ml) was added to the cells. After incubation for another 4 h, cells were lysed and the reduced intracellular formazan product was dissolved by replacing 150 μl of culture medium with the same volume of DMSO. The absorbances at 570 and 690 nm were measured with a microplate reader. Results were expressed as the percentage of cytotoxicity.

$$\text{Percentage of cytotoxicity} = (\text{OD}_{\text{control group}} - \text{OD}_{\text{treatment group}}/\text{OD}_{\text{control group}}) \times 100\% (\text{OD} = \text{Opticaldensity}_{570\text{nm}} - \text{Opticaldensity}_{690\text{nm}})$$

[^{3}H]-thymidine incorporation assay

HUVEC (2×10^4 cells/well) were seeded in gelatin-coated 96-well plates and incubated with various concentrations of sinomenine (6.25–1000 μM) for 48 h. After incubation for 24 h, [^{3}H]-thymidine (0.5 μCi) was added to each well and the mixtures were incubated for another 24 h. Cells were then harvested onto glass fiber filters using a cell harvester. The incorporated [^{3}H]-thymidine was determined by liquid scintillation counting (Beckman). Results were expressed as the percentage of inhibition. IC_{50} (drug concentration cause 50% inhibition) was calculated based on the dose–response curve.

$$\text{Percentage of growth inhibition} = (\text{CPM}_{\text{control group}} - \text{CPM}_{\text{treatment group}}/\text{CPM}_{\text{control group}}) \times 100\%$$

Cell cycle analysis

Flow cytometry was used to analyze the cell cycle progression of sinomenine-treated HUVEC. Briefly, a total of 2×10^6 normal and sinomenine treated-HUVEC were harvested and fixed in 70% ethanol (4 °C) overnight. The fixed cells were washed with cold PBS (with Ca^{2+} and Mg^{2+}) twice, and then incubated for 30 min with RNase (1 mg/ml) and propidium iodide (40 μg/ml) in Triton X-100 (0.1%). The stained cells were analyzed using the FACScan Flow Cytometer (Becton Dickinson) with argon-ion laser (488 nm). Fluorescent signal was detected through FL2-H channel and the proportion of DNA in G1, S and G2/M phases was analyzed with the ModfitLT Version 3.0 software.

Chemotaxis assay

The chemotatic mobility of HUVEC was assessed using the 10-mm tissue culture insert (transwell) with polycarbonate membrane (8 μm pores) and 24-well plate. The lower side of the membrane was coated with 0.1% (v/v) gelatin for an hours before experiment. Then HUVEC (2×104 cells/well) were resuspended in medium (500 μl) containing 150 or 300 μM of sinomenine and seeded in the culture inserts. Then the inserts were immersed into 24-well plate containing equal volume of medium supplemented with bFGF (150 ng/ml). After 5 h, the non-migrated cells (inside the inserts) were swapped with cotton buds and the migrated cells on the opposite side of the membrane were stained with haematoxylin and eosin. The migrated cells were captured with an image analyzer (Zeiss) and counted with the software Metamorph.

In vitro *tube formation assay*

HUVEC were seeded at a density of 8×10^4 cells/well on the Matrigel-coated 24-well culture plates and incubated with 150 μM or 1 mM of sinomenine, the overview of the network-like structures were captured using inverted microscope (50×) with camera attachment at 18 h. The anti-angiogenic effect of sinomenine was averaged from 10 images (per each trial) by counting the branch points of the formed tube.

In vivo *Matrigel plug assay*

Growth factor-reduced Matrigel (500 μl) containing 150 ng/ml bFGF and 24 U heparin were co-injected subcutaneously with or without sinomenine (final concentration, 150 μM) into the right and left abdomen of Balb/c mice respectively. On day 5, the mice were sacrificed and the plugs were removed and fixed in 10% formalin in PBS. To quantitate the formation of neovascularization, the amount of hemoglobin was measured using the Drakin's reagent [26].

Ex vivo *aortic ring sprouting assay*

Aortas were obtained from 6-week old SD rats. The aortas were immediately transferred to a culture dish with serum-free medium. The fibroadipose tissue around the aortas was carefully removed by gentle scraping and the aortas were sliced into one-mm long aortic ring fragments. After three consecutive washes in serum-free medium, the aortic rings were placed in wells of 96-well plate that were pre-coated with 40 μl of growth factor reduced-Matrigel. Additional Matrigel (40 μl) was added onto the aortic rings and allowed to solidify. Serum-free endothelial growth medium (200 μl) supplemented with 200 μg/ml ECGS and sinomenine were added to the wells. Medium alone and medium containing ECGS (200 μg/ml) served as the medium and positive controls respectively. Microvascular outgrowths were distinguished from the fibroblasts based on their unique morphology (greater thickness, uniformly cohesive

pattern of growth and dichotomous branching of one sprout generated two new sprouts). The numbers of microvascular outgrowths were counted on day 4 under an inverted light microscope (Nikon TMS).

In vitro *trans-endothelial migration assay*

The differentiation of HL60 from monocytes to macrophages was performed in 0.5% (v:v) DMSO (TC grade, Sigma) for 4 days before the experiment. The trans-endothelial migration of leukocytes was determined using the 10-mm tissue culture insert with polycarbonate membrane (8 μm pores) and 24-wells plate, the upper side of the membrane was coated with 150 μl Matrigel, diluted with 1:30 in water and incubated at 37 °C for 24 h. HUVEC (5×10^5 /0.5 ml/well) were incubated with sinomenine for 24 h. The adhesion molecules of HUVEC were induced with IL-1β (5 ng/ml) for 4 h. Differentiated HL60 cells were fluorescent labeled (5 μM CFSE, 37 °C, for 15 min) and incubated in tissue culture inserts for 2 h. The fluorescent signal of the migrated cells was measured with the BMG Flurostar Galaxy microplate reader. (Ex wavelength: 492 nm, Em wavelength: 517 nm).

Statistics

Results are expressed as mean ± SEM and Student's *t*-tests were performed when appropriate. For Matrigel plug assay, the data were analyzed for statistical significance by Wilcoxon signed rank test.

Results

Inhibition of bFGF-induced HUVEC proliferation

The growth inhibition effect of sinomenine towards bFGF-induced HUVEC was evaluated by [^{3}H]-thymidine incorporation assay. At a concentration ranging from 125 to 1000 μM, sinomenine inhibited HUVEC proliferation at high dosage (≥250 μM) (Figure 1a). Besides, the growth inhibition effect of sinomenine on HUVEC is not due to its cytotoxicity as indicated by the MTT assay (Figure 1b).

Sinomenine arrests the cell cycle progression of HUVEC in G1 phase

To ask whether the inhibition of HUVEC proliferation is due to the arrest of cell cycle by sinomenine, HUVEC was incubated with sinomenine (1 mM) for 48 h followed by flow cytometric analysis. As shown in Figure 2c, 65% of HUVEC was found to accumulate in G1 phase, whereas the number of cells in S phase decreased significantly to 9%. The result indicated that sinomenine at high concentration inhibited the proliferation of EC and the cell cycle progression was arrested in the G1 phase.

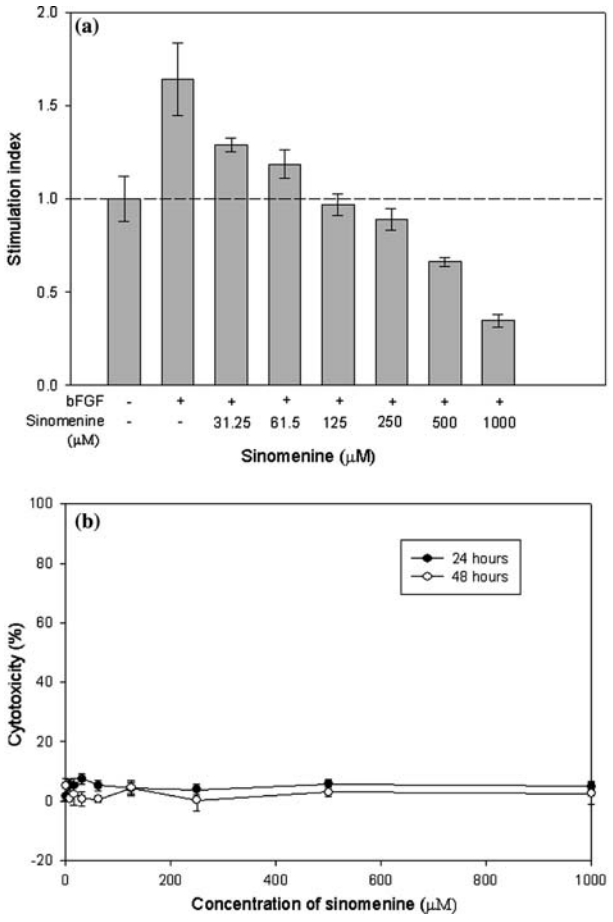

Figure 1. The inhibitory effect of sinomenine on bFGF-induced HUVEC proliferation. HUVEC seeded at a density of 2×10^4 cells/well in 96-well plate were incubated with bFGF (25 ng/ml, positive control) in the presence of sinomenine (6.25–1000 μM) with 1% FBS for 48 h. Then the anti-proliferative effect of sinomenine was determined by 3H-thymidine incorporation assay (a) Result was expressed as mean with standard error of mean of triplicates. The cytotoxicity on HUVEC was determined by MTT assay (b) Result was expressed as mean with standard error of mean of triplicates. Sinomenine is not toxic to HUVEC even at a concentration of 1 mM as determined by MTT assay.

Sinomenine suppressed bFGF-induced chemotaxis

The chemotatic mobility of HUVEC under the influence of sinomenine was demonstrated using the Transwell culture insert. Results showed that cell migration toward the chemo-attracting agent, bFGF, was suppressed by sinomenine in a dose-dependent manner. In Figure 3, sinomenine at the concentration of 300 μM reduced HUVEC migration toward bFGF by about 50% ($^*P < 0.05$) when compared with the positive control.

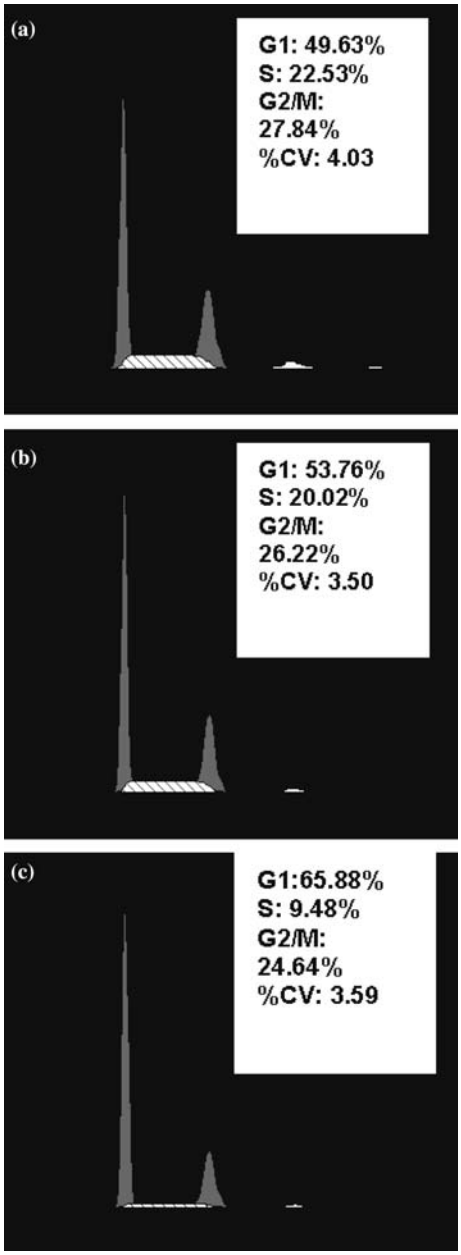

Figure 2. Flow cytometric analysis of sinomenine-treated HUVEC. HUVEC (2×10^6 cells/well) were seeded in 6-well plate and incubated with medium alone (a) 150 μM sinomenine in medium (b) and 1 mM sinomenine in medium (c) for 48 h. The proportion of DNA in G1, S and G2/M phases was determined by flow cytometry.

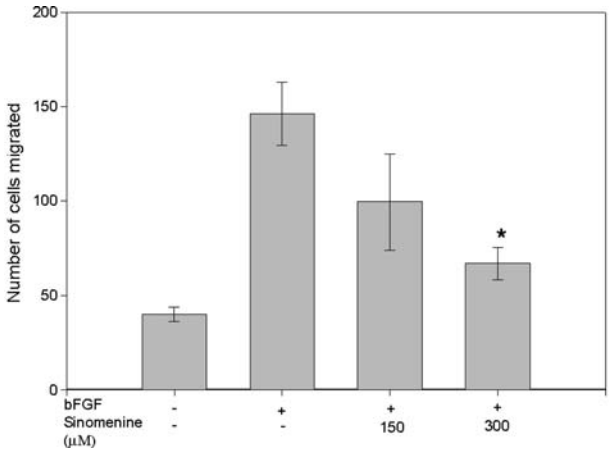

Figure 3. Effect of sinomenine on bFGF-induced chemotaxis. HUVEC (2×10^4 cells/well) were seeded in the tissue culture inserts containing culture medium alone, or different concentrations of sinomenine (150 and 300 μM). For each insert, the lower side of the membranes (8 μM pore size) were pre-coated with 0.1% gelatin. Then the inserts were immersed into 24-well plate containing either medium alone (medium control) or 150 ng bFGF. Under the chemo-attraction of bFGF, HUVEC migrated to the lower side of the membrane. The migrated cells were stained with Haematoxylin and Eosin and captured with image analyzer and counted. The result showed that sinomenine (300 μM) inhibited the migration of HUVEC towards the bFGF (*$P < 0.05$ by Student's *t*-test). Result was expressed as mean with standard error of mean of triplicates.

Sinomenine reduces tube formation of HUVEC on Matrigel

The three-dimensional (3D) tube formation assay of HUVEC that mimics the angiogenic process was used to demonstrate the anti-angiogenic activities of sinomenine. After plating HUVEC onto Matrigel, the cells quickly attached to the matrix and morphologically differentiated to a capillary-like network in about 8 h. When compared with the medium control, sinomenine-treated HUVEC formed relatively incomplete and narrow tube-like structures (Figures 4a–c). The anti-angiogenic activities of sinomenine on tube formation were quantitated by counting the number of branch points. In the presence of 150 μM and 1 mM sinomenine, the extent of tubular formation of HUVEC was found to reduce significantly and in a dose-dependent manner (*$P < 0.05$) (Figure 4d).

Sinomenine inhibits angiogenesis in vivo

Sinomenine was tested for its anti-angiogenic activity by subcutaneous injection of Matrigel containing bFGF (150 ng/ml) and heparin (24 U) with or without sinomenine (150 μM) into the right and left abdomen of Balb/c mice respectively. After 5 days, the Matrigel plugs were removed and the effect of sinomenine on *in vivo* angiogenesis was examined. Plugs containing bFGF and heparin were red in color indicating that occurrence of angiogenesis (Figure 5a). In the presence of sinomenine, plugs showed a clear and pale yellow appearance indicating the absence of angiogenesis (Figure 5a). Furthermore, the extending of vessel formation in the Matrigel plug was quantified by measuring the hemoglobin content. The hemoglobin content in the sinomenine containing Matrigel plug was lower in comparison to bFGF alone, the result indicating that sinomenine inhibited the neovascularization in Matrigel plug assay (Figure 5b).

Sinomenine inhibits endothelial sprouts formation

In the aortic ring sprouting assay, endothelial sprouts developed from the aortic ring segments starting on

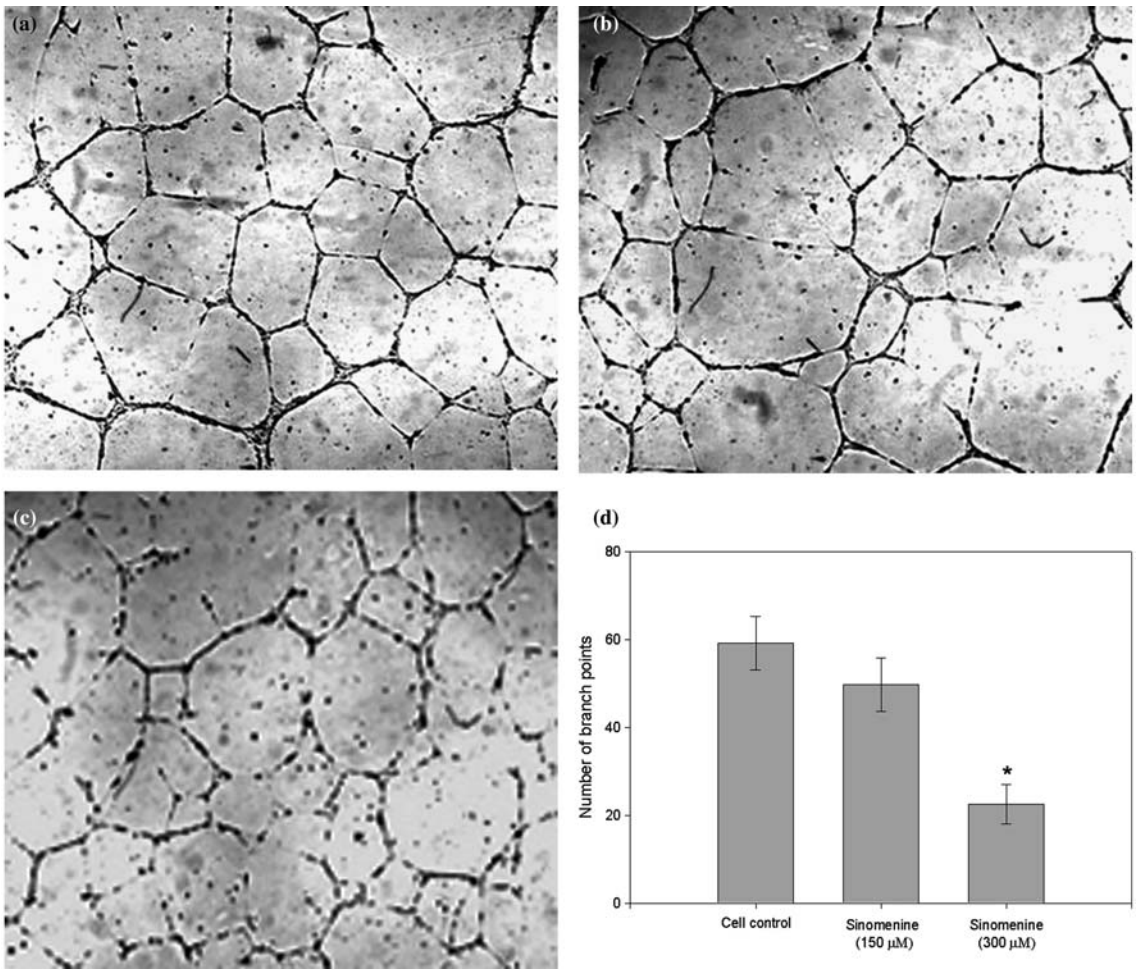

Figure 4. The effect of sinomenine on tube formation of HUVEC. HUVEC were pre-treated with culture medium (a), 150 μM sinomenine (b), 1 mM sinomenine (c) for 24 h. Then the pre-treated cells were plated on the Matrigel coated 24-wells plate at a density of 8×10^4 cells/well. After 18 h incubation, different extents of capillaries-network were formed on the Matrigel surface by HUVEC and the number of branch points was counted for each treatment (d). Result was expressed as mean with standard error of mean (*$P < 0.05$ by Student's *t*-test).

day 3 and drastically increased in number from day 4 to day 6. The sprouts branched extensively and matured into network of microvessel both in the medium control and positive control experiments (200 μg/ml ECGS) (Figures 6a and b). At different concentrations of sinomenine (125, 250 and 500 μM) (Figures 6c–e), the growth of endothelial sprouts was inhibited by 50.8%, 56.2% and 68.8% respectively (*$P < 0.005$) (Figure 6f). This *ex vivo* assay further demonstrated the anti-angiogenic effect of sinomenine.

Sinomenine inhibited trans-endothelial migration of HL60 cells

The anti-inflammatory effect of sinomenine on leukocytes recruitment was investigated with *in vitro* trans-endothelial migration assay. When compared with the result between cytokine-stimulated and unstimulated HUVEC, data from Figure 7 indicated that HL60 cells exhibited a significantly higher level of transmigration across activated-HUVEC, the migration index increased up to 2.26 as compared with unstimulated EC. Furthermore, pre-incubation of HUVEC with sinomenine (100 μM) for 24 h significantly reduced the migration index from 2.26 to 1.87 (*$P < 0.05$ by Student's *t*-test).

Discussion

In this study, we demonstrated for the first time that sinomenine, an anti-rheumatic drug, possesses anti-angiogenic activity in several *in vitro* and *in vivo* assay system. *Sinomenum acutum* has been widely used in China for treatment of rheumatoid related diseases for over 2000 years. Sinomenine is an active compound extracted from this herb. Although sinomenine exhibits a wide range of pharmacological activities such as anti-inflammatory, anti-rheumatic, immunosuppressive, analgesic and anti-arrhythmic, mechanistic study on sinomenine is very limited. Our previous study demonstrated that sinomenine could reduce the production of PGE_2, LTC_4 and NO from macrophages [1] which provided a basis for its anti-inflammatory

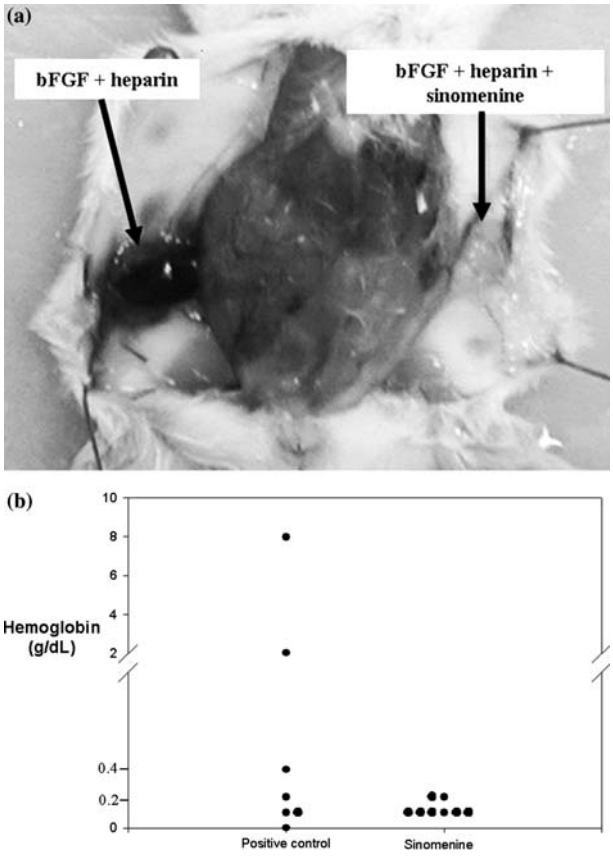

Figure 5. The anti-angiogenic activity of sinomenine in mouse Matrigel plug assay. Sinomenine (150 μM) in the presence or absence of bFGF (150 ng/ml) and heparin (24 U) were mixed with 400 μl growth-factor reduced Matrigel and injected subcutaneously into the right and left ventral abdominal to form a 'plug' (arrows). On day 5, the mice were sacrificed and the plug were examined (a). Matrigel plugs supplemented with bFGF and heparin showed a red color appearance. In the presence of sinomenine, clear and pale yellow Matrigel plug were found. The degree of angiogenesis was determined by measuring the hemoglobin contents of the Matrigel plugs (b) (*$P < 0.05$, compared with control by Wilcoxon signed rank test with small sample size).

activities. As the development of RA is related to the formation of new blood vessels – angiogenesis. It is envisaged that sinomenine may exert certain anti-angiogenic activities.

Our result indicated that sinomenine exhibited no cytotoxic effect on resting HUVEC even at high concentration (1mM). However, a significant inhibition on the proliferation of bFGF-induced HUVEC was observed and the cells were arrested in G1 phase by the action of sinomenine in a dose-dependent manner. Under normal physiological condition, EC are usually in a quiescent state, but can be activated by angiogenic factors, such as bFGF and VEGF [27]. Since endothelial proliferation is essential for sustained angiogenesis, the inhibition of activated EC clearly indicates the anti-angiogenic potential of sinomenine. As a result, the anti-angiogenic activities of sinomenine were further demonstrated by three different functional assays. The tube formation of EC on the Matrigel depends on migration and morphological differentiation which mimics the process of angiogenesis [28]. The incomplete and relatively narrow network-like structures formed by the sinomenine-treated HUVEC indicated that such *in vitro* behaviors of HUVEC would be altered by sinomenine. As the process of tube formation is highly relied on cell–cell adhesion and dissociation, there is a possibility that those adhesion molecules on HUVEC such as (ICAM-1 or E-selectin) would be modulated by sinomenine at the gene expression level. Besides, the effect of sinomenine on EC migration was also implicated by the chemotaxis assay. The chemotatic mobility of EC toward chemo-attracting agent – bFGF significantly reduced in the presence of sinomenine.

Similar effect of sinomenine was also observed in the *in vivo* Matrigel plug assay and rat aortic ring sprouting assay. The formation of neovessels in the Matrigel plugs was inhibited when sinomenine was co-injected with bFGF and heparin. Both bFGF and heparin can induce EC invasion and results in neovascularizaton [29]; histological studies showed that functional blood vessels were formed in these Matrigel plugs. The greatly reduced amount of hemoglobin in the sinomenine containing Matrigel plugs further confirmed its *in vivo* anti-angiogenic activities. Besides, the endothelial sprouting assay of rat aortic segments which involves binding of anastomosing microvessels develop from EC of aorta intima was tested [30, 31]. Results indicated that sinomenine can inhibit angiogenesis *ex vivo* as indicated by the reduced microvessels formation in the rat aorta ring sprouting assay.

It is well known that most primary EC used for investigation of the endothelial/leukocytes interactions in inflammatory processes differ in their response to cytokines. EC derived from umbilical vein was shown to induce the expression of adhesion molecules (VCAM-1 and E-selectin) in response to TNF that is essential in trans-endothelial migration of leukocytes. Differentiated HL60 cells exhibited a high level of transmigration across activated HUVEC when compared with the unstimulated EC. After incubation of HUVEC with sinomenine (100 μM) for 24 h, the percentage of migrated cells reduced significantly.

Trans-endothelial migration involved the induction of adhesion molecules, including, PECAM-1, VE-cadherin, integrins, and E-selectin for leukocyte recruitment [32], the use of antibodies against ICAM, VE-cadherin and E-selectin reduced the trans-endothelial migration [33]. E-selectin also involved in the early phase of endothelial–leukocytes interaction from rolling to arrest stage [34] which was mediated by the phosphorylation of E-selectin [35]. It is speculated that sinomenine inhibited the trans-endothelial migration of leukocytes through the down-regulation of adhesion molecules, but it should be confirmed in the future study. In addition, trans-endothelial migration is also important for leukocytes–endothelial interaction in leukocytes adhesion, recruitment and migration toward inflammatory site, the process is essential for the

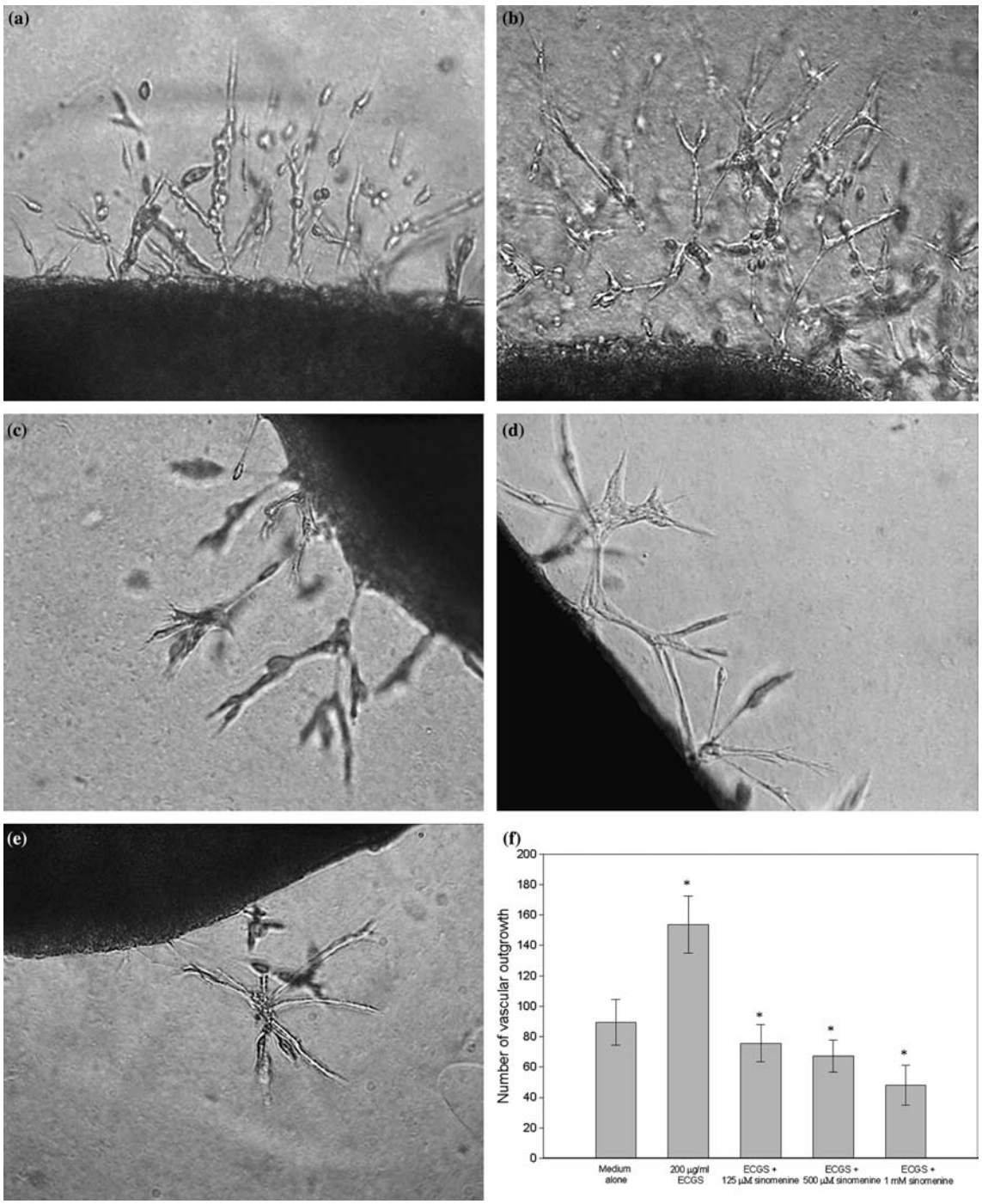

Figure 6. The inhibitory effect of sinomenine on *Ex vivo* aortic ring sprouting assay. Rat aorta was collected from 6-week-old SD rat, sectioned into 1 mm segments and embedded into Matrigel in 96-well plate. Aortic fragments were incubated with medium alone (a), culture medium supplemented with ECGS (200 μg/μl) (b), ECGS and sinomenine 125 (c), 250 (d), and 500 μM (e) respectively. Endothelial sprouts were observed to emerge from the aortic segments starting on day 3 and increased significantly afterwards. On day 4, endothelial sprouts were captured (a–e, 100×) and the number of vascular sprouts were determined (f). Experiments were performed at least in duplicates and repeated three times. (*$P < 0.05$ by Student's *t*-test as compared with the positive control).

development of inflammatory diseases [36]. Moreover, the increase of capillaries further enhances the recruitment and activation of leukocytes through the expression of adhesion molecules and angiogenic mediators by EC. Thus sinomenine may suppress inflammation through the suppression of leukocytes migration to the inflamed sites.

Taking all those data together, we found that sinomenine exerts the anti-angiogenic effect possibly through the inhibition or interruption of EC migration, proliferation, invasion and also morphological differentiation. These novel anti-angiogenic activities of sinomenine suggested that its pharmacological effects on treating RA might not be restricted to

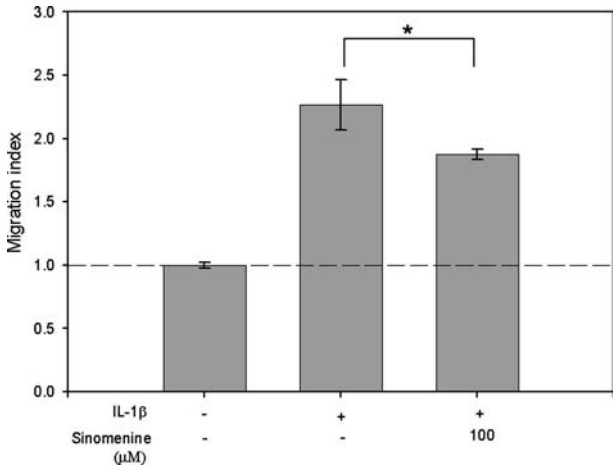

Figure 7. The trans-endothelial migration of differentiated HL60 cells across sinomenine-treated HUVEC. Matrigel (1:30 with water, 150 μl per well) was coated onto the tissue culture insert for 24 h. On the next day, HUVEC (1×10^6/0.5 ml/well) was incubated with or without sinomenine for 24 h, and the adhesion molecules of HUVEC was induced by IL-1β (5 ng/ml) for further 4 h. The fluorescent stained HL60 (5 μM CFSE, 37°C, for 15 min) was added into the tissue culture inserts and fluorescent signal of the migrated cells was measured after 2 h. Result was expressed as mean with standard deviation of duplicates (*$P < 0.05$ by Student's *t*-test).

anti-inflammation, in fact, the inhibition of angiogenesis might also be involved.

Our understanding of the anti-angiogenic role of sinomenine raises the exciting possibility of a novel therapeutic strategy for the treatment of RA [37]. Together with pathological study, the interruption of angiogenesis would not only minimize the nutritional supply to the inflammatory area [38] (e.g. pannus and synovial membrane) but also result in the regression of blood vessel and thus a new way to regulate the pathogenesis of RA [39, 40].

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