Inhibitory Effects of Tetrandrine on Angiogenesis in Adjuvant-Induced Chronic Inflammation and Tube Formation of Vascular Endothelial Cells

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The inhibitory effects of tetrandrine (an alkaloid isolated from the Chinese medicine Stepheilia tetrandrae S. Moore) were investigated in terms of the angiogenesis in an adjuvant-induced chronic inflammation model of mouse and tube formation of rat vascular endothelial cells (EC). Tetrandrine (7.5−30 mg/kg) reduced the carmine content, granuloma weight, inflammatory cell count and pouch fluid weight in the inflammation model in a dose−dependent manner. The inhibitory pattern of tetrandrine on these parameters was similar to that of hydrocortisone. The inhibitory effect of tetrandrine on carmine content was 0.56−fold smaller than that of hydrocortisone. Tetrandrine (0.1−10 μM) also inhibited 2% fetal bovine serum (FBS)−stimulated tube formation of vasucal EC. The inhibitory effect of tetrandrine on tube formation was more than 100−fold greater than that of hydrocortisone. Tetrandrine (10−30 mM) inhibited the tube formation stimulated by interleukin (IL)−1α and platelet−derived growth factor (PDGF)−BB to a greater extent than FBS−stimulated tube formation. The inhibitory effects of tetrandrine on the action of IL−1α and PDGF−BB were non−competitive. These results demonstrate that tetrandrine may reduce the tube formation of EC in the angiogenic process through inhibition on the post−receptor pathway of IL−1α and PDGF−BB in chronic inflammation.

Key words tetrandrine; anti−angiogenesis; anti−endothelial cell tube formation; adjuvant−induced mouse chronic inflammation model

Tetrandrine, a principal alkaloid isolated from the Chinese medicine Stepheilia tetrandrae S. Moore (root), has been used in China for several decades for the treatment of silicosis and arthritis. The alkaloid has been shown to be effective in animal models of experimental allergic encephalitis (multiple sclerosis),1 airway microvascular leakage (asthma),2 subcutaneous airpouch inflammation3 and adjuvant−induced arthritis.4 We have reported a quantitative method for determining granuloma angiogenesis in adjuvant−induced chronic inflammation in mice.5−7 The method is useful for the simultaneous measurement of angiogenesis, granuloma formation, infiltration of inflammatory cells and fluid exudation in chronic inflammation. The present study focused on the anti−angiogenesis of tetrandrine because little is known about its inhibitory effects on chronic inflammation.

Many sites of action of drugs are involved in angiogenic events, which include the production and release of inflammatory mediators, degradation of vascular basement membranes, migration and proliferation of vascular endothelial cells (EC) and the organization of capillaries, including tube formation of EC.8 Tetrandrine has been reported to inhibit the production and release of a broad range of inflammatory mediators such as histamine,9 prostaglandins, leukotrienes10 and platelet−activating factor,11 as well as cytokines such as tumor necrosis factor12 and interleukin (IL)−1.13 We have reported an assay model for the tube formation of EC in angiogenesis by culturing rat vascular EC in type I collagen gel with fetal bovine serum (FBS).14 Interferon−γ (IFN−γ) activates macrophages to release IL−1α to a greater extent than platelet−derived growth factor (PDGF)−BB for enhancing the tube formation of vascular EC.15 These results suggest that IL−1α plays a crucial role in the angiogenesis of chronic inflammation.

The aim of the present study was to determine the inhibitory effects of tetrandrine on granuloma angiogenesis in the chronic inflammation model and on the tube formation of vascular EC. The inhibitory effect of tetrandrine was further compared on IL−1α−induced and PDGF−BB−induced tube formation.

MATERIALS AND METHODS

Animals Male ddY mice (5−6 weeks of age) and male Wistar rats (9−10 weeks of age) were purchased from Japan Shizuoka Laboratory Center (Hamamatsu). These animals were maintained under a constant temperature (23±1°C) and humidity (55±5%), with lights on from 8 a.m. to 6 p.m., and were fed the usual laboratory diet (CA−1, Clea Japan, Tokyo) and tap water freely.

Adjuvant−Induced Pouch Granuloma Angiogenesis Air pouch granuloma of male ddY mice was prepared by the injection of Freund’s complete adjuvant (FCA) with 0.1% croton oil, as reported.5−7 Three ml of air was injected subcutaneously into the dorsum of the ddY mouse under ether anesthesia to produce a regular oval air pouch. The FCA emulsion was prepared using 2 mg heat−killed M. tuberculosis (from Professor I. Azuma, Hokkaido University, Hokkaido) per ml of Freund’s incomplete adjuvant, which was prepared from a mixture of 7.5% Arlacel−A, 42.5% paraffin liquid (Nacalai Tezuke, Kyoto) and 50% saline. The FCA emulsion (0.5 ml) containing 0.1% croton oil (Nacalai Tezuke) was injected into the air pouch under ether anesthesia. Mice were killed by the injection of 1 ml of 10% carmine solution (Merek, Darmstadt, Germany) containing 5% gelatin (Nacalai Tezuke), which was kept warm at 40°C, into the tail vein on day 5 after FCA injection. The dead mice were cooled at below 4°C for several hours. The granuloma tissues were excised, separated from the surrounding loose con-
nective tissue and weighed. All the exudative pouch fluid was harvested and weighed. The inflammatory cells in the pouch were counted with a hemocytometer. The carmine content of granuloma tissues was measured as follows: The granuloma tissues were cut, solubilized with 3 N NaOH and acidified with 36% HCl. After centrifugation, the supernatant was filtered. The carmine content in the supernatant was determined by measuring the optical density at 490 nm. The carmine content is an index of newly formed blood vessels in pouch granuloma.5-7

**Tube Formation by Cultured Vascular Endothelial Cells** Cultured EC in the thoracic aorta of male Wistar rats were prepared as reported.7,14,16 The EC were cloned from primary cells in Dulbecco’s modified Eagle medium (DMEM, Nissui, Tokyo) supplemented with 10% heat-inactivated FBS (Bioproduct, Walkersville, MD, U.S.A.), 160 μM benzyl penicillin potassium (Banyu Seiyaku, Tokyo) and 0.1 mg/ml streptomycin sulfate (Meiji Seika, Tokyo) on a 0.03% type I collagen (I-AC, Kokken, Tokyo)-coated 16 mm dish (Corning, Corning, NY, U.S.A.). The EC (the 5th-14th passages) were cultured 2-11 weeks after confluence in 10% FBS-DMEM without antibiotics under 5% CO₂ and 95% air. The post confluent cells were washed with Ca²⁺, Mg²⁺-free phosphate-buffered saline (PBS) and 0.02% EDTA in PBS, detached by 0.25% trypsin (Difco, Detroit, MI, U.S.A.)-0.02% EDTA in PBS, and harvested in 10% FBS-DMEM without antibiotics. The EC [(2.6±0.1)×10⁶ well] were cultured in 10% FBS-DMEM (0.5 ml) at 37°C for 20 to 24 h on a collagen gel that was prepared by solidifying 0.3 ml of 1.5% type I collagen solution in a 16 mm dish. The EC-cultured medium was aspirated, then the same volume of collagen solution was overlaid and solidified. The EC were cultured with 1% or 2% FBS-DMEM for 4 d, and the medium was changed every other day.

**Measurement of Tube Formation** Tubes that developed from EC were photographed with a Leitz Diavert camera equipped with a Wild photomaton MPM45 (Leitz, Germany). Four fields which were selected randomly from each 16 mm dish were photographed at ×36 magnification on day 4 after overlaying the collagen gel. Typical photographs of the tubes were presented in an earlier paper.15 These experiments were repeated at least three times. The lengths of all tubes on a photograph (×36) were measured and tabulated with Graphic Software MEAS I (Graphtec Corp., Tokyo) to provide the total tubular length, which is an index of tube formation.14

**Agents** Tetradrine was a gift from retired Prof. Zhang S.-Y. in Beijing Medical University, China. Tetradrine and hydrocortisone acetate (Nacalai Tesque) were suspended homogeneously in saline containing 1% Avicel (Asahi Chemical Industry, Tokyo), and injected intraperitoneally into mouse 2 h after FCA injection, and then into mouse once a day for 4 d. For the experiments involving the tube formation of EC, tetradrine and hydrocortisone acetate were dissolved in ethanol, and diluted more than 1000-fold with 1 or 2% FBS-DMEM (a final concentration of less than 0.1% ethanol). These drugs were administered into the medium of EC cultured in type I collagen gel in the presence or absence of various concentrations of recombinant mouse IL-1α (Genzyme, Cambridge, MA, U.S.A.) or recombinant human PDGF-BB homodimer (Amersham Japan, Tokyo).

**Statistical Analysis** Significant differences in the data were evaluated by a one-way analysis of variance followed by the multiple range tests of Scheffe and Tukey at p=0.05 or 0.01, respectively.

**RESULTS**

**Anti-angiogenic Effects of Tetradrine in Adjuvant-Induced Chronic Inflammation** The inhibitory effect of tetradrine on granuloma angiogenesis was compared with that of hydrocortisone in adjuvant-induced chronic inflammation. Tetradrine (7.5—30 mg/kg) and hydrocortisone (3.8—15 mg/kg) both inhibited the carmine content in granuloma tissues in a dose-dependent manner (Fig. 1). The value of the 50% inhibitory dose (ID₅₀; 95% confidence limit) of tetradrine was 13.1 mg/kg (9.9—17.4). The anti-angiogenic effect of tetradrine was 0.56-fold weaker than that of hydrocortisone. Tetradrine inhibited granuloma weight, inflammatory cell count and pouch fluid weight as well as carmine content. The inhibitory effects of tetradrine on granuloma weight and inflammatory cell count were also weaker than those of hydrocortisone. The effect of tetradrine on the pouch fluid weight was similar to that of hydrocortisone. These results demonstrate that the inhibitory pattern of tetradrine on these inflammatory parameters was similar to that of hydrocortisone.

**Inhibitory Effect of Tetradrine on FBS-Stimulated Tube Formation of Vascular Endothelial Cells** To investigate the inhibitory modes of action of tetradrine in the angiogenic process, the effect of tetradrine was compared with that of hydrocortisone on the tube formation of rat vascular EC which had been cultured in type I collagen gel with 2% FBS-DMEM for 4 d. Tetradrine (0.1—10 μM) and hydrocortisone (0.3—30 μM) inhibited 2% FBS-stimulated tube formation in a concentration-dependent manner (Fig. 2). The value of 50% inhibitory concentration (IC₅₀) with a 95% confidence limit of tetradrine was 1.72 μM (1.43—2.07). The effect of hydrocortisone did not exceed 50% inhibition of the control without drug, and its ID₅₀ value was estimated to be over 100 μM. The results demonstrate that the potency of tetradrine for the inhibition of tube formation was more

![Fig. 1. Inhibitory Effects of Tetradrine and Hydrocortisone on Carline Content, Granuloma Weight, Inflammatory Cell Count and Pouch Fluid Weight in Mouse Adjuvant-Induced Chronic Inflammation Model](image-url)
Fig. 2. Inhibitory Effects of Tetrandrine and Hydrocortisone on Tube Formation of Cultured Vascular EC

EC were cultured in type I collagen gel with 2% FBS-DMEM in the presence of tetrandrine (●: 0.1—10 μM) and hydrocortisone (○: 0.3—30 μM) for 4 d. Values are % tubular length relative to the control value without drug (2.96±0.14 mm/mm² dish, n=12) are expressed as means±S.E. of averages of 4 analyses in 3—6 dishes.

Fig. 3. Inhibitory Effects of Tetrandrine on IL-1α-Stimulated (A) and PDGF-BB-Stimulated Tube Formation (B)

EC were cultured in type I collagen gel with 1% FBS-DMEM containing IL-1α (A; 6.9—690 pM) or PDGF-BB (B; 1—100 pM) in the presence (●) or absence (○) of tetrandrine (10—30 nM) for 4 d. Absolute values are expressed as the means±S.E. of averages of 4 analyses in 3—10 dishes. C: Control without IL-1 or PDGF-BB.

way of IL-1α and PDGF-BB in chronic inflammation.

DISCUSSION

Tetrandrine has been reported to suppress chronic inflammation in subcutaneous air pouch inflammation and adjuvant-induced arthritis in rat. Tetrandrine inhibits the production and release of a broad range of inflammatory mediators, as well as the proliferation of synovial cells. However, the mechanism of its anti-inflammatory action is unknown. The present study demonstrates that tetrandrine inhibits granuloma angiogenesis as well as granuloma formation, inflammatory cell migration and pouch fluid exudation, in an adjuvant-induced chronic inflammation model with an inhibitory pattern similar to hydrocortisone. These results mean that the anti-angiogenic effect of tetrandrine depends on its inhibition of other inflammatory parameters. Tetrandrine also inhibited the FBS-stimulated tube formation of vascular EC in the angiogenic process. In this respect, the effect of tetrandrine was greater (over 100-fold) than that of hydrocortisone. This result indicates that tetrandrine has inhibitory action on vascular tube formation in the angiogenic process, as well as on the anti-inflammatory action, which is different from hydrocortisone.

In rheumatoid arthritis (RA) there is a chronic immune and inflammatory reaction which can lead to the destruction of the diseased joint. Macrophages within the rheumatoid tissues are believed to participate in the development of RA lesions. The synovial macrophages and synovial cells in RA patients release large amounts of IL-1 spontaneously. Murine peritoneal macrophage during the chronic exudative response to FCA produces maximal levels of IL-1 by days 4—7. We have previously reported that IL-1α plays a predominant role in synovial cell proliferation in lesions of RA models and patients. IFN-γ-activated peritoneal macrophage predominantly releases IL-1α and IL-1β, as well as PDGF-BB and basic fibroblast growth factor, stimulate the tube formation of EC. The present study demonstrates that tetrandrine potently inhibits both IL-1α-stimulated and PDGF-BB-stimulated tube formation in a non-competitive manner, suggesting that the site of action of tetrandrine may be in a common signal transduction in the post-receptor pathway of IL-1α and PDGF-BB. IL-1α and PDGF-BB are released from IFN-γ-activated macrophage and diabetic macrophage. The IFN-γ-activated macrophage releases a small amount of PDGF-BB, whereas the macrophage in spontaneously diabetic GK rat releases a great amount of PDGF-BB. This evidence suggests that the anti-angiogenic activity of tetrandrine may be associated with its interference with IL-1α-stimulated action rather than its effect on PDGF-BB-stimulated action in chronic inflammation.

The inhibitory effect of tetrandrine on EC tube formation was independent of its toxic action on EC. Tetrandrine (1—10 μM) did not damage EC, and less than 5% of total EC cultured on a collagen-coated dish for 4 d were stained with trypan blue by these concentrations of tetrandrine (data not shown). Tetrandrine is a blocker of the voltage-dependent, L-type and T-type Ca²⁺ channels in various cells. The decrease in intracellular Ca²⁺ level may be associated with the inhibition of cytokine-induced tube formation. However, a higher concentration (100 μM) of tetrandrine damaged EC
(data not shown). Tetrandrine’s toxic effects may be associated with the induction of apoptosis (programmed cell death). This suggestion is supported by reports that a high concentration of tetrandrine inhibits the proliferation of neoplastic lymphoid and myeloid cells via the induction of apoptosis.26,27

In conclusion, tetrandrine reduced angiogenesis by inhibiting inflammatory parameters and the tube formation of vascular EC in chronic inflammation. The anti-tube forming effect of tetrandrine may be associated with its inhibition of post-receptor pathway of IL-1α and PDGF-BB.

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