

# Monocyte Adhesion and Spreading on Human Endothelial Cells Is Dependent on Rho-regulated Receptor Clustering

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**Abstract.** The GTPase Rho is known to mediate the assembly of integrin-containing focal adhesions and actin stress fibers. Here, we investigate the role of Rho in regulating the distribution of the monocyte-binding receptors E-selectin, ICAM-1, and VCAM-1 in human endothelial cells. Inhibition of Rho activity with C3 transferase or N19RhoA, a dominant negative RhoA mutant, reduced the adhesion of monocytes to activated endothelial cells and inhibited their spreading. Similar effects were observed after pretreatment of endothelial cells with cytochalasin D. In contrast, dominant negative Rac and Cdc42 proteins did not affect monocyte adhesion or spreading. C3 transferase and cytochalasin D did not alter the expression levels of monocyte-binding receptors on endothelial cells, but did inhibit clustering of E-selectin, ICAM-1, and

VCAM-1 on the cell surface induced by monocyte adhesion or cross-linking antibodies. Similarly, N19RhoA inhibited receptor clustering. Monocyte adhesion and receptor cross-linking induced stress fiber assembly, and inhibitors of myosin light chain kinase prevented this response but did not affect receptor clustering. Finally, receptor clusters colocalized with ezrin/moesin/radixin proteins. These results suggest that Rho is required in endothelial cells for the assembly of stable adhesions with monocytes via the clustering of monocyte-binding receptors and their association with the actin cytoskeleton, independent of stress fiber formation.

**Key words:** Rho • actin cytoskeleton • intercellular adhesion molecule-1 • E-selectin • monocyte adhesion

**T**HE adhesion of monocytes to the vascular endothelial lining and their subsequent diapedesis constitutes one of the earliest changes detectable in inflammation, immune responses, and atherosclerosis (Luscinskas et al., 1996; O'Brien et al., 1996; Raines and Ross, 1996; McEvoy et al., 1997). Monocyte adhesion to endothelial cells can be significantly upregulated by activating the endothelium with inflammatory cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ),<sup>1</sup> interleukin 1 (IL-1), and IL-4 (Raines and Ross, 1996). Activated endothelial cells express several monocyte-binding proteins including intercellular adhesion molecule-1 (ICAM-1), ICAM-2, and

vascular cell adhesion molecule-1 (VCAM-1) of the immunoglobulin superfamily of adhesion molecules as well as members of the selectin family of adhesion molecules, E- and P-selectins (Bevilacqua and Nelson, 1993; Yoshida et al., 1996). E-Selectin mediates the initial steps of monocyte adhesion to endothelial cells, a process described as leukocyte rolling (Hogg and Landis, 1993; Rice et al., 1996). It is absent in unstimulated endothelial cells and is rapidly expressed de novo in response to inflammatory cytokines reaching a peak concentration after  $\sim$ 4 h of stimulation (Bevilacqua et al., 1987; Bevilacqua and Nelson, 1993; Rice et al., 1996). ICAM-1 and VCAM-1 are required for stable adhesion, subsequent spreading, and diapedesis of leukocytes through endothelium (Bevilacqua and Nelson, 1993; Luscinskas et al., 1996). Although the expression kinetics of these three molecules have been studied extensively, the signaling mechanisms leading to the formation of stable adhesions between endothelial cells and leukocytes are still poorly understood.

The interactions between intracellular cytoskeletal components and cell surface adhesion molecules have been studied extensively because of their potential impact on cell-cell and cell-substratum adhesion as well as receptor internalization (Pavalko and Otey, 1994). The best charac-

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; ERM, ezrin/radixin/moesin; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; IL, interleukin; MLCK, myosin light chain kinase; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; VCAM, vascular cell adhesion molecule.

terized model for the association of integral membrane proteins with the actin cytoskeleton is the focal adhesion, found in places of integrin-mediated cell attachment to the extracellular matrix (Burridge and Chrzanowska-Wodnicka, 1996; Hemler, 1998). Two of the leukocyte-binding receptors on endothelial cells, E-selectin and ICAM-1, have also been reported to associate with components of the actin cytoskeleton (Carpen et al., 1992; Yoshida et al., 1996). Clustering of E-selectin after leukocyte binding was observed on IL-1-activated human umbilical vein endothelial cells (HUVECs), and the cytoplasmic domain of E-selectin was found to interact with a number of actin-associated proteins, including  $\alpha$ -actinin, vinculin, filamin, paxillin, and focal adhesion kinase (Yoshida et al., 1996). The association of E-selectin with the cytoskeleton increased the mechanical resistance of clustered E-selectin to shear stress. Based on these observations, it was postulated that the transmembrane anchoring of a cluster of E-selectin molecules could serve as a physical nidus for counteraction during the spreading and migration of leukocytes to intercellular junctions that follows their stable arrest. In addition, the intracellular signals transmitted to the cytoskeleton during E-selectin clustering could influence the function of other endothelial adhesion molecules such as ICAM-1 and VCAM-1 (Yoshida et al., 1996). Interestingly, ICAM-1 can associate via its cytoplasmic domain with  $\alpha$ -actinin, an actin-binding cytoskeletal protein (Carpen et al., 1992). Similar associations were also observed with the adhesion proteins, ICAM-2, L-selectin, and  $\beta$ 1 and  $\beta$ 2 integrins (Heiska et al., 1996, 1998). The functional implications of this binding have not been fully elucidated but it may be that a cytoskeletal anchorage which immobilizes ICAM-1 on endothelial cells could provide a firm adhesive substratum for migrating leukocytes (Carpen et al., 1992).

The three small GTP-binding proteins, Cdc42, Rac, and Rho, are key mediators of actin cytoskeletal remodeling induced by extracellular signals and also regulate the formation of cell-cell and cell-substratum adhesions (Ridley, 1996; van Aelst and D'Souza-Schory, 1997). In particular, Rho is responsible for the formation and maintenance of integrin-containing focal adhesions (Ridley and Hall, 1992), whereas Rac and Cdc42 mediate the assembly of smaller adhesive complexes associated with lamellipodia and filopodia (Nobes and Hall, 1995). The observation that E-selectin and ICAM-1 associate with components of the actin cytoskeleton suggested that Rho family proteins could be involved in regulating adhesion mediated by these receptors. Therefore, we have investigated the roles of these proteins in regulating monocyte binding to TNF- $\alpha$ -activated human endothelial cells and clustering of the monocyte-binding receptors, E-selectin, ICAM-1, and VCAM-1. We have found that Rho but not Rac or Cdc42 is required for the initiation and maintenance of monocyte adhesion to endothelial cells. In addition, Rho regulates receptor clustering, most likely by mediating linkage of the actin cytoskeleton to the membrane receptors. Clustering of membrane receptors is independent of the activity of the myosin light chain kinase (MLCK) and stress fibers, and is therefore clearly different from the clustering of integrins in focal contacts (Chrzanowska-Wodnicka and Burridge, 1996).

## Materials and Methods

### Materials

Reagents were obtained from the following sources: medium 199 modified Earle's salt solution (Gibco Life Technologies); Clonetics EGM-2 medium (TCS Biologicals Ltd.); Nutridoma NS (Boehringer Mannheim Ltd.); human fibronectin, heparin, endothelial cell growth supplement, bromodeoxyuridine (BrdU), cytochalasin D, 2,3-butanedione 2-monoxime, TRITC-phalloidin, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid), and mouse monoclonal anti-human HLA class I antigen antibody (Sigma Chemical Co.); TNF- $\alpha$  (Insight Biotechnology); mouse monoclonal anti-CD45 and anti-CD14 antibodies (Leucogate; Becton Dickinson); mouse monoclonal anti-CD58 antibody and FITC-labeled goat anti-rabbit IgG (Southern Biotechnology Associates); mouse monoclonal anti-CD14 (SH-M1) antibody (Autogen Bioclear UK Ltd.); tetramethylrhodamine dextran with a molecular weight of 10,000 (Molecular Probes); mouse monoclonal anti-E/P-selectin (clone BBIG-E6), mouse monoclonal anti-ICAM-1 (function blocking, clone BBIG-I1), mouse monoclonal anti-VCAM-1 (function blocking, clone BBIG V1), mouse monoclonal anti-human E-selectin (function blocking, clone BBIG-E4), mouse monoclonal anti-human P-selectin (function blocking, clone 9E1), goat anti-human VCAM-1 polyclonal antibody, and goat polyclonal anti-ICAM-1 antibody (R&D Systems); mouse monoclonal anti-myc (9E10) antibody (Santa Cruz Biotechnology); FITC- and TRITC-labeled goat anti-mouse and donkey anti-goat antibodies (Jackson ImmunoResearch Laboratories); ML-7 (Calbiochem); Limulus Amoebocyte Lysate test (Biowhittaker Inc.); protein assay kit (Bio-Rad); enhanced chemiluminescence kit (Amersham International plc); and polystyrene plates (Costar Corp.). Rabbit polyclonal antiezzrin, antimosin, and antiradixin antibodies were generously provided by Paul Mangeat (Montpellier, France); pcDNA3 N19RhoA (myc epitope-tagged) was a kind gift from Alan Hall (London, UK).

### Cell Culture

HUVECs were a kind gift from Ruggero Pardi (Milano, Italy). The cells were cultured in TC Nunclon flasks coated with 10  $\mu$ g/ml human fibronectin in medium 199 modified Earle's salt solution containing 1.25 g/liter NaHCO<sub>3</sub> and Glutamax and supplemented with 20% FCS, 100  $\mu$ g/ml endothelial cell growth supplement, 1% Nutridoma NS, and 100  $\mu$ g/ml heparin. Cells were cultured at 37°C in humidified air containing 5% CO<sub>2</sub>. For experiments cells were used between 3 and 7 passages.

MMVE-tsLT cells (kindly provided by Catherine Clarke, London, UK) were human mammary microvascular endothelial cells expressing a temperature-sensitive SV-40 large T antigen to extend their life span in culture. MMVE-tsLT cells were cultured in EGM-2 medium and used for experiments up to 15 passages.

For microinjection experiments cells were grown on glass coverslips coated with 10  $\mu$ g/ml human fibronectin until confluent. Glass coverslips were marked with a cross using a diamond pen to facilitate the localization of microinjected cells. To obtain quiescent cells, the culture medium was replaced by medium containing 10% FCS but no heparin or other growth factors. Cells were incubated in this medium for 72 h and tested for DNA synthesis by incubation with 10  $\mu$ M BrdU for 24 h. The cells that had incorporated BrdU were visualized as described previously (Wójciak-Stothard et al., 1998). Over 97% of cells were quiescent and did not incorporate BrdU.

### Isolation of Peripheral Blood Monocytes

Single donor plateletpheresis residues were purchased from the North London Blood Transfusion Service. Mononuclear cells were isolated by Ficoll-Hypaque centrifugation (specific density, 1.077 g/ml) preceding monocyte separation in a Beckman JE6 elutriator. Monocyte purity was assessed by flow cytometry using directly conjugated anti-CD45 and anti-CD14 antibodies and was routinely >85%. All media and sera were routinely tested for endotoxin using the Limulus Amoebocyte Lysate test and rejected if the endotoxin concentration exceeded 0.1 U/ml.

### Measurement of Monocyte Adhesion and Spreading

To activate endothelial cells, TNF- $\alpha$  was added at 100 ng/ml for 4 or 24 h. Cell viability was tested after treatment with TNF- $\alpha$  by a trypan blue exclusion test. After 4 h endothelial cells were washed four times in culture

medium to remove TNF- $\alpha$  and then purified human monocytes were added at  $5 \times 10^5$  cells/ml and incubated for a further 2 h before fixation. The monocyte/endothelial cell ratio in cocultures was  $3.2 \pm 0.2$ .

Where indicated, cytochalasin D at 0.05  $\mu$ g/ml was added to cell cultures 3 h after addition of TNF- $\alpha$  and incubated for 1 h. C3 transferase was added to culture medium at 15  $\mu$ g/ml 1 h after the addition of TNF- $\alpha$  and incubated together with TNF- $\alpha$  for a further 3 h. To determine the contribution of each monocyte-binding receptor to monocyte adhesion, endothelial cells were incubated for 1 h with function-blocking antibodies against E-selectin, P-selectin, ICAM-1, and VCAM-1 at 10  $\mu$ g/ml before the addition of monocytes.

The number of adherent monocytes and monocyte spread area was determined using a confocal laser scanning microscope (MRC500; Bio-Rad). To determine changes in the spread area of monocytes, images of the basal aspect of the cells were collected and the area was measured using the software integrated to the MRC500 (SOM, version 6.42 a) against a Geller MRS-2 magnification reference standard 131 R3 (Agar Scientific Ltd.). To estimate the extent of monocyte adhesion, the number of monocytes bound per endothelial cell was calculated. Cell cultures were stained for F-actin with TRITC-phalloidin and incubated with mouse monoclonal anti-CD14 (SH-M1) antibody diluted 1:100 and FITC-labeled anti-mouse IgG diluted 1:100 in order to facilitate identification of the adherent monocytes. In each experiment >500 endothelial cells were scored to calculate monocyte adhesion, and experiments were performed in triplicate. In some experiments endothelial cells were stained for E- and P-selectin, ICAM-1, and VCAM-1 as described below.

### Introduction of Recombinant Proteins into Endothelial Cells

The recombinant proteins, V14RhoA, N17Rac1, N17Cdc42, and C3 transferase, were expressed in *Escherichia coli* from the pGEX-2T vector as glutathione S-transferase fusion proteins and purified as described previously (Ridley et al., 1992). Protein concentrations were estimated using a protein assay kit (Bio-Rad).

Proteins were microinjected into the cytoplasm of quiescent HUVECs 3.5 h after stimulation with TNF- $\alpha$ . After a 15-min incubation, the cells were washed four times in culture medium and monocytes were added to endothelial cell cultures. To identify injected cells, tetramethylrhodamine dextran (molecular weight of 10,000) at 5 mg/ml was microinjected together with recombinant proteins. C3 transferase was microinjected at a concentration of 4  $\mu$ g/ml, V14RhoA was microinjected at  $\sim$ 100  $\mu$ g/ml, N17Rac1 at 7 mg/ml, and N17Cdc42 at 2 mg/ml. In experiments involving receptor clustering C3 transferase was added to the culture medium at 15  $\mu$ g/ml, 1 h after the addition of TNF- $\alpha$ , and incubated together with TNF- $\alpha$  for a further 3 h.

To express N19RhoA, an expression vector containing myc epitope-tagged N19RhoA cDNA (pcDNA3-N19RhoA) was microinjected at 0.05 mg/ml together with tetramethylrhodamine dextran into cell nuclei at the same time as the addition of TNF- $\alpha$ , and cells were incubated for a further 3 h before adding antibodies to induce receptor clustering or for 4 h before assaying monocyte adhesion. Cells expressing N19RhoA were identified with the mouse monoclonal anti-myc epitope antibody 9E10 and FITC-labeled anti-mouse antibody: 84%  $\pm$  10% of microinjected cells expressed detectable levels of N19RhoA.

### Receptor Clustering, Immunofluorescence, and Affinity Fluorescence

To induce receptor clustering, TNF- $\alpha$  was added to endothelial cells and then after 3 h mouse monoclonal antibodies to E-selectin, ICAM-1, VCAM-1, HLA class I antigen, or CD58/LFA-3 were added to cells at a final concentration of 10  $\mu$ g/ml and incubated for 1 h at 37°C. The mouse monoclonal anti-human E/P-selectin antibody used here recognizes both E- and P-selectin on the surface of endothelial cells. Using mouse monoclonal antibodies that specifically recognized only E- or P-selectin, we determined that TNF- $\alpha$ -activated HUVECs expressed predominantly E-selectin and only very low levels of P-selectin, and therefore the results obtained with the anti-E/P-selectin antibody relate to E-selectin.

After incubation with primary antibodies, TNF- $\alpha$  and the primary antibodies were removed from the cell medium and 10  $\mu$ g/ml of FITC-labeled goat anti-mouse antibody was added to the cells for 30 min. Cells were then washed three times in PBS, fixed with 4% formaldehyde dissolved in PBS for 10 min at room temperature, permeabilized for 6 min with 0.2% Triton X-100, and then incubated with 1  $\mu$ g/ml TRITC-phalloidin for 45

min to stain actin filaments, or for 1 h with rabbit polyclonal antiezzrin, antimoiesin, or antiradixin antibodies diluted 1:200, followed by 5  $\mu$ g/ml TRITC-labeled goat anti-rabbit antibody for 1 h. The specimens were mounted in moviol. To examine the extent of spontaneous receptor clustering upon the addition of the primary antibodies only, TNF- $\alpha$ -stimulated HUVECs were incubated for 1 h with the primary antibodies as described above, and then fixed. Fixed cells were then incubated with the secondary antibody for 30 min, washed, permeabilized, and stained for actin filaments. For controls, nonstimulated HUVECs or HUVECs that were stimulated with TNF- $\alpha$  for 4 h were used. The cells were then fixed, incubated with primary and secondary antibodies, and then permeabilized and stained for actin as described above.

In experiments with MLCK inhibitors, ML-7 (40  $\mu$ M) and 2,3-butanedione 2-monoxime (BDM) (5 mM) were added to cell cultures together with the primary antibody, 3 h after stimulation with TNF- $\alpha$ . After a 1-h incubation, the cells were washed as described before, and the inhibitors were added again together with the secondary antibody and incubated for 30 min. The cells were then washed, fixed, and stained for actin filaments as described above.

Confocal laser scanning microscopy was carried out with an LSM 310 or 510 (Zeiss) mounted over an infinity corrected Axioplan microscope (Zeiss) fitted with a  $\times$ 10 eyepiece, using either a  $\times$ 40 NA 1.3 or a  $\times$ 63 NA 1.4 oil immersion objective. Image files were collected as a matrix of  $1024 \times 1024$  pixels describing the average of 8 frames scanned at 0.062 Hz where FITC and TRITC were excited at 488 nm and 543 nm and visualized with a  $540 \pm 25$  and a  $608 \pm 32$  nm bandpass filters, respectively, where the levels of interchannel cross-talk were insignificant.

### Cell Surface Immunoassay

To measure the cell surface expression of E-selectin, ICAM-1, and VCAM-1 we used an ELISA assay as described by Zund et al. (1996) with some modifications. In brief, HUVECs were plated onto fibronectin-coated 96-well polystyrene plates at a density of  $2 \times 10^4$  cells/well and grown for 48 h. The cells were then incubated in starvation medium (10% FCS) for 24 h. The cells were stimulated with TNF- $\alpha$  for 4 or 24 h and, where indicated, cytochalasin D at 0.05  $\mu$ g/ml was added to cell cultures 3 or 23 h after addition of TNF- $\alpha$  and incubated for 1 h. C3 transferase was added to the culture medium at 15  $\mu$ g/ml either 1 or 21 h after the addition of TNF- $\alpha$  and incubated together with TNF- $\alpha$  for a further 3 h. In controls, C3 transferase and cytochalasin D were added to nonstimulated cells. Cells were washed three times in PBS and then fixed in 1% paraformaldehyde at 4°C for 15 min. The cells were washed three times in PBS and then incubated overnight with 1% BSA solution in PBS at 4°C. All wells were then washed three times in PBS and incubated for 2 h at room temperature with 200  $\mu$ l/well of 0.5% BSA solution containing 5  $\mu$ g/ml of mouse monoclonal anti-human E/P-selectin antibody, mouse monoclonal anti-human ICAM-1 antibody or goat anti-human VCAM-1 antibody. Subsequently, cells were washed three times in PBS and incubated with 200  $\mu$ l of HRP-conjugated rabbit anti-mouse IgG or donkey HRP-conjugated anti-goat IgG solution at 1:1,000 in 0.5% BSA for 1 h at room temperature. After washing, plates were developed by addition of peroxidase substrate, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid) and OD at 650 nm was determined on a microtiter plate spectrophotometer. Data are presented as mean  $\pm$  SD (background subtracted).

### Western Blotting

HUVECs were grown to confluence in 40-mm plastic petri dishes pre-coated with human fibronectin and starved for 24 h in 10% FCS as described above. TNF- $\alpha$  was added as indicated and incubated with cells for 4 or 24 h. Cytochalasin D and C3 transferase were added as described above (cell surface immunoassay). Cells were washed, lysed, and debris was removed by centrifugation at 14,000 rpm. The protein concentration was measured using a Bio-Rad protein assay. Equal amounts of protein were separated by SDS-PAGE on 7.5% polyacrylamide gels under non-reducing conditions, and transferred to nitrocellulose membranes, which then were blocked overnight with 5% nonfat dry milk powder in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.05% Tween 20. This was followed by incubation with anti-E/P-selectin, mouse monoclonal anti-ICAM-1, or goat anti-VCAM-1 antibodies diluted 1:500 in TBS-Tween containing 1% dried milk powder for 2 h. Blots were then washed three times in TBS-Tween and incubated for 1 h at room temperature with HRP-conjugated sheep anti-mouse antibodies diluted 1:2,000. Membranes were developed using an enhanced chemiluminescence kit (Amersham International).

## Results

### C3 Transferase and Cytochalasin D Inhibit Monocyte Adhesion and Spreading on Endothelial Cells

Human monocytes showed low levels of adhesion to unstimulated, quiescent HUVECs (Fig. 1 a). The few adherent cells retained a regular, rounded morphology similar to that observed in a suspension of freshly isolated monocytes (Fig. 2 a). Monocyte adhesion increased sevenfold after stimulation of endothelial cells with TNF- $\alpha$  (Figs. 1 a and 2 c).

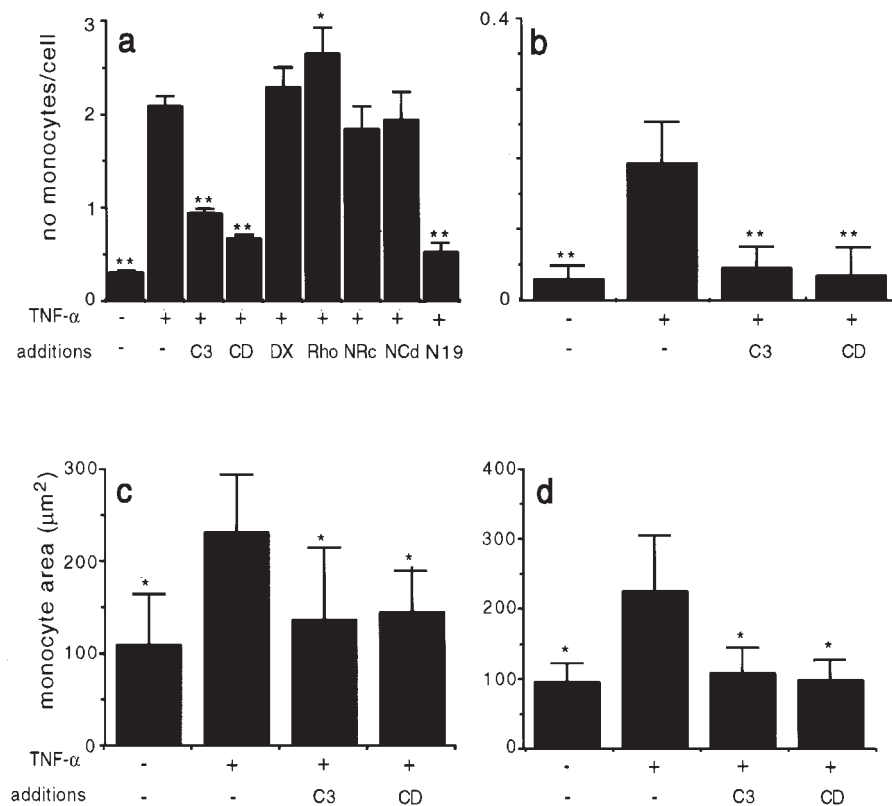
To determine whether Rho in endothelial cells plays a role in monocyte adhesion, we treated cells with C3 transferase, an exoenzyme produced by *Clostridium botulinum* which inhibits Rho by ADP ribosylation (von Eichel-Streiber et al., 1996). The TNF- $\alpha$ -induced increase in monocyte adhesion was reduced by 55% in HUVECs incubated with C3 transferase (Fig. 1 a). Monocyte adhesion was also inhibited in TNF- $\alpha$ -stimulated HUVECs which were microinjected with C3 transferase 30 min before the addition of monocytes (Fig. 2 d). Under the conditions used here, C3 transferase induced loss of stress fibers but did not induce cell rounding (Fig. 2 d). To provide further evidence for the involvement of Rho in monocyte adhesion, HUVECs were microinjected with a plasmid encoding N19RhoA, a dominant negative RhoA protein. In cells expressing N19RhoA, monocyte adhesion was reduced by 75% compared with control-injected cells (Fig. 1 a).

As Rho is known to induce actin reorganization, we specifically investigated the involvement of the endothelial cell actin cytoskeleton in monocyte adhesion by pretreat-

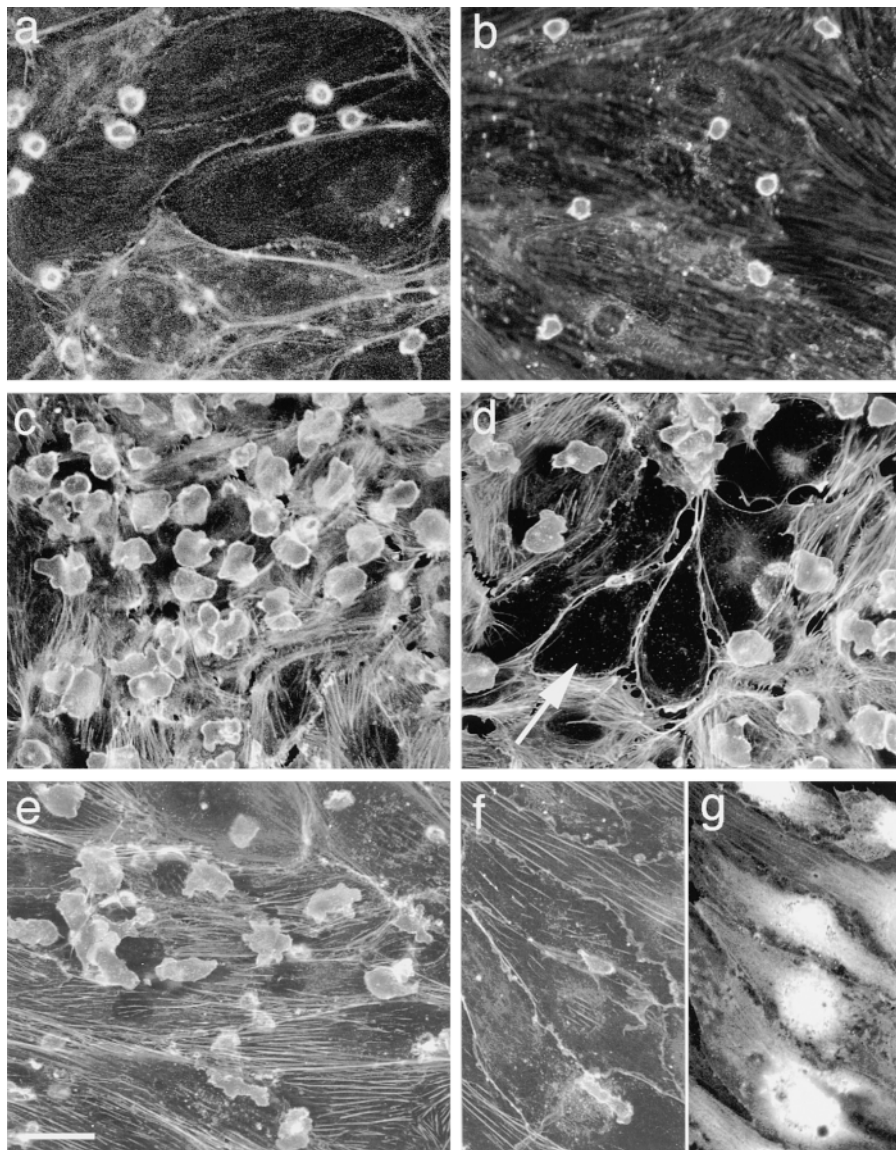
ing endothelial cells with cytochalasin D before addition of monocytes. Cytochalasin D inhibits addition of actin monomers to the barbed ends of actin filaments (Cooper, 1987), and as expected it induced a decrease in actin cables in HUVECs (see Fig. 7 i). It was necessary to wash out cytochalasin D from the medium before addition of monocytes, to prevent it acting on the monocytes. Control experiments showed that although the effects of cytochalasin D on the actin cytoskeleton of HUVECs were reversible, few actin cables reappeared during the 2-h incubation with monocytes. By 4 h after cytochalasin D removal, however, the actin cytoskeleton was essentially indistinguishable from that of untreated cells (data not shown). Monocyte adhesion was substantially inhibited in HUVECs pretreated with cytochalasin D (Fig. 1 a), indicating that the actin cytoskeleton in endothelial cells is important for promoting monocyte adhesion.

Monocytes attached to TNF- $\alpha$ -treated HUVECs showed an approximately twofold increase in their spread area relative to monocytes on unstimulated endothelial cells (Fig. 1 c). Many monocytes become elongated and extended lamellipodia, characteristic of a migratory phenotype (Fig. 2, c and e). The few monocytes that did attach to C3 transferase- or cytochalasin D-treated activated HUVECs did not spread significantly (Fig. 1 c).

As with HUVECs, adhesion of monocytes to nonactivated MMVE-tsLT cells was low and adherent monocytes remained rounded and unspread (Fig. 1, b and d, and Fig. 2 b). The addition of TNF- $\alpha$  to MMVE-tsLT cells increased the number of adherent monocytes fivefold (Fig. 1 b). This effect was almost completely inhibited by the ad-



**Figure 1.** Rho but not Rac or Cdc42 are required for the adhesion and spreading of monocytes on TNF- $\alpha$ -activated endothelial cells. Where indicated, endothelial cells were activated with TNF- $\alpha$  for 4 h. During this incubation time, cells were treated with C3 transferase (C3) for 3 h; cytochalasin D (CD) for 1 h; microinjected with V14RhoA (Rho), N17Rac (NRc), N17Cdc42 (NCd), or fluorescent dextran (DX) 3.5 h after TNF- $\alpha$  addition; or microinjected with pcDNA3-N19RhoA (N19) immediately after TNF- $\alpha$  addition. Monocytes were then added to endothelial cells for 2 h before fixation. a and b show the number of attached monocytes per endothelial cell in HUVEC and MMVE-tsLT cultures, respectively. c and d show the spread area of monocytes adherent to HUVEC and MMVE-tsLT cell cultures, respectively. Each experiment was performed in triplicate. All results are expressed as means  $\pm$  1 SD. The unpaired Student's *t* test was used to compare differences between control TNF- $\alpha$ -activated cells and cells treated or microinjected with the indicated substances. \* $P \leq 0.05$ ; \*\* $P < 0.01$ .



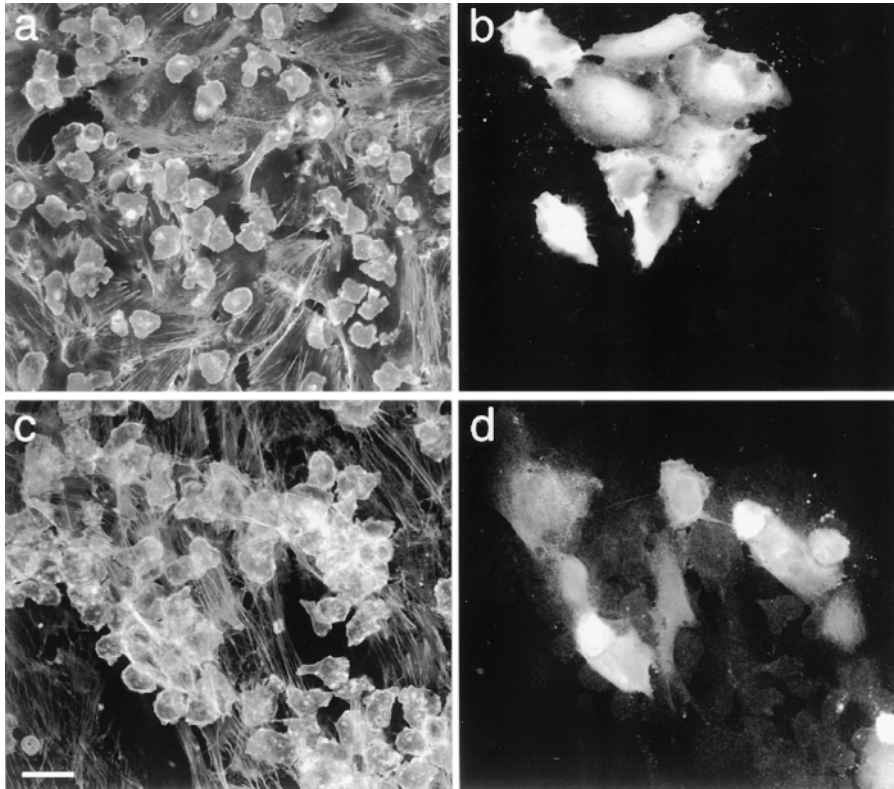
**Figure 2.** Inhibition of monocyte attachment and spreading on activated endothelial cells microinjected with C3 transferase. Monocytes were incubated for 2 h with control, unstimulated HUVECs (a), control, unstimulated MMVE-tsLT cells (b), TNF- $\alpha$ -activated HUVECs (c), and C3 transferase-microinjected HUVECs activated with TNF- $\alpha$  for 4 h (d) (the arrow points to the area of microinjected cells); TNF- $\alpha$ -activated MMVE-tsLT cells (e); and C3 transferase-microinjected TNF- $\alpha$ -activated MMVE-tsLT cells (f). Cells were then fixed and stained with FITC-phalloidin to show F-actin. The image in g corresponds to f and shows TRITC-dextran, which was coinjected with C3 transferase to visualize the injected cells. Bar, 10  $\mu$ m.

dition of C3 transferase or cytochalasin D to endothelial cells stimulated with TNF- $\alpha$  (Fig. 1 b, see also Fig. 2, e and f). Similar to HUVECs, the treatment of MMVE-tsLT cells with C3 transferase or cytochalasin D inhibited monocyte spreading on endothelial cells (Fig. 1 d).

As these results indicate that endothelial cell Rho is involved in regulating monocyte adhesion, we investigated whether the related proteins, Rac and Cdc42, also affect this process. Microinjection of TNF- $\alpha$ -activated HUVECs with dominant inhibitory Rac or Cdc42 protein, N17Rac1 or N17Cdc42, did not significantly change monocyte adhesion or spreading (Figs. 1 a and 3 a). The N17Cdc42 and N17Rac1 protein preparations were active as they were able to inhibit the formation of stress fibers in TNF- $\alpha$ -activated HUVECs (see Wójciak-Stothard et al., 1998). We conclude that Rho but not Rac or Cdc42 in endothelial cells is involved in the formation and maintenance of intercellular adhesions between monocytes and endothelial cells.

### ***V14RhoA Increases the Attachment of Monocytes to Endothelial Cells***

As our results indicate that Rho is required in endothelial cells for monocyte adhesion, we investigated the effects of introducing activated Rho into endothelial cells. Microinjection of constitutively activated Rho protein, V14RhoA, into quiescent, unstimulated cells did not enhance monocyte adhesion (Fig. 1 a), whereas in TNF- $\alpha$ -treated HUVECs V14RhoA induced a small but significant increase in monocyte adhesion (Fig. 1 a). Monocytes tended to form clusters around the cells microinjected with V14RhoA (Fig. 3 c). This was not a nonspecific consequence of microinjection because injection of fluorescent dextran into TNF- $\alpha$ -activated endothelial cells did not have a significant effect on monocyte adhesion (Fig. 1 a). These results provide further evidence for a role of Rho in promoting monocyte adhesion.



**Figure 3.** Effects of N17Cdc42 and V14RhoA on monocyte adhesion to HUVECs. TNF- $\alpha$ -activated HUVECs were microinjected with N17Cdc42 (a and b) or V14RhoA (c and d) 30 min before addition of monocytes for 2 h. Cells were then fixed and stained with FITC-phalloidin to show actin filaments (a and c); microinjected cells were visualized by coinjection of TRITC-dextran (b and d). Bar, 10  $\mu$ m.

### ***E-Selectin, ICAM-1, and VCAM-1 Are Major Monocyte-binding Receptors in TNF- $\alpha$ -activated HUVECs***

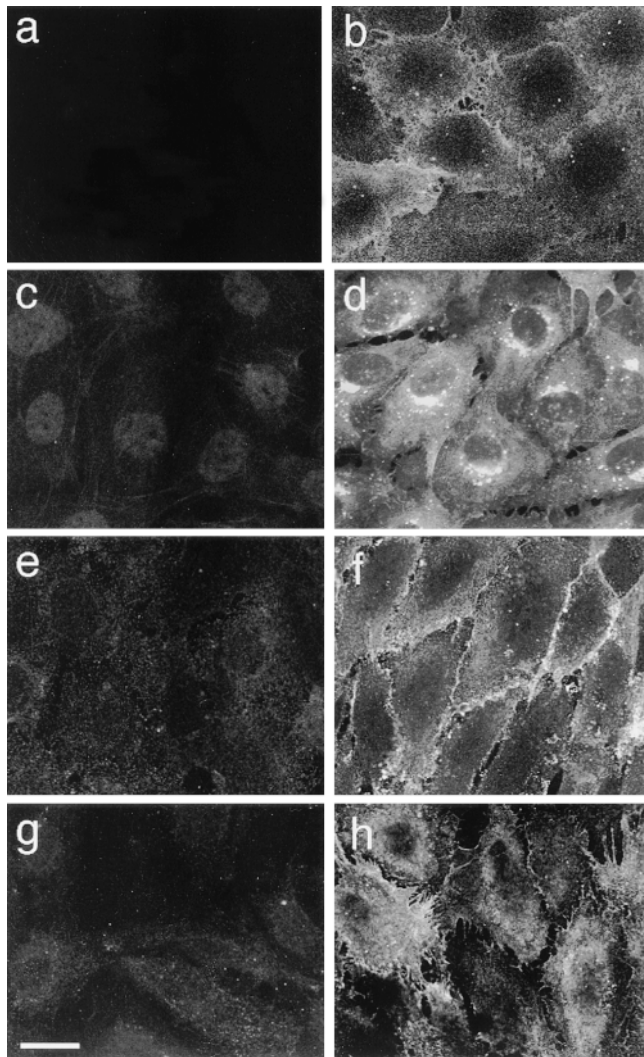
To investigate how Rho regulates monocyte adhesion and spreading, we first determined the involvement of different receptors for monocytes in mediating monocyte adhesion to TNF- $\alpha$ -activated monocytes. We focused specifically on E-selectin, ICAM-1, and VCAM-1, as these are the three major monocyte-binding receptors on HUVECs known to be upregulated by TNF- $\alpha$  (Luscinskas et al., 1996; May et al., 1996; Schleiffenbaum and Fehr, 1996; Melrose et al., 1998). As expected from previous studies (Beekhuizen and van Furth, 1993; Luscinskas et al., 1996; May et al., 1996), quiescent endothelial cells showed very low surface expression levels of E-selectin, ICAM-1, and VCAM-1 (Fig. 4, a, e, and g). TNF- $\alpha$  increased the surface expression of these three proteins by 4 h after stimulation (Fig. 4, b, f, and h). The levels of spontaneous receptor clustering were relatively low. ICAM-1 in TNF- $\alpha$ -treated cells tended to accumulate in the region of intercellular junctions (Fig. 4 f). The first changes in the expression of E-selectin were detectable by immunofluorescence 1 h after cell activation (data not shown). An increase in intracellular levels of E-selectin indicating de novo synthesis of the protein was also observed 2 h after stimulation with TNF- $\alpha$  (Fig. 4 d) (Weller et al., 1992).

Addition of function-blocking antibodies against E-selectin, ICAM-1, and VCAM-1 showed that each of these receptors contributed to monocyte adhesion to TNF- $\alpha$ -activated HUVECs. Antibodies against E-selectin, ICAM-1, and VCAM-1 inhibited monocyte adhesion by  $17\% \pm 5\%$ ,

$34\% \pm 9\%$ , and  $24\% \pm 8\%$ , respectively. Anti-P-selectin antibodies did not significantly reduce monocyte adhesion, reflecting the low level of P-selectin expression in HUVECs (see Materials and Methods). Inclusion of all four antibodies against E-selectin, P-selectin, ICAM-1, and VCAM-1 inhibited monocyte adhesion by  $61\% \pm 12\%$ . Similar results with adhesion-blocking antibodies carried out in stationary (nonflow) conditions have been reported previously (Luscinskas et al., 1996). These results indicate that E-selectin, ICAM-1, and VCAM-1 are major monocyte-binding receptors on TNF- $\alpha$ -activated HUVECs, although this does not rule out a contribution of other receptors such as ICAM-2, CD31/PECAM-1, or  $\alpha$ v $\beta$ 3 in monocyte-endothelial cell interactions (Schleiffenbaum and Fehr, 1996; Brown, 1997).

### ***C3 Transferase and Cytochalasin D Do Not Alter the Expression Levels of E-Selectin, ICAM-1, or VCAM-1***

One possible explanation for the decreased binding of monocytes to endothelial cells treated with C3 transferase or cytochalasin D is that there are lower levels of monocyte-binding receptors on the cell surface. To investigate this possibility, a cell surface immunoassay was performed on fixed monolayers of TNF- $\alpha$ -activated HUVECs (Fig. 5). Endothelial cells were stimulated with TNF- $\alpha$  for 4 or 24 h either with or without addition of C3 transferase for the last 3 h or cytochalasin D for 1 h before fixation. Analysis of receptor levels by ELISA showed that neither C3 transferase nor cytochalasin D induced any significant changes in the surface expression of E-selectin (Fig. 5 a), ICAM-1 (Fig. 5 b), or VCAM-1 (Fig. 5 c). Western blot

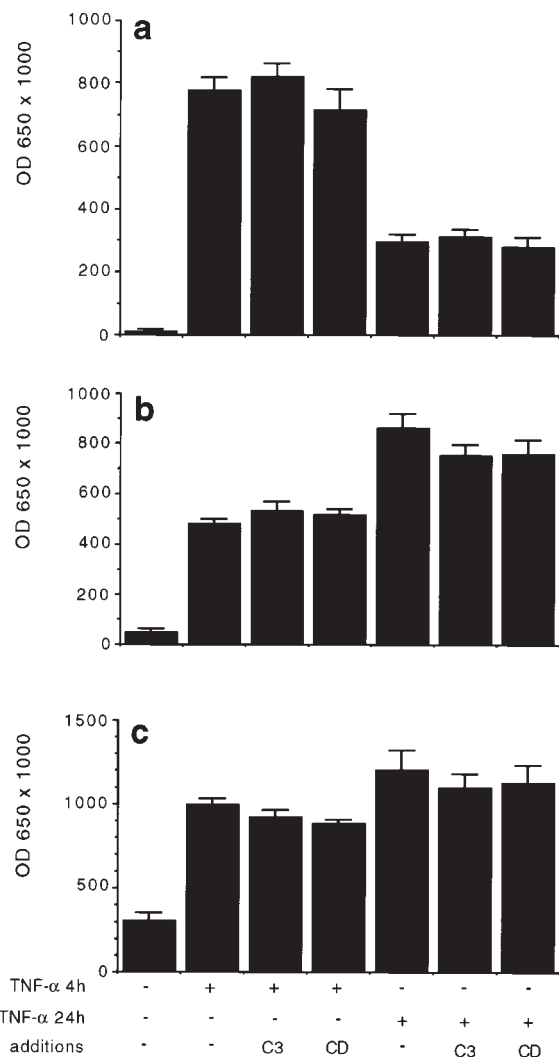


**Figure 4.** Expression of the monocyte-binding receptors E-selectin, ICAM-1, and VCAM-1 is increased in TNF- $\alpha$ -activated HUVECs. Surface expression in unstimulated, quiescent HUVECs is shown for E-selectin (a), ICAM-1 (e), and VCAM-1 (g). Increase in surface expression after treatment for 4 h with TNF- $\alpha$  is shown for E-selectin (b), ICAM-1 (f), and VCAM-1 (h). In c (no TNF- $\alpha$ ) and d (TNF- $\alpha$  for 2 h), cells were permeabilized after fixation to reveal intracellular E-selectin. Bar, 10  $\mu$ m.

analysis of TNF- $\alpha$ -treated HUVECs showed that the overall level of expression of E-selectin, ICAM-1, or VCAM-1 in HUVECs was also not altered by treatment with C3 transferase or cytochalasin D (data not shown).

#### **Monocyte Adhesion Induces Rho-mediated Changes in the Distribution of E-Selectin, ICAM-1, and VCAM-1 in HUVECs**

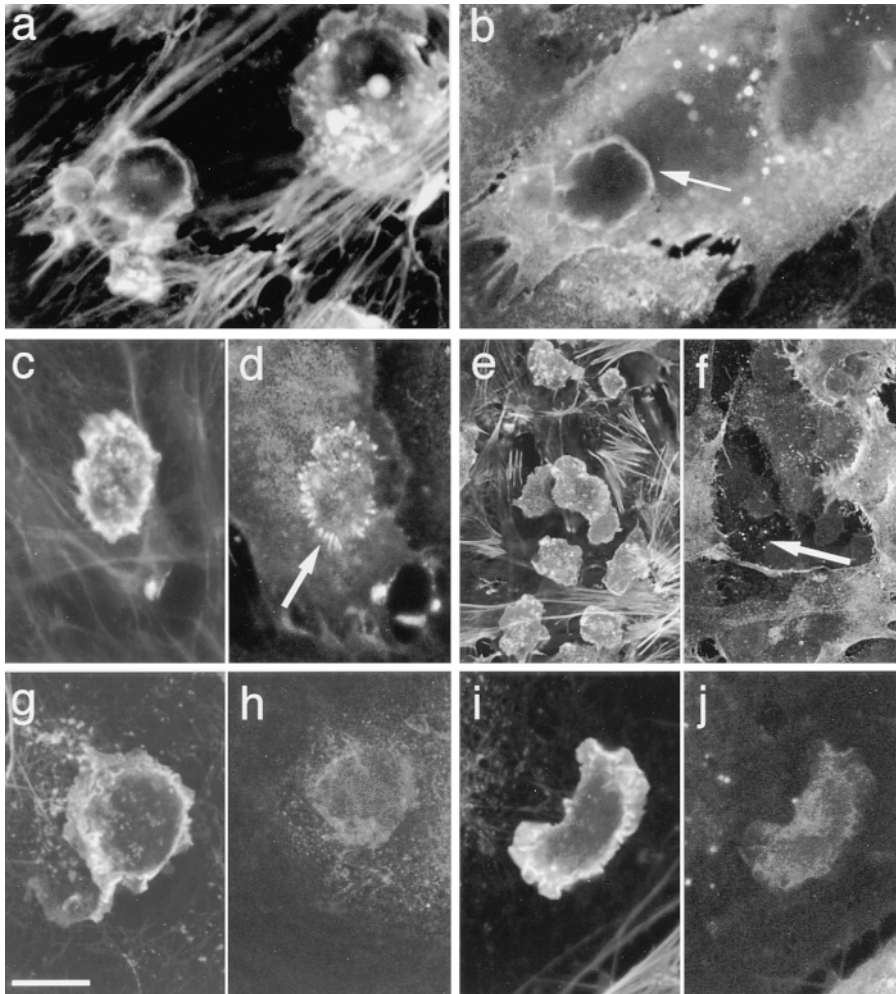
Clustering of E-selectin in endothelial cells has been postulated to be an initial step leading to the linkage of receptors to the cytoskeleton and stabilization of cell adhesion (Yoshida et al., 1996). We observed an accumulation of E-selectin on the surface of activated HUVECs around the margin of adhering monocytes (Fig. 6 b, arrow) and an



**Figure 5.** C3 transferase and cytochalasin D do not alter surface expression levels of E-selectin, ICAM-1, and VCAM-1 on HUVECs. Quiescent HUVECs were stimulated with TNF- $\alpha$  for 4 or 24 h and treated as indicated with C3 transferase (C3) or cytochalasin D (CD) for the last 3 or 1 h, respectively, of this incubation period. Cells were then fixed and the surface expression levels of E-selectin (a), ICAM-1 (b), and VCAM-1 (c) were determined by ELISA assay. All results are expressed as means  $\pm$  1 SD, and the unpaired Student's *t* test was used to compare differences between groups ( $n = 4$ ). The differences in surface expression of receptors in activated HUVECs compared with C3- or CD-treated activated HUVECs were statistically insignificant ( $P > 0.1$ ).

overall increase in receptor clustering often unrelated to the position of a monocyte (Fig. 6 b). The position of adhering monocytes was determined by F-actin staining (Fig. 6 a). ICAM-1 also accumulated along the margin of attached monocytes outlining fine protrusions formed by the monocyte membrane (Fig. 6, c and d).

In contrast, VCAM-1 did not significantly change its distribution upon the adhesion of monocytes. Clustering of receptors was seen only in a few places where monocytes were attached and the positions of clusters were unrelated to monocyte margins (Fig. 6, e and f). In endothelial cells



**Figure 6.** Adhesion of monocytes induces Rho-dependent clustering of E-selectin and ICAM-1 on HUVECs. HUVECs were treated with TNF- $\alpha$  for 4 h, then TNF- $\alpha$  was washed out and monocytes were added for 2 h. Cells were stained in a, c, e, g, and i for actin filaments to show the position of attached monocytes on the surface of endothelial cells and correspond to images b, d, f, h, and j, respectively. Clustering of E-selectin at the margin of an adhering monocyte is shown in b, ICAM-1 in d, and clustering of VCAM-1, unrelated to the position of monocytes, is shown in f (arrow); E-selectin (h) and ICAM-1 (j) clustering at the margin of monocytes is inhibited in HUVECs preincubated with C3 transferase for 3 h before addition of monocytes. Bar, 5  $\mu$ m.

that were treated with C3 transferase, E-selectin accumulation (Fig. 6, g and h) and clustering of ICAM-1 (Fig. 6, i and j) at the margin of the few adhering monocytes were much reduced. This lack of clustering could be a consequence of weaker monocyte adhesion which is unable to signal sufficiently to induce clustering, or represent a requirement for Rho in the process of clustering, which in turn is required for stable adhesion.

### ***C3 Transferase and Cytochalasin D Inhibit Clustering of Monocyte-binding Receptors on HUVECs***

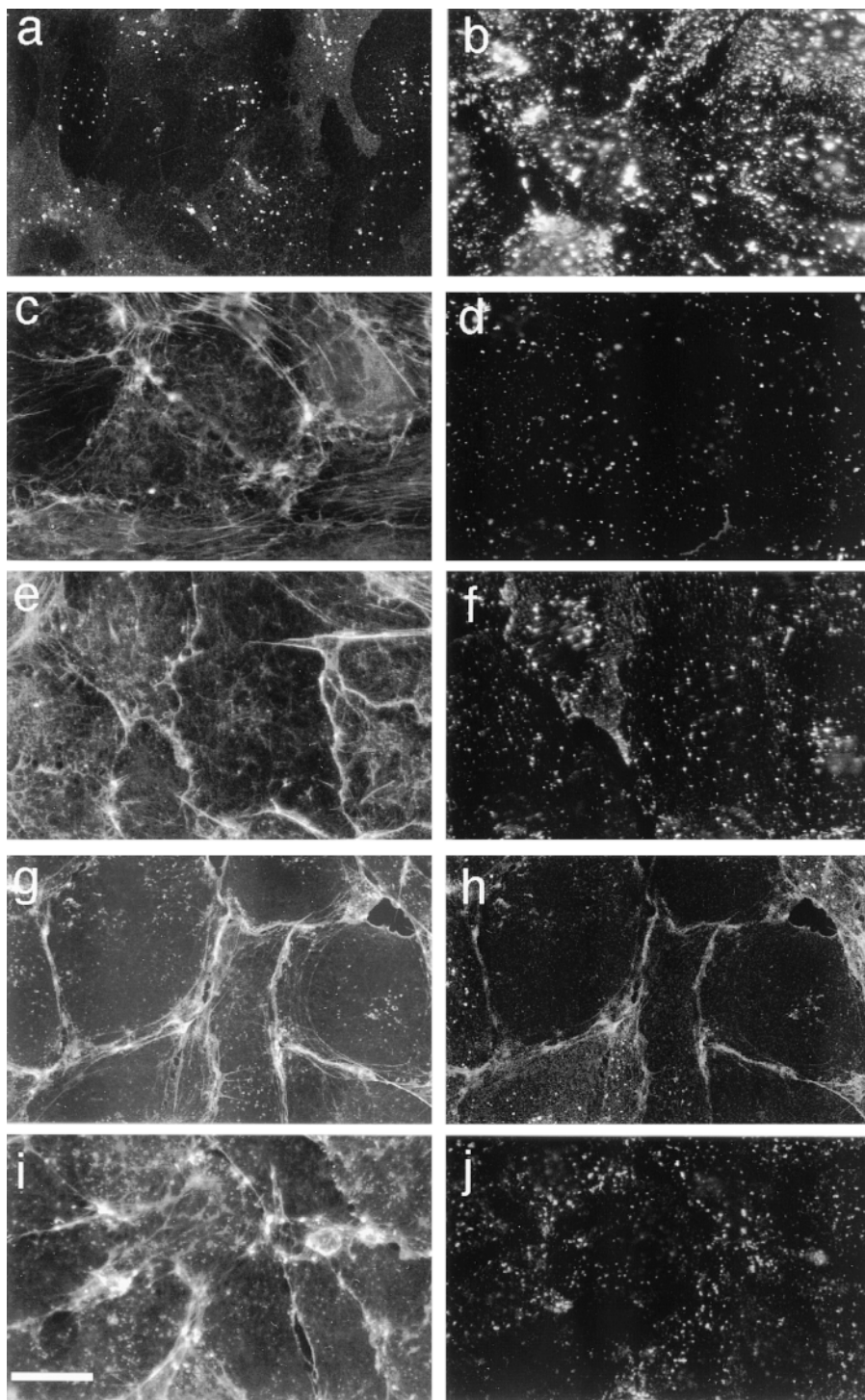
We have shown that although C3 transferase and cytochalasin D did not change the expression levels of monocyte-binding receptors, they inhibited the binding and spreading of monocytes on endothelial cells and inhibited receptor clustering around the adherent monocytes. To investigate further the role of Rho in regulating receptor clustering we mimicked clustering induced by adhering monocytes by incubating HUVECs with primary antibodies against E-selectin, ICAM-1, and VCAM-1 and then cross-linking them with fluorescently labeled secondary antibody. This technique of clustering membrane receptors with specific antibodies has been described previously

(Kornberg et al., 1991, 1992; Jewell et al., 1995; Yoshida et al., 1996).

Treatment of TNF- $\alpha$ -activated HUVECs with antibodies against E/P-selectin induced spontaneous clustering of E-selectin (Fig. 7 a) which was enhanced by the addition of secondary, cross-linking antibodies (Fig. 7 b). Clustering of E-selectin with primary antibodies alone or with primary antibodies followed by secondary antibodies was significantly reduced by the treatment of endothelial cells with C3 transferase (Fig. 7, d and f) or cytochalasin D (Fig. 7, h and j). Both C3 transferase and cytochalasin D also inhibited stress fiber formation, but under the conditions used did not induce cell rounding or detachment (Fig. 7, c, g, and i).

ICAM-1 in TNF- $\alpha$ -treated HUVECs incubated with only the primary antibody was localized mainly in the intercellular junctions (Fig. 8 a). Addition of secondary (cross-linking) antibodies caused a disappearance of ICAM-1 from the junctions and clustering of the receptors on the cell surface (Fig. 8 b). This was not a consequence of loss of intercellular junctions, as VE-cadherin localization to junctions was not altered by ICAM-1 cross-linking (data not shown). C3 transferase significantly inhibited antibody-induced clustering of ICAM-1 on the cell surface



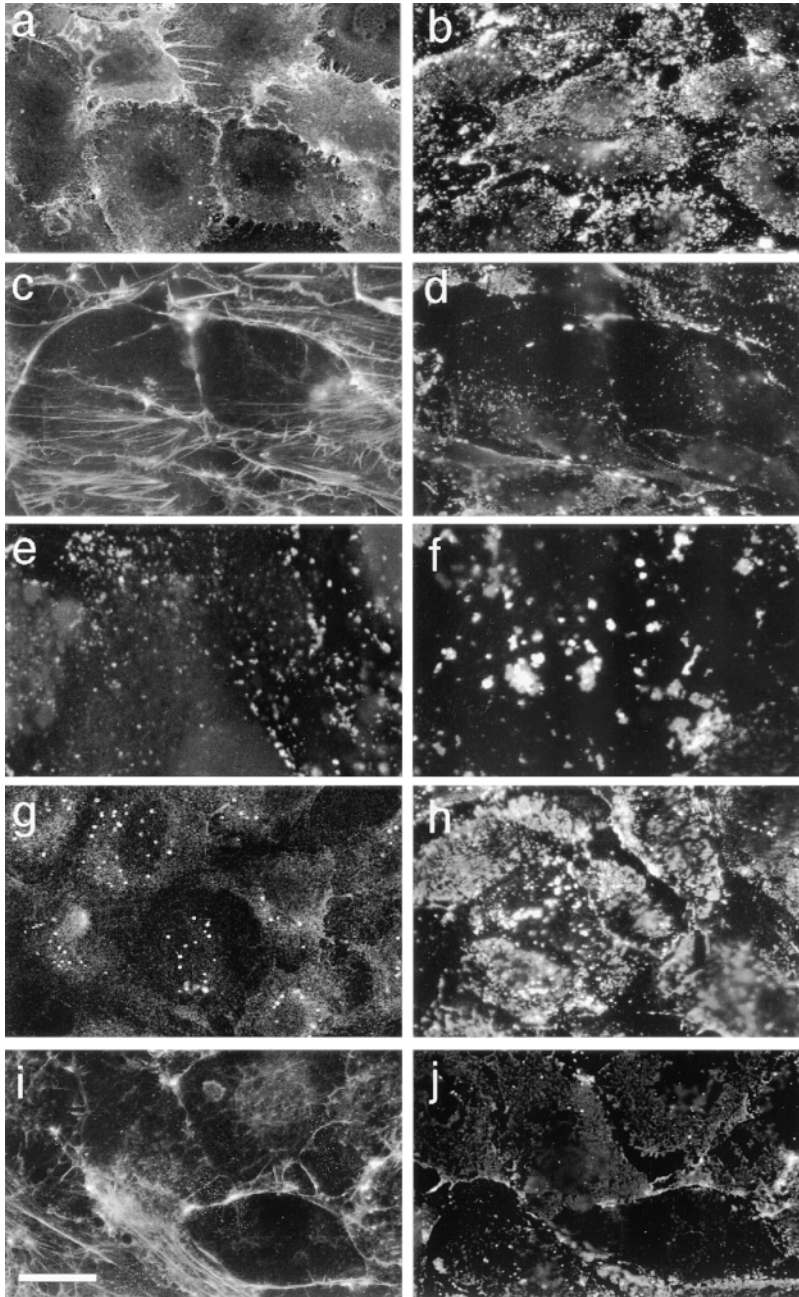


*Figure 7.* E-selectin clustering is inhibited by C3 transferase and cytochalasin D in HUVECs. HUVECs were activated with  $\text{TNF-}\alpha$  for 4 h then incubated with mouse anti-E-selectin antibody for 1 h. In a, d, and h, they were then fixed and stained with FITC-labeled goat anti-mouse IgG; in b, f, and j, they were incubated with FITC-labeled goat anti-mouse IgG for 30 min before fixation. In d and f, cells were incubated for 3 h with C3 transferase before addition of antibodies; in h and j cells were incubated with cytochalasin D for 1 h before antibody addition. F-Actin distribution is shown in c, e, g, and i; these images correspond to d, f, h, and j, respectively. Bar, 10  $\mu\text{m}$ .

(Fig. 8 d). It also reduced the localization of ICAM-1 to cell junctions, although again VE-cadherin localization was not altered (data not shown). Expression of dominant negative N19RhoA protein in endothelial cells also inhibited antibody-induced ICAM-1 clustering (Fig. 8, e and f) and E-selectin clustering (data not shown), providing further evidence that Rho plays a specific role in regulating receptor clustering.

Some clusters of VCAM-1 were present on the surface of  $\text{TNF-}\alpha$ -activated HUVECs treated with anti-VCAM-1

antibody (Fig. 8 g), but addition of secondary antibodies caused a marked increase in VCAM-1 clustering (Fig. 8 h). Pretreatment of the cells with C3 transferase (Fig. 8 j) or expression of N19RhoA (data not shown) inhibited clustering of VCAM-1 induced by the secondary antibodies. Clustering induced by the primary anti-VCAM-1 or anti-ICAM-1 antibodies alone was also inhibited by C3 transferase, and ICAM-1 and VCAM-1 clustering was similarly inhibited by cytochalasin D treatment (data not shown).

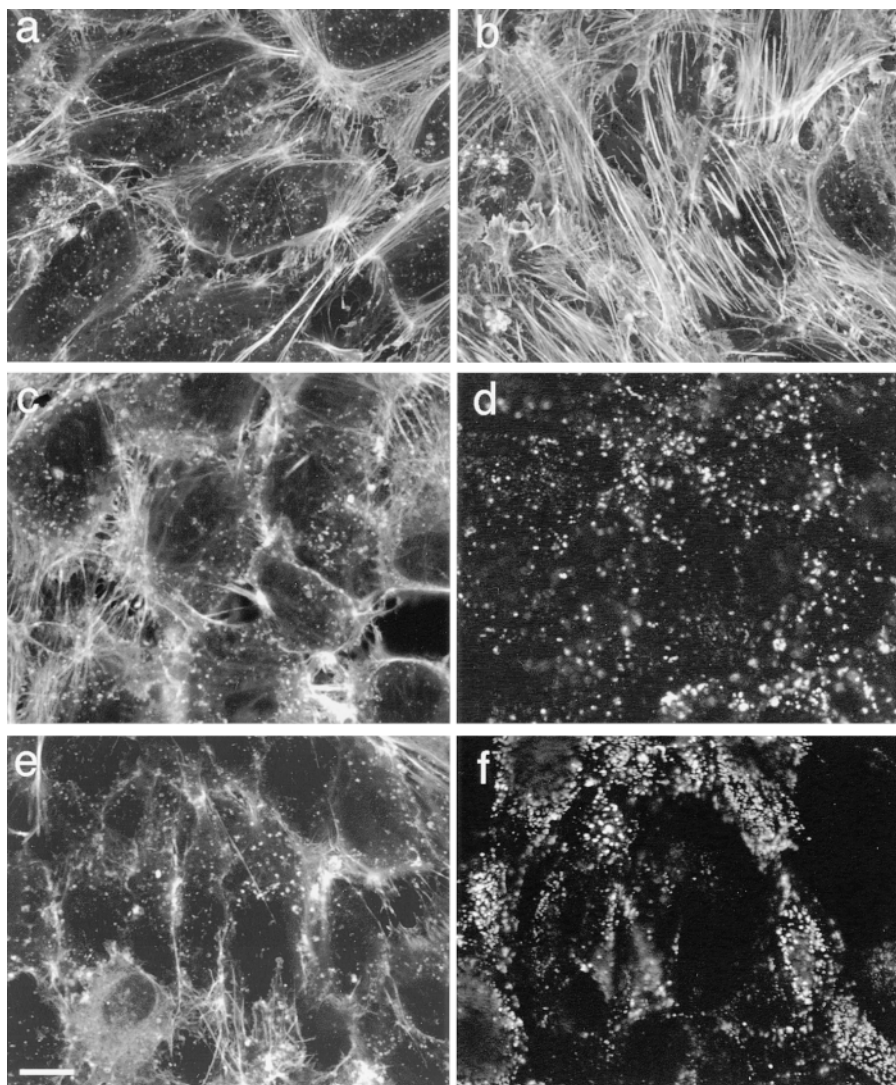


**Figure 8.** Rho is required for ICAM-1 and VCAM-1 clustering in HUVECs. In a–f, TNF- $\alpha$ -activated HUVECs were incubated for 1 h with mouse anti-ICAM-1 antibody. In a, cells were fixed and stained with FITC-labeled goat anti-mouse IgG; in b–f, they were incubated for 30 min with secondary, FITC-labeled goat anti-mouse IgG before fixation. In d, E-selectin distribution is shown in cells that were incubated with C3 transferase for 3 h before addition of antibodies; c corresponds to d and shows F-actin; in e, cells were microinjected with pcDNA3-N19RhoA plasmid 3 h after TNF- $\alpha$  addition, then incubated for a further 3 h before antibody addition; f shows control cells for e incubated with TNF- $\alpha$  for 6 h before antibody addition. In g–j, TNF- $\alpha$ -activated HUVECs were incubated with goat anti-VCAM-1 antibody for 1 h. In g, they were then fixed and stained with FITC-labeled anti-goat IgG; in h–j, they were incubated for 30 min with FITC-labeled anti-goat IgG before fixation. In j, VCAM-1 distribution is shown in cells incubated with C3 transferase for 3 h before antibody addition; i corresponds to j and shows F-actin distribution. Bar, in a–d and g–j, 10  $\mu$ m; in e and f, 3  $\mu$ m.

### ***Clustering of E-Selectin, ICAM-1, and VCAM-1 Is Not Dependent upon the Activity of MLCK and Does Not Require the Presence of Stress Fibers***

Cross-linking of E-selectin, ICAM-1, and VCAM-1 with antibodies was accompanied by increased stress fiber accumulation and appearance of intercellular gaps, indicative of increased contractility (Fig. 9 b and data not shown). Adhesion of monocytes to TNF- $\alpha$ -activated endothelial cells also induced stress fiber formation (compare Fig. 2 c with Fig. 9 a), consistent with previous observations (Lorenzon et al., 1998). Antibody-induced clustering of HLA class I antigen or CD58/LFA-3, a receptor of the Ig superfamily (Springer, 1990), did not induce stress fiber formation, showing that this is not a general re-

sponse to receptor clustering but is restricted to specific receptors (data not shown). C3 transferase inhibited the formation of stress fibers induced by the use of cross-linking antibodies (Fig. 7, c and e, and Fig. 8, c and i) or by monocyte adhesion (Fig. 2 d), indicating that Rho is required for this response. To investigate whether the mechanism of receptor clustering on HUVECs is linked to stress fiber formation and/or is dependent on the activity of MLCK, as was suggested for the Rho-mediated assembly of integrins into focal contacts (Chrzanowska-Wodnicka and Burridge, 1996), we used two MLCK inhibitors ML-7 and BDM. ML-7 is a potent and selective inhibitor of both Ca<sup>2+</sup>-dependent and -independent MLCKs (Saitoh et al., 1986). BDM acts as an inhibitor of muscle myo-



**Figure 9.** Inhibitors of MLCK have no effect on receptor clustering in HUVECs. In all cases, quiescent HUVECs were activated with TNF- $\alpha$  for 4 h. Cells were then fixed (a); or treated with primary, anti-E-selectin antibody and secondary, cross-linking antibody before fixation (b-f). In a and b, F-actin localization is shown. The images in c and d show F-actin (c) and E-selectin localization (d) in cells treated with ML-7; e and f show F-actin (e) and ICAM-1 (f) localization in cells treated with BDM (f). Bar, 10  $\mu$ m.

sin ATPase activity (Higuchi and Takemori, 1989). Both inhibitors caused a loss of stress fibers in HUVECs, as previously reported (Wójciak-Stothard et al., 1998) (Fig. 9, c and e), but did not inhibit the antibody-induced clustering of E-selectin (Fig. 9 d), ICAM-1 (Fig. 9 f), or VCAM-1 (data not shown).

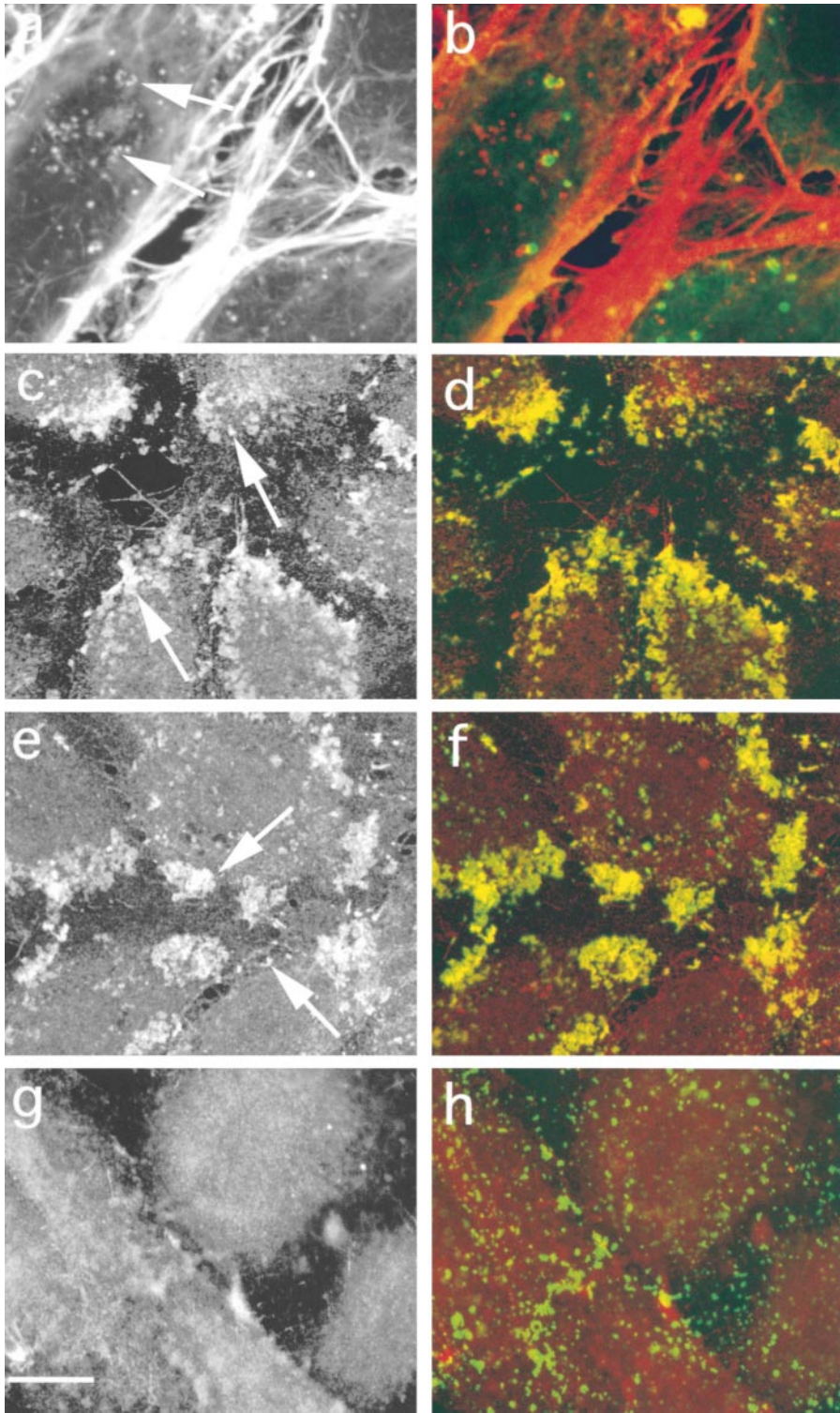
These results show that neither Rho-mediated stress fiber formation nor myosin-dependent contractility is necessary for receptor clustering, implying that Rho independently induces the clustering of monocyte-binding receptors and stress fiber formation. Therefore, we sought to determine whether F-actin or associated proteins were detectably linked with clusters of monocyte-binding receptors. The localization of E-selectin (Fig. 10 b), ICAM-1, and VCAM-1 (data not shown) after antibody-induced receptor clustering was not related to stress fibers in HUVECs (Fig. 10 a). Some large clusters of E-selectin (Fig. 10 a), ICAM-1, and VCAM-1 (data not shown) colocalized with F-actin, and in these cases the F-actin often appeared to form a ring around the cluster (Fig. 10 a, arrow). However, F-actin did not detectably colocalize with most clusters of receptors, suggesting that if they are linked with the

actin cytoskeleton this does not require the presence of large actin-containing structures.

Ezrin/radixin/moesin (ERM) proteins can provide a link between some membrane receptors and the actin cytoskeleton, and in particular ICAM-1 has been reported to interact with ezrin *in vitro* (Heiska et al., 1998). Antibody-induced clusters of ICAM-1 (Fig. 10 d), VCAM-1 (Fig. 10 f), and E-selectin (data not shown) often colocalized with moesin (Fig. 10, c and e), ezrin, and radixin (data not shown). This was observed with both small and large clusters of receptors (Fig. 10, arrows). This colocalization of ERM proteins with monocyte-binding receptors was not a nonspecific consequence of antibody-induced receptor clustering, as antibody-induced clusters of HLA class I antigen did not colocalize with moesin (Fig. 10, g and h), ezrin, or radixin (data not shown). Similarly, clusters of CD58/LFA-3 did not colocalize with ERM proteins (data not shown).

## Discussion

*In vivo*, leukocyte adhesion to endothelial cells is a prereq-



**Figure 10.** Colocalization of clusters of E-selectin, ICAM-1, and VCAM-1 with F-actin and ERM proteins. HU-VECs were activated with TNF- $\alpha$  for 4 h, then receptors were cross-linked with receptor-specific antibodies followed by FITC-labeled secondary antibodies (see Materials and Methods). In a, F-actin distribution is shown in HU-VECs treated with cross-linking antibodies against E-selectin. The image in b is the F-actin image in a (red) merged with the corresponding image showing E-selectin (green) localization. Large clusters of E-selectin colocalize with F-actin, as indicated by arrows in a. Moesin distribution is shown in cells treated with cross-linking antibodies against ICAM-1 (c), VCAM-1 (e), or HLA class I antigen (g). The merged images in d, f, and h are the same moesin images (red) merged with corresponding (green) images showing ICAM-1 (d), VCAM-1 (f), and HLA class I antigen (h) localization. Yellow color in these merged images indicates colocalization of moesin with the respective receptors. Arrows indicate examples of colocalization of moesin with receptors. Bar, a and b, 5  $\mu$ m; in c-h, 10  $\mu$ m.

uiseite for subsequent transmigration across the endothelium into underlying tissues. In this paper we have demonstrated that Rho in endothelial cells is required for the adhesion and spreading of monocytes, and modulates the clustering of the monocyte-binding receptors, E-selectin, ICAM-1, and VCAM-1, induced by monocyte adhesion or by cross-linking antibodies. This clustering is dependent on the actin cytoskeleton, as it is prevented by cytochala-

sin D, an inhibitor of actin polymerization, which also inhibits monocyte adhesion. These results demonstrate that responses in endothelial cells activated by receptor engagement are crucial for stable monocyte adhesion, and suggest that Rho may regulate the linkage between monocyte-binding receptors and the actin cytoskeleton to allow the formation of adhesion foci between monocytes and endothelial cells.

The precise links between leukocyte-binding receptors and the actin cytoskeleton have not been identified, but ICAM-1, ICAM-2, and E- and L-selectins have all been reported to associate with actin-binding proteins (Carpen et al., 1992; Yoshida et al., 1996; Brenner et al., 1997; Heiska et al., 1998). Clustering of adhesion receptors has been suggested to play a mechanical role in strengthening cell-cell or cell-extracellular matrix adhesion. In fibroblasts, tension transmitted via extracellular matrix proteins to integrins can strengthen their linkage to the cytoskeleton, and lead to further clustering of integrins (Choquet et al., 1997). Similar responses may occur after leukocyte binding to endothelial cells. E-Selectin clusters and associates with the actin cytoskeleton during leukocyte adhesion; this linkage increases the stress resistance of the ligand-receptor binding and can be inhibited by cytochalasin D (Yoshida et al., 1996). We have observed clustering of both E-selectin and ICAM-1 at the margin of adhering monocytes, and in addition found that F-actin colocalized with large clusters of antibody cross-linked receptors, suggesting association of these clusters with the actin cytoskeleton. Clusters of E-selectin, ICAM-1, and VCAM-1 also colocalized with ERM proteins, which are known to interact with F-actin (Heiska et al., 1996, 1998; Serrador et al., 1997; Tsukita et al., 1997; Yonemura et al., 1998). Association of ERM proteins with the clustered receptors is receptor specific as we did not observe any colocalization of ERM proteins with clusters of HLA class I antigen or with CD58/LFA-1, a receptor for CD2 (Springer, 1990). The involvement of F-actin in the cross-linking of monocyte-binding receptors and strengthening of monocyte-endothelial adhesion is further supported by the observation that clustering is inhibited by C3 transferase and cytochalasin D. VCAM-1 did not localize at the margins of adherent monocytes, although its clustering by cross-linking antibodies was also dependent on Rho activity. Therefore, it is likely that monocyte adhesion and spreading on endothelial cells depends initially on Rho-regulated clustering of E-selectin and ICAM-1, and that VCAM-1 plays a role at later stages of monocyte migration.

Recent evidence suggests that clustering of leukocyte-binding receptors plays a signaling as well as a mechanical role in endothelial cells. For example, adhesion of monocytes has been reported to induce a transient increase in the cytosolic free calcium concentration and also stress fiber assembly in HUVECs, and these responses are mimicked by incubation with antibodies against E-selectin, VCAM-1, or platelet/endothelial cell adhesion molecule (PECAM-1) (Lorenzon et al., 1998). In addition, cross-linking of ICAM-1 on brain endothelial cells was reported to activate Rho and to induce Rho-dependent tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130<sup>cas</sup> (Etienne et al., 1998). Our observation that stress fiber formation is stimulated in HUVECs after monocyte adhesion or antibody-induced clustering of E-selectin, ICAM-1, and VCAM-1 suggests that these events also activate Rho. Although we have reported previously that TNF- $\alpha$  itself induces stress fiber formation in quiescent HUVECs (Wójciak-Stothard et al., 1998), this is a transient response and by 4 h after TNF- $\alpha$  addition, when monocytes or cross-linking antibodies were added, the

background level of stress fibers was low. As the cross-linking of HLA class I antigen and CD58/LFA-3 did not result in increased stress fiber formation, this response appears to be limited to receptors involved in leukocyte interaction.

The mechanisms whereby monocyte-binding receptors transduce signals in endothelial cells have not been established, but interestingly E-selectin can interact via its cytoplasmic domain with paxillin and focal adhesion kinase (Yoshida et al., 1996), which are known to be involved in integrin-mediated signaling and are activated via a Rho-regulated pathway (Hemler, 1998). E-Selectin itself is a target for protein phosphorylation: its cytoplasmic domain contains several potential phosphorylation sites, at least one of which becomes phosphorylated in cytokine-activated HUVECs and may therefore act to recruit signaling proteins (Smeets et al., 1993).

The cross-linking of E-selectin, ICAM-1, and VCAM-1 induces stress fiber formation, but MLCK inhibitors which are known to prevent the formation of stress fibers (Chrzanowska-Wodnicka and Burridge, 1996; Wójciak-Stothard et al., 1998) do not inhibit receptor clustering. This suggests that occupancy of these receptors leads to Rho activation, which then activates at least two separate signaling pathways, one leading to stress fiber assembly and another to receptor clustering. As stress fiber formation is not required for receptor clustering, receptor clustering represents a new response mediated by Rho signaling. Interestingly, introduction of activated V14RhoA into endothelial cells only slightly increased monocyte adhesion, suggesting that endogenous Rho is strongly activated during monocyte binding and that this is sufficient to induce near-maximal monocyte adhesion. Some of the downstream signaling partners involved in Rho-induced stress fiber formation have been identified (Sahai et al., 1998; reviewed in van Aelst and D'Souza-Schory, 1997), and it will be interesting to determine which of these are involved in receptor clustering on endothelial cells. In some circumstances, Rho can be activated indirectly via Cdc42 and Rac (Nobes and Hall, 1995; Wójciak-Stothard et al., 1998), but as the binding of monocytes to endothelial cells was not inhibited by dominant negative inhibitors of Cdc42 and Rac, clustering of receptors is likely to be an effect of direct activation of Rho by receptor engagement.

The effect of inhibiting Rho on monocyte binding and receptor clustering closely resembled that caused by cytochalasin D, suggesting that Rho regulates the linkage of the actin cytoskeleton to the surface receptors. Incubation of cells with cytochalasin D leads to gradual loss of actin filaments, as cytochalasin D acts by binding to barbed ends of actin filaments and preventing them from further polymerization or shortening (Cooper, 1987). Interestingly, C3 transferase and cytochalasin D have also been reported to inhibit the clustering of Fc $\gamma$  receptors on macrophages induced by opsonized particles, and concomitantly inhibit Fc $\gamma$  receptor-induced protein tyrosine phosphorylation, calcium release, and actin cup formation (Hackam et al., 1997). Together with our results, this suggests that Rho may be more generally involved in mediating the linkage of receptors to the actin cytoskeleton, and that this linkage and resultant receptor clustering is important for cellular signaling. Precisely how Rho alters receptor clustering is

not known. It could be required for the formation of links between receptors and the actin cytoskeleton, once receptors have diffused in the plasma membrane, and thereby stabilize transient clusters of receptors. Alternatively, it could be actively involved in regulating the diffusion of receptors in the plasma membrane.

The mechanism by which Rho regulates links between receptors and the actin cytoskeleton could well involve ERM proteins, which interact with both F-actin and several adhesion receptors, including ICAM-1 (Heiska et al., 1996, 1998; Serrador et al., 1997; Tsukita et al., 1997; Yonemura et al., 1998), and which we have found colocalize with clusters of ICAM-1, VCAM-1, and E-selectin. ERM proteins also interact with RhoGDI, which is normally found in the cytoplasm in complex with Rho proteins, and may thereby facilitate Rho targeting to cell adhesion receptors and subsequent activation (Takai et al., 1995; Hirao et al., 1996; Takahashi et al., 1997).

In conclusion, our data indicate that Rho regulates the clustering of the monocyte-binding receptors E-selectin, ICAM-1, and VCAM-1 on the surface of endothelial cells, probably by enhancing their association with the actin cytoskeleton. The assembly of cytoskeletal connections with the clusters of membrane receptors could then provide "footholds" for attached monocytes and create the tension required for their spreading and migration, thereby mimicking the more static nature of extracellular matrix. A similar requirement for association with the actin cytoskeleton has been reported for integrins in focal adhesion complexes and for L-selectin- and LFA-1-mediated adhesion in leukocytes (Pavalko et al., 1995; Chrzanoska-Wodnicka and Burridge, 1996; Lub et al., 1997). Clustering of monocyte-binding receptors is not dependent on stress fibers, in contrast to their involvement in focal adhesion assembly (Burridge and Chrzanoska-Wodnicka, 1996). The formation of stress fibers that accompanies cross-linking of membrane receptors may instead serve to provide a more rigid cell structure to facilitate monocyte migration.

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