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# Effects of protein tyrosine kinase inhibitors on cytokine-induced adhesion molecule expression by human umbilical vein endothelial cells

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1 Endothelial cells can be stimulated by the pro-inflammatory cytokines interleukin (IL)-1α and tumour necrosis factor (TNF)α to express the leukocyte adhesion molecules E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 but the intracellular signalling mechanisms leading to this expression are incompletely understood. We have investigated the role of protein tyrosine kinases (PTK) in adhesion molecule expression by cytokine-activated human umbilical vein endothelial cells (HUVEC) using the PTK inhibitors genistein and herbimycin A, and the protein tyrosine phosphatase (PTP) inhibitor sodium orthovanadate.

2 Maximal E-selectin expression induced by incubation of HUVEC for 4 h with IL-1α (100 u $ml^{-1}$) and TNFα (100 u $ml^{-1}$) was dose-dependently inhibited by genistein and herbimycin A. Although similar effects were seen on phorbol 12-myristate, 13-acetate (PMA)-induced expression, this was not due to inhibition of protein kinase C (PKC) activity as the selective inhibitors of PKC, bisindolylmaleimide (BIM), Ro31-7549 or Ro31-8220 did not affect IL-1α- or TNFα-induced E-selectin expression at concentrations which maximally inhibited PMA-induced expression.

3 Genistein inhibited VCAM-1 expression induced by incubation of HUVEC for 24 h with TNFα or IL-1α whereas it did not affect ICAM-1 expression induced by 24 h incubation with either of these cytokines. Herbimycin A inhibited both VCAM-1 and ICAM-1 expression induced by TNFα.

4 Basal expression of E-selectin, VCAM-1 and ICAM-1 was dose-dependently enhanced by sodium orthovanadate. In contrast, vanadate differentially affected TNFα-induced expression of these molecules with maximal E-selectin and ICAM-1 expression being slightly enhanced and VCAM-1 expression dose-dependently reduced.

5 We also studied the effects of PTK and PTP inhibitors on adhesion of the human pre-myeloid cell line U937 to TNFα-stimulated HUVEC. Adhesion of U937 cells to HUVEC pretreated for 4 or 24 h with TNFα was dose-dependently inhibited by genistein and herbimycin A but unaffected by daidzein. Adhesion of U937 cells after 4 h was partially inhibited by blocking antibodies against both E-selectin and VCAM-1 but after 24 h was only inhibited by anti-VCAM-1.

6 Sodium orthovanadate had no effect on TNFα-induced U937 adhesion but dose-dependently enhanced adhesion to unstimulated HUVEC. Vanadate-induced adhesion was inhibited by an antibody against VCAM-1.

7 These results demonstrate that PTK-mediated phosphorylation events are important for the regulation of adhesion molecule expression by human endothelial cells, and additionally show that PTK inhibitors differentially affect upregulation of different adhesion molecules, implicating divergent regulatory pathways for cytokine-induced adhesion molecule expression.

**Keywords:** Adhesion molecules; cytokines; protein tyrosine kinases; protein tyrosine phosphatases; endothelium

## Introduction

Leukocyte adhesion to endothelial cells and migration into tissues at sites of inflammation requires the interaction of specific adhesion molecules expressed by leukocytes with complementary adhesion molecules expressed by the endothelium (reviewed Springer, 1994; Carlos & Harlan, 1994). Endothelial cells in culture can be stimulated by the cytokines interleukin (IL)-1α and tumour necrosis factor (TNF)α to express the adhesion molecules E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 which can mediate the adhesion and migration of leukocytes across cell monolayers (Dustin *et al.*, 1986; Pober *et al.*, 1986; Osborn *et al.*, 1989; Carlos *et al.*, 1990). Many *in vivo* studies have confirmed the role of these molecules in the recruitment of leukocytes from the blood into tissues (reviewed Carlos & Harlan, 1994) and together with elegant *in vitro* experiments, a multistep process of leukocyte/endothelial cell interactions has been described (Springer, 1994; Carlos & Harlan, 1994). First, leukocytes roll along the luminal surface of endothelial cells via low-affinity interactions between selectin molecules (including E-selectin) and their carbohydrate counter-ligands (Lawrence & Springer, 1993; Ley *et al.*, 1995). Recently VCAM-1 has also been shown to facilitate leukocyte rolling (Alon *et al.*, 1995). Following this, leukocytes firmly adhere to the endothelium via integrins, including $\alpha_4\beta_1$ (VLA-4) and $\alpha_L\beta_2$ (LFA-1), which recognize members of the Ig-superfamily of molecules (VCAM-1 and ICAM-1 respectively) (Dustin *et al.*, 1986; Chan *et al.*, 1992). Leukocytes are then activated by several mechanisms including crosslinking of surface receptors (Dustin & Springer, 1989), binding of chemokines such as MIP-1β or IL-8 (Kuijpers *et al.*, 1992; Tanaka *et al.*, 1993) or by the action of PAF expressed on the endothelial cell surface (Zimmerman *et al.*, 1990; Kuijpers *et al.*, 1992). This activation step leads to an increase in leukocyte integrin avidity and subsequent transendothelial migration (Dustin & Springer, 1989; Oppenheimer-Marks *et al.*, 1991; Hourihan *et al.*, 1993). Clearly, regulation of adhesion molecule expression by endothelial cells is a crucial step in this process and although the kinetics of expression of E-selectin,

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VCAM-1 and ICAM-1 are well documented (Dustin *et al.*, 1986; Pober *et al.*, 1986; Osborn *et al.*, 1989; Carlos *et al.*, 1990; Springer, 1994; Carlos & Harlan, 1994), the signalling mechanisms evoked by cytokines leading to their expression by endothelial cells are poorly understood.

Activation of rat endothelial cells with IL-1α causes increased levels of the intracellular second messenger cyclicAMP and this has been shown to regulate IL-1α-induced leukocyte binding and migration across these cells (Turunen *et al.*, 1990). In human umbilical vein endothelial cells (HUVEC), TNFα also enhances cyclicAMP levels (Galéa *et al.*, 1993), as does treatment with forskolin (Galéa *et al.*, 1993; Pober *et al.*, 1993), but forskolin treatment leads to inhibition of TNFα-induced E-selectin and VCAM-1 but not ICAM-1 expression (Pober *et al.*, 1993). In various cell types IL-1α and TNFα activate phosphatidylcholine-specific phospholipase C (PC-PLC) leading to the generation of inositol 1,4,5-triphosphate ($IP_3$) and 1,2-diacylglycerol (DAG) and subsequent activation of protein kinase C (PKC) (Schütze *et al.*, 1990; 1994). In bovine pulmonary artery endothelial cells TNFα-induced PKC activation (Ferro *et al.*, 1993) leads to decreased nitric oxide secretion (Johnson *et al.*, 1994) and it has been proposed that PKC plays a role in the increased leukocyte adhesion to HUVEC induced by IL-1 and TNFα (Magnuson *et al.*, 1989). However, although direct activation of PKC with PMA upregulates the expression of E-selectin, VCAM-1 and ICAM-1 by endothelial cells (Lane *et al.*, 1989; Mattila *et al.*, 1992; Deisher *et al.*, 1993a,b), TNFα- and IL-1α-induced expression of E-selectin and ICAM-1 occur independently of PKC activation (Ritchie *et al.*, 1991; Myers *et al.*, 1992). In contrast, PKC activation has been proposed to be involved in TNFα-induced VCAM-1 expression (Deisher *et al.*, 1993a,b).

A separate class of protein kinase enzymes known to be activated by IL-1α and TNFα are the protein tyrosine kinases (PTK) (Fuortes *et al.*, 1993; Joshi-Barve *et al.*, 1993; Marczin *et al.*, 1993; Corbett *et al.*, 1994; Lee *et al.*, 1994; Tiisala *et al.*, 1994). Studies in which selective pharmacological inhibitors of PTK have been used have shown that these enzymes are involved in a range of responses to these cytokines in many cells including NO synthesis in smooth muscle cells (Marczin *et al.*, 1993) and β-cells prepared from islets of Langerhans (Corbett *et al.*, 1994), mitogen-activated protein kinase (MAPK) activation in keratinocytes (Lee *et al.*, 1994), NF-κB activation in melanoma cells (Joshi-Barve *et al.*, 1993) and adhesion of neutrophils (Fuortes *et al.*, 1993; Tiisala *et al.*, 1994). In endothelial cells, studies with PTK inhibitors have demonstrated a role for these enzymes in cell proliferation (Koroma & de Juan, 1994), IL-1α-induced endothelin production (Katabami *et al.*, 1992) and TNFα-induced plasminogen activator inhibitor (PAI)-1 production (van Hinsbergh *et al.*, 1994). Inhibition of PTK activity has also been shown to inhibit the adhesion of neutrophils, monocytes and lymphocytes to TNFα-treated endothelial cells although the mechanism of this inhibition has not been investigated (McGregor *et al.*, 1994).

In this study we have used cultured HUVEC to investigate the role of PTK in TNFα- and IL-1α-induced expression of E-selectin, VCAM-1 and ICAM-1 with the PTK inhibitors genistein (Akiyama *et al.*, 1987) and herbimycin A (Uehara *et al.*, 1989). We have also examined the role of protein tyrosine phosphatases (PTP) by using the PTP inhibitor sodium orthovanadate (Swarup *et al.*, 1982). In addition, we have studied the effects of these inhibitors on cytokine-induced adhesion of the pre-myeloid cell line U937 to HUVEC.

## Methods

### *Cell culture*

Unless otherwise noted, all cell culture reagents were purchased from Sigma. Endothelial cells from human umbilical veins were isolated as previously described (Gimbrone *et al.*, 1974). Primary cultures of HUVEC were grown in M199 medium supplemented with 10% foetal calf serum (FCS), 10% newborn calf serum, 4 mM glutamine, 100 u $ml^{-1}$ penicillin, 100 u $ml^{-1}$ streptomycin and 20 mM $NaHCO_3$ at 37°C in 5% $CO_2$/95% air atmosphere in 25 $mm^2$ tissue culture flasks (Becton Dickinson UK.Ltd., Plymouth) which had been pre-coated with 1% gelatin. When confluent ($10^6$ cells/flask), primary cultures were trypsinized with phosphate buffered saline (PBS) containing 0.1% trypsin/0.025% EDTA, plated into 75 $cm^2$ tissue culture flasks (Becton Dickinson UK.Ltd.) and grown in the above medium containing 90 μg $ml^{-1}$ heparin and 20 μg $ml^{-1}$ endothelial cell growth factor. For experiments, confluent first passage HUVEC ($3 \times 10^6$ cells/flask) were plated into the middle 60 wells of 96-well tissue culture trays (6 mm diameter; Becton Dickinson UK.Ltd) at a cell density of $1.7 \times 10^4$/well in growth factor-containing medium. To prevent evaporation during incubation, 200 μl of PBS were placed in each of the outer wells of the plates. All experiments were performed with these second passage HUVEC up to 120 h after reaching confluence. The human pre-myeloid cell line U937, a gift from Dr Lindsey Needham, British Biotechnology Ltd. (Oxford), was grown in RPMI medium supplemented with 5% FCS, 4 mM glutamine, 100 u $ml^{-1}$ penicillin, 100 u $ml^{-1}$ streptomycin and 20 mM $NaHCO_3$ at 37°C in 5% $CO_2$/95% air atmosphere. U937 cells were maintained in suspension culture and fed every two days.

### *Cell viability assay*

The viability of HUVEC after treatment with cytokines, phorbol esters, PKC- and PTK-inhibitors or vanadate was determined with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) by using a modification of a method described by Mosmann (Mosmann, 1983). Briefly, 10 μl of MTT (5 mg $ml^{-1}$ in PBS) was added to 100 μl of medium in each well of a 96-well plate containing a confluent monolayer of HUVEC and incubated at 37°C in 5% $CO_2$/95% air for 2–3 h. The formazan crystals formed were then solubilized by adding 100 μl of 0.01 M HCl/10% SDS to each well and incubating at 37°C for a further 4 h. Absorbance was measured at 540 nm by a Titertek Multiskan PLUS MkII microtitre reader (ICN Flow, Irvine).

### *Enzyme linked immunosorbent assays (ELISA) to measure adhesion molecule expression*

Expression of E-selectin, ICAM-1 or VCAM-1 by confluent monolayers of HUVEC in 96-well tissue culture trays was measured by ELISA. Cells were washed three times with prewarmed (37°C) PBS'B' (PBS containing 20 mg $ml^{-1}CaCl_2$ and 26.4 mg $ml^{-1}MgCl_2$) and then fixed with cold glutaraldehyde (0.1% v/v in PBS) for 10 min at 4°C. Brief fixation does not alter leukocyte adhesion to cytokine treated endothelial cells (eg. Bevilacqua *et al.*, 1985). After a further three washes with PBS, plates were either used immediately or air dried overnight and stored for up to one week at 4°C. Cells were incubated for 60 min in four changes of a blocking solution of PBS containing 10% powdered milk (Marvel). This was followed by incubation for 60 min with 1 μg $ml^{-1}$ of primary antibody diluted in blocking solution. Cells were then washed (five × 5 min) with blocking solution and incubated a further 60 min with a 1:5000 dilution of the secondary antibody (peroxidase conjugated goat anti-mouse IgG). Cells were washed four times with PBS and then incubated with substrate (0.36 mg $ml^{-1}$o-phenylenediamine [OPD]; Sigma) in buffer (pH 5, containing 7.3 g $l^{-1}$citric acid and 23.8 g $l^{-1}$ $Na_2HPO_412H_2O$) and 0.0002% $H_2O_2$ (v/v). Substrate was developed and absorbance (at 450 nm) measured at intervals between 5 and 30 min after incubation. Sample wells were compared with wells incubated in the absence of primary and secondary antibodies (blanks) and the results calculated as means ± s.e.mean of triplicate observations from three separate experiments.

### U937 adhesion assay

The adhesion assays were performed as previously described (Simmons & Needham, 1991). Briefly, U937 cells were radiolabelled in suspension for 24 h with 0.075 $\mu$Ci [$^3$H]-thymidine per $2 \times 10^5$ cells ([methyl-$^3$H]-thymidine, 5 Ci mmol$^{-1}$, Amersham International plc, Amersham). The cells were then washed twice by adding 200 $\mu$l/well of pre-warmed PBS containing 2% FCS and then incubated at 37°C for 15 min in assay medium (RPMI containing 2% FCS, 4 mM glutamine, 100 u ml$^{-1}$ penicillin, 100 u ml$^{-1}$ streptomycin, 20 mM $NaHCO_3$ and 5 mM HEPES). HUVEC monolayers in 96-well trays were also pre-incubated at 37°C in assay medium before addition of 100 $\mu$l of radiolabelled U937 cells ($2 \times 10^6$ ml$^{-1}$) to each well. For assays in which blocking antibodies were included, HUVEC were pre-incubated for 1 h at room temperature with 100 $\mu$l assay medium containing antibodies at a final concentration of 50 $\mu$g ml$^{-1}$ and U937 cells were pre-incubated at 37°C for 30 min in assay medium containing 20 mg ml$^{-1}$ Gammagard to prevent binding via Fc receptors. HUVEC and U937 cells were incubated together for 40 min at 37°C in 5% $CO_2$/95% air and then unbound U937 cells were removed by a standardized washing procedure (five times with pre-warmed PBS containing 2% FCS). The HUVEC monolayers and adherent U937 cells were then solubilized in 100 $\mu$l 90% formic acid per well, suspended in Optiphase 'Safe' (LKB, Loughborough) liquid scintillant and counted on a Packard Canberra TRI-CARB 1900 TR $\beta$-counter. Results were calculated as either the percentage of radioactivity plated (% U937 adhesion) or as a percentage of the maximally induced level of adhesion (% maximal U937 adhesion).

### Reagents

The human recombinant cytokines IL-1$\alpha$ and TNF$\alpha$ were purchased from R&D Systems Ltd. (Oxford). The phorbol esters phorbol 12-myristate, 13-acetate (PMA) and 4$\alpha$-phorbol 12,13-didecanoate ($\alpha$PDD) were from Sigma (Poole). The monoclonal antibodies to E-selectin (BBIG-E4), ICAM-1 (BBIG-11) and VCAM-1 (BBIG-VI) were purchased from R&D Systems Ltd., an isotype matched (IgG1) control antibody was purchased from DAKO Ltd. (High Wycombe), the peroxidase-conjugated goat anti-mouse IgG secondary antibody was purchased from Pierce (Rockford, IL, U.S.A.) and Gammagard human immunoglobulin was from Baxter (Lessines, Belgium). The PKC inhibitors, 3-[1-(3-aminopropyl)-3-indolyl]-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione hydrochloride (Ro31-7549) and 3-[1-[3-(amidinothio)propyl]-3-indolyl]-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione mesylate (Ro31-8220) (Davis *et al.*, 1989) were generously provided by Dr Trevor Hallam (Roche Products Ltd.). 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7) and bisindolylmaleimide (BIM) were from Calbiochem (La Jolla, CA, U.S.A.) as were genistein, daidzein, herbimycin A and methyl 2,5-dihydroxy cinnamate (MDHC). Sodium orthovanadate was purchased from Sigma.

### Statistical analysis

Statistical analysis of the dose-dependent effects of inhibitors on cytotoxicity, adhesion molecule expression and U937 cell adhesion was carried out on raw data by use of the Peritz F multiple means comparison test (Harper, 1984). Student's *t* test on unpaired raw data was used to compare means of groups in antibody blocking experiments. $P<0.05$ was considered statistically significant.

## Results

### High concentrations of tyrosine kinase inhibitors are cytotoxic for HUVEC

In this study HUVEC were treated for either 4 or 24 h with the tyrosine kinase inhibitors herbimycin A or genistein or daidzein, a structural analogue of genistein with much lower inhibitory activity for tyrosine kinases (Akiyama *et al.*, 1987). To determine whether these compounds were cytotoxic for HUVEC we employed a viability assay using MTT as previously described (Mosmann, 1983). As shown in Figure 1 treatment of HUVEC for either 4 or 24 h with 100 $\mu$M genistein, 100 $\mu$M daidzein or 10 $\mu$M herbimycin A did not affect cell viability. Similarly, treatment with up to 400 $\mu$M daidzein or 20 $\mu$M herbimycin A for 4 h had no cytotoxic effects. Genistein at concentrations of 200 $\mu$M and above was cytotoxic after both 4 and 24 h of treatment as were similar concentrations of daidzein after 24 h. Cytotoxic effects of herbimycin A were only observed after 24 h with the highest concentration tested (20 $\mu$M) although this effect was not significantly different from the effects of vehicle alone. In light of these data, the maximal concentrations of inhibitors used in all subsequent experiments were 100 $\mu$M for genistein and daidzein and 10 $\mu$M for herbimycin A.

### Genistein and herbimycin A inhibit IL-1$\alpha$-, TNF$\alpha$- and PMA-induced E-selectin expression

At rest, HUVEC did not express E-selectin. Incubation with either 100 u ml$^{-1}$ IL-1$\alpha$, 100 u ml$^{-1}$ TNF$\alpha$ or 100 nM PMA (but not 100 nM $\alpha$PDD) time-dependently induced E-selectin expression with maximal effects occurring at 4–6 h and expression returning to basal levels after 24 h (data not shown). When HUVEC were pre-incubated with a range of concentrations of genistein or herbimycin A for 15 min followed by 4 h incubation with either IL-1$\alpha$, TNF$\alpha$ or PMA in the continued presence of the inhibitor, E-selectin expression was dose-dependently inhibited (Figure 2). The dose-dependence of inhibition of IL-1$\alpha$-(Figure 2a), TNF$\alpha$- (Figure 2b) and PMA-(Figure 2c) induced E-selectin expression was similar. Daidzein did not affect E-selectin expression induced by IL-1$\alpha$ or TNF$\alpha$ at concentrations up to 100 $\mu$M (30 $\mu$g ml$^{-1}$); however, 100 $\mu$M daidzein partially inhibited PMA-induced expression (24 $\pm$ 6% inhibition, $P<0.001$). An additional tyrosine kinase inhibitor, methyl-2,5-dihydroxy-cinnamate (MDHC), which is a stable analogue of erbstatin (Umezawa *et al.*, 1990) failed to inhibit IL-1$\alpha$-, TNF$\alpha$- or PMA-induced E-selectin expression at concentrations up to 20 $\mu$M (data not shown). At concentrations exceeding 20 $\mu$M MDHC was cytotoxic. None of the PTK inhibitors tested induced expression of E-selectin in the absence of TNF$\alpha$ or PMA (data not shown).

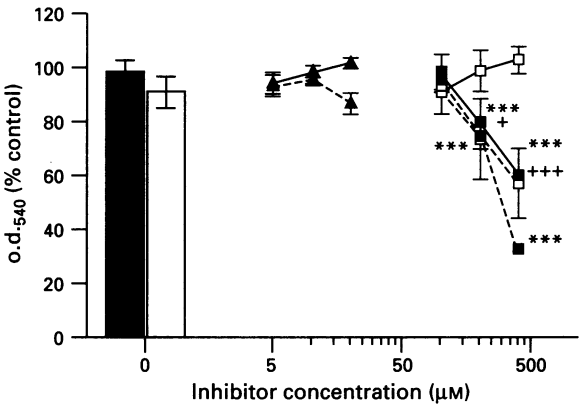


**Figure 1** Cytotoxicity of tyrosine kinase inhibitors measured with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). HUVEC were treated with a range of concentrations of either genistein (■), daidzein (□) or herbimycin A (▲) for 4 (solid lines) or 24 (dashed lines) h. Cytotoxicity was measured with MTT as described in Methods section. Data were calculated as a percentage of o.d.$_{540}$ in the absence of inhibitors (solid bar) and are means $\pm$s.d. ($n=3$) from one experiment representative of three performed. $^{+}P<0.05$; $^{+++}$/***$P<0.001$ compared with vehicle alone (open column) for genistein (*) and daidzein ($^{+}$).

Inhibition of PMA-induced E-selectin expression by genistein and herbimycin A suggests that they may act, at least in part, via inhibition of PKC. In an *in vitro* PKC assay we found that 100 $\mu$M genistein significantly inhibited (31 ± 9%) phospholipid-dependent kinase activity; however, the selective inhibitors of PKC, bisindolylmaleimide (BIM), Ro31-7549 and Ro31-8220, did not affect IL-1α- or TNFα-induced E-selectin expression at concentrations which maximally inhibited PMA-induced expression (Table 1). One exception was the non-selective PKC/PKA inhibitor H7 which inhibited PMA-, IL-1α- and TNFα- induced expression equally (Table 1). Downregulation of PKC activity by treatment of HUVEC for 48 h with PMA prevented re-induction of E-selectin expression by PMA (Table 2). Pretreatment with the inactive PMA analogue αPDD did not affect the subsequent expression induced by PMA or IL-1α (data not shown). PMA pretreatment did not

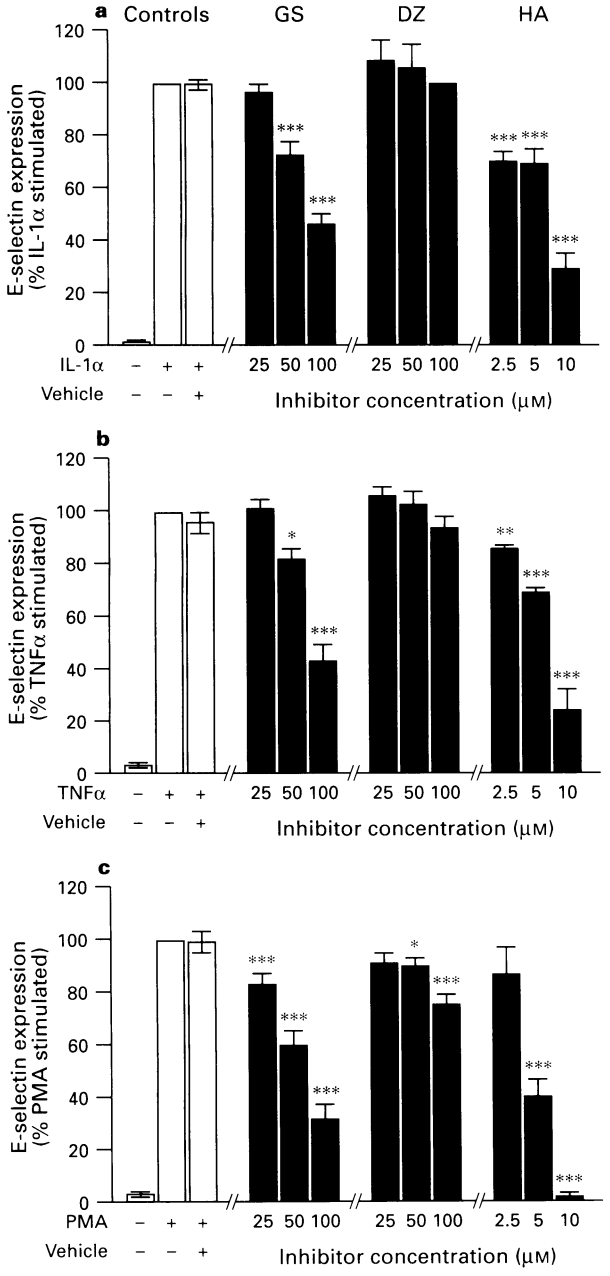


**Figure 2** Effects of tyrosine kinase inhibitors on interleukin-1α (IL-1α)-, tumour necrosis factorα (TNFα)- and phorbol 12-myristate, 13-acetate (PMA)-induced E-selectin expression. HUVEC were treated for 15 min with either genistein (GS), daidzein (DZ) or herbimycin A (HA) at the concentrations shown and then incubated for a further 4 h with either 100 u ml$^{-1}$ IL-1α (a), 100 u ml$^{-1}$ TNFα (b) or 100 nM PMA (c) in the continued presence of inhibitors. E-selectin expression was measured by ELISA. In separate experiments the o.d. values for basal E-selectin expression ranged from 0.01–0.04 and the values for cytokine- and PMA-induced maximal expression ranged from 0.38–0.75. The inactive PMA analogue αPDD did not induce E-selectin expression (data not shown). The data are presented as percentage of maximally induced expression in the absence of inhibitors and represent means ± s.e.mean from three experiments. $*P<0.05$; $**P<0.01$; $***P<0.001$ compared with maximal expression induced by IL-1α, TNFα or PMA in the presence of vehicle (0.1% DMSO).

**Table 1** Effects of protein kinase C (PKC) inhibitors on E-selectin expression induced by phorbol 12-myristate, 13-acetate (PMA), interleukin-1α (IL-1α) or tumour necrosis factor α (TNFα)

| | | *E-selectin expression (% maximal)* | | |
|---|---|---|---|---|
| *Inhibitor* | *Conc.* | *PMA* | *IL-1α* | *TNFα* |
| BIM | 1 $\mu$M | 24 ± 2 | 99 ± 7 | 102 ± 7 |
| | 10 $\mu$M | 6 ± 2 | 100 ± 4 | 97 ± 6 |
| Ro31-7549 | 1 $\mu$M | 102 ± 3 | 104 ± 7 | 95 ± 6 |
| | 10 $\mu$M | 20 ± 4 | 100 ± 9 | 107 ± 3 |
| Ro31-8220 | 10 nM | 44 ± 5 | 95 ± 4 | ND |
| | 100 nM | 6 ± 1 | 95 ± 1 | 96 ± 8 |
| H7 | 25 $\mu$M | 57 ± 16 | 83 ± 6 | 53 ± 7 |
| | 50 $\mu$M | 9 ± 1 | 16 ± 4 | 6 ± 3 |
| | 100 $\mu$M | 7 ± 2 | 2 ± 1 | 1 ± 1 |

HUVEC were treated for 15 min with either bisindolylmaleimide (BIM), Ro31-7549, Ro31-8220 or H7 at the concentrations shown and then incubated for a further 4 h with 100 nM PMA, 100 u ml$^{-1}$ IL-1α or 100 u ml$^{-1}$ TNFα in the continued presence of inhibitors. E-selectin expression was measured by ELISA. In separate experiments the o.d. readings for basal E-selectin expression ranged from 0.01–0.05 and for IL-1α-, TNFα- and PMA-induced expression ranged from 0.83–1.15. The data are presented as percentage of maximally induced expression in the presence of vehicle. No effects of vehicle (DMSO for BIM, Ro31-7549 and Ro31-8220; distilled water for H7) were observed and at the concentrations used, none of these inhibitors was cytotoxic (data not shown). Data are means ± s.d. ($n = 3$) from one experiment representative of at least three performed with each inhibitor.

**Table 2** Effects of protein kinase C (PKC) downregulation on subsequent inhibition of tumour necrosis factor α (TNFα)-induced E-selectin expression by genistein or H7

| | | *E-selectin expression (% maximal) 48 hours treatment* | |
|---|---|---|---|
| *4 hour treatment* | | *Medium* | *PMA* |
| Untreated ($n = 6$) | | 11 ± 5 | 10 ± 4 |
| PMA ($n = 6$) | | 50 ± 6 | 13 ± 5** |
| TNFα + GS | 50 $\mu$M | 76 ± 7 | 86 ± 4 |
| | 100 $\mu$M | 46 ± 5 | 54 ± 3 |
| TNFα + DZ | 50 $\mu$M | 91 ± 3 | 94 ± 5 |
| | 100 $\mu$M | 84 ± 2** | 93 ± 0 |
| TNFα + H7 | 25 $\mu$M | 52 ± 11 | 56 ± 11 |
| | 50 $\mu$M | 19 ± 6 | 14 ± 5 |

HUVEC which had been incubated for 48 h with either medium alone or 100 nM PMA were treated for a further 4 h with either 100 nM PMA or 100 u ml$^{-1}$ TNFα in the presence of genistein (GS), daidzein (DZ) or H7 at the concentrations shown. E-selectin expression was measured by ELISA. The o.d. readings for basal expression ranged from 0.01–0.08 and for maximal expression ranged from 0.31–1.13. The data are presented as a percentage of maximal TNFα- induced expression in the absence of inhibitors and represent means ± s.e.mean from three separate experiments. $**P<0.01$ comparing data from medium and PMA pretreated cells. For key to abbreviations used see Table 1.

affect the ability of either genistein or H7 to inhibit subsequent TNFα-induced E-selectin expression. As shown in Table 2, there were no significant differences observed in the inhibitions of TNFα-induced expression by genistein or H7 between untreated or PMA-treated cells. Daidzein did not affect TNFα-induced E-selectin expression on PMA-treated HUVEC although slight inhibition was observed with 100 μM daidzein on untreated HUVEC (16±2% inhibition, $P<0.01$).

### *Genistein inhibits VCAM-1 but not ICAM-1 expression induced by TNFα and IL-1α*

As previously described, untreated HUVEC expressed little or no VCAM-1 but did express significant levels of ICAM-1 (Pober *et al.*, 1986; Dustin *et al.*, 1986; Osborn *et al.*, 1989; Carlos *et al.*, 1990). Expression of these molecules was dose- and time-dependently increased by TNFα with maximal levels observed after 24 h incubation with 100 u ml$^{-1}$ TNFα (data not shown). Preincubation of HUVEC for 15 min with genistein followed by incubation for a further 24 h with TNFα resulted in significant inhibition of VCAM-1 (34±5% inhibition, $P<0.01$; Figure 3a) but not ICAM-1 (Figure 3b) expression. Similar inhibition by 100 μM genistein of VCAM-1 (30±9%, $P<0.01$) but not ICAM-1 expression was observed when HUVEC were treated with 100 u ml$^{-1}$ IL-1α (Table 3). Daidzein had no effect on either VCAM-1 or ICAM-1 expression at concentrations up to 100 μM.

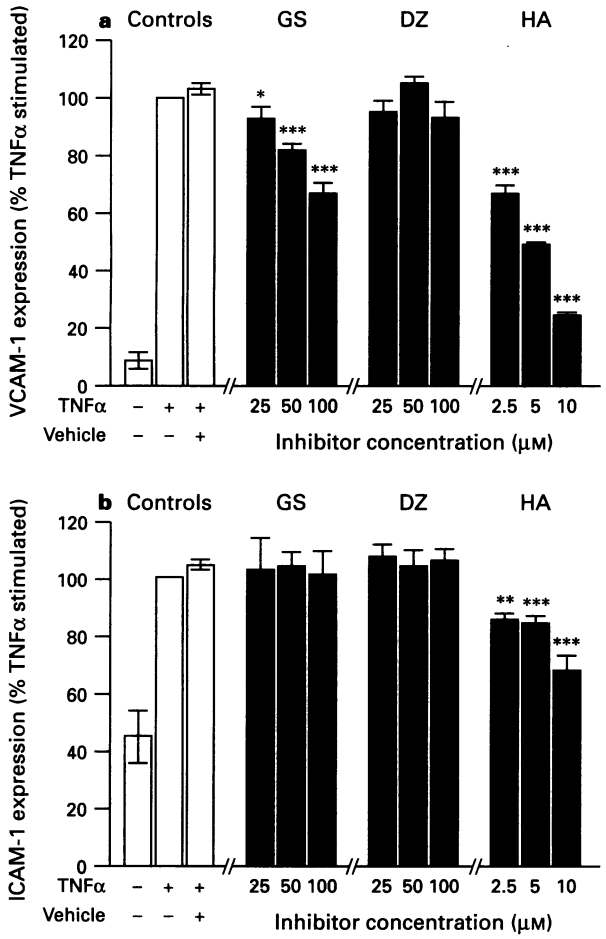


**Figure 3** Effects of tyrosine kinase inhibitors on tumour necrosis factorα (TNFα)-induced VCAM-1 and ICAM-1 expression. HUVEC were treated for 15 min with either genistein (GS), daidzein (DZ) or herbimycin A (HA) at the concentrations shown and then incubated for a further 24 h with 100 u ml$^{-1}$ TNFα in the continued presence of inhibitors. VCAM-1 (a) and ICAM-1 (b) expression were measured by ELISA. The o.d. readings for basal VCAM-1 and ICAM-1 expression ranged from 0.02–0.08 and 0.28–0.79 respectively. Maximal TNFα-induced expression had o.d. readings ranging from 0.22–0.61 for VCAM-1 and 0.83–1.41 for ICAM-1. The data are presented as percentage of maximally induced expression in the absence of inhibitors and represent means±s.e.mean from three experiments performed for each adhesion molecule. *$P<0.05$; ***$P<0.001$ compared with maximal expression in the presence of TNFα+ vehicle.

In contrast to the selective effect of genistein, herbimycin A potently inhibited both VCAM-1 and ICAM-1 expression induced by TNFα (Figure 3). Inhibition of VCAM-1 and ICAM-1 expression was observed with 2.5 μM herbimycin A and at 10 μM, expression of either molecule in the presence of TNFα was not significantly different from basal levels. Basal expression of VCAM-1 or ICAM-1 was unaffected by either genistein, daidzein or herbimycin A (data not shown).

### *The tyrosine phosphatase inhibitor sodium orthovanadate differentially regulates expression of E-selectin, VCAM-1 and ICAM-1*

To investigate further the role of tyrosine phosphorylation in adhesion molecule expression we examined the effects of the tyrosine phosphatase inhibitor sodium orthovanadate on expression of E-selectin, VCAM-1 and ICAM-1. We first attempted to use peroxyvanadate which enters cells more rapidly than sodium orthovanadate but this was toxic for HUVEC at the incubation times and concentrations tested (data not shown). As shown in Figure 4a incubation for 4 h with a range of concentrations of sodium orthovanadate dose-dependently induced E-selectin expression by HUVEC. Maximal expression was observed with 50 μM (10 μg ml$^{-1}$) vanadate but the level of expression was only 41±12% of that induced by 100 u ml$^{-1}$ TNFα. When HUVEC were co-incubated with vanadate and TNFα, statistically significant increased E-selectin expression was only observed with 25 μM (110±1%, $P<0.01$) vanadate. Incubation of HUVEC for 4 h with concentrations of vanadate higher than 200 μM was cytotoxic (data not shown).

Treatment of HUVEC with vanadate for 24 h dose-dependently induced basal VCAM-1 expression and enhanced basal ICAM-1 expression (Figure 4b and c). Maximal effects on VCAM-1 expression were observed with 12.5 μM vanadate and at this concentration expression was 22±2% of that induced by TNFα. Maximum effects on ICAM-1 expression were seen with 50 μM vanadate which increased expression from a basal level (56±4% of TNFα-induced expression) to 87±1%. Co-incubation of HUVEC for 24 h with vanadate and 100 u ml$^{-1}$ TNFα had opposite effects on maximal levels of VCAM-1 and ICAM-1 expression. As observed with maximal E-selectin expression (Figure 4a), vanadate slightly enhanced maximal

**Table 3** Effects of genistein and daidzein on interleukin-1α (IL-1α)-induced VCAM-1 and ICAM-1 expression

| | | *% maximal expression* | |
|---|---|---|---|
| *Inhibitor* | *Conc.* | *VCAM-1* | *ICAM-1* |
| Genistein | 50 μM | 81±6* | 106±2 |
| | 100 μM | 70±9** | 101±6 |
| Daidzein | 50 μM | 95±4 | 107±3 |
| | 100 μM | 99±7 | 103±4 |

HUVEC were treated for 24 h with 100 u ml$^{-1}$ IL-1α in the presence of inhibitors and expression of VCAM-1 and ICAM-1 was measured by ELISA. In separate experiments the o.d. values for basal VCAM-1 and ICAM-1 ranged from 0.02–0.04 and 0.42–0.57 respectively. Maximal o.d. readings for IL-1α-induced VCAM-1 and ICAM-1 expression ranged from 0.25–0.58 and 1.02–1.44 respectively. Data were calculated as a percentage of maximal expression in the absence of inhibitors. Corresponding dilutions of vehicle alone (DMSO) did not effect VCAM-1 or ICAM-1 expression (data not shown). Data are presented as means of six observations from two separate experiments ±s.e.mean. *$P<0.05$, **$P<0.01$ compared with maximal expression in the presence of vehicle.

ICAM-1 expression (Figure 4c) but significant effects were only observed with 50 $\mu$M ($112 \pm 1\%$, $P<0.05$). In contrast, TNF$\alpha$-induced VCAM-1 expression was dose-dependently reduced by vanadate such that in the presence of 50 $\mu$M vanadate, expression was $82 \pm 9\%$ of the maximal level. Treatment of HUVEC for 24 h with concentrations of vanadate greater than 50 $\mu$M was cytotoxic (data not shown).

### *Genistein and herbimycin A inhibit TNFα-induced U937 cell adhesion to HUVEC*

Having established a role for tyrosine kinases in the regulation of adhesion molecule expression by endothelial cells, we examined the effects of genistein, daidzein and herbimycin A on adhesion of the monocytic cell line U937 to TNF$\alpha$-treated HUVEC. In twenty separate experiments U937 adhesion to untreated HUVEC was between 3 and 15% of the total number of cells added. Incubation of HUVEC for 4 or 24 h with TNF$\alpha$ increased adhesion to between 36% and 54% of added cells. None of the inhibitors affected adhesion to untreated HUVEC (data not shown). Figure 5a shows that maximal adhesion induced by TNF$\alpha$ at 4 h was dose-dependently inhibited by pre-incubation of HUVEC with either genistein or herbimycin A whereas daidzein had no effect at concentrations up to 100 $\mu$M. In the presence of 100 $\mu$M genistein and 10 $\mu$M herbimycin A adhesion was $65 \pm 6\%$ and $49 \pm 7\%$ respectively of that induced by TNF$\alpha$ alone. After 24 h, daidzein did not affect TNF$\alpha$-induced U937 cell adhesion but 100 $\mu$M genistein and 10 $\mu$M herbimycin A inhibited adhesion by $21 \pm 9$ and $19 \pm 1\%$, respectively (Figure 5b).

Previous studies have shown that U937 cells adhere to E-selectin and VCAM-1 expressed by cytokine-treated HUVEC (Simmons & Needham, 1991). We have used available blocking antibodies to investigate the contribution of E-selectin and VCAM-1 to U937 cell adhesion to HUVEC after treatment for 4 and 24 h with 100 u ml$^{-1}$ TNF$\alpha$ (Figure 6). Pre-incubation of HUVEC with anti-E-selectin inhibited TNF$\alpha$-induced U937 cell adhesion after 4 h by $28 \pm 10\%$ but did not affect adhesion after 24 h. In contrast, anti-VCAM-1 inhibited adhesion after both 4 ($23 \pm 1\%$ inhibition) and 24 ($35 \pm 3\%$ inhibition) h. A combination of both antibodies inhibited adhesion at 4 h to a

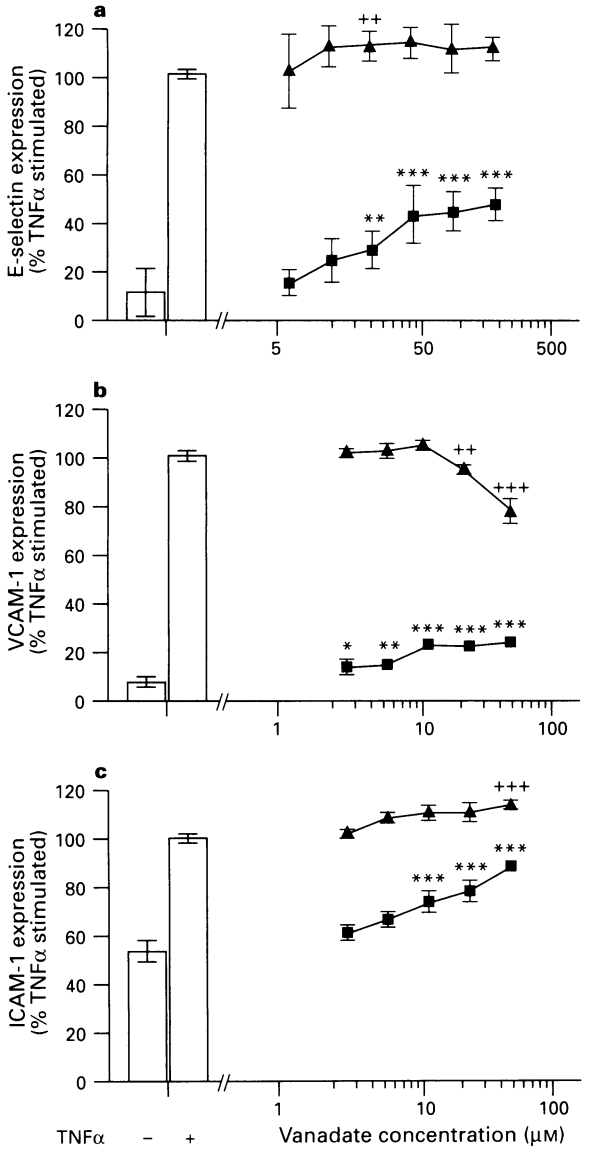


**Figure 4** Dose-dependent effects of vanadate on adhesion molecule expression by untreated and tumour necrosis factor$\alpha$ (TNF$\alpha$)-treated HUVEC. HUVEC were treated for 15 min with a range of concentrations of vanadate and then incubated for a further 4 (a) or 24 (b and c) h with either medium alone (■) or 100 u ml$^{-1}$ TNF$\alpha$ (▲) in the continued presence of vanadate. E-selectin (a), VCAM-1 (b) and ICAM-1 (c) expression were measured by ELISA. The o.d. readings for basal expression of the adhesion molecules ranged from 0.01–0.10 for E-selectin, 0.02–0.08 for VCAM-1 and 0.31–0.84 for ICAM-1 and the maximum TNF$\alpha$-induced values were 0.44–0.75, 0.45–0.80 and 0.74–1.20 for each molecule respectively. The data are presented as a percentage of TNF$\alpha$-induced expression and represent means ± s.e.mean from three experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ compared with basal expression (no TNF$\alpha$) and $^{++}P<0.05$; $^{+++}P<0.001$ compared with TNF$\alpha$-induced expression.

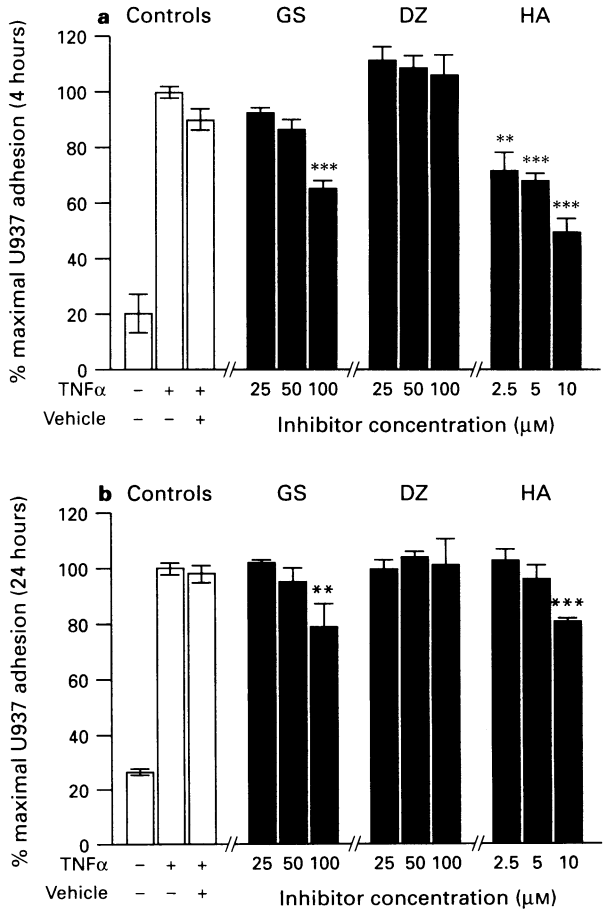


**Figure 5** Effects of tyrosine kinase inhibitors on U937 cell adhesion to tumour necrosis factor$\alpha$ (TNF$\alpha$)-treated HUVEC. Cells were treated with inhibitors and TNF$\alpha$ for 4 (a) or 24 (b) h as described for Figure 2 and U937 cell adhesion was measured as described in the Methods section. The data are presented as percentage of maximally induced adhesion in the absence of inhibitors and represent means ± s.e.mean from three experiments. $^{**}P<0.01$; $^{***}P<0.001$ compared with maximal adhesion in the presence of vehicle.

greater extent than either of the antibodies individually (49±5% inhibition) but at 24 h the effects of the combination were only slightly greater than anti-VCAM-1 alone (43±3% inhibition). An isotype matched control antibody did not inhibit U937 cell adhesion to HUVEC.

### *Sodium orthovanadate enhances U937 cell adhesion to untreated but not TNFα-treated HUVEC*

The effects of vanadate on basal and TNFα-induced U937 cell adhesion to HUVEC were also measured (Figure 7). Treatment of HUVEC for 4 h with a range of concentrations of vanadate alone resulted in a dose-dependent increase in U937 cell adhesion. Maximum increased adhesion was observed with 100 μM vanadate which caused a two fold increase in adhesion over basal levels (31±1% compared with 15±5% of TNFα-induced adhesion). Maximal U937 adhesion induced by TNFα was unaffected by vanadate at concentrations up to 100 μM. To investigate the mechanism of U937 cell adhesion to vanadate-treated HUVEC, we pre-incubated cells with anti-E-selectin and anti-VCAM-1 blocking antibodies (Figure 8). Adhesion was not affected by incubation with a control antibody and was not significantly inhibited by anti-E-selectin (85±6% of control). Anti-VCAM-1 inhibited vanadate-induced adhesion by 44±11% to 11±2% of plated U937 cells which was not significantly different from the basal level of adhesion to untreated HUVEC (9±1% plated U937 cells). The effect of a combination of anti-E-selectin and anti-VCAM-1 was not significantly different from the effect of anti-VCAM-1 alone.

## Discussion

The PTK inhibitor genistein has been shown to inhibit TNFα- and IL-1α-induced responses in a number of cell types (Fuortes *et al.*, 1993; Joshi-Barve *et al.*, 1993; Katabami *et al.*, 1993; Corbett *et al.*, 1994; Koroma & de Juan, 1994; Lee *et al.*, 1994; McGregor *et al.*, 1994; van Hinsbergh *et al.*, 1994). In this study we have demonstrated that in HUVEC, genistein differentially affects the expression of the leukocyte adhesion molecules E-selectin, VCAM-1 and ICAM-1 induced by TNFα and IL-1α. Genistein inhibited TNFα- and IL-1α-induced expression of E-selectin and VCAM-1 but did not affect maximal levels of ICAM-1 expression. Inhibition was not due to non-specific effects of genistein as the structural analogue daidzein, which has much lower PTK inhibitory activity than genistein (Uehara *et al.*, 1989), did not affect expression of either molecule. By using a colorimetric cytotoxicity assay, employing the tetrazolium salt MTT which is converted to formazan by

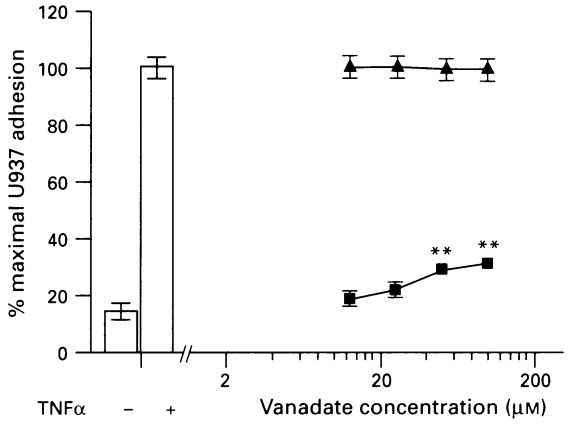


**Figure 7** Dose-dependent effect of vanadate on U937 cell adhesion to untreated and tumour necrosis factorα (TNFα)-treated HUVEC. HUVEC were treated for 15 min with a range of concentrations of vanadate and then a further 4 h in the presence (▲) or absence (■) of 100 u ml$^{-1}$ TNFα. Subsequently U937 cell adhesion was measured as described in the Methods section. The data are presented as percentage of maximally induced adhesion in the absence of vanadate and represent means±s.e.mean of triplicate observations from two separate experiments. $^{**}P<0.01$ compared with basal adhesion (no TNFα).

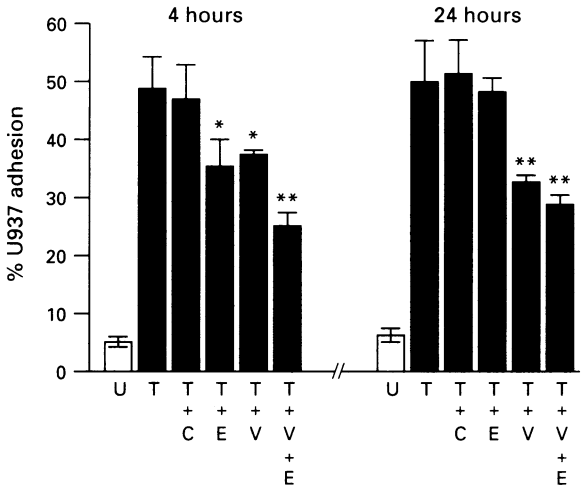


**Figure 6** Effects of blocking antibodies on adhesion of U937 cells to tumour necrosis factorα (TNFα)-treated HUVEC. HUVEC were treated for 4 or 24 h with either medium alone (U; open bars) or 100 u ml$^{-1}$ TNFα (T; solid bars). HUVEC were then incubated for a further 1 h in the presence of either medium alone or the following antibodies (all 50 μg ml$^{-1}$); C, an isotype matched control antibody; E, anti-E-selectin; V, anti-VCAM-1; V+E, both V and E. Adhesion of U937 cells was measured as described in the Methods section. The data are presented as a percentage of the U937 cells plated and are means±s.d. of quadruplicate samples from one experiment representative of three performed. $^{*}P<0.05$; $^{**}P<0.01$ compared with adhesion to TNFα-treated cells in the presence of the control antibody.

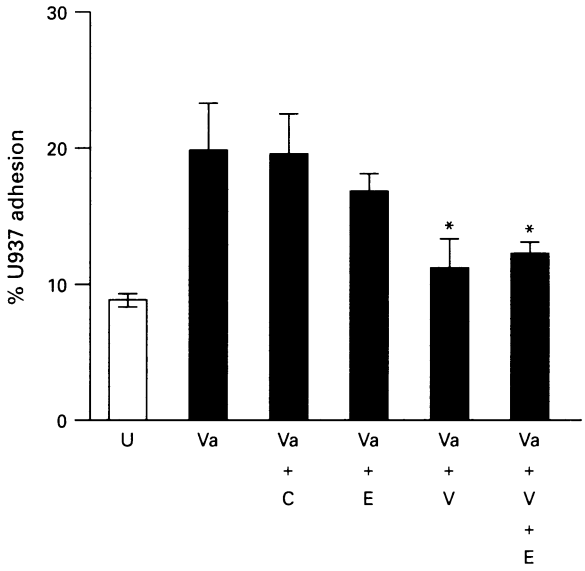


**Figure 8** Effects of blocking antibodies on vanadate-induced U937 cell adhesion to HUVEC. HUVEC were treated for 4 h with either medium alone (U; open columns) or 100 μM vanadate (Va; solid columns). HUVEC were then treated with antibodies as described in Figure 6. Adhesion of U937 cells was measured as described in the Methods section. The data are presented as a percentage of the U937 cells plated and are means±s.d. of quadruplicate samples from one experiment representative of two performed. $^{*}P<0.05$ compared with adhesion to vanadate-treated cells in the presence of the control antibody.

mitochondrial enzymes only active in live cells (Mosmann, 1983), we showed that at the concentrations and times used in this study, neither genistein nor daidzein were cytotoxic for HUVEC. Furthermore, van Hinsbergh *et al.* (1994) have demonstrated that at concentrations up to 300 μM genistein does not affect overall RNA synthesis in HUVEC. Thus, the data presented in this paper indicate that the signalling pathways evoked by IL-1α or TNFα to induce the expression of E-selectin and VCAM-1 involve genistein-inhibitable tyrosine phosphorylation, whereas upregulation of ICAM-1 occurs independently of genistein sensitive PTK activation.

In a recent study, Weber *et al.* (1995) demonstrated partial inhibition of TNFα-induced ICAM-1 expression by genistein at the same concentration shown here to inhibit E-selectin and VCAM-1 expression. One explanation for these contrasting effects may be that Weber *et al.* (1995) pre-incubated HUVEC with genistein for 1 h whereas in this study HUVEC were pre-incubated for 15 min. This longer pre-incubation time may have caused inhibition of non-PTK protein kinases responsible for ICAM-1 expression. Alternatively, it is possible that inhibition of cytokine-activated PTK by genistein alters the timecourse of cytokine-induced ICAM-1 expression such that the effects described by Weber *et al.* (1995), who measured expression after 6 h, were not detected in the present study in which measurements were made after 24 h incubation. Interestingly Tiisala *et al.* (1994) showed that expression of ICAM-1 by the endothelial cell line Ea.Hy 926 induced by sub-optimal concentrations of TNFα was enhanced by genistein. It is possible that this synergistic effect of genistein was not detected in the presence of maximal cytokine concentrations used in the present study. However, the contrasting results of this study and that of Tiisala and co-workers may reflect differences in the requirement for PTKs in cytokine-induced ICAM-1 expression by HUVEC and Ea.Hy 926 cells. Selective inhibitory effects of genistein on TNFα-induced responses in HUVEC have been obtained previously by van Hinsbergh *et al.* (1994), who showed that at concentrations similar to those described in this study, genistein inhibited TNFα-induced type 1 plasminogen activator inhibitor production but did not affect TNFα-induced production of urokinase. Together with our results, these findings suggest that in HUVEC, signalling via the TNFα and IL-1α receptors leads to activation of at least two separate signalling pathways, one being genistein-sensitive and the other genistein-insensitive.

In parallel studies, we have examined the effects of IL-1α and TNFα on tyrosine phosphorylation in HUVEC by Western blot analysis with a specific anti-phosphotyrosine antibody and have observed tyrosine phosphorylation of at least two proteins with molecular masses of approximately 65–70 kDa and 42–44 kDa (manuscript in preparation). We have identified the 42–44 kDa protein as the known cytokine-activated signalling molecule p42-MAP kinase (Bird *et al.*, 1991) and observed that its phosphorylation is inhibitable by genistein. It is not known, however, whether p42-MAP kinase has any role in adhesion molecule expression by HUVEC.

High concentrations of genistein have also been shown to inhibit serine/threonine specific protein kinases (Akiyama *et al.*, 1987). One such kinase is PKC, which is involved in a number of responses to IL-1α and TNFα (Schütze *et al.*, 1994), although it is not believed to play a role in E-selectin or ICAM-1 expression (Ritchie *et al.*, 1991; Myers *et al.*, 1992). We found that genistein at concentrations $\geqslant 100$ μM partially inhibited the ability of PKC purified from HUVEC to phosphorylate the substrate histone IIIs *in vitro*. Because genistein inhibited E-selectin expression induced by PMA we used the selective PKC inhibitors BIM, Ro31-7549 and Ro31-8220 and found that although they could inhibit PMA-induced E-selectin, they had no effect on expression induced by either TNFα or IL-1α. Furthermore, treatment of HUVEC for 48 h with PMA, which downregulates PKC (Ritchie *et al.*, 1991; Deisher *et al.*, 1993a), did not prevent re-induction of E-selectin by TNFα, nor did it affect the potency of genistein to inhibit this re-induced expression. Taken together these results confirm that cytokine-induced E-selectin expression is PKC-independent (Ritchie *et al.*, 1991) and show that inhibition of expression by genistein is not via PKC.

The serine/threonine protein kinase inhibitor H7 reduced equally PMA and cytokine-induced expression of E-selectin. Although H7 is frequently used as a PKC inhibitor it is almost equally active as an inhibitor of PKA (Hidaka *et al.*, 1984) and is a relatively non-specific serine/threonine kinase inhibitor. The potency of inhibition of E-selectin expression by H7, like genistein, was unaffected by PKC downregulation, suggesting that H7 inhibits an as yet undefined PMA-insensitive kinase involved in E-selectin expression. H7 also inhibits ICAM-1 expression (Myers *et al.*, 1992). Genistein and H7 may inhibit the same kinase(s), but the fact that H7 completely inhibits E-selectin expression whereas the effects of genistein are only partial suggests that this is not the case. We conclude that induction of E-selectin by TNFα and IL-1α involves both PTK activity inhibitable by genistein and an unidentified serine/threonine kinase inhibitable by H7. It is possible that PKC activity induced by PMA replaces this kinase which acts upstream of a genistein-sensitive PTK in the signalling pathway leading to E-selectin expression.

Our conclusion that tyrosine kinase activity controls the expression of VCAM-1 by HUVEC in response to IL-1α and TNFα is consistent with the recent demonstration by Palmer-Crocker & Pober (1995) that VCAM-1 expression induced by IL-4 involves tyrosine phosphorylation and is inhibited by herbimycin A, a tyrosine kinase inhibitor structurally unrelated to genistein (Uehara *et al.*, 1989). We examined the effects of herbimycin A on TNFα-induced expression of E-selectin, VCAM-1 and ICAM-1 and found that unlike genistein, it potently inhibited expression of all three adhesion molecules. These effects were not due to cytotoxicity of herbimycin A as judged by the MTT assay. Inhibition of ICAM-1 expression by herbimycin A but not genistein may implicate a requirement for a different pattern of PTK activation for upregulation of ICAM-1. Alternatively, the similar effect of herbimycin A on all three adhesion molecules may be due to its ability to modify a thiol group on, and inactivate the p50 subunit of, the transcription factor NFκB (Mahon & O'Neill, 1995) which is required for transcriptional activation of the E-selectin, VCAM-1 and ICAM-1 genes (Collins, 1993), rather than to inhibition of PTKs. The possible effect of herbimycin A on NFκB prevents definitive conclusions being drawn without direct identification of PTKs in the signalling pathways.

To investigate further the role of tyrosine phosphorylation in adhesion molecule expression, we used the potent PTP inhibitor sodium orthovanadate (Swarup *et al.*, 1982). Treatment of many cell types with vanadate has been shown to increase the overall intracellular phosphotyrosine content and mimic the effects of PTKs (Hunter, 1995). In HUVEC, vanadate causes increased production of PAI-1 in response to TNFα, opposing the effects of genistein (van Hinsberg *et al.*, 1994). Furthermore, vanadate alone markedly enhances the level of phosphotyrosine in several endogenous HUVEC proteins (manuscript in preparation). We found that basal expression of E-selectin, VCAM-1 and ICAM-1 was enhanced by vanadate suggesting that protein tyrosine phosphorylation alone is sufficient to induce expression of these molecules and further indicating a role for PTK in TNFα- and IL-1α-induced signalling in endothelial cells. A previous study has demonstrated that, at the concentrations used here, vanadate does not affect overall levels of protein synthesis in HUVEC (van Hinsbergh *et al.*, 1994). The fact that vanadate-induced expression did not reach the levels induced by TNFα, along with the observation that genistein did not completely inhibit E-selectin and VCAM-1 expression, further suggests that a non-PTK signal is also required for maximal expression of these molecules.

Although ICAM-1 induction was genistein-insensitive, the ability of vanadate to enhance significantly ICAM-1 expression suggests a role for tyrosine phosphorylation. In addition to IL-1α and TNFα, ICAM-1 expression can be upregulated by

the immunoregulatory cytokine interferon (IFN)-γ (Dustin *et al.*, 1986). The signalling mechanisms evoked by IFN-γ have been the subject of intense study recently (reviewed by Ihle *et al.*, 1994). Although little is known about the mechanism of action of IFN-γ in endothelial cells, in many cell types IFN-γ has been shown to activate members of the Janus kinase (JAK) family of PTKs. These kinases in turn tyrosine phosphorylate members of a family of transcription factors named STATs (signal transducers and activators of transcription) which bind to specific sequences in gene promoters and activate transcription and at least one of which (p91) is tyrosine phosphorylated in response to vanadate (David *et al.*, 1993; Lamb *et al.*, 1994; Look *et al.*, 1994). It has been shown that binding of a protein, which may be pp91, to an IFN-γ response element (IRE) within the promoter for ICAM-1, controls expression of ICAM-1 by epithelial cells (Look *et al.*, 1994). Thus, the up-regulated expression of endothelial ICAM-1 by vanadate may be due to activation of this IFN-γ responsive transcriptional mechanism. Interestingly, although E-selectin expression cannot be induced by IFN-γ alone, IFN-γ enhances and prolongs IL-1α- and TNFα-induced expression (Leeuwenberg *et al.*, 1990). Activation of a IFN-γ signalling pathway by vanadate may account for the partially enhanced expression of E-selectin we observed with TNFα-treated HUVEC.

TNFα-induced E-selectin and VCAM-1, but not ICAM-1, expression by HUVEC is inhibited by forskolin, which raises intracellular adenosine 3′:5′-cyclic monophosphate (cyclicAMP) levels (Pober *et al.*, 1993) and a cyclicAMP responsive element (CRE) within the promoter region of the E-selectin gene has been shown to be involved in E-selectin down-regulation (De Luca *et al.*, 1994; Ghersa *et al.*, 1994). We found that VCAM-1 expression induced by TNFα was inhibited by vanadate, suggesting that PTKs are involved not only in the induction of VCAM-1, but also in its down-regulation. Alternatively the inhibitory effect of vanadate on maximal VCAM-1 expression may be due to direct effects on the signalling pathway evoked by TNFα. Although vanadate alone induces partial VCAM-1 expression, it may additionally cause phosphorylation of unrelated proteins or kinases which interfere with the TNFα-induced signalling mechanism for VCAM-1.

In addition to investigating the role of PTK in adhesion molecule expression by HUVEC we examined the effects of PTK and PTP inhibitors on adhesion of the pre-myeloid cell line U937 to HUVEC treated for 4 h and 24 h with TNFα. By using blocking antibodies we found that up to 30% of U937 cell adhesion at 4 h was inhibited by either anti-E-selectin or anti-VCAM-1 whereas after 24 h anti-E-selectin was without effect. These inhibitions are consistent with the time courses of adhesion molecule expression by HUVEC in response to TNFα. Treatment of HUVEC for 4 h with TNFα induces maximal expression of E-selectin and partially enhances VCAM-1 and ICAM-1, although maximal expression of the latter molecules occurs after 16–24 h. We did not investigate the effects of genistein or herbimycin A on VCAM-1 expression after 4 h incubation with TNFα but the magnitude of inhibition of U937 cell adhesion by anti-VCAM-1 antibodies at this time suggests that even partial expression of VCAM-1 plays an important role in U937 adhesion to HUVEC. McGregor *et al.* (1994) have shown that adhesion of neutrophils or monocytes to TNFα- and IL-1α-treated HUVEC is inhibited by herbimycin A and genistein. We also found that genistein and herbimycin A inhibited maximal U937 cell adhesion induced by TNFα whereas basal adhesion was unaffected by the PTK inhibitors, results which are consistent with the effects of the PTK inhibitors on adhesion molecule expression. Unlike the PTK inhibitors, sodium orthovanadate did not affect maximal U937 cell adhesion induced by TNFα. The small increase by vanadate in enhanced E-selectin expression induced by TNFα after 4 h thus seems insufficient to enhance adhesion. This may be due to all available space on the endothelial surface being occupied by U937 cells leaving no space for extra binding, even in the presence of supermaximal expression of adhesion molecules. Although we observed a decrease in VCAM-1 expression after 24 h treatment with TNFα and vanadate, the levels of VCAM-1 expression after 4 h may be too low to affect overall adhesion maintained by the other adhesion molecules. However, when we measured the effects of treatment of HUVEC for 4 h with vanadate in the absence of TNFα, U937 cell adhesion was dose-dependently enhanced. Blocking studies showed that vanadate-induced U937 cell adhesion at 4 h was reduced to basal levels by anti-VCAM-1 antibodies.

In conclusion, the results of this study demonstrate that PTK are differentially involved in TNFα- and IL-1α-induced adhesion molecule expression by HUVEC. Inhibition of E-selectin and VCAM-1, but not ICAM-1, expression by genistein, together with the differential effects of sodium orthovanadate indicate a diversity of signalling pathways involving tyrosine phosphorylation in the expression of these molecules by HUVEC.

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