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Gold Sodium Thiomalate Down-regulates Intercellular Adhesion Molecule-1 and Vascular Cell Adhesion Molecule-1 Expression on Vascular Endothelial Cells

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SUMMARY

We examined whether antirheumatic drugs alter cytokine- or lipopolysaccharide-induced expression of adhesion molecules on vascular endothelial cells. Human umbilical cord vein endothelial cells were co-cultured with various antirheumatic drugs in the presence of inflammatory cytokines, and adhesion molecule expression was measured by cell enzyme-linked immunosorbent assay and Northern blot analysis. Among these antirheumatic drugs, gold sodium thiomalate significantly inhibited intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression on vascular endothelial cells and suppressed cellular binding between human monocytic cell lines, including U937 and HL-60 cells, and interleukin-1β-stimulated vascular endothelial cells. It is speculated that down-regulation of adhesion molecules might be one of the novel mechanisms of action of gold sodium thiomalate.

Gold therapy (chrysotherapy) has been used as an effective treatment for patients with RA. It is capable of inducing definite remissions in some patients with RA, resulting in decreased serum levels of immunoglobulins and rheumatoid factors (1), although its precise mechanisms remain to be clarified. *In vitro*, gold compounds inhibit antigen- and mitogen-induced lymphocyte proliferative responses (2) and suppress proliferative responses of cultured T cells to IL-2 (3). It is also known that the actions of gold compounds are directed against monocytes and macrophages (4–6). Gold compounds inhibit the capacity of monocytes to produce superoxide anions (4) and complement components (5), and they hamper the differentiation of monocytes into effector cells (6). Furthermore, their effects are also directed to other type of cells, incuding neutrophils and endothelial cells (7, 8).

Up-regulated expression of adhesion molecules, including ICAM-1, VCAM-1, and E-selectin (endothelial leukocyte adhesion molecule-1), in RA synovial tissues has been reported (9–11). These adhesion molecules are known to be up-regulated by proinflammatory cytokines such as IL-1 and TNF-α (12, 13), which are abundantly produced in inflammed synovium (14). Furthermore, cells with up-regulated expression of adhesion molecules could be further activated by the binding of corresponding receptors or ligands (15), which would in turn result in constitutive production of inflammatory cytokines and other soluble mediators such as prostaglandins and collagenase. We therefore examined in this study whether antirheumatic drugs including gold compounds exert their effects upon the cytokine- or LPS-induced expression of adhesion molecules in vascular endothelium. Our study clearly demonstrates that GST not only significantly down-regulates expression of adhesion molecules, including ICAM-1 and VCAM-1, on HUVECs but also inhibits cellular binding between human monocytic cell lines, such as U937 and HL-60 cells, and HUVECs.

Materials and Methods

Cell cultures. The human monocytic cell lines U937 and HL-60 were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 100 units/ml penicillin, and 100 μg/ml streptomycin. HUVECs were isolated from umbilical cords within 12 hr after delivery, by the method

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ABBREVIATIONS: RA, rheumatoid arthritis; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; HUVEC, human umbilical cord vein endothelial cell; GST, gold sodium thiomalate; IL, interleukin; TNF, tumor necrosis factor; DMARD, disease-modifying antirheumatic drug; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; BCECF, 2′,7′-bis(carboxyethyl)-5(6′)-carboxyfluorescein; LFA, leukocyte function-associated molecule; VLA, very late activation antigen; LAM, leukocyte adhesion molecule.

of Jaffe *et al.* (16). The cells were grown to confluence in RPMI 1640 medium supplemented with 20% fetal calf serum, 30 μg/ml endothelial cell growth supplement (Collaborative Research, Bedford, MA), and 10 units/ml porcine mucosal heparin, in gelatin-coated plastic dishes, and were harvested (17). No cells were used for the study after more than three serial passages. The viability of cells was always examined morphologically with an inverted microscope and by the trypan blue dye exclusion test.

Reagents and drugs. Hydrocortisone sodium phosphate (Banyu Pharmaceuticals, Tokyo, Japan), prednisolone (Takeda Pharmaceuticals, Osaka, Japan), dexamethasone (Banyu Pharmaceuticals), cyclophosphamide (Shionogi Pharmaceuticals, Osaka, Japan), methotrexate (Lederle, Tokyo, Japan), mizoribine (Asahikasei Co., Tokyo, Japan), cisplatin (Bristol Pharmaceuticals, Tokyo, Japan), cyclosporin (Sandoz Pharmaceuticals, Basle, Switzerland), salazosulfapyridine (Midorijuji, Tokyo, Japan), bucillamine (Santen Pharmaceuticals, Osaka, Japan), lobenzarit disodium (Chugai Pharmaceuticals, Tokyo, Japan), GST (Shionogi Pharmaceuticals), and auranofin (SmithKline Beecham, Osaka, Japan) were all obtained from the manufacturers. All drugs were dissolved in RPMI 1640 medium except for cyclosporin, which was dissolved in dimethylsulfoxide, and all drugs were diluted to appropriate concentrations with RPMI 1640 medium. Human recombinant IL-1β was a gift of Otsuka Pharmaceuticals. Human recombinant TNF-α was purchased from Genzyme (Boston, MA).

Cell ELISA for measurement of ICAM-1 expression. Twenty thousand HUVECs were seeded into each well of flat-bottomed, 96-well, culture plates and stimulated with either 200 pg/ml recombinant IL-1β, 10 units/ml TNF-α, or 1 μg/ml LPS (*Escherichia coli* 0127:B8) for 24 hr, to form confluent monolayers, in the presence or absence of various drugs. Cells were subsequently fixed with 3% paraformaldehyde/8% saccharose/PBS. Nonspecific binding was blocked by the sequential addition of Blockace (Yukijirushi, Sapporo, Japan) (processed skim milk)/PBS and 5% goat serum/PBS (for 1 hr each). Either anti-ICAM-1 (CD54) monoclonal antibody (1/4000 dilution; British Bio-technology Ltd., Oxon, England) or anti-VCAM-1 monoclonal antibody (1/4000; British Bio-technology Ltd.) and alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Tago Inc., Burlingame, CA) were used as the first and second antibodies, respectively, followed by the addition of phosphatase substrate (Sigma Chemical Co., St. Louis, MO). The absorbance of each well was determined with a microplate reader (Bio-Rad Laboratories, Richmond, CA) at 405 nm (17). To measure E-selectin expression, HUVECs were stimulated with the aforementioned stimulants for 4 hr and reacted with anti-E-selectin monoclonal antibody (British Bio-technology Ltd.).

Cell binding assay. Ten thousand HUVECs were seeded into each well of gelatin-coated, 96-well, culture plates and stimulated with 100 pg/ml IL-1β and various concentrations of drugs for 48 hr. Confluence of the cells was always confirmed with the inverted microscope. U937 cells or HL-60 cells were labeled with 10 μM BCECF tetraacetoxymethyl ester (Dojindo Laboratories, Kumamoto, Japan), as reported previously (17). Twenty thousand labeled cells were added to each well and incubated with HUVECs for 1 hr. Cells that were nonspecifically bound to HUVECs were removed by inverting the plates for 30 min. Wells were subsequently washed once with RPMI 1640 medium, and the remaining cells were lysed with 1% Nonidet P-40 (Nakarai Tesque, Kyoto, Japan). Fluorescence was measured with an automated microplate fluorometer (Nihon Bunko, Tokyo, Japan) at 490 nm.

Northern blot analysis. HUVECs were cultured in six-well plates using the same conditions as described for the cell ELISA method. Total RNA was isolated from HUVECs by guanidinium isothiocyanate extraction (18). Twenty micrograms/lane of RNA were separated in 1.2% agarose gels and transferred to nylon membranes. Hybridization was carried out using a ^{32}P-labeled cDNA probe for ICAM-1 (British Bio-technology Ltd.) or VCAM-1 (1.5-kilobase cDNA in pUC119) (19) in 5× SSPE (sodium chloride/sodium phosphate/EDTA buffer), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, 50% formamide, at 42° overnight. Filters were washed with 6× SSPE/0.1% sodium dodecyl sulfate at 42° for 90 min.

Statistical analysis. Statistical analysis was performed with Student's *t* test.

Results

Cytokine-mediated ICAM-1 expression on HUVECs was time and dose dependent. We first performed time course and dose-response studies of cytokine-mediated adhesion molecule expression on HUVECs. Recombinant IL-1β induced time-dependent ICAM-1 and VCAM-1 expression that reached a maximum after 24 hr. In contrast, E-selectin expression reached its maximum at 6 hr (Fig. 1A). There was also a dose-dependent increase in cytokine-induced ICAM-1 expression, with 100 pg/ml IL-1β, 10 units/ml TNF-α, and 1 μg/ml LPS yielding the maximal response (Fig. 1B). VCAM-1 expression was also maximally induced by the same concentrations of IL-1β, TNF-α, and LPS. E-selectin was also up-regulated by

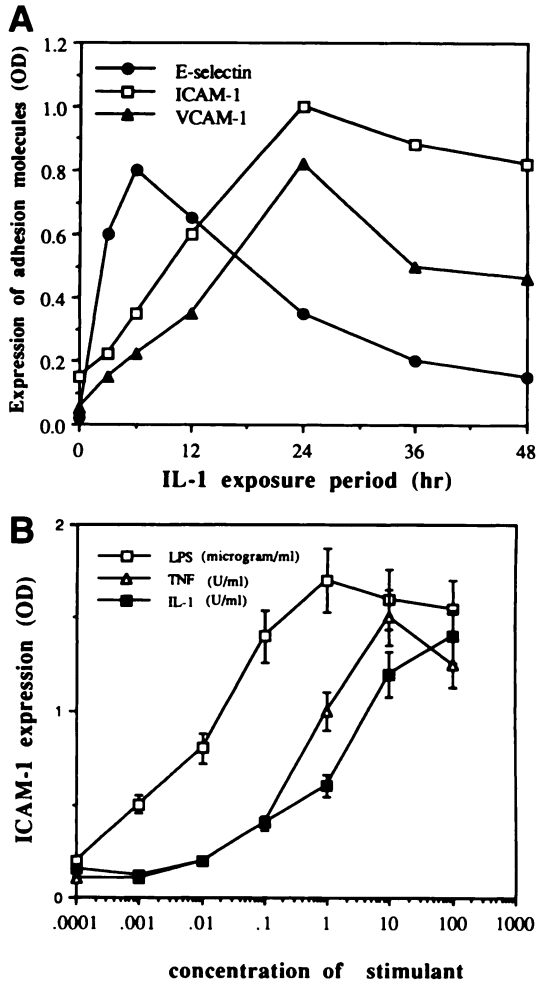


Fig. 1. A, Time-dependent increase in ICAM-1, VCAM-1, and E-selectin expression on HUVECs induced by IL-1β. HUVECs were cultured with 100 pg/ml IL-1β for various periods, and adhesion molecule expression was assayed by cell ELISA as described in Materials and Methods. *Vertical axis*, expression of adhesion molecules, as measured by absorbance. Representative data are shown. B, Dose-dependent increase in ICAM-1 expression induced by either IL-1β, TNF-α, or LPS (five experiments). HUVECs were cultured with various concentrations of stimulants for 24 hr, and ICAM-1 expression was assayed by cell ELISA. *Vertical axis*, ICAM-1 expression, as measured by absorbance; *bars*, standard errors.

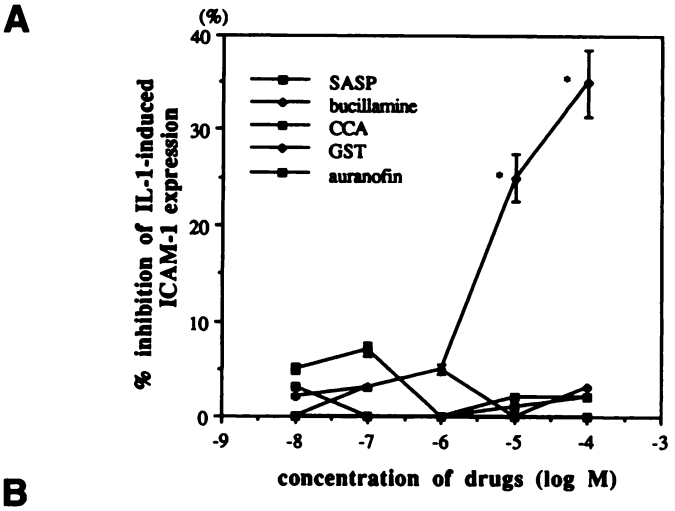
IL-1β, TNF-α, and LPS in a dose-dependent fashion (data not shown). We therefore used these conditions for the later experiments.

Corticosteroids did not inhibit cytokine-induced adhesion molecule expression on HUVECs. Three different corticosteroids, i.e., prednisolone, dexamethasone, and hydrocortisone, were tested for their ability to alter adhesion molecule expression on HUVECs. Each of the corticosteroids was added to the culture together with IL-1β and cultured for various periods. However, none of them suppressed IL-1β-induced or basal ICAM-1 expression in our assay system (data not shown). Stimulation with either TNF-α or LPS instead of IL-1β gave the same results (data not shown). In addition, corticosteroids did not alter VCAM-1 or E-selectin expression.

Immunosuppressive agents did not affect cytokine-induced adhesion molecule expression on HUVECs. We selected five immunosuppressive agents, i.e., cyclophosphamide, methotrexate, mizoribine, cisplatin, and cyclosporin, that are currently being used in or may be applied to antirheumatic therapy. None of the immunosuppressants examined in this study, except for a high dose of cisplatin, affected IL-1β-induced expression of adhesion molecules, including ICAM-1, VCAM-1, and E-selectin, on HUVECs (data not shown). However, $>10^{-6}$ M cisplatin was apparently cytotoxic to HUVECs, and this suppression could be ascribed to decreased viability of HUVECs and not to down-regulation of adhesion molecule expression. Stimulation with either TNF-α or LPS instead of IL-1β produced the same results (data not shown).

GST significantly suppressed ICAM-1 and VCAM-1 expression on HUVECs without affecting their viability. We next examined five DMARDs, i.e., sulfasalazopyrine, bucillamine, lobenzarit disodium, GST, and auranofin, in our assay. Among them, only GST down-regulated IL-1β-induced ICAM-1 expression on HUVECs in a dose-dependent manner without showing cytotoxicity (Fig. 2a). Cell viability was >98% by the trypan blue dye exclusion test, and this inhibition could not be explained by detachment of the cells from the culture plates (Fig. 2b). However, decreased viability of HUVECs was observed when $>10^{-4}$ M GST was used (70% viability, on average). Either TNF-α or LPS stimulation resulted in the same tendency (data not shown). No other DMARDs altered ICAM-1 expression. Cytokine-stimulated VCAM-1 expression was more profoundly suppressed with high concentrations of GST (Fig. 3). Furthermore, GST (10^{-5} M) significantly inhibited ICAM-1 and VCAM-1 mRNA expression of IL-1β-stimulated HUVECs (Fig. 4). However, cytokine-induced E-selectin expression was hardly affected by any of these DMARDs (Fig. 5).

GST inhibited the cellular binding of human monocytic cell lines to IL-1β-stimulated HUVECs. When U937 cells were co-cultured with nonstimulated HUVECs for 1 hr, their mutual binding was <3%. In contrast, U937 cells efficiently bound to IL-1β-stimulated HUVECs, with binding increasing up to 2-fold in a time-dependent manner. This binding was shown to be partly mediated by both ICAM-1 and VCAM-1 in our preliminary experiments, inasmuch as pretreatment of IL-1-stimulated HUVECs with monoclonal antibodies against either ICAM-1 or VCAM-1 partially inhibited their mutual binding (30% on average). GST dose-dependently inhibited this IL-1β-mediated enhancement of cellular binding (Fig. 6), although 4-hr incubation of HUVECs with GST (10^{-5} M) and



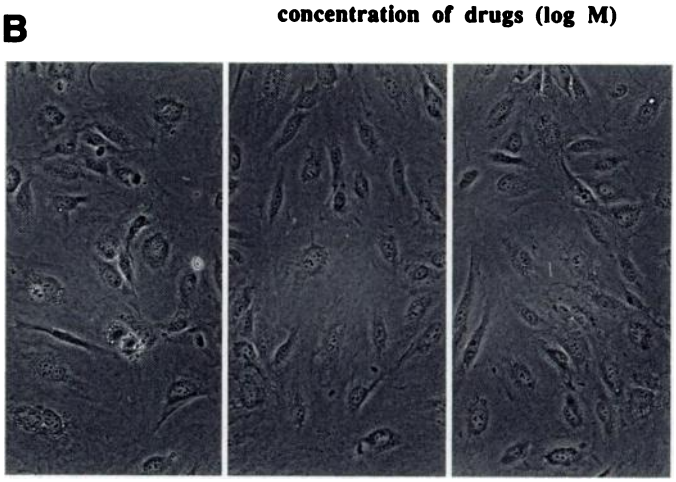


Fig. 2. a, Effect of DMARDs on IL-1β-stimulated ICAM-1 expression. Sulfasalazopyrine (*SASP*), bucillamine, lobenzarit disodium (*CCA*), GST, or auranofin was added to HUVECs together with IL-1β, and ICAM-1 expression was assayed by cell ELISA (five experiments). High concentrations of GST ($>10^{-5}$ M) inhibited IL-1β-induced ICAM-1 expression. *, $p < 0.01$. *Vertical axis*, percentage inhibition of IL-1-induced ICAM-1 expression; *bars*, standard errors. b, Cellular morphology of nonstimulated HUVECs (A) or IL-1-stimulated HUVECs cultured for 24 hr in the absence (B) or presence (C) of GST (10^{-5} M). No morphological changes were observed with GST treatment (magnification, ×100).

IL-1β did not induce significant inhibition (data not shown). The same tendency was observed when HL-60 cells were used instead of U937 cells (data not shown). Dexamethasone did not alter cellular binding between U937 cells and IL-1β-stimulated HUVECs.

Discussion

Our study demonstrated that GST down-regulated ICAM-1 and VCAM-1 expression on HUVECs not only at the protein level but also at the level of mRNA. However, E-selectin expression was hardly affected by GST. GST also suppressed cellular binding between human monocytic cell lines, such as U937 and HL-60 cells, and IL-1β-stimulated HUVECs.

Gold compounds have been used with marked efficacy as DMARDs in the treatment of patients with RA. They can alter the natural history of RA by protecting against joint destruction. They selectively accumulate in the lysosomes of macrophages, including type A synovial cells, resulting in their de-

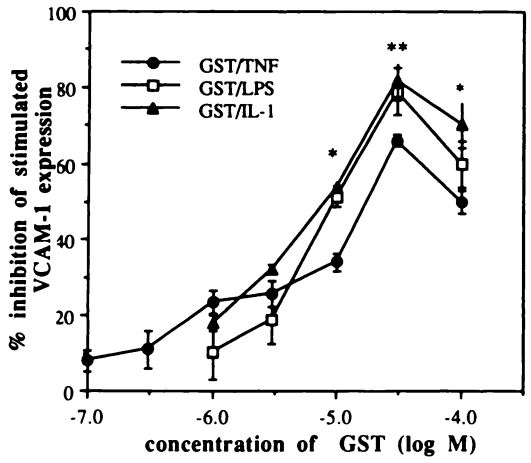


Fig. 3. Inhibition of VCAM-1 expression by GST. HUVECs were stimulated with either IL-1β (200 pg/ml), TNF-α (10 units/ml), or LPS (1 μg/ml) for 24 hr, in the presence or absence of various concentrations of GST, and VCAM-1 expression was measured by cell ELISA (five experiments). GST inhibited cytokine- or LPS-induced VCAM-1 expression in a dose-dependent manner. *, $p < 0.01$; **, $p < 0.001$. *Vertical axis*, percentage inhibition of stimulated VCAM-1 expression; *bars*, standard errors.

creased phagocytic capacity (20). Although it has been suggested that they act as immunoregulators, the molecular mechanisms of action remain to be clarified. Recent studies have indicated that GST inhibits adenylyl cyclase activity of lymphocyte membranes (21) and protein kinase C activity of both neutrophils (22) and T cells (23). However, there has been no *in vitro* study examining the effects of GST on adhesion molecule expression. Corkill *et al.* (24) recently reported that gold treatment of RA decreased synovial expression of E-selectin without affecting the degree of lymphocytic infiltration. However, it was not clear from their study whether gold acted directly on endothelial cells to inhibit E-selectin expression or modified augmented production of proinflammatory cytokines such as IL-1. In our study, GST inhibited IL-1β-induced, but not basal, expression of both ICAM-1 and VCAM-1 on HUVECs, suggesting that GST inhibits the transcription of both genes induced by IL-1β. In addition, only GST, and not auranofin, inhibited ICAM-1 and VCAM-1 expression in our experiments. Furthermore, our preliminary experiments showed that gold thioglucose did not show any inhibitory effect. It is therefore likely that the inhibitory effects of GST could be ascribed to thiomalate.

Adhesion of leukocytes to vascular endothelium is a crucial step in inflammatory cell infiltration in the lesion (25). Leukocyte adhesion to endothelial cells involves multiple receptor-ligand interactions, such as the interactions of LFA-1 with ICAM-1 and ICAM-2, Mac-1 with ICAM-1, and VLA-4 with VCAM-1, E-selectin, L-selectin (LAM-1), and CD44 (25). Homing receptors on lymphocytes and ligands (addressins) might also be involved in the interactions of the cells (26). The use of multiple receptor-ligand interactions amplifies the strength of adhesion and also involves a sequential process termed the "adhesion cascade" (27). GST significantly suppressed cellular binding between human monocytic cell lines, such as U937 and HL-60 cells, and IL-1β-stimulated HUVECs in our study. The rationale for using human monocytic lines for the binding assay is that one of the hallmarks of RA is the activation of the monocyte-macrophage system (28). Infiltra-

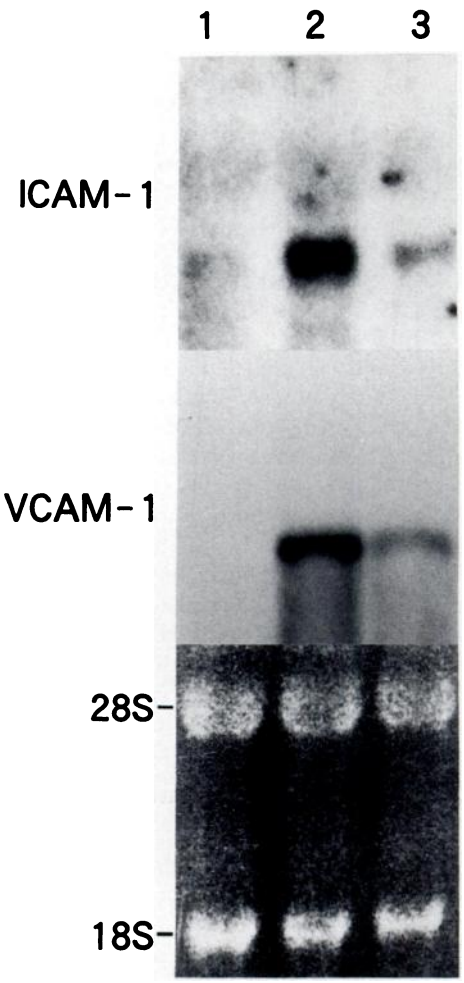


Fig. 4. Northern blot analysis of ICAM-1 and VCAM-1 mRNA. *Lane 1*, basal expression of either ICAM-1 or VCAM-1 is shown. *Lanes 2* and *3*, HUVECs were stimulated for 24 hr with 100 pg/ml IL-1β in the absence (*lane 2*) or presence (*lane 3*) of GST (10^{-5} M). RNA was extracted and the same amount of RNA (20 μg) was applied to each lane for Northern blot analysis, as confirmed by measurement of rRNA.

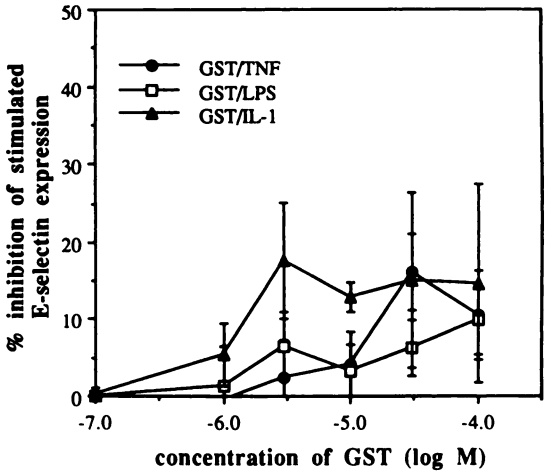


Fig. 5. Effect of GST on E-selectin expression. The same stimulants as shown in Fig. 3 were used to induce E-selectin expression (five experiments). *Vertical axis*, percentage inhibition of stimulated E-selectin expression; *bars*, standard errors.

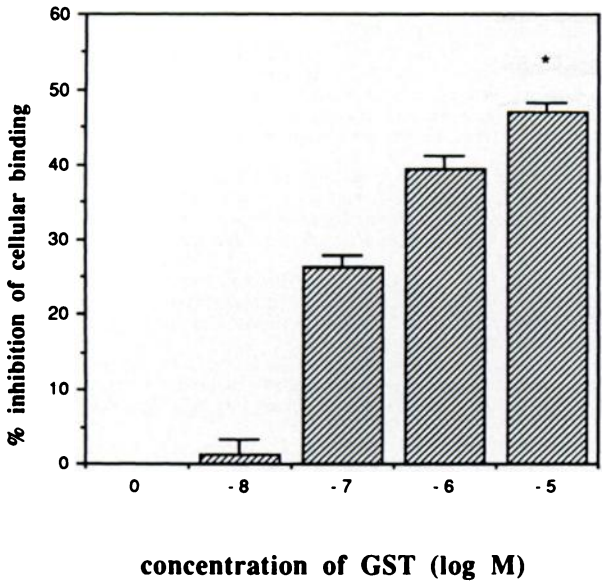


Fig. 6. Effect of GST on cellular binding between U937 cells and IL-1β-stimulated HUVECs. HUVECs were cultured for 48 hr with 100 pg/ml IL-1β, in the presence or absence of 10^{-5} M GST, and assayed for binding to U937 cells (five experiments). *Vertical axis*, percentage inhibition of cellular binding; *bars*, standard errors. *, $p < 0.01$.

tion of activated macrophages in RA synovium, resulting in constitutive production of inflammatory cytokines, can frequently be observed (29). Monocyte adhesion to cytokine-activated HUVECs is reported to be inhibited by various monoclonal antibodies against CD18, CD11b, CD11c, ICAM-1, VCAM-1, E-selectin, and P-selectin (30–32), suggesting that multiple pathways are operative in binding. We treated HUVECs with GST plus IL-1β for 48 hr before assay. This resulted in decreased binding, indicating that GST exerts its effect on HUVECs and not U937 cells or HL-60 cells. Furthermore, GST treatment of HUVECs for 4 hr did not produce significant inhibition, suggesting that the involvement of E-selectin is negligible. Regarding this aspect, 3-deazaadenosine, a structural analog of adenosine with immunomodulatory and anti-inflammatory activity, might have actions similar to those of GST (33, 34). This agent inhibited both the binding of polymorphonuclear cells to TNF-α-stimulated HUVECs and ICAM-1 synthesis by HUVECs (33). It also suppressed the binding of U937 cells to thrombin-stimulated human aortic endothelial cells and platelet-derived growth factor production by endothelial cells (34). E-selectin expression was reduced by 3-deazaadenosine, which is the difference between GST and 3-deazaadenosine (34). Further study will be necessary to clarify the adhesion molecules modulated by GST in the cellular binding between U937 cells and HUVECs.

ICAM-1 is strongly expressed not only on vascular endothelium but also on macrophage-like type A synovial cells and synovial fibroblasts in RA (9). Increased expression of E-selectin and VCAM-1 in RA synovium (10) and augmented binding of RA synovial T cells to these molecules have also been reported (11). These findings suggest that up-regulated expression of adhesion molecules in RA synovium plays an important role in the pathophysiology of RA. Furthermore, a recent study by Iigo *et al.* (35) clearly demonstrated that ICAM-1-dependent pathways are critically involved in the induction and progression of adjuvant arthritis in rats. In their study, *in vivo* administration of anti-ICAM-1 antibody effectively abrogated the development of adjuvant arthritis. Regulation of adhesion molecule expression has also been proven to be effective for treatment of allograft rejection (36) and airway eosinophilia in asthma (37). It is therefore possible that part of the therapeutic efficacy of GST could be ascribed to the down-regulation of adhesion molecules on vascular endothelial cells. We stress that the concentration range of GST used in this study is easily attainable both in serum and in synovial tissue of patients receiving GST injections (38).

Corticosteroids did not alter either ICAM-1, VCAM-1, or E-selectin expression on HUVECs in our assay. However, others have reported that corticosteroids down-regulate the expression of ICAM-1 on human carcinoma cells (39) and that of ICAM-1 and E-selectin on IL-1-stimulated HUVECs (40). Cronstein *et al.* (40) further mentioned that dexamethasone did not block ICAM-1 or E-selectin expression induced by TNF-α. The discrepancy between our findings and those of others may be explained by differences in the target cells used, the timing of cytokine treatment of the target cells, or the culture system used. It is also possible that corticosteroids inhibited cytokine production from target cells in their system, which resulted in the inhibition of adhesion molecule expression.

This is the first study to show that GST down-regulates adhesion molecule expression by HUVECs. The development of therapeutic agents to regulate the expression of adhesion molecules on vascular endothelium may create a new mode of treatment for intractable inflammatory diseases such as RA.

Acknowledgments

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