Cross-Linking of Brain Endothelial Intercellular Adhesion Molecule (ICAM)-1 Induces Association of ICAM-1 With Detergent-Insoluble Cytoskeletal Fraction

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Abstract—Intercellular adhesion molecule (ICAM)-1 plays a vital role in the process of leukocyte transmigration through endothelial cell (EC) barriers and has been shown to mediate signal transduction events in ECs induced either by its cross-linking or by the binding of T lymphocytes. Immunoblotting of ICAM-1 of Triton X-100 detergent fractions demonstrated that the majority of ICAM-1 was contained within the detergent-soluble fraction (noncytoskeletal associated) under basal conditions. After cross-linking of endothelial ICAM-1 with monoclonal antibody or coculture with T lymphocytes, EC ICAM-1 was observed to partition with a Triton X-100–insoluble (cytoskeletal associated) fraction in a dose- and time-dependent manner. Redistribution of ICAM-1 was specific, inasmuch as no association with the Triton X-100–insoluble fraction was observed after cross-linking of vascular cell adhesion molecule-1, nor did cross-linking of ICAM-1 result in a redistribution of the platelet and EC adhesion molecule. ICAM-1 association with the endothelial cytoskeleton after cross-linking was unaffected after treatment of the cells with cytochalasin D, C3-transferase, removal of extracellular calcium ions, or chelation of intracellular calcium ions. These data show that ICAM-1 colocalizes with the endothelial cytoskeleton and associates with a detergent-insoluble fraction after cross-linking. (Arterioscler Thromb Vasc Biol. 2001;21:810-816.)

Key Words: endothelium • intercellular adhesion molecule-1 • cytoskeleton

Lymphocytes migrating across the central nervous system vasculature have to overcome impermeable tight junctions that are characteristic of the endothelial cells (ECs) forming the blood–central nervous system barriers. However, it is becoming apparent that in this context, the barrier is not passive, inasmuch as brain ECs are intimately and actively involved in facilitating lymphocyte diapedesis into the central nervous system. The expression of intercellular adhesion molecule (ICAM)-1 on ECs is pivotal in supporting lymphocyte migration across the vascular endothelium. Neutralization of ICAM-1/lymphocyte function–associated antigen (LFA)-1, but not of vascular cell adhesion cell (VCAM)-1/very late antigen-4, leads to a decreased migration of interleukin-2–activated antigen-specific T lymphocytes through unstimulated brain EC monolayers.

Recent evidence has shown that brain EC ICAM-1 is capable of evoking signal transduction events after ICAM-1 cross-linking or coculture with T lymphocytes bearing the ICAM-1 counterreceptor. Mimicking lymphocyte attachment, through cross-linking EC ICAM-1 molecules in vitro, leads to an induction of actin stress fibers and an enhanced tyrosine phosphorylation of cortactin in the rat brain EC lines GP8/3.9 and RBE4. In addition, focal adhesion kinase, paxillin, and p130

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signal transduction initiated through ICAM-1 in response to leukocyte adhesion may be propagated via the endothelial cytoskeleton. The present study demonstrates that after ICAM-1 ligation, ICAM-1 specifically associates with an endothelial cytoskeletal fraction. We also show that ICAM-1 association with the endothelial cytoskeleton after cross-linking is unaffected after inhibition of downstream signaling pathways, suggesting that ICAM-1 redistribution is an early event in the signaling cascade.

Methods

Chemicals

Recombinant rat interferon (IFN)-γ was obtained from Genzyme. 1,2-Bis(2-amino-5-methylphenoxy)ethane-N,N,N′,N′-tetraacetic acid tetraethyl ester (MAPTAM) and BAPTA acetoxyethyl ester (BAPTA) were obtained from Calbiochem. C3-transferase protein was expressed and purified from *Escherichia coli.*

Cells

The rat brain microvascular EC line GP8/3.9 was grown as previously described. Peripheral lymph node T lymphocytes, which are adhesive but nonmigratory, were isolated and purified as previously described.1

Antibodies

Anti-rat ICAM-1 monoclonal antibody (mAb, clone 1A29) and anti-rat major histocompatibility complex (MHC) class I (RT1a) were obtained from Serotec. Anti-rat ICAM-1 mAb (clone 3H8) and anti-rat platelet and EC adhesion molecule (PECAM)-1 mAb (clone 3A12) were kind gifts from Dr W. Hickey, Dartmouth Medical School, Hanover, NH. Anti-rat VCAM-1 mAb (5F10) was a gift from Dr Roy Lobb, Biogen, Boston, Mass. Anti-caveolin polyclonal antibody, FITC-conjugated anti-rabbit IgG, and TRITC-conjugated anti-mouse IgG were obtained from Dr Roy Lobb, Biogen, Boston, Mass. Anti-caveolin polyclonal antibody was obtained from Transduction Laboratories. FITC and TRITC-conjugated anti-mouse IgG and FITC conjugated anti-rabbit IgG were obtained from Jackson Immunchemicals.

Immunoprecipitation and Western Blotting

Cell lysates and sucrose density fractions were added to SDS-PAGE sample buffer and resolved on 8% to 12.5% SDS-PAGE under nonreducing conditions as previously described.8

Fluorescence Microscopic Analysis of ICAM-1 and Caveolin-1 Colocalization

In all cases, GP8/3.9 ECs were grown on 8-well plastic chamber slides and treated with 100 U/mL rat recombinant IFN-γ for 48 hours to upregulate the surface expression of ICAM-1 on ECs. Cells were fixed, permeabilized, and blocked as previously described.1 Cells were incubated with mouse monoclonal anti–ICAM-1 antibody (IgG, supernatant from 3H8 hybridoma) and rabbit polyclonal anti–caveolin-1 (IgG, 1:500) overnight at 4°C. Cells were washed and incubated with secondary antibody, FITC-conjugated anti-rabbit IgG (1:50), and TRITC-conjugated anti-mouse IgG (1:50). Cells were washed, mounted in Vectashield (Vector Laboratories, Burlingame, Calif), and viewed on a scanning confocal microscope. Selected fields were scanned at high resolution (1024×1024) with sequential Z sections acquired per field for TRITC (543 nm) or FITC (488 nm). The intensity of expression for each fluorochrome in the field was then plotted against the x-y position.

Detergent Fractionation of Cellular Proteins

Cells (1×10⁶) were washed in RPMI containing 1% FCS and replaced with ice-cold HBSS containing 5 mmol/L EDTA. Lysates were centrifuged at 10 000g for 5 minutes, supernatant was removed, and 100 μL of ice-cold PHEM buffer (consisting of 60 mmol/L Pipes, pH 6.9, 25 mmol/L Hepes, pH 6.9, 10 mmol/L EGTA, 2 mmol/L MgCl₂, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L/β-mercaptoethanol, and 5 mmol/L iodoacetamide) containing 0.1% Triton X-100 were added to the pellet. Pellets were extracted for 2 minutes on ice and recentrifuged at 10 000g for 30 minutes. Supernatants were removed and added to 3× nonreducing sample buffer (detergent-soluble fraction). Pellets were washed with PHEM buffer containing 0.1% Triton X-100 and were added directly to 3× nonreducing Laemmli sample buffer (detergent-insoluble fraction). Detergent phases were subsequently vortexed and passed through a 21-gauge needle before resolution of proteins on 8% to 10% SDS-PAGE.14,15

Subcellular Fractionation of ECs

Cells (2×10⁶) were washed with ice-cold PBS and scraped into 2 mL of 500 mmol/L sodium carbonate (pH 11). Cells were lysed by homogenization and sonication (3 times, 20 seconds). Lysates were mixed with an equal volume of 80% sucrose, and 4 mL of the solution was placed in the bottom of an ultracentrifuge tube. The sucrose gradient was achieved by the addition of 4 mL of a 30% sucrose solution in sodium carbonate 250 mmol/L (pH 11), followed by 4 mL of 5% sucrose in the same buffer. Sucrose gradients were then centrifuged at 100 000g for 16 hours at 4°C. Sucrose gradients were fractionated into 1-mL fractions, and the protein content was determined by the Bradford method (Bio-Rad). Proteins from each fraction were precipitated with 10% trichloroacetic acid, and samples were centrifuged at 10 000g for 15 minutes. Proteins were solubilized in sample buffer and resolved on 8% to 12.5% SDS-PAGE.

Results

ICAM-1 Associates With a Triton X-100–Insoluble Cytoskeletal Fraction After ICAM-1 Cross-Linking or Adhesion of T Lymphocytes

GP8/3.9 cells were treated with 50 U/mL IFN-γ for 48 hours to upregulate the surface expression of ICAM-1 on ECs. Cells were extracted by detergent fractionation to produce a Triton X-100–soluble fraction and a Triton X-100–insoluble fraction. These fractions have previously been demonstrated as containing noncytoskeletal and cytoskeletal elements, respectively.14,15 Under basal conditions, ICAM-1 was almost exclusively associated with the Triton X-100–soluble fraction (Figure 1A). Partitioning of ICAM-1 within the Triton X-100–soluble fraction differed from that in the rat epithelial cell line IE6, in which ICAM-1 was distributed equally between phases (data not shown). ICAM-1 was apparent as an immunostaining band at ~90 kDa. On ligation of ICAM-1 with anti-ICAM-1 and goat anti-mouse IgG (GAM), there was a significant shift in the solubility of ICAM-1 from the Triton X-100–soluble to the –insoluble fraction (Figure 1A). There was no change in the Triton X-100 solubility of ICAM-1 when cells were incubated with primary or secondary antibodies alone (Figure 1A). The redistribution of ICAM-1 was dependent on the concentration of primary anti–ICAM-1 mAb (Figure 1A), with maximal effects occurring at ~10 μg/mL anti–ICAM-1 mAb. Maximal effects were also observed to occur at 20 μg/mL (data not shown). The effect of cross-linking ICAM-1 with mAb on its Triton X-100 solubility was time dependent but occurred rapidly, being maximal within ~5 minutes (Figure 1C). Identical results were obtained if the anti–ICAM-1 mAb clone 1A29 was substituted with anti–ICAM-1 clone 3H8 (data not shown) or if GAM was substituted with rabbit anti-mouse IgG (data not shown). In addition, identical results were also obtained when experiments were conducted in the absence of IFN-γ (data not shown) because of the presence of basal ICAM-1 expression. Cross-linking of an alternative immunoglobulin superfamily molecule such as MHC class I (RT1a) did not increase its association with the Triton X-100–
Insoluble fraction after cross-linking with anti-RT1a/GAM antibodies (data not shown).

To demonstrate that cross-linking ICAM-1 with anti-ICAM-1/GAM was physiologically significant in mimicking leukocyte adhesion to ECs, the detergent solubility of EC ICAM-1 was determined after adhesion of T lymphocytes. T lymphocytes were incubated with ECs for 45 minutes, followed by a wash in ice-cold PBS, which is effective in removing 99.9% of adherent and nonadherent T lymphocytes. Adhesion of T lymphocytes resulted in a significant shift of ICAM-1/GAM to the Triton X-100–insoluble fraction (Figure 1B). EC lysates showed no contamination with T-lymphocyte proteins, inasmuch as all lysates were negative for T-cell receptor protein by Western blot analysis (data not shown).

Cross-Linking of Endothelial VCAM-1 Does Not Induce ICAM-1 Redistribution

GP8/3.9 cells display no basal expression of VCAM-1 but can be induced to express high levels of VCAM-1 after treatment with IFN-γ. IFN-γ–treated GP8/3.9 cells, which were treated with either 10 μg/mL anti–VCAM-1 (clone 5F10) mAb alone for 30 minutes or 10 μg/mL isotype control mAb alone for 30 minutes, or 10 μg/mL GAM alone for 15 minutes. Alternatively, cells were cross-linked by using 10 μg/mL anti–ICAM-1 (1A29) for 30 minutes, followed by 10 μg/mL GAM for 15 minutes, 10 μg/mL anti–VCAM-1 (5F10) for 30 minutes, followed by 10 μg/mL GAM for 15 minutes, or 10 μg/mL anti–ICAM-1 plus 10 μg/mL anti–VCAM-1 for 30 minutes, followed by 10 μg/mL GAM for 15 minutes. Cells were detergent-extracted with TX-100, and TX-100–soluble and –insoluble proteins were resolved by nonreducing SDS-PAGE. The distribution of ICAM-1 between detergent phases was visualized after immunoblotting. Position of ICAM-1 is shown by arrow. Lower molecular weight bands represent anti–ICAM-1 IgGα bands. B, Cross-linking of ICAM-1 on GP8/3.9 cells does not cause association of PECAM-1 with the TX-100–insoluble fraction. GP8/3.9 cells (10^6) were untreated or cocultured with 10^6 T lymphocytes for 45 minutes. C, GP8/3.9 cells (10^6) were treated with 10^6 g/mL isotype-matched control mAb alone for 30 minutes, 10 μg/mL isotype-matched control mAb alone for 30 minutes, or 10 μg/mL GAM alone for 15 minutes. Alternatively, cells were cross-linked by using 10 μg/mL anti–ICAM-1 (1A29) for 30 minutes, followed by 10 μg/mL GAM for 15 minutes, 10 μg/mL anti–VCAM-1 (3A12) alone for 30 minutes, or 10 μg/mL GAM alone for 15 minutes. Alternatively, cells were cross-linked by using 10 μg/mL anti–ICAM-1 (1A29) for 30 minutes, followed by 10 μg/mL GAM for 15 minutes, 10 μg/mL isotype control mAb for 30 minutes, followed by 10 μg/mL GAM for 15 minutes, 10 μg/mL isotype control mAb for 30 minutes, followed by 10 μg/mL GAM for 15 minutes. Cells were detergent-extracted with TX-100, and TX-100–soluble and –insoluble proteins were resolved by nonreducing SDS-PAGE. The distribution of PECAM-1 between detergent phases was visualized after immunoblotting.

Figure 2A, Cross-linking of VCAM-1 on GP8/3.9 cells does not cause association of ICAM-1 with the TX-100–insoluble fraction. GP8/3.9 cells (10^6) were treated with 10 μg/mL anti–ICAM-1 (1A29) alone for 30 minutes, 10 μg/mL anti–VCAM-1 (5F10) alone for 30 minutes, or 10 μg/mL GAM alone for 15 minutes. Alternatively, cells were cross-linked by using 10 μg/mL anti–ICAM-1 (1A29) for 30 minutes, followed by 10 μg/mL GAM for 15 minutes, 10 μg/mL anti–VCAM-1 (5F10) for 30 minutes, followed by 10 μg/mL GAM for 15 minutes, or 10 μg/mL anti–ICAM-1 plus 10 μg/mL anti–VCAM-1 for 30 minutes, followed by 10 μg/mL GAM for 15 minutes. Cells were detergent-extracted with TX-100, and TX-100–soluble and –insoluble proteins were resolved by nonreducing SDS-PAGE. The distribution of ICAM-1 between detergent phases was visualized after immunoblotting.
Cross-Linking of EC ICAM-1 Does Not Induce Redistribution of PECAM-1

GP8/3.9 cells constitutively express PECAM-1, which on immunoblotting with anti-PECAM mAb (3A12) detected 1 isoform with an apparent molecular mass of $\approx$120 kDa. Analysis of unstimulated cells showed that PECAM-1 was predominantly associated with a Triton X-100–soluble fraction. Treatment of cells with 10 $\mu$g/mL anti–ICAM-1 alone, 10 $\mu$g/mL isotype-matched control IgG alone, or 10 $\mu$g/mL anti–PECAM-1 (3A12) alone showed no ability to shift PECAM-1 into a Triton X-100–insoluble fraction (Figure 2B). When cells were treated with anti–ICAM-1, isotype-matched control, or anti–PECAM-1 and were subsequently cross-linked with anti–ICAM-1, only the anti–PECAM-1/GAM combination was effective in changing the Triton X-100 solubility of PECAM-1 (Figure 2B). Taken together, the inability of VCAM-1/GAM to alter the detergent solubility of ICAM-1 and the inability of ICAM-1/GAM to alter the detergent solubility of PECAM-1 suggests that these cross-linking strategies are specific and do not result in large nonspecific aggregations of integral membrane proteins.

Redistribution of ICAM-1 Is Not Dependent on Actin Polymerization or Activation of Rho Proteins

Treatment of GP8/3.9 cells with 2 $\mu$mol/L cytochalasin D for 30 minutes, which has previously been shown to inhibit actin polymerization and inhibit ICAM-1–mediated signaling events, including transendothelial lymphocyte migration, did not inhibit the ability of ICAM-1/GAM to shift the Triton X-100 solubility of ICAM-1 (Figure 3B). Similarly, treatment of cells with 50 $\mu$g/mL C3-transferase for 8 hours (which has previously been shown to be effective in inhibiting cellular Rho proteins in these cells) was also incapable of affecting the ICAM-1/GAM–induced redistribution of ICAM-1 to the Triton X-100–insoluble fraction (Figure 3A).

Redistribution of ICAM-1 to Cytoskeletal Fraction Is Not Inhibited After Removal of Extracellular Calcium or Chelation of Cytosolic Calcium

Recent data from this laboratory have suggested that intracellular calcium fluxes resulting from ICAM-1 cross-linking are important for transendothelial lymphocyte migration and that the treatment of cells with intracellular calcium chelators is effective in inhibiting transendothelial lymphocyte migration. The presence of 2 mmol/L EGTA (which will chelate the available calcium ions in the extracellular medium) had no effect on the altered Triton X-100 solubility of ICAM-1 caused by cross-linking with ICAM-1/RAM (Figure 4). In a similar manner, cells treated with 20 $\mu$mol/L BAPTAM or 20 $\mu$mol/L MAPTAM for 30 minutes, which inhibits transendothelial lymphocyte migration, had no effect on Triton X-100 solubility of ICAM-1 either basally or when cross-linked (Figure 4).

ICAM-1 and Caveolin-1 Cofractionate on Sucrose Density Gradients but Do Not Colocalize in GP8/3.9 Cells

Although Triton X-100–soluble and –insoluble fractions have previously been interpreted as noncytoskeletal and...
cytoskeletal cellular fractions, respectively. The insoluble fraction also contains distinct membrane domains formed by the clustering of glycosphingolipids and cholesterol, along with glycosyl-phosphatidylinositol–anchored proteins and acylated proteins. Caveolae are low density membrane microdomains that have been implicated in initiating the signaling events induced by growth factors, such as epidermal growth factor and endothelin-1, and receptors for these ligands are enriched within caveolae. As expected, caveolin-1, a characteristic caveolar marker protein in the brain, appeared to be retained in the Triton X-100–insoluble fraction under basal conditions and after ICAM-1 cross-linking (Figure 5D). Under basal (Figure 5A) and ICAM-1 cross-linking conditions (data not shown), ICAM-1 and caveolin-1 showed no significant colocalization in GP8/3.9 cells, although data from sucrose density gradient fractionation of GP8/3.9 cell lysates showed that caveolin-1 and ICAM-1 cofractionated (Figure 5C). Cofractionation was also not affected after ICAM-1 cross-linking. Western blotting analysis showed that ICAM-1 and caveolin-1 were contained within fractions 5 to 8, which correspond to 30% sucrose (Figure 5D).

Discussion

We have previously demonstrated that ICAM-1 is pivotal in controlling the transendothelial migration of T lymphocytes and have shown that intracellular signals initiated through lymphocyte binding to ECs are mediated through the LFA-1/ICAM-1 interaction. Indeed, in a large number of experiments, ligation of ICAM-1 using cross-linking antibodies have been confirmed by T-lymphocyte adhesion. T-lymphocyte adhesion–mediated events have also been demonstrated to be LFA-1 dependent (data not shown). Such observations suggest that ICAM-1 cross-linking experiments have a physiological relevance. Almost all the ICAM-1–mediated signals so far identified, as well as transendothelial lymphocyte migration, are inhibited after treatment of cells with either cytochalasin D or C3-transferase. These agents are effective in inhibiting transendothelial migration without affecting leukocyte-endothelium adhesion. Therefore, it is likely that the endothelial actin cytoskeleton and endothelial Rho proteins are essential in transducing intracellular signals in ECs initiated through leukocyte adhesion to ICAM-1 and are necessary for the efficient migration of lymphocytes through the EC barrier.
After endothelial ICAM-1 cross-linking strategies, ICAM-1 was found to specifically associate with a Triton X-100–insoluble fraction, whereas in non–cross-linked cells, ICAM-1 was primarily associated with the Triton X-100–soluble fraction. These detergent fractions have previously been shown to contain noncytoskeletal (soluble) and cytoskeletal (insoluble) cellular fractions.\textsuperscript{14,15} The association of ICAM-1 with the Triton X-100 fraction was time dependent and dependent on the concentration of primary and secondary antibodies. The association of ICAM-1 with the Triton X-100–insoluble fraction occurred only when ICAM-1 was cross-linked and not when either isotype-matched control or antibodies directed against other immunoglobulin superfamily molecules were used (eg, anti–VCAM-1, Figure 2A). Studies in which anti–PECAM-1 mAb was used to cross-link PECAM resulted in a similar shift of PECAM-1, but no redistribution of PECAM-1 was observed when anti–ICAM-1 was used as the primary antibody (Figure 2B). Thus, shifting of ICAM-1 or PECAM-1 to the detergent-insoluble fraction is a specific effect of cross-linking and not the result of nonspecific membrane aggregates formed through antibody cross-linking. In support of this, cross-linking MHC class I (RT1a) with a specific mAb did not result in the redistribution of this molecule.

The observation that the association of ICAM-1 with the Triton X-100–insoluble fraction still occurred in the presence of cytochalasin D or C3-transferase demonstrates that the partitioning of ICAM-1 is not dependent on actin polymerization or the activity of endothelial Rho proteins. Although both of these treatments result in a dramatic inhibition of ICAM-1 signaling, how ICAM-1 is retained with the actin cytoskeleton, particularly after treatment of the cells with cytochalasin D, is presently unknown. However, experiments have shown that the cytoskeletal linker protein ezrin colocalizes with F-actin and ICAM-1 in control and cytochalasin D–treated cells (data not shown). The data also strongly suggest that the association of ICAM-1 with the Triton X-100–insoluble fraction occurs before the induction of actin stress fibers that follows ICAM-1 cross-linking in these cells.\textsuperscript{1} The inability of C3-transferase to prevent cytoskeletal association of ICAM-1 also implies that this association is an upstream event, occurring before Rho protein activation. It is also interesting to note that C3-transferase does not inhibit ICAM-1–induced tyrosine phosphorylation of cortactin, whereas this is effectively inhibited after pretreatment of the cells with cytochalasin D.\textsuperscript{1} This supports the idea that alterations in the endothelial cytoskeleton after ICAM-1 cross-linking is an early event and upstream from Rho activation.

It has also been demonstrated that intracellular calcium-chelating agents are effective in inhibiting ICAM-1–generated signal transduction events in ECs with concomitant abrogation of transendothelial lymphocyte migration.\textsuperscript{16} In addition, these data show that the activation of endothelial Rho proteins and calcium mobilization within ECs after ICAM-1 cross-linking are distinctly separate pathways that are both induced after ICAM-1 ligation. Induction of src activation, by ICAM-1 ligation, is a calcium-dependent event, which is inhibited by cytochalasin D, demonstrating that cytoskeletal integrity is essential for calcium-mediated signals after ICAM-1 cross-linking.\textsuperscript{16} The observation that intracellular calcium-chelating agents or removal of extracellular calcium ions is ineffective in preventing the cross-linking–induced association of ICAM-1 with the Triton X-100–insoluble fraction also indicates that this association is an early event in the mechanism of ICAM-1–induced signal transduction, with cortactin phosphorylation and Rho protein and intracellular calcium mobilization occurring consequentially.

Caveolin-1 is retained in the detergent-soluble fraction under basal and cross-linking conditions, demonstrating that caveolae do not associate with the cytoskeleton after ICAM-1 cross-linking. Confocal microscopy confirmed that despite the cosedimentation of ICAM-1 and caveolin-1 in the membrane fraction of the cells, there is no obvious association of ICAM-1 and caveolin-1.\textsuperscript{17} In contrast, confocal images have previously demonstrated that under control and ICAM-1–cross-linked conditions, there is significant colocalization of ICAM-1 and F-actin.\textsuperscript{1}

ICAM-1 has previously been reported to interact with the cytoskeletal linker protein ezrin, and this interaction is enhanced after exposure to phosphatidylinositol 4,5-bisphosphate.\textsuperscript{19} These studies suggest that ICAM-1 signal transduction events may be propagated via ezrin to the actin cytoskeleton. In addition, because ICAM-1 signaling is highly dependent on functional endothelial Rho proteins, it is interesting to note that ezrin and other ERM proteins are effectors of Rho signaling pathways,\textsuperscript{20} controlling stress fiber and focal adhesions. Moreover, Rho proteins have been shown to regulate phosphatidylinositol 4-phosphate 5-kinase,\textsuperscript{21} which may therefore affect the interaction of ICAM-1 and ezrin, thereby altering ICAM-1–mediated signaling responses. Our results suggest that the endothelial cytoskeleton is an important initial component in propagating signals within ECs after the adhesion of leukocytes and is therefore consistent with the translocation of ICAM-1 to a detergent-insoluble fraction on ICAM-1 cross-linking.

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