E-selectin and ICAM-1 are incorporated into detergent-insoluble membrane domains following clustering in endothelial cells

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Received 6 June 2002; revised 3 July 2002; accepted 3 July 2002

First published online 16 July 2002

Edited by Beat Imhof

Abstract Here we present data supporting the role of lipid rafts in endothelial cells during leukocyte adhesion. Following adhesion of THP-1 cells or antibody-mediated clustering, both E-selectin and intercellular adhesion molecule-1 (ICAM-1) partitioned into the detergent-insoluble portion of the endothelial cellular lysate. Sucrose gradient centrifugation revealed the partitioning of clustered E-selectin and ICAM-1 with the low-density fraction where they co-fractionated with src family kinases, markers of lipid rafts. Depleting the plasma membrane of cholesterol inhibited clustering of adhesion molecules following their antibody-induced crosslinking and inhibited their association with src kinases. Thus, our data suggest that E-selectin and ICAM-1 associate with lipid rafts in human endothelial cells following leukocyte adhesion.

Key words: Endothelial cell; Lipid raft; Tyrosine kinase; Adhesion

1. Introduction

Leukocyte recruitment involves leukocyte adhesion to the endothelium, which is mediated by specific adhesion molecules such as E-selectin and intercellular adhesion molecule-1 (ICAM-1) expressed by the endothelial cells at sites of tissue damage [1,2]. While the role of these adhesion molecules in leukocyte recruitment is well established, the signals that are transduced into endothelial cells following leukocyte adhesion are not well characterized. Such signaling events could be necessary to prime the endothelial cells for leukocyte transmigration by regulating endothelial barrier properties and cellular contraction. Previously, we have shown that leukocyte adhesion to endothelial cells results in the association of endothelial cell adhesion molecules with at least one member of the src family of non-receptor tyrosine kinases, and the phosphorylation of cortactin, an actin binding protein and substrate of src family kinases [3]. Src family kinase activity was necessary for adhesion molecule clustering at the site of leukocyte contact. The mechanism whereby adhesion molecules associate with these kinases, however, is unclear. It is known that src family kinases such as Lck, Lyn, and Fyn, because of their doubly acylated N-termini, preferentially associate with microdomains of the plasma membrane that consist of tightly packed sphingolipids [4,5]. These microdomains, termed 'lipid rafts', are enriched in cholesterol and are insoluble in non-ionic detergents at 4°C [6,7]. Lipid rafts are believed to be important in protein trafficking and signal transduction [8,9], and they would also provide a mechanism whereby adhesion molecules could associate with src family kinases. Here, we report that, following their engagement, the endothelial cell adhesion molecules E-selectin and ICAM-1 become incorporated into the detergent-insoluble, low-density portion of the cell lysate, consistent with their association with lipid rafts. In addition, depletion of the plasma membrane of cholesterol led to an inhibition of adhesion molecule clustering and a disruption of the adhesion molecule–src complex. These results support a model whereby endothelial adhesion molecules, upon adhesion to a leukocyte, will incorporate into lipid rafts, which mediate their association with src family kinases.

2. Materials and methods

2.1. Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords as described previously [10]. Endothelial cells were maintained in M199+15% iron-supplemented calf serum, 5% Nu serum, 1 U/ml penicillin/streptomycin, 1 U/ml fungizone, 1 U/ml heparin, and 10 μl/ml endothelial cell growth supplement (purified from bovine hypothalami as described previously [11]). HUVECs were routinely split 1:3 and were not used after passage 5. The hybridoma cell lines and the THP-1 monocytic cell line [12] were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI+10% fetal bovine serum (FBS). THP-1 cells were fixed by washing twice with Hanks' balanced salt solution (HBSS, pH 7.4) followed by an incubation with 1% fresh paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 5 min at 37°C. Fixation was stopped by adding 1 M ice-cold glycylglycine. The cells were washed twice with PBS and incubated for 1 h in RPMI+10% FBS at 37°C to quench any remaining formaldehyde. The cells were then washed twice in HBSS before use.

2.2. Antibodies and reagents

Anti-ICAM-1 and anti-E-selectin goat polyclonal antibodies were purchased from R&D Systems (Minneapolis, MN, USA). M199, HBSS, RPMI, penicillin/streptomycin, and fungizone were from Gibco. Serum was purchased from Summit Biotechnologies (Fort Collins, CO, USA). Human recombinant tumor necrosis factor-α (TNFα) was obtained from Calbiochem (La Jolla, CA, USA), and methyl-β-cyclodextrin (BCD) was from Sigma.

Monoclonal antibodies to E-selectin (clone H18/7) and ICAM-1 (clone R6′5′) were purified from hybridoma supernatants using a protein A/G column (Pierce) as per the manufacturer's instructions.

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Abbreviations: HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; TNFα, tumor necrosis factor-α; BCD, β-cyclodextrin

0014-5793/02/$22.00
PII: S0014-5793(02)03070-3

Antibody concentration was quantitated with the D_c Protein Assay Kit (Bio-Rad). Each antibody was determined to be >95% pure by SDS–PAGE followed by Coomassie blue staining. Purified antibodies were stored frozen at −20°C in PBS with 0.1% bovine serum albumin. Rabbit anti-src (SRC 2), a polyclonal antibody that detects multiple members of the src kinase family, was from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Unconjugated rabbit anti-mouse antibodies were purchased from ICN (Costa Mesa, CA, USA).

2.3. Detergent fractionation, sucrose gradient fractionation, and Western blotting

The HUVEC monolayers were washed twice with HBSS, and 300 μl cold lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM Na_3VO_4, 10 mM $NaPO_4$, and 10 mM NaF, containing either 1% or 0.1% Triton-X) was added to the dish for 1 min on ice. The lysis buffer was then removed from the dish and collected as the soluble portion of the lysate. The dish was then washed twice with lysis buffer without Triton-X, and the remaining insoluble cellular structures were scraped into 300 μl lysis buffer and collected as the insoluble portion. Both the soluble and insoluble samples were diluted 1:1 in SDS–PAGE sample buffer. For sucrose gradient fractionation, cells were lysed and scraped in 1 ml lysis buffer containing 1% Triton-X. 1 ml of 80% sucrose in lysis buffer was added to the lysates to achieve a final concentration of 40% sucrose. The samples were placed at the bottom of a centrifuge tube, and a discontinuous gradient was achieved by layering 4 ml of 30% sucrose above the sample followed by 4 ml of 5% sucrose. The gradients were centrifuged in a Beckman SW-41 rotor at 31 000 rpm for 18 h at 4°C. Samples were taken from the top of the gradient, and proteins were precipitated by the addition of cold trichloroacetic acid to a final concentration of 10%. The pellets were washed twice with cold ethanol/acetone (1:1) and resuspended in 200 μl SDS–PAGE sample buffer.

Samples were separated on a 7.5% polyacrylamide gel, and proteins were transferred to nitrocellulose membranes and probed with anti-E-selectin, anti-ICAM-1, or anti-src antibodies. Bound horseradish peroxidase-conjugated secondary antibodies were detected using a chemiluminescent substrate (Santa Cruz).

2.4. Immunofluorescence microscopy

HUVECs were grown on fibronectin-coated glass coverslips until confluent and treated for 4 h with TNFα. Adhesion molecules were clustered as above, using a fluorescein-conjugated secondary antibody (Jackson Laboratories). The coverslips were then washed twice with HBSS and then fixed with 3.7% formaldehyde. After three additional washes with PBS, the coverslips were mounted on glass microscope slides and examined under a fluorescence microscope. Photographs were taken of the fluorescent images using a Nikon FX-35a camera.

2.5. Bead assay

Paramagnetic beads (Dynal, Oslo, Norway) were coated with a monoclonal antibody against either E-selectin or ICAM-1 and were allowed to adhere to TNFα-treated HUVECs for 15 min at 37°C. The cells were then washed twice with HBSS and lysed with lysis buffer containing 1% Triton-X. The beads were separated from the remaining cell lysate by a side-pull magnetic system (Dynal) washed three times with lysis buffer. The beads were resuspended in 50 μl SDS–PAGE sample buffer and analyzed by Western blotting as above.

3. Results

3.1. E-selectin and ICAM-1 partition into the detergent-insoluble portion of the cell lysate following engagement

A hallmark characteristic of lipid rafts is their resistance to solubilization by non-ionic detergents at 4°C [7]. Both E-selectin and ICAM-1 were present in the 0.1% Triton-X-soluble portion of lysates from TNFα-treated endothelial cells as detected by Western blot analysis (Fig. 1, lane 1). A substantial amount of ICAM-1 was present in the insoluble portion of the lysate (Fig. 1, lane 2, bottom panel), indicating that ICAM-1 may constitutively associate with lipid rafts or cytoskeletal proteins. Adhesion of THP-1 cells to the endothelial cells prior to cellular lysis caused both E-selectin and ICAM-1 to become insoluble in 0.1% Triton-X (Fig. 1, lanes 3 and 4). To specifically engage either E-selectin or ICAM-1, monoclonal antibodies directed against these proteins were allowed to bind to TNFα-pretreated endothelial cells, followed by cross-linking with a polyclonal secondary antibody to cluster the adhesion molecules. The cells were lysed with 1% Triton-X and the detergent-soluble and detergent-insoluble portions of the lysates were analyzed by Western blotting for E-selectin and ICAM-1 as above. In 1% Triton-X, both E-selectin and ICAM-1 were primarily in the detergent-soluble portion of the lysate (Fig. 2, lanes 1 and 2). However, following clustering with antibodies, E-selectin and ICAM-1 partitioned into the detergent-insoluble portion of the lysate (Fig. 2, lanes 3 and 4). This was not inhibited by cytochalasin B, even though cytochalasin disrupted the actin cytoskeleton as determined by fluorescent microscopy of endothelial cells stained with phalloidin (data not shown). These data suggest that, upon their engagement, both E-selectin and ICAM-1 associate with detergent-insoluble structures other than the actin cytoskeleton in endothelial cells.

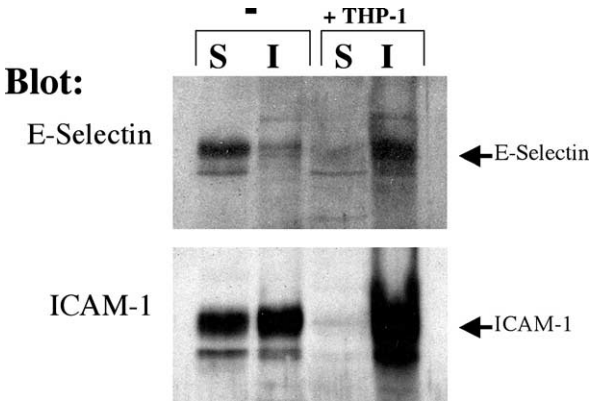

Fig. 1. E-selectin and ICAM-1 partition into the detergent-insoluble fraction following THP-1 cell adhesion. THP-1 cells were allowed to adhere to activated HUVECs and the cells were lysed as in Section 2. The detergent-soluble (S) and -insoluble (I) fractions of the cell lysate were analyzed by Western blotting for E-selectin (top) or ICAM-1 (bottom).

3.2. E-selectin and ICAM-1 fractionate into the low-density portion of the cell lysates following engagement

To determine if endothelial cell adhesion molecules become associated with detergent-insoluble portions of the plasma membrane ('lipid rafts') following engagement, endothelial cell lysates were fractionated by sucrose density gradient centrifugation and fractions were analyzed by SDS–PAGE and Western blotting. In TNFα-treated endothelial cells, both E-selectin and ICAM-1 were primarily in the detergent-soluble, high-density (40% sucrose) portion of the cell lysate (Fig. 3A). The blot was stripped and reprobed for src family kinases as markers for lipid rafts, using a polyclonal antibody that reacts with members of the src family, including c-Src, Yes, and Fyn, which were predominantly detected in the raft portion of the gradient (Fig. 3A, bottom). Following adhesion molecule clustering with antibodies, E-selectin (Fig. 3B) and ICAM-1 (Fig. 3C) both shifted into the low-density fraction of the gradient, consistent with the association of these molecules with lipid rafts following adhesion molecule engage-

ment. Src family kinases were predominantly maintained in the lipid raft fraction regardless of adhesion molecule clustering (Fig. 3B,C, bottom panels). These results suggest that, upon engagement, E-selectin and ICAM-1 are incorporated into lipid rafts where they can associate with src family kinases.

3.3. Membrane cholesterol is required for adhesion molecule clustering and association with src family kinases

Clustering E-selectin (data not shown) and ICAM-1 (Fig. 4A) with a fluorescently labeled secondary antibody allowed visualization of the adhesion molecule clusters, apparent as punctate markings on the surface of endothelial cells (Fig. 4A, right panel). To determine if these punctate structures represented lipid rafts, we treated endothelial cells with the cholesterol-sequestering drug BCD prior to antibody-mediated clustering of adhesion molecules. Plasma membrane depletion of cholesterol resulted in an inhibition of clustering as determined by fluorescence microscopy (Fig. 4B, right panels), suggesting that cholesterol is required for this clustering event and further implying an active role for lipid rafts in the clustering of adhesion molecules.

We previously showed that at least one member of the src family of non-receptor tyrosine kinases is associated with engaged adhesion molecules in endothelial cells [3]. To determine if lipid rafts were required for this association, TNFα-treated endothelial cells were incubated with BCD prior to addition of beads coated with an antibody against E-selectin or ICAM-1. The beads were allowed to adhere to the endothelial cells, mimicking leukocyte adhesion. The cells were then lysed and proteins bound to the beads were analyzed by SDS–PAGE and Western blotting. BCD did not significantly inhibit binding of the beads to E-selectin and ICAM-1 (Fig. 5, top and middle panels). However, BCD did inhibit the association of src family kinases with these molecules (Fig. 5, bottom panel). This suggests that lipid rafts mediate the association between engaged adhesion molecules and src family kinases.

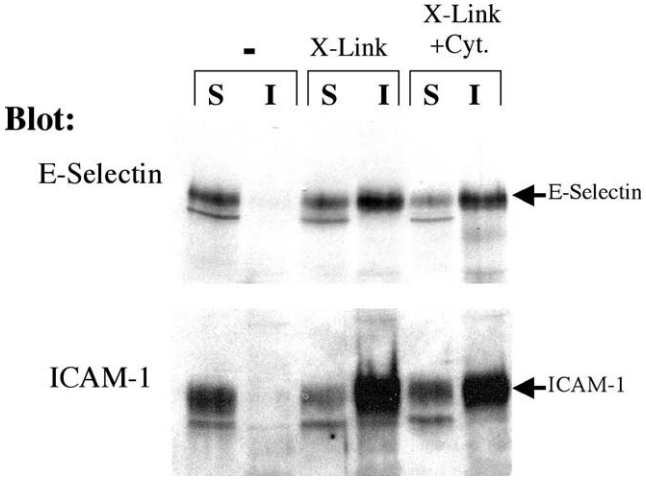

Fig. 2. Adhesion molecule partitioning into the insoluble fraction following antibody-induced clustering is not inhibited by cytochalasin B. Either E-selectin (top) or ICAM-1 (bottom) was clustered on HUVECs by antibody crosslinking (X-Link) in the absence or presence of cytochalasin B (Cyt.). The cellular lysate was separated into detergent-soluble (S) or -insoluble (I) fractions and analyzed by Western blotting for E-selectin (top) or ICAM-1 (bottom).

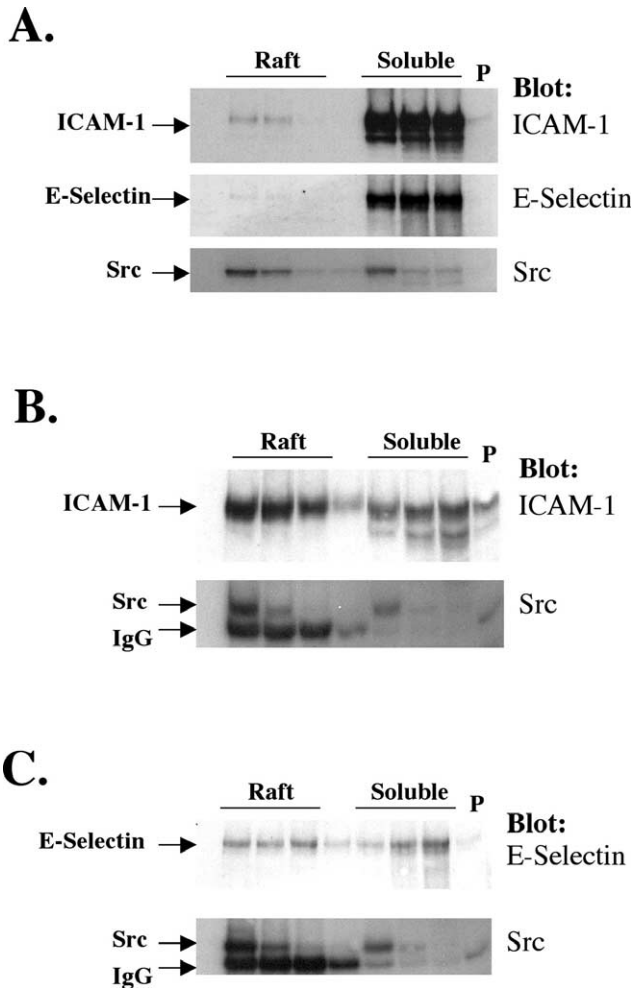

Fig. 3. Endothelial adhesion molecule partition with lipid rafts following antibody-mediated clustering. HUVEC whole cell lysates were fractionated in a discontinuous sucrose gradient by equilibrium centrifugation. Fractions were taken from the top of the gradient and the pellet (P) and analyzed by Western blot. A: HUVEC lysates with unclustered adhesion molecules. Sucrose gradient fractions were analyzed by Western blotting for ICAM-1 (top), E-selectin (middle), or src family kinases (bottom). ICAM-1 (B) or E-selectin (C) was clustered by antibodies prior to cell lysis and equilibrium centrifugation, and samples were analyzed by Western blotting for ICAM-1 (B, top), E-selectin (C, top), or src family kinases (A and B, bottom). The IgG band in B and C represents the heavy chain of the rabbit polyclonal antibody that was used to cluster the adhesion molecules.

4. Discussion

Tyrosine phosphorylation is known to play an important role in cell adhesion. Phosphorylation of focal adhesion proteins accompanies cellular adhesion to the extracellular matrix, and tyrosine phosphorylation has also been implicated in the regulation of cytoskeletal rearrangements [13,14]. Thus, it is not surprising that src family kinases are activated following E-selectin and ICAM-1 engagement [3,15]. However, exactly how these adhesion molecules – which have very little homology to each other – become associated with these kinases was unresolved. Here, we show that both E-selectin and ICAM-1 become incorporated into lipid rafts upon engagement where they co-fractionate with src family kinases in a sucrose gradient. This would provide a mechanism whereby these adhe-

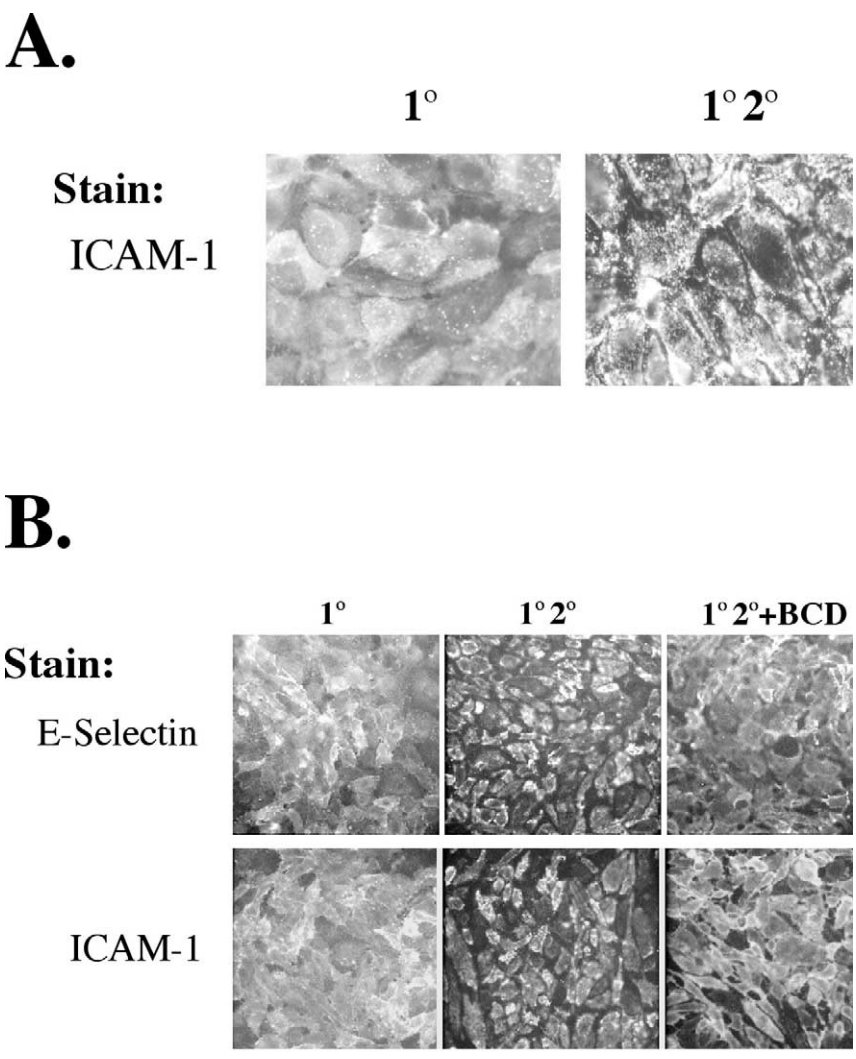

Fig. 4. Cholesterol depletion inhibits adhesion molecule clustering. A: ICAM-1 staining pattern on cells treated with the primary antibody (1°, left panel) and both the primary and the secondary crosslinking antibodies (1° 2°, right panel). Magnification, 400×. B: E-selectin (top panels) or ICAM-1 (bottom panels) were treated with a primary monoclonal antibody only (1°), or the primary antibody followed by an FITC-conjugated secondary antibody (1° 2°), either in the absence or in the presence of 10 mM BCD. Magnification, 200×.

sion molecules are coupled to a tyrosine kinase. Exactly how these kinases become activated remains unknown, although it has been shown that clustering of lipid rafts in cells is sufficient to activate src family kinases such as Lck [16,17]. Thus, adhesion molecule clustering as a result of leukocyte adhesion could also induce the clustering of lipid rafts, resulting in src family kinase activation and cortactin phosphorylation. Phosphorylated cortactin is known to bind to the SH2 domains of several scaffolding proteins and kinases, including c-src [18]. The SH2 domains of other, raft-associated src family members were not examined, however it is likely that they also would then bind to phosphorylated cortactin, resulting in linkage of the raft to the actin cytoskeleton and further adhesion molecule clustering.

Another possible function of lipid raft incorporation would be the maintenance of the adhesion molecules as a cluster. The lipid rafts would serve to restrict lateral diffusion of the adhesion molecules in the plasma membrane once they have been incorporated into the cluster, thereby maintaining a high localized concentration of the adhesion molecule at the site of adhesion. This would result in a higher affinity for the leukocyte due to the increased number of adhesion molecules at that site. A similar role has been proposed for lipid rafts in B cells during antigen presentation, where rafts would serve to concentrate the MHC molecule in patches along with its bound peptide on the surface of the cell [19]. In endothelial cells, incorporation into lipid rafts could represent the initial step in the larger-scale clustering of adhesion molecules, an event that is necessary for efficient leukocyte adhesion.

One issue that remains unresolved is the precise mechanism whereby E-selectin and ICAM-1 become incorporated into lipid rafts. Several other transmembrane proteins have been reported to associate with lipid rafts; however, a specific amino acid sequence that mediates this association has yet to be identified [19–22]. A strong possibility is that these transmembrane proteins have a basal affinity for lipid rafts that is substantially increased upon clustering [23]. This notion is supported by the data showing the differences in the amount of unclustered E-selectin and ICAM-1 in the 0.1% Triton-X-insoluble portion of the cellular lysate (Fig. 1). This may be due to differences in the amino acid sequence of their transmembrane domains.

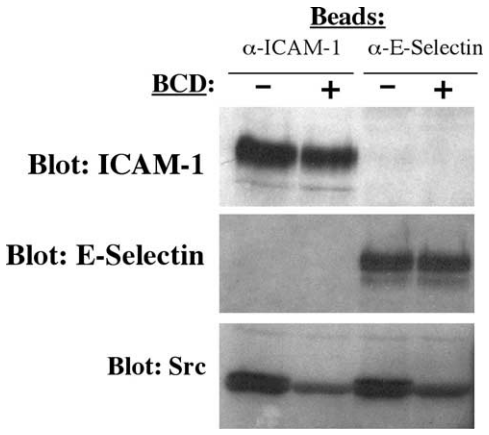

Fig. 5. The association of src family kinases with adhesion molecules requires cholesterol. Beads coated with either E-selectin (lanes 1and 2) or ICAM-1 (lanes 3 and 4) were allowed to adhere to TNFα-activated HUVECs in the presence (+) or absence (−) of 10 mM BCD. The beads were isolated and bound proteins were analyzed by Western blotting for ICAM-1 (top panel), E-selectin (middle panel), or src family kinases (bottom panel).

The results reported here provide a mechanism that links endothelial cell adhesion molecules to mediators of signal transduction. Lipid rafts may thus represent an additional target for therapies designed to attenuate leukocyte recruitment in inflammatory diseases.

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