Effects of anti-rheumatic herbal medicines on cellular adhesion molecules

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Abstract

Objective—To test the hypothesis whether herbal medicines ameliorate inflammatory diseases via the modulation of cellular adhesion molecules (CAMs).

Methods—Human neutrophils, synovial fibroblasts, and endothelial cells were incubated with different concentrations of Tripterygium Wilfordii Hook-f (TWH-f) or Tetrandrine in the presence or absence of interleukin 1 (IL1). The amount of soluble E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) secreted by cells were determined by ELISA. The cell surface expression of these three CAMs was detected by flow cytometry.

Results—TWH-f at high concentration (50 ng/ml) has a significant ($p < 0.05$) inhibitory effect on both the secretion and the expression of the cellular adhesion molecules. However, Tetrandrine did not demonstrate the same effects.

Conclusions—The cellular adhesion molecules of the endothelium and leucocytes may constitute excellent targets for the development of new anti-inflammation medicines. These results indicate that TWH could be a potential therapeutic agent in the treatment of inflammatory diseases.

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Inflammation is characterised by the accumulation of leucocytes and other mesenchymal cells in response to attractant molecules at sites of injury or infection.¹ Persistent inflammation without an identifiable antigen such as rheumatoid arthritis and systemic lupus erythematosus is often as destructive to the host as any invaded micro-organism.² Once exposed to chemoattractants within the vasculature, leucocytes become activated and capable of adhering tightly to endothelium. In 1985, Bevilacqua and coworkers demonstrated that cytokines and endotoxin stimulated the endothelium to become more adhesive for leucocyte.³

Many of the adhesion molecules that mediate the interaction between endothelium and leucocyte and that are expressed on both these cell types have been isolated.⁴ There are three general classes of adhesion molecules present on leucocytes and endothelium: integrins, selectins, and members of the immunoglobulin superfamily of cell surface protein.² Integrins and selectins on leucocytes mediate the adhesion of circulating cells to endothelium,

whereas selectins and members of the immunoglobulin superfamily on the endothelium mediate their adherence for leucocyte.²

The CD11/CD18 glycoproteins are exclusively expressed on circulating leucocytes, and play an important part in the rapid adhesion of leucocytes to endothelial cells. Intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1), are well characterised endothelial cell ligands for the CD11/CD18 glycoproteins.⁴ E-selectin (ELAM-1) was the first inducible adhesive molecule that was originally shown to mediate the adhesion of neutrophils to cytokine or lipopolysaccharide activated endothelial cells.⁵ The explosion of information on the adhesion molecules that govern the emigration of leucocytes from blood vessels and their accumulation at loci of inflammation has completely changed our understanding of inflammation and may lead to new strategies in the treatment of acute and chronic inflammation.

Previously published animal studies demonstrated that antibodies directed against adhesion molecules prevent experimental arthritis in animals.⁶ The results of new studies also suggest that measures designed to interfere with selectin mediated adhesion may diminish experimental arthritis.⁷

Tripterygium Wilfordii Hook-f (TWH) is a vine-like plant that grows in a wide area of south China. The structures of various constituents of TWH have been identified and include diterpenes, triterpenes, alkaloids, glycosides, b-sitosterol, daucosterol, dulcitol, and wilforonide.⁸ Some of these have anti-inflammatory and immunosuppressive properties and are concentrated in the roots of the TWH plant.⁹ A refined extract from the root xylem of TWH, available in the Chinese pharmacopoeia as tablets for the long term treatment of rheumatoid arthritis⁹ and systemic lupus erythematosus,¹⁰ has recently been demonstrated to be effective in suppression of renal disease and arthritis in MRL-lpr mice.¹¹ In addition, tetrandrine (TTD), an extract of the tuberous root of the creeper *Stephania tetrandra*, a benzyloquinoline with the empirical formula C₃₈H₄₂O₆N₂ and molecular weight 622.73 daltons,¹² is used in traditional Chinese medicine for the treatment of rheumatic diseases.¹³ However, both these two root extracted, historically famous, and clinical effective anti-rheumatic medicines have an unclear mechanism of action.

Previously, we have demonstrated that both TWH and TTD significantly inhibited interleukin 1 β (IL1 β) and tumour necrosis factor α (TNF α) release from monocyte.¹⁴ In this study,

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we observed the herbal medicine effects on the secretion and expression of adhesion molecules on neutrophils, synovial cells, and endothelial cells.

Methods

DRUG PREPARATION

TWH was obtained as tablets (containing 33 µg pure extract) from Yellow Stone, Hu-Pei, China. A stock solution was prepared by dissolving the tablets in ethanol. TTD, a pure form of isolated alkaloid from han-fang-chi,¹⁵ was obtained from Gin-Hwa, Chekiang, China. These agents were further diluted in RPMI 1640 medium for the experiments: the final concentration of ethanol (0.5%) has been shown to have no effect on cell function in preliminary experiments. When 0.5% ethanol was used as side by side control comparing with medium in the current experiments, it also had no effect on the results of either secretion or expression of the cellular adhesion molecules.

EFFECT OF HERBAL MEDICINES ON CELL VIABILITY

The drug effect on cellular viability was monitored by the 0.5% trypan blue exclusion method at the end of the culture periods. TWH concentrations of 5 mg/ml or lower, TTD concentrations of 10 µg/ml or lower were not toxic to human neutrophils, synovial fibroblasts or endothelial cells. For further assessment of the drug safety, herbal medicine treated cells (4–48 hour incubation) were incubated with acridine orange and ethidium bromide (AO/EB) fluorescent staining solution for 15 minutes at room temperature in the dark room. Viable (green) and dead (red) cells could be differentiated with a fluorescence microscope (excitation filter 450/490 nm). TWH concentrations of 0.05 µg/ml and TTD concentrations of 10 µg/ml had no effect on cell viability, we thus used this concentrations in our current experiments. Ten mg/ml or less of TTD is the achievable concentrations in serum during treatment.¹⁶ There is no information indicating the therapeutic level of TWH.

NEUTROPHILS PREPARATION

Blood, donated by healthy volunteers, was aseptically collected into sterile preservative free, EDTA Vacutainer tubes. The blood was mixed with 6% dextran in saline in a 4:1 ratio and incubated at 37°C for one hour. The supernatant was removed and subsequently spun at 275 g for 10 minutes. The resulting cell pellet was saved and the supernatant spun at 20 000 rpm for 10 minutes to cause sedimentation of the platelets. The cell pellet was resuspended in the platelet free plasma and was layered over Ficoll-Hypaque (Pharmacia, Piscataway, NJ, USA), which was spun at 450 g for 30 minutes at 22°C. The cell pellet that contained the neutrophils was subsequently resuspended in 10 ml of platelet free plasma, and 30 ml of erythrocyte lysing solution (0.87% NH₄Cl) was added. The neutrophils were washed three times and 5 × 10⁶ cells/ml were resuspended in RPMI 1640 (GIBCO, Grand Island, NY) and were tested immedi-

ately. The cell purity of neutrophils (>98%) was monitored by Wright-Giemsa staining.

ENDOTHELIAL CELL PREPARATION

Endothelial cells were obtained from human umbilical cord veins (HUVEC) by an adaptation of the method of Maruyama.¹⁷ A sterile technique was used in all manipulations of the cord. Briefly, the cord was severed from the placenta soon after birth, placed in a sterile container filled with cord buffer (0.14M NaCl, 0.004 M KCl, 0.001 M phosphate buffer, pH 7.4, 0.011 M glucose), and held at 4°C until processing. HUVEC were obtained immediately by infusion and incubation the cord with 0.1% collagenase at 37°C for 15 minutes. Isolated cells were cultured in medium E-199 (GIBCO, Grand Island, NY) supplemented with 25 µg/ml heparin (Sigma, St Louis, MO), 10% heat inactivated pooled human AB and 20% fetal calf serum (GIBCO), penicillin (200 U/ml), streptomycin (200 µg/ml), 2 mM L-glutamine, and 0.1 mg/ml EC growth supplement (ECGS; Collaborative Research Inc, MA). HUVEC was cultured in plastic culture dishes (Falcon no 3080, Becton-Dickinson Labware, Oxnard, CA) in a 5% carbon dioxide incubator at 37°C for 3–5 days until confluence was achieved. For subculture, cells were harvested with 0.01% EDTA. HUVEC origin was identified by the characteristic cobblestone morphology observed in tissue culture and by positive immunofluorescence with factor VIII antibodies (DAKO, USA) (>95% positive). We routinely used confluent cells (105 cells/2 cm² culture well, three replicates/culture) at the third to fourth passage maintained in E199 medium with 20% newborn calf serum supplemented with ECGS (50 µg/ml) and heparin (100 µg/ml). When cells were confluent, the HUVEC were stimulated with recombinant IL1α (10 ng/ml for 48 hours; Genzyme, MA, USA) in the presence or absence of serial dilutions of herbal medicines.

SYNOVIAL FIBROBLAST CULTURES

Cultures were established from explant outgrowths of synovial tissue obtained from either healthy people undergoing surgery for meniscal tear or rheumatoid hip or knee joints undergoing joint replacement surgery.^{18 19} For experiments, human synovial fibroblasts (HFB) were grown in 150 cm² flasks with L-15 medium (Biological Industries, Beth Ha-Emek, Israel), supplemented with 1% fetal calf serum (FCS), 2 mM L-glutamine, 3 mM NaHCO₃, 10 mM HEPES, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. After three days of incubation at 37°C in a humidified atmosphere, the culture medium was replaced with fresh medium, and test materials (different concentrations of herbal medicines and IL1α) were added to confluent cultures. For measurement of the herbal medicine inhibitory effects on IL1 mediated ICAM-1 expression, the cells were washed with PBS, and the cell layers were trypsinised. Samples were analysed on a flow cytometer.

Figure 1 Effect on secretion of soluble cellular adhesion molecules by HUVEC. Cells (5×10^6) were incubated with IL1 α (10 ng/ml) in the presence or absence of herbal medicines for 4–48 hours. Data were presented as mean (SD) (ng/ml) of duplicate assay in triplicate experiments.

SOLUBLE ADHESION MOLECULES DETERMINATION

HUVEC (5×10^6) or HFB (5×10^6) were incubated with recombinant IL1 α (10 ng/ml) in the presence or absence of different concentrations of TWH (0.5–50 ng/ml) or TTD (0.1–10 µg/ml) for 4–48 hours. The supernatants were collected, filtered through 0.2 micro disc (Acrodisc, Gelman Sciences, Ann Arbor, MI), and then determined by ELISA (R and D Systems, MN, USA) for soluble ICAM-1, E-selectin, and VCAM-1.

FLOW CYTOMETRIC ANALYSIS

The expression of ICAM-1 (CD54) was measured on both HUVEC and HFB. Cells (1×10^6) were incubated with recombinant IL1 α (10 ng/ml) in the presence or absence of different concentrations of herbal medicines for 4–48 hours. Cells were then removed with a rubber policeman and resuspended in culture medium. In addition, the expression of CD18 was measured on neutrophils. Cells (1×10^6) were incubated with IL1 α (10 ng/ml) in the presence or absence of different concentrations of herbal medicines for 1–8 hours.

Cells in 50 µl of medium were stained with 20 µl of monoclonal antibodies of either CD-54 PE or CD18 PE (Becton Dickinson, Mountain View, CA, USA) in the dark at 4°C for 30 minutes. Mouse IgG1 PE was used as fluorescence controls. The cell surface adhesion molecules of the incubated cells were then analysed with a FACScan (Becton Dickson, Mountain View, CA, USA) using 488 nm wavelength laser excitation.

STATISTICAL ANALYSIS

Data analysis was performed using the Power Macintosh Computer software StateWorkTMs. The level of statistical significance was $p < 0.05$ by Mann-Whitney U test.

Results

EFFECT ON SECRETION OF SOLUBLE CELLULAR ADHESION MOLECULES BY HUVEC AND HFB

Figures 1 and 2 show that 10 ng/ml IL1 stimulated adhesion molecules secretion from HUVEC (including ICAM-1, E selectin, and

VCAM-1) and HFB (including ICAM-1 and VCAM-1 but not E-selectin). TWH in high concentrations (50 ng/ml) significantly inhibited both ICAM-1 and VCAM-1 secretion. The percentage of decrease was expressed by (mean of the former one–mean of the latter one)/ mean of the former one $\times$ 100%. Secretory ICAM-1 and VCAM-1 from HUVEC were decreased from (mean (SD)) 0.73 (0.12) to 0.51 (0.04) ng/ml (30% reduction) and from 27.2 (2.3) to 20 (1.5) ng/ml (26% reduction) respectively after 24 hour incubation, and from 1.9 (0.3) to 0.8 (0.2) ng/ml (58% reduction) and from 36.0 (5.1) to 24.2 (1.3) ng/ml (33% reduction) respectively after 48 hour incubation. The secretion of these two adhesion molecules (ICAM-1 and VCAM-1) from HFB also were inhibited from 0.71 (0.09) to 0.50 (0.08) ng/ml (30% reduction) and from 26 (3) to 14 (2) ng/ml (46% reduction) respectively after 48 hour incubation (fig 2A and 2B). E-selectin secretion from HUVEC could be detected after 8–48 hour IL1 stimulation. TWH at the concentration of 0.5 ng/ml could significantly inhibited E-selectin secretion (fig 1B). TTD, however, did not demonstrate the same effect.

EFFECT OF HERBAL MEDICINES ON THE EXPRESSION OF CAM BY HUVEC AND HFB

Furthermore, IL1 significantly stimulated ICAM-1 expression on both HUVEC and HFB (tables 1 and 2). TWH and TTD in high concentrations (50 ng/ml and 10 µg/ml respectively) inhibited ICAM-1 expression on both HUVEC (table 1) and HFB (table 2). The expression of CD18 on neutrophils could be increased under recombinant IL1 α (10 ng/ml) stimulation. However, reproducible experiments demonstrated that high concentrations of TWH (50 ng/ml) and TTD (10 µg/ml) have no effect on CD18 expression by IL1 α stimulated neutrophils (data not shown).

Table 1 Effect on expression of ICAM-1 by human umbilical venous endothelial cells (HUVEC)

Conditions	ICAM-1			
	4 hours	8 hours	24 hours	48 hours
Mouse IgG1 PE control	16 (6)	16 (9)	25 (7)	20 (8)
HUVEC+ media	747 (56)	655 (44)	542 (38)	555 (41)
HUVEC+IL1	1732 (176)	2922 (176)	1081 (99)	1213 (109)
HUVEC+IL1+TWH 50 ng/ml	1107 (58)*	474 (27)***	647 (45)*	553 (39)**
HUVEC+IL1+TWH 5 ng/ml	1335 (186)	2047 (162)	882 (117)	876 (107)
HUVEC+IL1+TWH 0.5 ng/ml	1547 (181)	2103 (197)	1307 (124)	1218 (98)
HUVEC+IL1+TTD 10 µg/ml	1086 (95)*	1695 (133)*	780 (62)*	636 (58)**
HUVEC+IL1+TTD 1 µg/ml	1236 (199)	1973 (194)	1453 (182)	1348 (97)
HUVEC+IL1+TTD 0.1 µg/ml	1465 (98)	1635 (181)	1199 (108)	1209 (104)

Cells (5×10^6) were incubated with IL1 α (10 ng/ml) in the presence or absence of herbal medicines for 4–48 hours. TWH indicates Tripterygium Wilfordii Hook-f, and TTD indicates tetrandrine. Data (fluorescence intensity) were presented as mean (SD) of duplicate assay in triplicate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared with HUVEC+IL1 by Mann-Whitney U test.

Table 2 Effect on expression of ICAM-1 by human fibroblast (HFB)

Conditions	ICAM-1			
	4 hours	8 hours	24 hours	48 hours
Mouse IgG1 PE control	23 (6)	16 (9)	25 (7)	20 (8)
HUVEC+ media	262 (12)	656 (44)	52 (38)	555 (41)
HUVEC+IL1	1033 (176)	2922 (176)	871 (99)	1213 (109)
HUVEC+IL1+TWH 50 ng/ml	266 (38)***	474 (27)***	206 (25)**	553 (39)*
HUVEC+IL1+TWH 5 ng/ml	841 (166)	2048 (162)	794 (87)	876 (107)
HUVEC+IL1+TWH 0.5 ng/ml	778 (181)	2104 (197)	556 (124)	1218 (98)
HUVEC+IL1+TTD 10 µg/ml	660 (115)*	1695 (133)*	554 (62)*	636 (58)**
HUVEC+IL1+TTD 1 µg/ml	854 (149)	1973 (194)	588 (182)	1348 (97)
HUVEC+IL1+TTD 0.1 µg/ml	1046 (98)	1936 (181)	787 (108)	1209 (104)

Effect on expression of ICAM-1 by HFB. Cells (5×10^6) were incubated with IL1 α (10 ng/ml) in the presence or absence of herbal medicines for 4–24 hours. Data (fluorescence intensity) were presented as mean (SD) of duplicate assay in triplicate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared with HFB+IL1 by Mann-Whitney U test. Abbreviations as in table 1.

Discussion

Our results suggested that herbal medicine could inhibit the IL1 stimulated secretion and expression of cellular adhesion molecules, and thus may play a part in the course of anti-inflammation.

Although the inciting event in the development of most rheumatic diseases remains a matter of speculation, the vascular endothelium interacts with circulating leucocytes by virtue of a complex of adhesion molecules present on the surface of both the endothelium and leucocytes play active parts in the pathogenesis of inflammation.^{2–5} Studies of animal models of inflammation indicate that

E-selectin is expressed on the surface of endothelium at inflamed sites.²⁰ Belmont *et al* have demonstrated statistically significant up-regulation of the endothelial cellular adhesion molecules E-selectin, VCAM-1 and ICAM-1 on skin biopsy specimens from non-light exposed skin in patients with active systemic lupus erythematosus (SLE) compared with patients with inactive SLE and normal controls.²¹ During disease flares circulating neutrophils are also activated as indicated by up regulation of the surface 2 integrin CD11b/CD18.^{21–22} In patients with rheumatoid arthritis (RA), peripheral blood neutrophils express greater numbers of CD11b/CD18 on their surface.²³ Another study demonstrates that there is increased expression of endothelial adhesion molecules (E-selectin, ICAM-1, and VCAM-1) in the microvascular of rheumatoid but not osteoarthritic synovium.²⁴

Over recent years it has become recognised that there is heterogeneity not only between endothelial cells from different species but also between those from different sites within a species.²⁵ Abbot *et al* isolated endothelial cells from the synovium of rheumatoid hip and knee joints, and compared the characteristics of these cells with HUVEC.²⁶ The synovial cells had characteristic endothelial morphology and function, which were stable in culture. Similar basal patterns of expression of ICAM-1 and E-selectin were observed in both synovial cells and HUVEC. However, E-selectin expression was significantly increased in the synovial endothelial cells in response to a range of concentrations of IL1 whereas there was no comparable effect on the HUVEC. Although it is possible that this represents an artefactual effect from the isolation and culture techniques used, this observation implies that information from modelling the synovial microvasculature using endothelial cells of non-synovial origin may be limited for interpretation.

Heino *et al* demonstrated that the potentially cytotoxic mononuclear cells were bound equally well to rheumatoid and normal synovial fibroblast culture.²⁷ Other investigations dem-

Figure 2 Effect on secretion of soluble cellular cellular adhesion molecules by HFB. Cells (5×10^6) were incubated with IL1 α (10 ng/ml) in the presence or absence of herbal medicines for 4–48 hours. Data were presented as mean (SD) (ng/ml) of duplicate assay in triplicate experiments.

onstrated that synovial ICAM level in RA is significantly higher than in non-inflammatory arthritis.²⁸ We obtained HFB from either traumatic or RA joints, and demonstrated no significant difference of cell response to IL1 from these two origins for the secretion and expression of cellular adhesion molecules (data not shown). Probably, the local inflammation in our RA patients who received joint replacement and in the acute traumatic joint was similar.

E-selectin is not constitutively expressed by endothelial cells, but is rapidly induced and reaches maximal levels after 4–6 hours of IL1 treatment. Both in vitro and in vivo expression is usually transient.²⁹ ICAM-1 is expressed at low levels on resting endothelium but is markedly up regulated by IL1, with maximal expression 24 hours after activation, which is maintained for > 72 hours.³⁰ VCAM-1 is normally present minimally on non-activated endothelial cells, but is induced with a similar time course of ICAM-1.³¹ Our study demonstrated that these soluble CAM could be detected quickly by ELISA after IL1 stimulation. The surge of E-selectin at 48 hours supported more recent data that human endothelial cells are capable of persistent E-selectin expression in vitro^{32–33} and that factors in human serum or plasma are critical in preventing cytokine refractoriness and loss of E-selectin expression.³³

An understanding of the molecules that mediate the initial events of inflammation may eventually lead to newer modes of treatment for the rheumatic diseases, and it seems useful to determine whether agents currently used in treating rheumatic diseases have an effect on adhesive molecules. Indeed, most non-steroidal anti-inflammatory drugs affect the capacity of neutrophils by diminishing their adhesiveness mediated by CD11b/CD18.³⁴ The anti-inflammatory effects of methotrexate probably result from increased adenosine release and thus inhibited leucocyte adhesion mediated by CD11b/CD18³⁵ and synovial expression of cellular adhesion molecules such as E-selectin, ICAM-1, and VCAM-1.³⁶ Treatment of patients with RA with gold salt diminish expression of E-selectin on the endothelium of synovial blood vessels.²³ Corticosteroids also have the similar effect.³⁷ Colchicine, an old anti-inflammatory agent, markedly diminishes expression of E-selectin on the surface of leucocyte, but has no effect on the expression of ICAM-1 on endothelium.³⁸ Our studies also demonstrated that TWH-f in high concentrations significantly inhibited both the secretion and the expression of the adhesion molecules, and high concentration of TTD significantly inhibited ICAM-1 expression, which suggested that the currently available and effective anti-rheumatic medicines may ameliorate inflammatory diseases via their effects on the expression or activation of adhesive molecules.

In response to such inflammatory agents as IL1 or TNF, the vascular endothelium expresses a variety of adhesion molecules to which circulating leucocytes adhere.² Our previous studies¹⁴ demonstrated that TWH-f

inhibited both IL1 and TNF α secretion. Thus, a possible hypothesis as to mechanism of action of TWH-f is that this agent affects the secretion and expression of adhesion molecules on cells by diminishing the secretion of these cytokines. However, there is no evidence that, in this study, they have modified IL1 activity or secretion. Further studies will be needed to verify this point.

This demonstrated that TWH significantly inhibited the secretion of E-selectin, ICAM-1, and VCAM-1. In addition, TWH and TTD also inhibited the expression of ICAM-1 on both endothelial cells and synovial fibroblasts. These potent anti-adhesion molecule properties may account for some of their potential anti-inflammatory effects.

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