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Tumour necrosis factor-α-induced ICAM-1 expression in human vascular endothelial and lung epithelial cells: modulation by tyrosine kinase inhibitors

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1 Tumour necrosis factor-α (TNFα) increases the expression of the adhesion molecule intercellular adhesion molecule-1 (ICAM-1) on cultured endothelial and epithelial cells and modulation of this may be important in controlling inflammation. Activation of tyrosine kinase(s) is known to be involved in the signal transduction pathways of many cytokines. In this study we have investigated the effects of the tyrosine kinase inhibitors, ST638, tyrphostin AG 1288 and genistein, on TNFα-induced ICAM-1 expression in human alveolar epithelial (A549) and vascular endothelial (EAhy926) cell lines and also normal human lung microvascular endothelial cells (HLMVEC).

2 ICAM-1 expression on cultured cells was determined by a sensitive enzyme-linked immunosorbant assay (ELISA). Endothelial or epithelial monolayers were exposed to increasing doses of TNF-α (0.01–10 ng ml^{-1}), in the presence or absence of either ST638 (3–100 μM), AG 1288 (3–100 μM) or genistein (100 μM) and ICAM-1 expression was measured at 4 and 24 h. Control experiments examined the effect of ST638 on phorbol 12-myristate 13-acetate (PMA, 20 ng ml^{-1}, 4 h)-stimulated ICAM-1 and compared it to that of a specific protein kinase C inhibitor, Ro31-8220 (10 μM). Also, functional consequences of changes in ICAM-1 expression were assessed by measuring adhesion of ^{111}In-labelled human neutrophils to EAhy926 endothelial and A549 epithelial monolayers treated with TNFα, in the presence or absence of ST638.

3 ST638 caused a concentration-dependent reduction in TNFα- (0.1–10 ng ml^{-1})-induced ICAM-1 on EAhy926 endothelial (at 4 h) and A549 epithelial monolayers (at 4 and 24 h). In contrast, ST638 caused a concentration-dependent increase in TNFα- (0.1–10 ng ml^{-1})-induced ICAM-1 on EAhy926 endothelial cells at 24 h. Similar effects were seen with AG 1288 or genistein. ST638 (100 μM) significantly ($P<0.01$) inhibited ICAM-1 expression on HLMVEC endothelial cells induced by 0.01 ng ml^{-1} TNFα at 4 or 24 h or 0.1 ng ml^{-1} at 4 h, but increased ICAM-1 expression induced by 0.1 ng ml^{-1} TNFα at 24 h. ST638 did not significantly change the expression of PMA-stimulated ICAM-1 on either A549 epithelial, EAhy926 or HLMVEC endothelial cells. However, PMA-induced ICAM-1 expression was inhibited by Ro31-8220. Also, treatment of epithelial or endothelial monolayers with TNFα and ST638 altered adhesion of human neutrophils to A549 epithelial or EAhy926 endothelial cells in a manner that corresponded to the alteration in ICAM-1 expression.

4 These results show that tyrosine kinase inhibitors alter TNFα-induced ICAM-1 expression, but that the cell type, concentration of TNFα and time of exposure to this cytokine determine whether expression is decreased or increased by the inhibitor. An increased understanding of the signal transduction pathway(s) involved in TNFα-induced ICAM-1 expression on lung epithelial and vascular endothelial cells may be of potential therapeutic value in the treatment of inflammatory disease.

Keywords: Endothelial cell; epithelial cell; tyrosine kinase; ICAM-1; ST638; genistein; AG1288

Introduction

Regulation of adhesion molecule expression is important in the control of inflammation. Adhesion of leukocytes to endothelial cells is the first step in the recruitment of leukocytes to sites of inflammation. This process is controlled by adhesion molecules on both cell types (reviewed by Carlos & Harlan, 1994). Similar processes govern the adhesion of leukocytes to lung airway epithelial cells (Look *et al.*, 1992; Tosi *et al.*, 1992; Bloemen *et al.*, 1993) and may contribute to the damage to these cells seen in asthma (Montefort *et al.*, 1992). Intercellular adhesion molecule-1 (ICAM-1) has been shown, by *in vivo* studies with blocking anti-ICAM-1 monoclonal antibodies, to play a key role in inflammation (Barton *et al.*, 1989; Wegner *et al.*, 1991; Mulligan *et al.*, 1995). Although ICAM-1 is constitutively expressed on endothelial and epithelial cells, its contribution to the inflammatory process is thought to be due to upregulation by cytokines such as tumour necrosis factor-α (TNFα; Pober *et al.*, 1986; Tosi *et al.*, 1992).

The signal transduction pathway of TNF, and in particular the mechanism by which it elevates ICAM-1 expression, is not well understood. Activation of protein kinase C (PKC), sphingomyelinase, phospholipase A_2 (PLA_2) and the transcription factors nuclear transcription factor-κB (NFκB) and activator protein (AP-1) are known to be involved in TNF-mediated responses (reviewed by Heller & Krönke, 1994; Kolesnick & Golde, 1994; Schutze *et al.*, 1994). PKC-dependent (Lane *et al.*, 1990; Wuthrich *et al.*, 1992; Sung *et al.*, 1994) and -independent pathways (Ritchie *et al.*, 1992; Myers *et al.*, 1992; Wertheimer *et al.*, 1992; Eissner *et al.*, 1994; Ishizuka *et al.*, 1994) and also activation of NFκB (Ledebur & Parks, 1995) have been implicated in TNFα-induced ICAM-1 expression on endothelial and/or epithelial cells.

It has recently been shown that some TNFα-mediated responses in endothelial cells and colonic epithelial cells involve tyrosine phosphorylation (van Hinsbergh *et al.*, 1994; Gross *et al.*, 1995; Weber *et al.*, 1995). Although phosphorylation of proteins on tyrosine constitutes less than 0.01% of total intracellular phosphorylation this process may be the primary or exclusive indicator of signal transduction in multicellular or-

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ganisms (Ullrich & Schlessinger, 1990). It is unlikely that the TNF receptor has a tyrosine kinase enzyme as an integral part of its structure, unlike many growth factor receptors (Ullrich & Schlessinger, 1990; Cadena & Gill, 1992; Kishimoto *et al.*, 1994). However, tyrosine kinase(s) may be non-covalently associated with TNF receptor(s), as is the case for the homologous nerve growth factor receptor (Berg *et al.*, 1991) or other cytokines such as interleukin-2 (IL-2; Hatakeyama *et al.*, 1991).

A number of tyrosine kinase inhibitors have been used to investigate the role of tyrosine kinase enzymes in cell signalling. These inhibitors vary in specificity/selectivity and mechanism of action. For example, genistein, a widely used tyrosine kinase inhibitor, inhibits (at concentrations similar to those used to inhibit tyrosine kinase) topoisomerase I and II, phosphatidylinositol turnover and histidine kinase (Okura *et al.*, 1988; Imoto *et al.*, 1988; Huang *et al.*, 1992), by competing with these enzymes for adenosine 5'-triphosphate (ATP) (Akiyama *et al.*, 1987). Erbstatin, another widely used tyrosine kinase inhibitor, has been shown to inhibit PKC with an IC_{50} of 19.8 ± 3.2 μM (Bishop *et al.*, 1990) by competing for ATP. The tyrphostin family of tyrosine kinase inhibitors also inhibit PKC, protein kinase A (PKA) or Ca^{2+}/calmodulin-dependent kinases by competing with ATP, although they are 100 to 10,000 fold more potent at inhibiting tyrosine kinase (Levitzki, 1992). Tyrphostin AG 1288 has also been shown to inhibit TNFα-mediated cytotoxicity *in vitro* (Novogrodsky *et al.*, 1994), a feature of some non-selective tyrosine kinase inhibitors. In contrast, ST638 (α-cyano-3-ethoxy-4-hydroxy-5-phenyl-thiomethylcinnamide), originally identified as an inhibitor of tyrosine specific protein kinase activity of the epidermal growth factor (EGF) receptor, competes with substrate protein for tyrosine kinase binding sites (Shiraishi *et al.*, 1987). ST638 has been shown to have no effect on PKA, PKC or casein kinase I and II at concentrations of up to 100 μM (Shiraishi *et al.*, 1987; 1989; Hirst *et al.*, 1995). Also, the inhibitory effect described for ST638 on phospholipase D (PLD) activity is due to an indirect effect on a tyrosine kinase component of the inflammatory stimuli receptor coupling to PLD and not a direct effect on the enzyme (Uings *et al.*, 1992). To our knowledge, ST638 has not been used to investigate a role for tyrosine kinase(s) in TNFα-mediated endothelial or epithelial cell functions, although it has been shown to inhibit tyrosine phosphorylation in neutrophils treated with TNFα (Fuortes *et al.*, 1993). ST638 has also been shown to inhibit tyrosine phosphorylation triggered by a variety of inflammatory stimuli, for example zymosan or thrombin, or other cell types including human platelets, mouse bone marrow-derived macrophages, human leukaemic cells, rat basophil leukaemic cells and rabbit tracheal smooth muscle cells (Asahi *et al.*, 1992; Green *et al.*, 1992; Adunyah 1993; Kumada *et al.*, 1993; Hirst *et al.*, 1995).

A recent study showed that genistein acted on endothelial cells to potentiate the increase in ICAM-1 triggered at 24 h by TNFα or interferon γ (IFNγ) (Tiisala *et al.*, 1994). In contrast McGregor *et al.* (1994) showed a decrease in adhesion of leukocytes to TNFα-treated endothelial cells (2 h) in the presence of genistein. In our study, we investigated the effect of ST638 on TNFα-induced ICAM-1 expression on a human alveolar epithelial cell line (A549), a vascular endothelial cell line (EAhy926) and normal human lung microvascular (HLMVEC) endothelial cells. Finally, we examined human neutrophil adhesion to A549 epithelial cells and EAhy926 endothelial cells, treated with TNFα and ST638, to investigate whether modulation of ICAM-1 expression was mimicked by similar effects on neutrophil adhesion.

Methods

Cell culture

The human umbilical vein endothelial cell (HUVEC) line, EAhy926, was a gift from Dr C-J. Edgell, Department of Pathology, School of Medicine, University of North Carolina, U.S.A. This cell line, a hybridoma of HUVEC and the human epithelial cell line A549, has been shown to retain a number of properties of EC including the production of human Factor-VIII-related antigen (Edgell 1983). The cells were maintained in Dulbecco's modified Eagles medium (DMEM; high-glucose) containing 10% heat-inactivated foetal bovine serum (FBS), 4 mM glutamine, 200 u ml^{-1} penicillin, 200 μg ml^{-1} streptomycin and HAT supplement (medium supplemented with hypoxanthine, aminopterin and thymidine). Confluent cells were trypsinized (with 0.05% trypsin + 0.02% EDTA) and seeded at a density of 4×10^4 cells per well onto 1%-gelatin-coated flat-bottomed Nunclon 96-well microtitre plates.

A549 cells (type II alveolar epithelial cell carcinoma) were obtained from ATCC (Rockville, Maryland, U.S.A.). Cells were maintained and cultured as for EAhy926 except that the DMEM medium was not supplemented with HAT and the cells were seeded onto gelatin-coated 96-well plates at a density of 3×10^4 cells per well.

HLMVEC, prepared by Clonetics (California, U.S.A.), were obtained as cryopreseved tertiary (3°) cultures from TCS Biologicals Ltd. (Buckingham). HLMVEC were maintained in microvascular endothelial growth medium (EGM-MV), based on the MCDB 131 formulation and supplemented with 10 ng ml^{-1} human recombinant epidermal growth factor, 1 μg ml^{-1} hydrocortisone, 5% heated-inactivated FBS, 50 μg ml^{-1} gentamicin, 50 ng ml^{-1} amphotericin-B and bovine brain extract containing 12 μg ml^{-1} protein and 10 μg ml^{-1} heparin. HLMVEC were cultured for up to 12° without significant alteration in magnitude of TNFα-induced ICAM-1 expression (Blease, Hellewell & Burke-Gaffney; unpublished observation). HLMVEC used in this study were between 5°–8°. Confluent cells were washed with Hanks balanced salt solution (HBSS), trypsinized with 0.025% trypsin + 0.01% EDTA and collected into tyrpsin neutralizing solution. Cells were seeded at a density of 3.2×10^3 cells per well onto gelatin-coated 96-well plates.

Enzyme-linked immunosorbant assay (ELISA) for ICAM-1 expression

ICAM-1 was detected at 2 (EAhy926, A549) or 4 days (HLMVEC) after seeding by an ELISA method (Pigott *et al.*, 1991) by use of a mouse anti-human ICAM-1 (RR1/1) IgG_1 primary monoclonal antibody (Rothlein *et al.*, 1988) and a peroxidase-linked goat anti-mouse secondary antibody. Briefly, confluent EAhy926, HLMVEC or A549 epithelial cells in Nunclon 96 well plates were incubated for the times indicated with various concentrations of TNFα, IFNγ or phorbol 12-myristate 13-acetate (PMA) in their respective serum-supplemented complete culture media, with or without other additions (including DMSO-vehicle for tyrosine kinase inhibitors) as noted in the text. All agents were filter sterilized (pore size: 0.22 μm) before addition to cells under sterile conditions. Following removal of stimuli, cells were washed three times (with phosphate buffered saline (PBS) containing Ca^{2+} and Mg^{2+} and 0.1% bovine serum albumin (BSA)) and incubated for 45 min with a saturating (1 μg ml^{-1}) concentration of RR1/1. The antibody was then removed by washing and a 1:1000 dilution (in PBS + 10% goat serum) of the developing antibody (goat anti-mouse peroxidase conjugate) was applied (45 min) followed by incubation with the substrate, 2,2'-azinobis (3-ethylbenz-thiazoline-6-sulphonic acid (ABTS: 1 mg ml^{-1}, in 0.2 M citrate/phosphate buffer, pH 5, containing 0.1% hydrogen peroxide) for 30 min. The reaction was then terminated by addition of 0.2 M citrate. All incubations were carried out at room temperature. Chromophore development was determined by measuring optical density (O.D.) at 405 nm (OD_{405}) with a Titretec MCC/340 Multiscan microplate reader. Background absorbance was determined from monolayers incubated without primary antibody. This value was then subtracted from the absorbance readings. The data presented were derived from O.D. readings which fell along the linear portion of the development

curve. ICAM-1 expression is calculated as a percentage of O.D. for TNFα-, PMA- or IFNγ-stimulated ICAM-1, where this represents 100%. We have previously shown, by substituting a control antibody (MOPC2) for RR1/1, that binding of this anti-ICAM-1 mAb was specific (Burke-Gaffney & Hellewell, 1996).

Tyrosine kinase activity

Protein tyrosine kinase activity of A549 and EAhy926 cell lysates and the effect of tyrosine kinase inhibitors was determined by use of a photometric enzyme immunoassay kit. Confluent monolayers of A549 and EAhy926 grown in 50 mm dishes were incubated for 15 min at 37°C with TNFα (1 ng ml^{-1}) alone or with the addition of ST638 (3–100 μM), AG 1288 (100 μM) or genistein (100 μM). Cells were then washed with PBS and lysed in 1 ml pre-chilled detergent lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, 1.0% IGEPAL CA-630, 0.5% deoxycholate, 0.1% Sodium dodecyl sulphate (SDS), 100 μg ml^{-1} phenylmethyl-sulphonyl fluoride (PMSF), 1 μg ml^{-1} aprotinin, 2 μg ml^{-1} leupeptin and 100 μg ml^{-1} Na_3VO_4). The lysate was centrifuged at 10,000 × *g* for 10 min at 4°C and tyrosine kinase activity was determined according to the kit instructions.

Measurement of cell viability

Cell respiration, as an indicator of cell viability, was assessed by use of a modification of the method of Mosmann (1983). This depends on mitochondrial reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Cells were treated with TNFα ± dimethylsulphoxide (DMSO) or ± ST638 for 4 or 24 h. Stimuli were removed and, before or after subjection to ELISA washing/incubation steps, viability was assessed by incubating cells for 1 h (37°C) with MTT (0.2 mg ml^{-1}) dissolved in culture medium. Medium was removed and formazan crystals were solubilized in DMSO (100 μl). Reduction of MTT to formazan within the cells was assessed by measurement of O.D. at 540 nm. Cell viability was >94% for any treatment or condition used.

Separation of human peripheral blood neutrophils and assessment of their adhesion to epithelial or endothelial monolayers

Neutrophils were isolated from peripheral blood of adult donors by the methods of Haslett *et al.* (1985). Briefly, blood was collected to a total volume of 40 ml into 3.8% citrate and spun for 20 min at 300 *g*. The platelet-rich plasma was removed, underlayed with 90% Percoll and spun at 2000 *g* for 20 min at room temperature (temperature used unless otherwise stated) to produce platelet-poor plasma (PPP). To the lower buffy coat produced by the first spin, 6% dextran was added and the volume made up to 50 ml with 0.9% saline. This was allowed to stand for 30 min for erythrocyte sedimentation to occur. The leukocyte-rich supernatant was removed and centrifuged at 275 *g* for 6 min. The pellet was resuspended in PPP and layered onto freshly prepared discontinuous Percoll-plasma gradients (42 and 51% Percoll in PPP) and centrifuged for 10 min at 260 *g*. The neutrophil-rich band (>98% pure) was collected, washed, resuspended in 1–2 ml PPP and radiolabelled by incubating (15 min, room temperature) $5-15 \times 10^7$ cells with approximately 100 μCi of $^{111}InCl_3$ chelated to 2-mercaptopyridine-N-oxide (400 μg ml^{-1} in 50 mM PBS, pH 7.4). Cells were washed twice in PPP and resuspended at 2.5×10^6 cells per ml, in PBS containing Ca^{2+}, Mg^{2+}, glucose and 0.25% BSA (low endotoxin) for the adhesion assay.

A549 epithelial or EAhy926 endothelial monolayers grown on 96-well plates were pretreated with TNFα ± DMSO or ST638 as indicated in the text and washed three times with PBS to remove ST638/TNFα, before carrying out the adhesion assay. One hundred microlitres of ^{111}In-labelled neutrophils were added per well and the plate was incubated at 37°C for 30 min after which nonadherent neutrophils were removed by three gentle washes with PBS. Adherent cells were lysed with formic acid and 100 μl aliquots counted in a gamma counter. Results are expressed as a % of TNFα-stimulated adhesion (100%) and calculated from the following equation:

$$\frac{(\text{stimulated adhesion} + \text{ST638}) - (\text{basal adhesion})}{(\text{stimulated adhesion} + \text{DMSO}) - (\text{basal adhesion})} \times 100$$

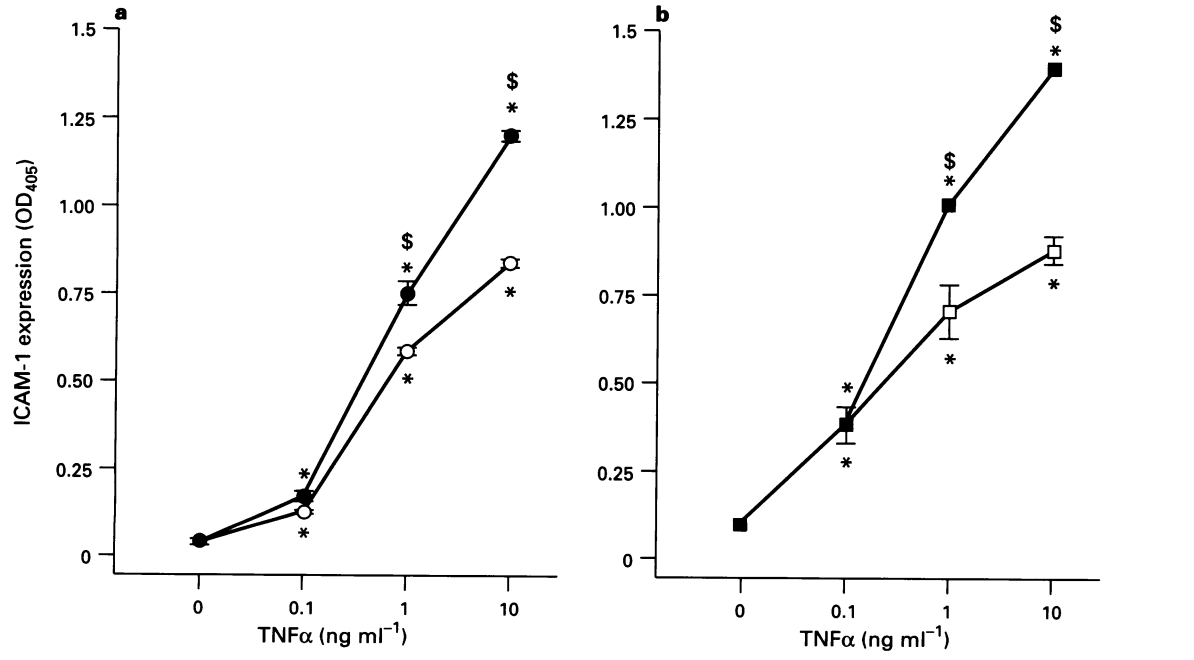

Figure 1 Concentration-dependent increase in ICAM-1 expression following exposure of (a) A549 epithelial or (b) EAhy926 endothelial monolayers to tumour necrosis factor-α (TNFα; 0.1–10 ng ml^{-1}) for 4 h (open symbols) or 24 h (solid symbols). Data are expressed as the mean ± s.e.mean of triplicate determinations from 3 separate experiments ($n=9$). $^*P<0.01$, $^\$P<0.01$, denote significant differences from basal ICAM-1 expression, or ICAM-1 expression at 4 h, respectively.

Materials

DMEM (high glucose), 0.05% trypsin + 0.02% EDTA, PBS (with and without Ca^{2+} and Mg^{2+}), FBS, penicillin-streptomycin, HAT supplement were purchased from Life Technologies (Paisley, Scotland). The following products were purchased from Sigma Chemical Company Ltd. (Poole, Dorset): ABTS, gelatin, goat serum, H_2O_2, MTT, DMSO, daidzein, dithiothreitol, IGEPAL, CA-630, deoxycholate, SDS, PMSF, aprotinin, leupeptin and 2-mercaptopyridine-N-oxide. ST638, tyrphostin, AG 1288 and genistein were obtained from Calbiochem/Novabiochem (Nottingham). Affinity isolated goat anti-mouse peroxidase conjugate gamma and light chain specific, EGM-MV, 0.025% trypsin + 0.01% EDTA, HBSS, trypsin neutralizing solution and HLMVEC endothelial cells were obtained from TCS Biologicals Ltd. (Buckingham). Percoll and dextran were obtained from Pharmacia Biotech Ltd. (St. Albans, Herts.), 0.9% saline from FL (Manufacturing) Ltd. Fresenius Health Care Group (Basingstoke, Hants). and very low endotoxin BSA from Bayer Ltd (Basingstoke, Hants). Citric acid, di-sodium hydrogen orthophosphate, glucose and propan-2-ol were obtained from BDH Chemicals Ltd. (Poole, Dorset). $^{111}InCl_3$ was obtained from Amersham (Bucks). Human recombinant TNF-α (specific activity $>1\times10^8$ u ml^{-1}) and the Tyrosine Kinase Assay Kit were obtained from Boehringer Mannheim U.K. (Lewes, East Sussex) and IFNγ (1×10^7 u mg^{-1}) from R&D Systems (Oxford, U.K.). Mouse anti-human ICAM-1 (RR1/1) IgG_1 mAb (Rothlein *et al.*, 1986) was provided by Dr R. Rothlein (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, U.S.A.). Ro31-8220 (1-[3-(amidinothio)propyl-1H-indoyl-3-yl]-3-(1-methyl-1H-indoyl-3-yl)-maleimide-methane sulphate) was a kind gift from Dr D. Bradshaw, Roche, Welwyn Garden City.

Statistics

Results are expressed as mean ± s.e.mean of n determinations. Statistical analysis was carried out by one-way ANOVA followed by Dunnett's multiple comparison test (where all test values are compared to control) unless otherwise stated. Results were deemed significant if $P<0.05$.

Results

Basal ICAM-1 expression was detected by ELISA on A549 epithelial (OD_{405} 0.040 ± 0.006) or EAhy926 endothelial (OD_{405} 0.101 ± 0.005) monolayers (Figure 1). TNFα (0.1 – 10 ng ml^{-1}) caused a concentration-dependent increase in ICAM-1 expression on these cell monolayers at 4 or 24 h (Figure 1). In both cell types, ICAM-1 expression was significantly greater ($P<0.01$) at 24 h, compared to 4 h, following incubation with 1 or 10 but not with 0.1 ng ml^{-1} TNFα (Figure 1).

Effects of genistein or AG 1288 on the expression of ICAM-1 on TNFα-treated EAhy926 endothelial and A549 epithelial cell monolayers

Thirty microgram per ml (110 μM) genistein, a concentration used in previous studies (Tiisala *et al.*, 1994), significantly inhibited ($P<0.01$) TNFα- (1 ng ml^{-1}) induced ICAM-1 expression on A549 epithelial cells at 4 h (67 ± 4%, $n=9$), but not 24 h, compared with DMSO vehicle (1% v/v; Figure 2a). Since DMSO alone significantly ($P<0.05$) inhibited ICAM-1 expression at 4 h (25 ± 1%, $n=9$; Figure 2a), the specific inhibition contributed by genistein was 42 ± 5% ($n=9$). In contrast, genistein (110 μM) incubated with EAhy926 endothelial cells and TNFα (1 ng ml^{-1}) significantly ($P<0.01$) increased ICAM-1 expression at 24 h (45 ± 1%, $n=9$), but had no effect at 4 h, when compared with DMSO vehicle (Figure 2b). DMSO (1%) alone also significantly increased ($P<0.01$) ICAM-1 expression (17 ± 2%, $n=9$, Figure 2b) at 24 h; hence, the increase contributed by genistein was 28 ± 3% ($n=9$). Since

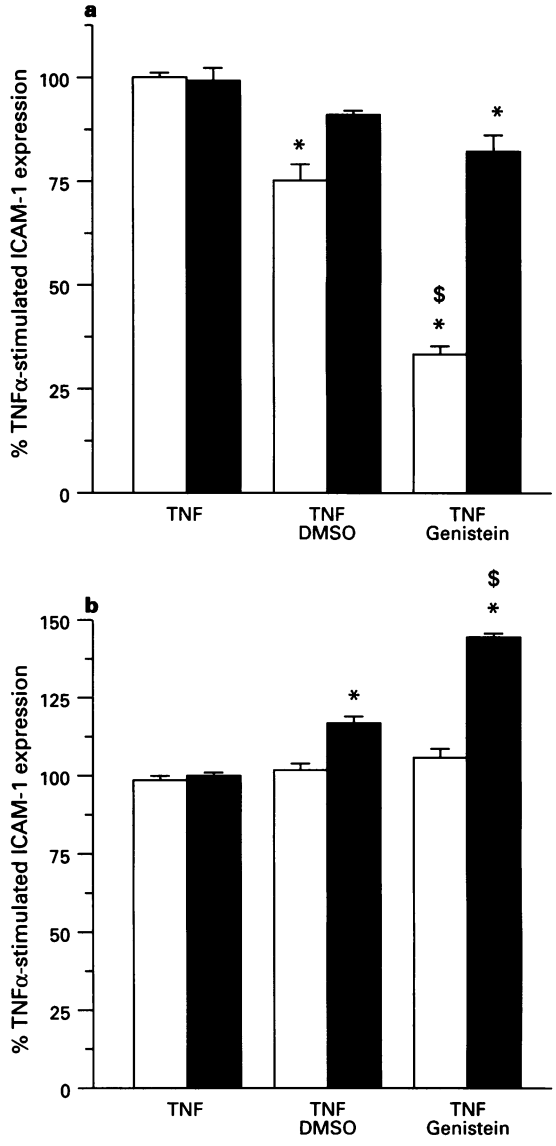

Figure 2 ICAM-1 expression on tumour necrosis factor-α (TNFα, 1 ng ml^{-1})-activated monolayers of (a) A549 epithelial or (b) EAhy926 endothelial cells, in the presence of DMSO (1%) or genistein (30 μg ml^{-1}) at 4 h (open columns) or 24 h (solid columns). Data are expressed as the mean ± s.e.mean of 9 replicate measurements from 3 separate experiments. Statistical analysis was performed by one-way ANOVA followed by the Tukey-Kramer multiple comparison post test (compares all values to each other). $^*P<0.01$, $^\$P<0.01$. denote significant differences from TNFα or TNFα + DMSO, respectively (at 4 or 24 h).

DMSO alone altered ICAM-1 expression, subsequent results (Figures 3 and 4) were expressed as a % of TNFα-induced ICAM-1 expression corrected for the inhibition contributed by the appropriate concentration of DMSO (ie. 0.03 – 1%). Daidzein, the inactive analogue of genistein, at a concentration of 100 μM had no significant effect on TNFα-induced ICAM-1 expression at either 4 or 24 h (data not shown).

The effects of AG 1288 on ICAM-1 expression induced by TNFα (1 ng ml^{-1}) are shown in Table 1. On A549 cells, tyrphostin AG 1288 concentration-dependently inhibited ICAM-1 expression at 4 h with only a small effect at 24 h. In contrast, it had little inhibitory effect on TNFα-induced ICAM-1 on EAhy926 at 4 h and significantly increased expression at 24 h. Thus, the effects of AG 1288 were similar to genistein.

Tyrosine kinase activity in A549 cells was reduced by 61 ± 16% by genistein (100 μM) and 54 ± 19% (mean ± s.e.mean, 4 replicates in two experiments) by AG 1288

(100 μM). In TNFα-stimulated EAhy926 cells tyrosine kinase activity was reduced 92% and 76% by genistein (100 μM) and AG 1288 (100 μM), respectively.

Effects of ST638 on ICAM-1 expression on A549 epithelial and EAhy926 endothelial cells

ST638 (3–100 μM) incubated with A549 epithelial cells and TNFα (0.1 or 1 ng ml^{-1}) for 4 h produced a concentration-dependent inhibition of ICAM-1 expression (maximum inhibition of 54±7% and 30±1%, respectively, $n=12$; Figure 3a). ICAM-1 expression, induced with 10 ng ml^{-1} TNFα (4 h), was not affected by concentrations of ST638 up to 100 μM (Figure 3a). Also, ST638 (10–100 μM) inhibited TNFα (0.1 ng ml^{-1})-induced ICAM-1 expression at 24 h on epithelial cells (maximum inhibition, 39±4%, $n=12$), but only inhibited expression induced by 10 or 1 ng ml^{-1} TNFα by 7±1% ($P<0.05$, $n=12$) and 8±2% ($P<0.01$, $n=12$; Figure 3b).

ST638 (10–100 μM) incubated with EAhy926 endothelial cells and TNFα (0.1 ng ml^{-1}) for 4 h caused a significant ($P<0.01$) reduction in ICAM-1 expression (maximal inhibition of 50±5%, $n=9$; Figure 4a). Only 100 μM ST638 inhibited ICAM-1 expression induced at 4 h by 1 or 10 ng ml^{-1} TNFα (33±3% or 21±3%, respectively, $n=12$). In contrast, ST638 (10–100 μM) incubated with endothelial cells and TNFα (0.1–10 ng ml^{-1}) for 24 h produced a concentration-dependent increase in ICAM-1 expression (Figure 4b). A maximal increase (43±5%, $n=12$) was detected with 0.1 ng ml^{-1} TNFα and 100 μM ST638 (Figure 4b).

The effect of ST638 on IFNγ (40 ng ml^{-1})-induced ICAM-1 expression was also examined. This could be investigated in EAhy926 cells only, since A549 cells do not express significant amounts of ICAM-1 in response to this cytokine. A 24 h activation period was also required. ICAM-1 measured at 24 h in the presence of DMSO vehicle (OD_{405} 0.694±0.019, subtracted for basal) was not significantly altered by ST638 (100 μM; 0.688±0.015) or AG 1288 (100 μM; 0.631±0.019), but was significantly decreased by genistein (100 μM; 0.564±0.015).

Tyrosine kinase activity of A549 cells was reduced by 28%, 38%, 53% and 58% by ST638 at 3, 10, 30 and 100 μM, respectively (mean values from 2 experiments). In EAhy926 cells, activity was reduced by 11%, 67%, 63% and 88% by the same concentrations of ST638, respectively.

Effects of ST638 on normal lung microvascular endothelial cells

Basal ICAM-1 expression was detected by ELISA on HLMVEC monolayers (OD_{405} 0.410±0.014, $n=9$; Table 2). TNFα (0.01–10 ng ml^{-1}) caused a concentration-dependent increase in ICAM-1 expression at 4 or 24 h (data not shown). To investigate the effect of ST638 on TNFα-induced ICAM-1 expression on HLMVEC, concentrations of TNFα (0.01, 0.1 ng ml^{-1}) were used that gave increases in ICAM-1 expression above basal ICAM-1 (Table 2) similar to those measured on EAhy926 cells with 0.1 and 1 ng ml^{-1} TNFα respectively (see Figure 1). ST638 (100 μM) incubated with HLMVEC and 0.01 or 0.1 ng ml^{-1} TNFα for 4 h or 0.01 ng ml^{-1} for 24 h, significantly ($P<0.01$) inhibited TNFα-induced ICAM-1 expression (55±5, 48±2 or 51±5% respectively, $n=9$; Table 2). In contrast, ST638 (100 μM) incubated with HLMVEC and 0.1 ng ml^{-1} TNFα for 24 h significantly ($P<0.01$) increased ICAM-1 expression (14±3%, Table 2).

Effects of ST638 or Ro31-8220 on PMA-induced ICAM-1 on epithelial and endothelial cells

PMA (20 ng ml^{-1}) increased ICAM-1 expression (4 h) on A549 epithelial or EAhy926 endothelial cells was not significantly affected by ST638 (100 μM; Figure 5). Also, PMA-induced ICAM-1 expression (4 h) on HLMVEC (OD_{405}

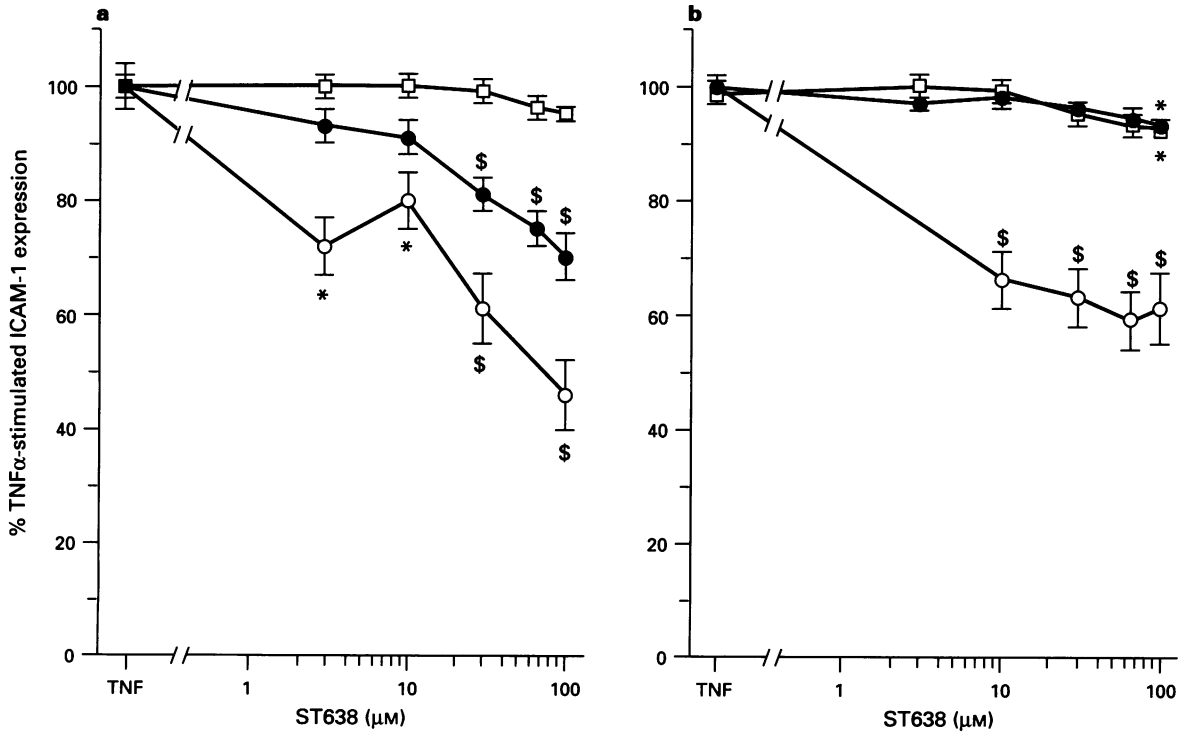

Figure 3 ICAM-1 expression at (a) 4 h or (b) 24 h on tumour necrosis factor-α (TNFα)-activated A549 epithelial cells in the presence of ST638 (3–100 μM). Concentrations of TNFα used were 0.1 (○), 1 (●), 10 (□) ng ml^{-1}. Data are expressed as the mean±s.e.mean of 12 replicate measurements from 4 separate experiments. *$P<0.05$, $^{\$}P<0.01$, denote significant differences from ICAM-1 expression on cells treated with TNFα alone.

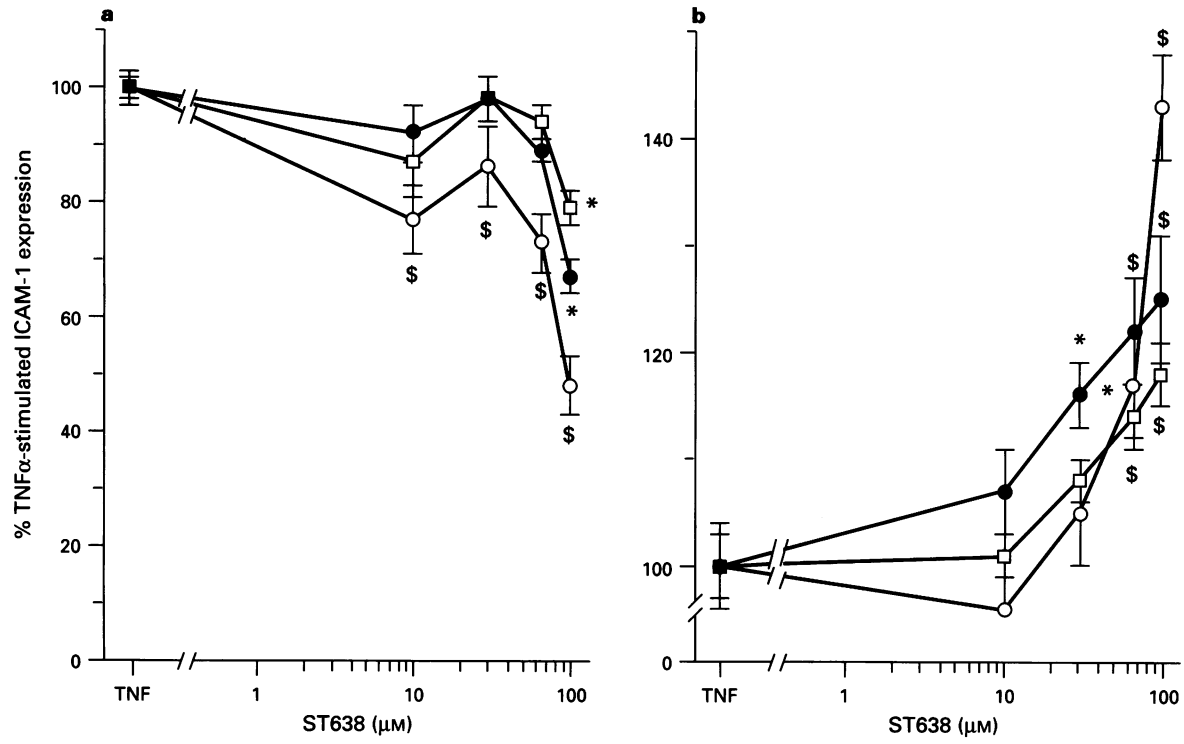

Figure 4 ICAM-1 expression at (a) 4 h or (b) 24 h on tumour necrosis factor-α (TNFα)-activated EAhy926 endothelial cells in the presence of ST638 (3–100 μM). Concentrations of TNFα used were 0.1 (○), 1 (●), 10 (□) ng ml^{-1}. Data are expressed as the mean ± s.e.mean of 12 replicate measurements from 4 separate experiments. *$P<0.05$, $^{\$}P<0.01$, denote significant differences from ICAM-1 expression on cells treated with TNFα alone.

Table 1 Effect of AG 1288 on tumour necrosis factor-α (TNFα)-induced ICAM-1 expression on A549 epithelial cells and EAhy926 endothelial cells

Cells	*AG1288* (μM)	*%TNFα-induced ICAM-1-expression in the presence of AG 1288*	
		4 h	*24 h*
A549	0	100.0 ± 1.6	100.0 ± 1.2
	3	104.0 ± 3.9	101.1 ± 1.2
	10	102.0 ± 4.1	98.4 ± 1.3
	30	79.1 ± 2.7*	95.3 ± 0.3
	66	54.2 ± 2.2*	94.0 ± 1.9
	100	39.5 ± 2.8*	78.1 ± 2.0*
EAhy926	0	100.0 ± 1.1	100.0 ± 2.6
	3	100.7 ± 1.1	103.8 ± 0.9
	10	93.8 ± 0.8	103.0 ± 0.6
	30	93.5 ± 1.7	102.5 ± 2.1
	66	89.4 ± 1.6*	101.0 ± 1.4
	100	85.0 ± 2.4*	114.0 ± 1.2*

A549 epithelial or EAhy926 endothelial cells were treated for 4 or 24 h with 1 ng ml^{-1} of TNF with or without AG1288 (3–100 μM) and ICAM-1 expression was determined by ELISA. The effects of AG1288 are expressed as % of ICAM-1 expression induced by TNFα in the presence of vehicle. Data are means of 6 replicates from 2 different experiments ± s.e.mean. *$P<0.01$ compared with TNFα-induced ICAM-1 expression.

Table 2 Effect of ST638 on tumour necrosis factor-α (TNFα)-induced ICAM-1 expression on human normal lung microvascular endothelial cells

Time (h)	*TNFα* (ng ml^{-1})	*TNFα-induced ICAM-1 expression* (OD_{405})	*% TNFα-induced ICAM-1 expression in the presence of ST638*
4	0.01	0.269 ± 0.024	45 ± 5*
	0.1	0.602 ± 0.027	52 ± 2*
24	0.01	0.193 ± 0.026	49 ± 5*
	0.1	0.967 ± 0.043	114 ± 3*

Normal human lung microvascular endothelial cells were treated for 4, 24 h with 0.01 or 1 ng ml^{-1} of TNFα with or without ST638 (100 μM) and ICAM-1 expression was determined by ELISA. The OD value for basal ICAM-1 was 0.410 ± 0.014 ($n=9$ replicates from 3 experiments) and has been subtracted from the values shown. Concentrations of TNFα (0.01, 0.1 ng ml^{-1}) were used that gave increases in ICAM-1 expression similar to those measured on EAhy926 cells (0.1 and 1 ng ml^{-1} respectively, see Figure 1). DMSO vehicle (1%) did not signifcantly alter ICAM-1 expression (data not shown). The effects of ST638 (100 μM) are expressed as % of ICAM-1 expression induced by TNFα in the presence of vehicle. Data are means of 6 replicates from 2 different experiments ± s.e.mean. *$P<0.01$ compared with TNFα-induced ICAM-1 expression.

0.631 ± 0.006) was not significantly altered by ST638 (OD_{405} 0.657 ± 0.010). A similar lack of effect on PMA-induced ICAM-1 expression at 24 h on all three cell types was also observed with 100 μM ST638; data not shown. In contrast, a specific PKC inhibitor, Ro31-8220 (10 μM; Davis *et al.*, 1992) abolished PMA-induced ICAM-1 expression on A549 epithelial cells and inhibited expression on EAhy926 endothelial cells, by 82 ± 8% ($P<0.01$, $n=9$; Figure 5).

Effect of ST638 on human neutrophil adhesion to TNFα-treated A549 epithelial and EAhy926 endothelial monolayers

ST638 (100 μM) did not significantly alter adhesion of neutrophils to unstimulated A549 epithelial or EAhy926 endothelial monolayers (data not shown). ST638 (100 μM) incubated with A549 epithelial cells and TNFα (1 ng ml^{-1}) for

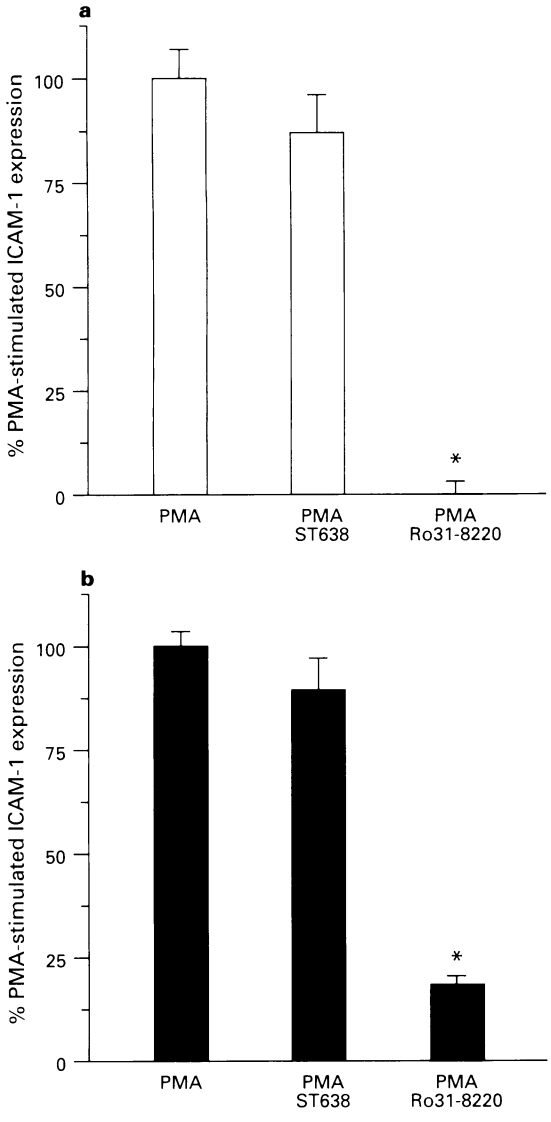

Figure 5 ICAM-1 expression on (a) A549 epithelial or (b) EAhy926 endothelial monolayers following incubation with phorbol 12-myristate 13-acetate (PMA, 20 ng ml^{-1}, 4 h) with or without ST638 (100 μM) or Ro31-8220 (10 μM). Data are expressed as % of the PMA response (OD_{405} = 0.132 ± 0.01 or 0.254 ± 0.01, n = 9, for epithelial or endothelial cells, respectively) and values are shown as the mean ± s.e.mean of 9 replicate measurements from 3 separate experiments. *P < 0.01, denotes a significant difference from PMA-treated cells.

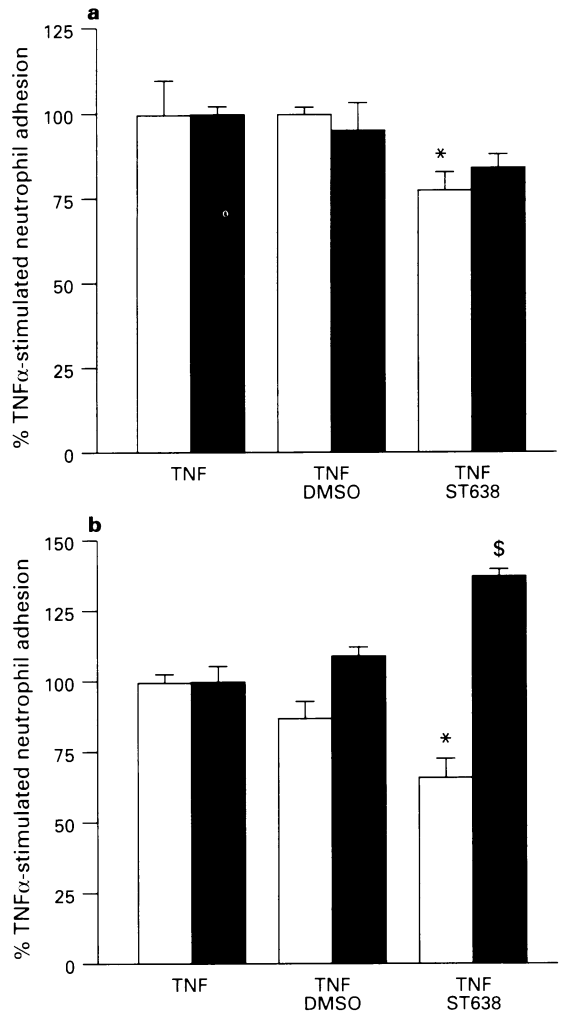

Figure 6 Adhesion of unstimulated human neutrophils to tumour necrosis factor-α (TNFα; 1 ng ml^{-1})-activated (a) A549 epithelial or (b) EAhy926 endothelial monolayers in the presence of ST638 (100 μM) at 4 (open columns) or 24 h (solid columns). Data are expressed as the mean ± s.e.mean of 4 replicate measurements from one of 3 similar experiments. Statistical analysis was performed by one-way ANOVA followed by the Tukey-Kramer multiple comparison post test. *P < 0.05, $^{\$}P$ < 0.01 denotes a significant difference from TNFα or TNFα/DMSO at 4 h or 24 h, respectively.

4 h, but not 24 h, significantly (P < 0.05) inhibited adhesion (23 ± 5%, n = 9), when compared to TNFα in the presence of DMSO (1%) vehicle (Figure 6a). Also, ST638 (100 μM) incubated with EAhy926 endothelial cells and TNFα (1 ng ml^{-1}) for 4 h significantly decreased adhesion (33 ± 6%, n = 12) but, in contrast, increased adhesion at 24 h (37 ± 3%, n = 9, Figure 6b). DMSO (1%) alone did not significantly alter adhesion under any of the conditions investigated (Figure 6).

Discussion

In this study we have shown that inhibition, or enhancement, of TNFα-induced ICAM-1 expression by tyrosine kinase inhibitors depends on the cell type and concentration of TNFα and time for which cells are exposed to this cytokine. ST638, at concentrations known to inhibit tyrosine phosphorylation in human platelets, mouse bone marrow-derived macrophages, human leukaemic cells, rat basophil leukaemic cells and rabbit tracheal smooth muscle cells (Asahi *et al.*, 1992; Green *et al.*, 1992; Adunyah, 1993; Kumada *et al.*, 1993; Hirst *et al.*, 1995) inhibited, in part, TNFα (0.1, 1, 10 ng ml^{-1})-induced ICAM-1 expression on A549 epithelial cells (4, 24 h) and on EAhy926 endothelial cells at 4 h. ICAM-1-induced on HLMVEC by TNFα 0.01 ng ml^{-1} (4, 24 h) or 0.1 ng ml^{-1} (4 h) was also inhibited. In contrast, at 24 h, ST638 increased ICAM-1 expression on EAhy926 endothelial cells induced by 0.1, 1 or 10 ng ml^{-1} TNFα, and on HLMVEC induced by 0.1 ng ml^{-1} TNFα (the highest concentration used with these cells). Since ST638 (100 μM) did not inhibit PMA- or IFNγ-induced ICAM-1 expression (on epithelial or endothelial cells) it is unlikely that this tyrosine kinase inhibitor alters ICAM-1 expression by a non-specific effect on PKC. A lack of ST638 effect on PMA- and IFNγ-induced ICAM-1 would also suggest that inhibition by this compound of TNFα-induced ICAM-1 is unlikely to result from a non-specific effect on protein synthesis. When the effects of ST638 or AG 1288 were compared to those of genistein, at similar concentrations, the latter was shown to be more effective than ST638 at inhibiting TNFα-induced epithelial ICAM-1 expression at 4 h, but less effective at increasing TNFα-induced endothelial ICAM-1 expression at 24 h. These differences may be due to an effect of genistein on other enzymes or signalling pathways as described in the In-

troduction (Okura *et al.*, 1988; Imoto *et al.*, 1988; Huang *et al.*, 1992).

Recent studies have shown that tyrosine kinase inhibitors (i) increase TNFα-induced ICAM-1 expression on EAhy926 endothelial cells at 24 h (Tiisala *et al.*, 1994) and (ii) decrease leukocyte adhesion to, or ICAM-1 expression on HUVEC at 2 or 4 h, respectively, with magnitudes similar to those in the present study (McGregor 1994; Weber *et al.*, 1995). Also, this two-phase response is similar to that in the present study following incubation of EAhy926 or HLMVEC with TNFα and ST638. It is not clear why tyrosine kinase inhibitors augment TNFα-induced ICAM-1 expression on endothelial cells, but not epithelial cells at 24 h, or why enhanced expression on HLMVEC is dependent on the TNFα concentration. It is possible that: (i) tyrosine kinases expressed by endothelial or epithelial cells differ, (ii) tyrosine kinase(s) are involved in the up- and down-regulation of TNFα-induced ICAM-1 expression in endothelial cells; (iii) normal HLMVEC are more susceptible to ST638 inhibition of ICAM-1 expression than the vascular endothelial cell line EAhy926. To date, little is known about which tyrosine kinase(s) are present in vascular endothelial or lung epithelial cells or which tyrosine kinase(s), in addition to the EGF-receptor tyrosine kinase, may be inhibited by ST638. It will be important in future studies to identify these and which tyrosine-phosphorylated protein(s) are inhibited by ST638 in epithelial and endothelial cells.

The degree to which TNFα-induced ICAM-1 expression was inhibited or enhanced depended on the level of ICAM-1 expression. This was determined by the concentration of, and time of exposure to, TNFα. For example, the amount of ICAM-1 expression induced on A549 epithelial cells by 0.1 ng ml^{-1} TNFα and the degree of inhibition caused by ST638 (100 μM), were similar at 4 and 24 h. In contrast, 1 ng ml^{-1} TNFα induced more ICAM-1 expression at 24 h than at 4 h on A549 epithelial cells and inhibition, by ST638, was correspondingly less at 24 than at 4 h. Since TNFα is known to activate several signal transduction pathways (Heller & Kronke, 1994; Kolesnick & Golde, 1994; Schutze *et al.*, 1994), it is possible that tyrosine kinase inhibition reduced only one of several components necessary for, or involved in, TNFα induction of ICAM-1 expression. A balance may also exist between tyrosine kinase-dependent and -independent pathways leading to TNFα induction of ICAM-1 expression. Tyrosine kinase-dependent pathways may predominate when ICAM-1 is induced by low concentrations of TNFα, but this may be overcome with increasing concentrations of TNFα, since other signalling pathways may now dominate. This explanation may also, in part, account for why tyrosine kinase inhibitors do not abolish TNFα-induced ICAM-1 expression.

It is not known at which point tyrosine kinase inhibitors act in the TNFα-ICAM-1 transduction pathway. A recent study showed that membrane-associated, rather than cytosolic, components sensitive to tyrosine kinase inhibitors were involved in TNFα activation of NFκB in U937 cells (Reddy *et al.*, 1994). Whether a membrane-associated tyrosine kinase is responsible for phosphorylation of the IκB inhibitory subunit, leading to dissociation of NFκB and subsequent translocation to the nucleus, is unclear. However, a putative tyrosine phosphorylation site on an IκB molecule has been identified (Inoue *et al.*, 1992). Also TNFα stimulation of fibroblasts or HL-60 cells has been shown to increase tyrosine phosphorylation of a p42 mitogen-activated protein (MAP) kinase (Raines *et al.*, 1993; Vietor *et al.*, 1993). A ras-dependent MAP kinase cascade has been shown to link IL-6 to activation of a C/EBP transcription factor (for a review see Kishimoto *et al.*, 1994) and C/EBP and NFκB binding sites have recently been shown to form a composite response element to TNFα in the ICAM-1 gene promoter region (Hou *et al.*, 1994). It is possible, therefore, that a MAP kinase cascade and activation of C/EBP may link TNFα-induced tyrosine kinase activity to regulation of ICAM-1 expression. Clearly, this is the subject of further investigation.

Finally, we showed that changes in human neutrophil adhesion to epithelial or endothelial cells, incubated with TNFα (1 ng ml^{-1}) and ST638 (100 μM), were similar to changes in ICAM-1 expression under these conditions. We have previously shown that neutrophil adhesion to TNFα-activated EAhy926 cells is inhibited by an anti-ICAM-1 blocking mAb (Burke-Gaffney & Hellewell, 1996). We have also shown that TNFα-treated A549 epithelial cells only express ICAM-1 (Burke-Gaffney & Hellewell, 1995). It is likely, therefore that the changes in neutrophil adhesion in this study are due to the ST638-induced changes in ICAM-1 expression.

Although cultured epithelial and endothelial cells are widely used model systems for studying neutrophil adhesion to endothelium and epithelium, extrapolation of results to human disease and/or possible therapeutic value(s) of tyrosine kinase inhibitors must be made with caution. Enhanced tyrosine kinase activity is associated with many inflammatory responses including septic shock (Weinstein *et al.*, 1991; Dong *et al.*, 1993). Also, a tyrphostin tyrosine kinase inhibitor, AG 126 has been used to prevent lipopolysaccharide (LPS)-induced lethal toxicity in a mouse model of sepsis (Novogrodsky *et al* 1994). However, questions such as: (i) 'do tyrosine kinase inhibitors alter neutrophil-induced tissue damage associated with advanced sepsis?'; (ii) 'could tyrosine kinase inhibitor-enhanced expression of ICAM-1 on endothelial cells be of therapeutic significance?' and; (iii) 'would inhibitors that selectively reduce lung epithelial ICAM-1 expression be beneficial in the treatment of diseases such as asthma?', remain to be answered.

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References

ADUNYAH, S.E. (1993). Granulocyte macrophage colony stimulating factor stimulates rapid phosphorylation of proteins on tyrosines in human U937 and HL-60 leukaemic cells. *Biochem. Biophys. Res. Commun.*, **193**, 890–896.

AKIYAMA, T., ISHIDA, J., NAKAGAWA, S., OGAWARA, H., WATANABE, S-I., ITOH, N., SHIBUYA, M. & FIKAMI, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.*, **262**, 5592–5595.

ASAHI, M., YANAGI, S., OHTA, S., INAZU, T., SAKAI, K., TAKEUCHI, F., TANIGUCHI, T. & YAMAURA, H. (1992). Thrombin-induced human platelet aggregation is inhibited by protein-tyrosine kinase inhibitors, ST638 and genistein. *FEBS Lett.*, **309**, 10–14.

BARTON, R.W., ROTHLEIN, R., KSIAZER, J. & KENNEDY, C. (1989). The effect of anti-intercellular adhesion molecule-1 on phorbol ester-induced rabbit lung inflammation. *J. Immunol.*, **143**, 1278–1282.

BERG, M.M., STERNBERG, D.W., HEMPSTEAD, B.L. & CHAO, M.V. (1991). The low-affinity p75 nerve growth factor (NGF) receptor mediates NGF-induced tyrosine phosphorylation. *Proc. Natl Acad. Sci. U.S.A.*, **88**, 7106–7110.

BISHOP, W.R., PETRIN, J., WANG, L., RAMESH, U. & DOLL, R.J. (1990). Inhibition of protein kinase C by the tyrosine kinase inhibitor erbstatin. *Biochem. Pharmacol.*, **40**, 2129–2135.

BLOEMEN, P.G.M., VAN DEN TWEEL, M.C., HENRICKS, P.A.J., ENGELS, F., WAGENAAR, S.S., RUTTEN, A.A.J.J.L. & NIJKAMP, F.P. (1993). Expression and modulation of adhesion molecules on human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.*, **9**, 586–593.

BURKE-GAFFNEY, A.C. & HELLEWELL, P.G. (1995). Regional differences in expression and regulation of airway epithelial cell adhesion molecules. *Am. J. Resp. Crit. Care Med.*, **151**, A192.

BURKE-GAFFNEY, A. & HELLEWELL, P.G. (1996). Regulation of ICAM-1 by dexamethasone in a human vascular endothelial cell line, EAhy926. *Am. J. Physiol.*, **270,** C552–C561.

CADENA, D.L. & GILL, G.N. (1992). Receptor tyrosine kinases. *FASEB J.*, **6,** 2332–2337.

CARLOS, T.M. & HARLAN, J.M. (1994). Leukocyte-endothelial adhesion molecules. *Blood*, **84,** 2068–2101.

DAVIS, P.D., ELLIOT, L.H., HARRIS, W., HILL, C.H., HURST, S.A., KEECH, E., KUMAR, M.K., LAWTON, G., NIXON, J.S. & WILKINSON, S.E. (1992). Inhibitors of protein kinase C. 2. Substituted bisindolylmaleimides with improved potency and selectivity. *J. Med. Chem.*, **35,** 994–1001.

DONG, Z., QI, X. & FIDLER, I.J. (1993). Tyrosine phosphorylation of mitogen-activated protein kinases is necessary for activation of murine macrophages by natural and synthetic bacterial products. *J. Exp. Med.*, **177,** 1071–1077.

EDGELL, C-J.S., MCDONALD, C.C. & GRAHAM, J.B. (1983). Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. U.S.A.*, **80,** 3734–3737.

EISSNER, G., KOLCH, W., MISCHAK, H., BORNKAMM, G.W. & HOLLER, E. (1994). Differential role of protein kinase C in cytokine induced lymphocyte-endothelium interactions *in vitro*. *Scand. J. Immunol.*, **40,** 395–402.

FUORTES, M., JIN, W-W. & NATHAN, C. (1993). Adhesion-dependent protein tyrosine phosphorylation in neutrophils treated with tumour necrosis factor. *J. Cell Biol.*, **120,** 777–784.

GREEN, S.P. & PHILLIPS, W.A. (1994). Activation of the respiratory burst by phorbol myristate acetate: evidence for both tyrosine-kinase-dependent and -independent pathways. *Biochem. Biophys. Acta*, **1222,** 241–248.

GROSS, V., ANDUS, T., DAIG, R., ASCHENBRENNER, E., SCHOLMERICH, J. & FALK, W. (1995). Regulation of interleukin-8 production in a human colon epithelial cell line (HT-29). *Gastroenterol.*, **108,** 653–661.

HASLETT, C., GUTHRIE, L.A., KOPANIAK, M.M., JOHNSON, Jr, R.B. & HENSON, P.M. (1985). Modulation of multiple neutrophil functions by preparative methods of trace amounts of bacterial lipopolysaccharide. *Am. J. Physiol.*, **119,** 101–110.

HATAKEYAMA, M., KOTO, T., KOBAYASHI, N., KAWAHARA, A., LEVIN, S.D., PERLMUTTER, R.M. & TANIGUCHI, T. (1991). Interaction of the IL-2 receptor with the Src-family kinase p56-lck: identification of novel intermolecular association. *Science*, **252,** 1523–1528.

HELLER, R.A. & KRONKE, M. (1994). Tumour necrosis factor receptor-mediated signalling pathways. *J. Cell Biol.*, **126,** 5–9.

HIRST, S.J., WEBB, B.L., GIEMBYCZ, M.A., BARNES, P.J. & TWORT, C.H. (1995). Inhibition of fetal calf serum-stimulated proliferation of rabbit cultured tracheal smooth muscle cells by selective inhibitors of protein kinase C and protein tyrosine kinase. *Am. J. Respir. Cell Mol. Biol.*, **12,** 149–161.

HOU, J., BAICHWAL, V. & CAO, Z. (1994). Regulatory elements and transcription factors controlling basal and cytokine-induced expression of the gene encoding intercellular adhesion molecule 1. *Proc. Natl Acad. Sci. U.S.A.*, **91,** 11641–11645.

HUANG, J., NASR, M., KIM, Y. & MATTHEWS, H.R. (1992). Genistein inhibits protein histidine kinase. *J. Biol. Chem.*, **267,** 15511–15515.

IMOTO, M., YAMASHITA, T., SAWA, T., KURASAWA, S., NAGANAWA, H., TAKEUCHI, T., BAO-QUAN, Z. & UMEZAWAM, K. (1988). Inhibition of cellular phosphatidylinositol turnover by psi-tectorigenin. *FEBS Lett.*, **230,** 43–46.

INOUE, J., KERR, L.D., KAKIZUKA, A. & VERMA, I.M. (1992). IκBγ, a 70 kd protein identical to the C-terminal half of p110 NFκB: a new member of the IκB family. *Cell*, **68,** 1109–1120.

ISHIZUKA, T., SUZUKI, K., KAWAKAMI, M., KAWAGUCHI, Y., HIDAKA, T., MATSUKI, Y. & NAKAMURA, H. (1994). DP-1904, a specific inhibitor of thromboxane A2 synthesizing enzyme, suppresses ICAM-1 expression by stimulated vascular endothelial cells. *Eur. J. Pharmacol.*, **262,** 113–123.

KISHIMOTO, K., TAGA, T. & AKIRA, S. (1994). Cytokine signal transduction. *Cell*, **76,** 253–262.

KOLESNICK, R. & GOLDE, D.W. (1994). The sphingomyelin pathway in tumour necrosis factor and interleukin-1 signalling. *Cell*, **77,** 325–328.

KUMADA, T., MIYATA, H. & NOZAWA, Y. (1993). Involvement of tyrosine phosphorylation in IgE receptor-mediated phospholipase D activation in rat basophilic leukemia (RBL-2H3) cells. *Biochem. Biophys. Res. Commun.*, **191,** 1363–1368.

LANE, T.A., LAMKIN, G.E. & WANCEWICZ, E.V. (1990). Protein kinase C inhibitors block the enhanced expression of intercellular adhesion molecule-1 on endothelial cells activated by interleukin-1, lipopolysaccharide and tumour necrosis factor. *Biochem. Biophys. Res. Commun.*, **172,** 1273–1281.

LEDEBUR, H.C. & PARKS, T.P. (1995). Transcription regulation of the intercellular adhesion molecule-1 gene by inflammatory cytokines in human endothelial cells. *J. Biol. Chem.*, **270,** 933–943.

LEVITZKI, A. (1992). Tyrphostins: tyrosine kinase blockers as novel antiproliferative agents and dissectors of signal transduction. *FASEB J.*, **6,** 3275–3282.

LOOK, D.C., RAPP, S.R., KELLER, B.T. & HOLTZMAN, M.J. (1992). Selective induction of intercellular adhesion molecule-1 by interferonγ in human airway epithelial cells. *Am. J. Physiol.*, **263,** L79–L87.

MCGREGOR, P.E., AGRAWAL, D.K. & EDWARDS, J.D. (1994). Attenuation of human leukocyte adherence to endothelial cell monolayers by tyrosine kinase inhibitors. *Biochem. Biophys. Res. Commun.*, **198,** 359–365.

MONTEFORT, S., HERBERT, C.A., ROBINSON, C. & HOLGATE, S.T. (1992). The bronchial epithelium as a target for inflammatory attack in asthma. *Clin. Exp. Allergy*, **22,** 511–520.

MOSMANN, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **65,** 55–63.

MULLIGAN, M.S., VAPORCIYAN, A.A., WARNER, R.L., JONES, M.L., FOREMAN, K.E., MIYASAKA, M., TODD, R.F. 3rd & WARD, P.A. (1995). Compartmentalised roles for leukocytic adhesion molecules in lung inflammatory injury. *J. Immunol.*, **154,** 1350–1363.

MYERS, C.L., WERTHEIMER, S.J., SCHEMBRI-KING, J., PARKS, T. & WALLACE, R.W. (1992). Induction of ICAM-1 by TNF-alpha, IL-1 beta, and LPS in human endothelial cells after down regulation of PKC. *Am. J. Physiol.*, **263,** C767–72.

NOVOGRODSKY, A., VANICHKIN, A., PATYA, M., GAZIT, A., OSHEROV, N. & LEVITZKI, A. (1994). Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors. *Science*, **264,** 1319–1322.

OKURA, A., ARAKAWA, H., OKA, H., YOSHINARI, T. & MONDEN, Y. (1988). Effect of genistein on topoisomerase activity and on growth of [VAL 12]Ha-ras-transformed NIH 3T3 cells. *Biochem. Biophys. Res. Commun.*, **157,** 183–189.

PIGOTT, R., NEEDHAM, L.A., EDWARDS, R.M., WALKER, C. & POWER, C. (1991). Structural and functional studies of the endothelial activation antigen endothelial leukocyte adhesion molecule-1 using a panel of monoclonal antibodies. *J. Immunol.*, **147,** 130–135.

POBER, J.S., GIMBRONE, Jr, M.A., LAPIERRE, L.A., MENDRICK, D.L., FIERS, W., ROTHLEIN, R. & SPRINGER, T.A. (1986). Overlapping patterns of activation of human endothelial cells by interleukin-1, tumour necrosis factor and immune interferon. *J. Immunol.*, **137,** 1893–1896.

RAINES, M.A., KOLESNICK, R.N. & GOLDE, D.W (1993). Sphingomyelinase and ceramide activated mitogen-activated protein kinase in myeloid HL-60 cells. *J. Biol. Chem.*, **266,** 16580–16586.

REDDY, S.A.G., CHATURVEDI, M.M., DARNAY, B.G., CHAN, H., HIGUCHI, M. & AGGARWAL, B.B. (1994). Reconstitution of nuclear factor κB-activation induced by tumour necrosis factor requires membrane-associated components. *J. Biol. Chem.*, **269,** 25369–25372.

RITCHIE, A.J., JOHNSON, D.R., EWENSTEIN, B.M. & POBER, J.S. (1991). Tumour necrosis factor induction of endothelial cell surface antigens is independent of protein kinase C activation or inactivation. Studies with phorbol myristate acetate and staurosporine. *J. Immunol.*, **146,** 3056–3062.

ROTHLEIN, R., DUSTIN, M.L., MARLIN, S.D. & SPRINGER, T.A. (1986). An intercellular adhesion molecule-1 distinct from LFA-1. *J. Immunol.*, **137,** 1270–1274.

ROTHLEIN, R., CZAJKOWSKI, M., O'NEIL, M.M., MARLIN, S.D., MAINOLFI, E. & MERLUZZI, V.J. (1988). Induction of intercellular adhesion molecule-1 on primary and continuous cell lines by pro-inflammatory cytokines: regulation by pharmacologic agents and neutralizing antibodies. *J. Immunol.*, **141,** 1665–1669.

SCHUTZE, S., MACHLEIDT, T. & KRONKE, M. (1994). The role of diacylglycerol and ceramide in tumour necrosis factor and interleukin-1 signal transduction. *J. Leuk. Biol.*, **56,** 533–541.

SHIRAISHI, T., DOMOTO, T., IMAI, N., SHIMADA, Y. & WATANABE, K. (1987). Specific inhibitors of tyrosine-specific protein kinase, synthetic 4-hydroxycinnamamide derivatives. *Biochem. Biophys. Res. Commun.*, **147,** 322–328.

SHIRAISHI, T., OWADA, M.K., TATSUKA, M., TAMASHITA, T., WATANABE, K. & KAKUNAGA, T. (1989). Specific inhibitors of tyrosine-specific protein kinases: properties of 4-hydroxycinnamide derivatives in vitro. *Cancer Res.*, **49,** 2371–2378.

SUNG, C.P., ARLETH, A.J. & NAMBI, P. (1994). Evidence for involvement of protein kinase C in expression of intracellular adhesion molecule-1 (ICAM-1) by human vascular endothelial cells. *Pharmacol.*, **48,** 143–146.

TIISALA, S., MAJURI, M-L., CARPEN, O. & RENKONEN, R. (1994). Genistein enhances the ICAM-mediated adhesion by inducing the expression of ICAM-1 and its counter-receptors. *Biochem. Biophys. Res. Commun.*, **203,** 443–449.

TOSSI, M.F., STARK, J.M., SMITH, C.W., HAMEDANI, A., GRUENERT, D.C. & INFELD, M.D. (1992). Induction of ICAM-1 expression on human airway epithelial cells by inflammatory cytokines: Effects on neutrophil-epithelial cell adhesion. *Am. J. Respir. Cell Mol. Biol.*, **7,** 214–221.

UINGS, I.J., THOMPSON, N.T., RANDALL, R.W., SPACEY, G.D., BONSER, R.W., HUDSON, A.T. & GARLAND, L.G. (1992). Tyrosine phosphorylation is involved in receptor coupling to phospholipase D but not phospholipase C in the human neutrophil. *Biochem. J.*, **281,** 597–600.

ULLRICH, A. & SCHLESSINGER, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell*, **61,** 203–212.

VAN HINSBERGH, V.W.M., VERMEER, M., KOOLWIJK, P., GRIMBERGEN, J. & KOOISTRA, T. (1994). Genistein reduces tumour necrosis factor α-induced plasminogen activator inhibitor-1 transcription but not urokinase expression in human endothelial cells. *Blood*, **84,** 2984–2991.

VIETOR, I.P., SCHWENGER, P., LI, W., SCHLESSINGER, J. & VILCEK, J. (1993). Tumour necrosis factor-induced activation and increased tyrosine phosphorylation of mitogen-activated protein (MAP) kinase in human fibroblasts. *J. Biol. Chem.*, **268,** 18994–18999.

WEBER, C., NEGRESCU, E., ERL, W., PIETSCH, A., FRANKENBERGER, M., ZIEGLER-HEITBROCK, H.W.L., SIESS, W. & WEBER, P.C. (1995). Inhibitors of protein tyrosine kinase suppress TNF-stimulated induction of endothelial cell adhesion molecules. *J. Immunol.*, **155,** 445–451.

WEGNER, C.D., ROTHLEIN, R. & GUNDEL, R.H. (1991). Adhesion molecules in the pathogenesis of asthma. *Agents Actions*, **34,** 529–544.

WEINSTEIN, S.L., GOLD, M.R. & DEFRANCO, A.L. (1991). Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. *Proc. Natl. Acad. Sci. U.S.A.*. **88,** 4148–4152.

WERTHEIMER, S.J., MYERS, C.L., WALLACE, R.W. & PARKS, T.P. (1992). Intercellular adhesion molecule-1 gene expression in human endothelial cells. Differential regulation by tumour necrosis factor-alpha and phorbol myristate acetate. *J. Biol. Chem.*, **267,** 12030–12035.

WUTHRICH, R.P. (1992). Tumour necrosis factor-alpha- and interleukin-1-stimulated intercellular adhesion molecule-1 expression by murine renal tubular epithelial cells is transcriptionally regulated and involves protein kinase C. *Renal Physiol. Biochem.*, **15,** 302–306.

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