Leukocyte Adhesion to Vascular Endothelium Induces E-Selectin Linkage to the Actin Cytoskeleton

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Abstract. We have examined functions of the cytoplasmic domain of E-selectin, an inducible endothelial transmembrane protein, especially its ability to associate with the cytoskeleton during leukocyte adhesion. Confocal microscopy of interleukin-1 β (IL-1β)–activated human umbilical vein endothelial cells (HUVEC) visualized clustering of E-selectin molecules in the vicinity of leukocyte-endothelial cell attachment sites. A detergent based extraction and Western blotting procedure demonstrated an association of E-selectin with the insoluble (cytoskeletal) fraction of endothelial monolayers that correlated with adhesion of leukocytes via an E-selectin–dependent mechanism. A mutant form of E-selectin lacking the cytoplasmic domain (tailless E-selectin) was expressed in COS-7 cells and supported leukocyte attachment (in a nonstatic adhesion assay) in a fashion similar to the native E-selectin molecule, but failed to become associated with the cytoskeletal fraction. To identify the cytoskeletal components that associate with the cytoplasmic domain of E-selectin, paramagnetic beads coated with the adhesion-blocking anti–E-selectin monoclonal antibody H18/7 were incubated with IL-1β–activated HUVEC, and then subjected to detergent extraction and magnetic separation. Certain actin-associated proteins, including α-actinin, vinculin, filamin, paxillin, as well as focal adhesion kinase (FAK), were copurified by this procedure, however talin was not. When a mechanical stress was applied to H18/7-coated ferromagnetic beads bound to the surface of IL-1β–activated HUVEC, using a magnetical twisting cytometer, the observed resistance to the applied stress was inhibited by cytochalasin D, thus demonstrating transmembrane cytoskeletal mechanical linkage. COS-7 cells transfected with the tailless E-selectin failed to show resistance to the twisting stress. Taken together, these data indicate that leukocyte adhesion to cytokine-activated HUVEC induces transmembrane cytoskeletal linkage of E-selectin through its cytoplasmic domain, a process which may have important implications for cell–cell signaling as well as mechanical anchoring during leukocyte-endothelial adhesive interactions.

THE selectin family of adhesion molecules is thought to play an important role during the initial interactions of circulating blood leukocytes with the endothelial lining of blood vessels in response to inflammatory stimuli such as injury and infection. The three members of this family (L-, P-, and E-selectin) share a similar complex mosaic molecular architecture, consisting of an amino-terminal lectin domain, an EGF domain, a variable number of complement regulatory repeats, a transmembrane domain, and a short cytoplasmic tail (Bevilacqua and Nelson, 1993). The extracellular lectin domains of each of these selectin molecules support reversible binding to a variety of complex carbohydrate ligands (Bevilacqua and Nelson, 1993). L-selectin, which is constitutively expressed on the surface of various blood leukocytes, appears to play a role in lymphocyte recirculation, as well as in the initial rolling on the vascular lining at sites of inflammation in vivo (Tedder et al., 1993), through interaction with both constitutive and inducible endothelial ligands (Spertini et al., 1991). E- and P-selectin are inducibly expressed on the surface of stimulated/activated endothelial cells via de novo protein synthesis or by redistribution from storage granules (in the case of P-selectin) (Bevilacqua and Nelson, 1993). Both P- and E-selectin molecules, in artificial bilayers or in transfected cells, can support rolling interactions with blood leukocytes under simulated physiologic

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flow conditions in vitro (Lawrence and Springer, 1991, 1993), and P-selectin–deficient mice exhibit a marked rolling defect (Norton et al., 1993). To date, most structure-function analyses of the selectins, using monoclonal antibodies and other adhesion inhibitors, have focused primarily on the function of the extracellular domains of these molecules (Rosen and Bertozzi, 1994).

Interactions between cell surface adhesion molecules and intracellular cytoskeletal components are receiving increased attention because of their potential implications for cell–cell and cell–matrix adhesion, receptor-ligand interactions including signal transduction, and receptor internalization (Pavalko and Otey, 1994). The cytoplasmic domains of adhesion molecules appear to play a key role in these processes, serving as sites for structural linkage and regulation between cell surface molecules (and their ligands) and internal cytoskeletal structures. At present, perhaps the most extensively studied model for adhesion molecule-cytoskeletal transmembrane linkage is the focal adhesion complex formed by integrin receptors at sites of cell attachment to extracellular matrix (Burridge et al., 1988). On their cytoplasmic aspect, these macromolecular assemblies consist of the cytoplasmic domains of the heterodimeric integrins in noncovalent association with cytoskeletal components such as talin, vinculin, α-actinin, and filamin, as well as regulatory proteins including focal adhesion kinase (FAK),[1] proteases, and several phosphoproteins (Burridge et al., 1988; Pavalko and Otey, 1994; Schaller and Parsons, 1994). This specialized "organelle", in addition to its adhesive/anchoring function, participates in the dynamic responses of cells to biomechanical forces (Wang, et al., 1993; Davies et al., 1994), cell shape regulation (Ingber, 1990), morphogenesis, and tissue architecture maintenance (Schwartz and Ingber, 1994), as well as chemical signaling and apoptotic cell death (Re et al., 1994). In addition to the heterodimeric integrins, the cadherins, a superfamily of single-chain, calcium-regulated cell–cell adhesion molecules also associate with certain cytoskeletal components including vinculin, α-actinin, and α-/β-catenin (Geiger and Ginsberg, 1991; Lampugnani et al., 1995). Intercellular adhesion molecule-1 (ICAM-1), a member of the single chain immunoglobulin superfamily of adhesion receptors, also has been shown to become associated with α-actinin, an actin-binding cytoskeletal protein, via its cytoplasmic domain (Carpen et al., 1992), but the functional implications for leukocyte-endothelial interactions have not been fully elucidated. Studies involving "domain swapping", and other molecular biological manipulations of the selectins, have indicated that their respective cytoplasmic domains appear to play a functional role in leukocyte homing in the case of L-selectin (Kansas et al., 1993) and in intracellular targeting to secretory or storage pathways in the case of P-selectin (Subramaniam et al., 1993). Recent biochemical studies have demonstrated that the cytoplasmic domain of L-selectin can interact directly with α-actinin (Pavalko et al., 1995). Although the cytoplasmic domain of E-selectin has been implicated in endocytosis of surface-expressed E-selectin in activated HUVEC (Kristakis et al., 1990; Von Asmuth et al., 1992; Subramaniam et al., 1993), specific molecular interactions and functional roles for this domain during leukocyte-endothelial interaction have not been elucidated.

In the current study, using a number of experimental approaches, we have found that E-selectin can become biochemically and physically associated with the endothelial cytoskeleton during leukocyte adhesion, and have identified certain cytoskeletal proteins involved in this process. These findings have important implications for E-selectin function during leukocyte-endothelial interactions, including the potential for outside-in signaling and transmembrane cytoskeletal anchoring, and point to a more complex role for this inducible endothelial cell surface molecule in inflammatory reactions.

Materials and Methods

Reagents

Medium199, RPMI-1640, Dulbecco's phosphate buffered saline (DPBS), and HBSS were obtained from M. A. Bioproducts (Walkersville, MD). FBS and DME were purchased from GIBCO BRL (Grand Island, NY). 15-cm and 35-mm-diam tissue culture plates were purchased from Costar Corp. (Cambridge, MA). Endothelial cell growth factor and acetylated low density lipoprotein (AcLDL) were obtained from Biomedical Technologies (Stoughton, MA). Paraformaldehyde was purchased from Fischer Scientific (Springfield, NJ). Recombinant human interleukin-1β (IL-1β) was a gift of Biogen (Cambridge, MA). Biscarboxyethyl-carboxyfluorescein acetoxymethyl ester (BCECF) and 5-chloromethylfluorescein diacetate (CMFDA) were purchased from Molecular Probes (Eugene, OR). BSA, cytochalasin D, leupeptin, iodoacetamide, Triton X-100, benzamidine, and PMSF were obtained from Sigma Chem. Co. (St. Louis, MO). 4-(2-aminoethyl) benzensulfonyl fluoride (AEBSF) and pepstatin-A were purchased from Calbiochem (San Diego, CA). Indocyanine (Cy3)-conjugated goat anti–mouse IgG immunoglobulin was purchased from Jackson ImmunoResearch Labs., Inc. (West Grove, PA). Monoclonal antibodies to α-actinin, vinculin, and talin were obtained from Harlan Bioproducts for Science, Inc. (Indianapolis, IN). Monoclonal antibodies to FAK and paxillin were purchased from Transduction Laboratories (Lexington, KY).

Cultured Cells

COS-7, a monkey kidney fibroblast cell line, was obtained from the Amer. Type Culture Collection (ATCC; Rockville, MD) and grown in DME supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM L-glutamine. HL-60 (Collins, 1987), a promyelocytic human leukocyte cell line, was also obtained from the ATCC and grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM L-glutamine. JY human lymphocytic cells (Rothlein and Springer, 1986), kindly provided by Dr. T.A. Springer (Center for Blood Research, Boston, MA), were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM glutamine. HUVEC were isolated and established in culture, as previously described (Bevilacqua et al., 1985). Primary cultures were serially passaged (≤1:3 split ratio) and maintained in Medium 199 buffered with 25 mmol/L Hepes and supplemented with 10% FBS, endothelial cell growth factor (25 μg/ml), and porcine intestinal heparin (50 μg/ml). For experimental use, subcultured (passage 2 or 3) endothelial cells were plated on gelatin-coated 35-mm tissue culture dishes, 15-cm dishes, or 10-mm glass coverslips.

Mutation of E-Selectin cDNA and COS Cell Expression

To generate a tail-less E-selectin (ΔCYTO-E-selectin) cDNA, which lacks the cytoplasmic domain (see Fig. 3 *a*), oligonucleotide-directed in vitro mutagenesis (Amersham Corp., Arlington Heights, IL) was used to change the codon for lysine at the second amino acid of the cytoplasmic

1. *Abbreviations used in this paper*: AcLDL, acetylated low density lipoprotein; AEBSF, 4-(2-aminoethyl) benzensulfonyl diacetate; BCECF, biscarboxyethyl-carboxyfluorescein; CLSM, confocal laser scanning microscope; CMFDA, 5-chloromethylfluorescein diacetate; Cy3, Indocyanine; FAK, focal adhesion kinase ($_{pp}125^{FAK}$); HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; IL-1β, interleukin-1 β; RGD, arginine-glycine-aspartic acid; WT, wild type.

domain in a full-length wild-type E-selectin (WT-E-selectin) cDNA (Bevilacqua et al., 1989) to a stop codon. Mutated sequence was confirmed by the dideoxy chain termination method (Sanger et al., 1977). Both WT- and ΔCYTO-E-selectin cDNAs were transfected into 10^6 COS-7 cells, using a modified calcium coprecipitation method (Sambrook et al., 1982) and plated in tissue culture plates.

72 h after transfection, the WT- and ΔCYTO-E-selectin–transfected COS-7 cells were studied, in parallel, in quantitative leukocyte adhesion assays and detergent fractionation analyses of cellular proteins (see below). In addition, single cell suspensions were generated for FACS analysis of surface E-selectin expression by washing with HBSS (without Ca^{2+}/Mg^{2+}), incubating with HBSS + 5 mM EDTA for 15 min at 37°C followed by gentle pipetting. Cells suspended in RPMI + 1% FBS were incubated with H18/7 IgG, a murine anti–human E-selectin mAb (10 μg/ml) (Bevilacqua et al., 1987), or medium alone for 25 min at 4°C. After three washes with RPMI + 1% FBS, the cell suspension was incubated with 1:100 dilution of a FITC-conjugated goat anti–mouse polyclonal IgG (Caltag, San Francisco, CA) for 25 min at 4°C in the dark, and then fixed in 2% paraformaldehyde in PBS (20 min at 4°C) and analyzed with a FACScan (Becton-Dickinson) flow cytometer. Data were collected for 10,000 cells and voltage and gain settings were adjusted to produce a normalized fluorescence of the control cells.

Quantitative Leukocyte Adhesion Assays

HL-60 cells, prelabeled with the fluorescent dye BCECF were added (1.6×10^6 cells/well in RPMI + 1% FBS) to monolayers of WT- and ΔCYTO-E-selectin–transfected COS-7 cells in 35-mm dishes. After incubation under nonstatic adhesion assay conditions (rotation at 64 rpm, 10 min, 22–25°C), nonadherent HL-60 cells were removed by washing three times with RPMI + 1% FBS, and the monolayer-associated HL-60 cells were collected into HBSS + 5 mM EDTA + 4 mM EGTA and their fluorescence measured in a Pandex plate reader (IDEXX Laboratories, Westbrook, ME), as previously described (Westlin and Gimbrone, 1993).

Detergent Fractionation of Cellular Proteins

Activated HUVEC (IL-1β 10 U/ml, 4 h), or WT- and ΔCYTO-E-selectin–transfected COS-7 cells, plated in 35-mm plates were incubated with HL-60 cells, or JY cells (1.6×10^6 cells per well) for 10 min at 37°C under static conditions. Alternatively, in some experiments, the cells were incubated with H18/7 or H4/18, an E-selectin–specific mAb, for 10 min at 4°C (10 μg/ml in 1 ml RPMI + 1% FBS), followed by incubation with a goat anti–mouse polyclonal IgG (Caltag) for 10 min at 37°C (1:200 dilution in 1 ml RPMI + 1% FBS) to cross-link the anti–E-selectin mAb. After three washes with RPMI + 1% FBS, HBSS + 5 mM EDTA were added and the monolayers were collected by scraping with a rubber policeman and centrifuged at 14,000 rpm for 5 min. After removing the supernatant, 100 μl of a cocktail consisting of 0.1% Triton X-100 in a PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$ containing 1 mM PMSF, 1 μg/ml leupeptin, 20 mM benzamidine, 5 mM iodoacetamide; pH 6.9) which was designed to minimize disruption of cytoskeletal network organization (Schliwa, 1982) was added to the cell pellet. The detergent extraction was carried out on ice for 2 min, and the samples then centrifuged at 14,000 rpm for 30 min. Nonreducing SDS sample buffer (3×) was added to the supernatant to a final volume of 150 μl, and this was used as the "detergent-soluble fraction." The pellet was washed with 0.1% Triton X-100 in PHEM buffer, dissolved in 100 μl of PHEM buffer and nonreducing SDS sample buffer (3×) was added to a final volume of 150 μl. After reducing the viscosity by extensive vortexing and trituration through a 21-G needle, this preparation was used as the detergent-insoluble fraction.

Western Blotting of Cellular Proteins

Fractionated samples were separated by SDS-PAGE using 8% polyacrylamide gels under nonreducing conditions, and transferred to PVDF membranes (Millipore, Bedford, MA), which then were blocked with 7.5% dry milk in TBS-T (20 mM Tris, 137 mM $NaCl_2$, 0.1% Tween-20, pH 7.6) for 90 min, and followed by incubation with the nonfunction blocking anti–E-selectin mAb H4/18 (1:1,000 diluted in blocking buffer) for 60 min. After three washes with TBS-T, the blots were incubated with an HRP-conjugated polyclonal goat-anti–mouse IgG (1:5,000) in TBS-T, again washed three times with TBS-T and the labeled proteins were visualized using an enhanced chemiluminescence kit (Amersham Corp.).

Confocal Immunofluorescence Microscopy

HL-60 and JY cells were prelabeled with CMFDA following the manufacturer's protocol, and then added to activated (IL-1β, 10 U/ml, 4 h) HUVEC monolayers grown on gelatin-coated glass coverslips (1.6×10^6 cells per coverslip) for 10 min at 37°C. After a brief wash with RPMI + 1% FBS, the monolayers and adherent leukocytes were fixed with 3.7% paraformaldehyde in PHEM buffer for 20 min at 4°C. In some experiments the cells were permeabilized with 0.1% Triton X-100 in PHEM buffer for 1 min at 22°C. After extensive washing (three times with PHEM buffer, 5 min each), the cells were incubated for 1 h with 1% BSA, 1% goat serum in PHEM buffer to block nonspecific binding, followed by incubation with anti–E-selectin mAb H4/18 diluted 1:1,000 in blocking buffer. Primary antibodies were visualized using 1:150 dilution of a Cy3-conjugated goat anti–mouse IgG. The samples were examined with a Sarasto 2000 Confocal Laser Scanning Microscope (CLSM; Molecular Dynamics, Sunnyvale, CA) fitted with a 25-mW Argon-Ion Laser attenuated to 10% transmittance and images recorded using a 60× (1.4 numeric aperture) oil-immersion objective lens. The fluorescent signals from the HUVEC and adherent cells were obtained using 488 nm excitation for the detection of CMFDA and 514 nm excitation for Cy3. The emission spectra were filtered with a high-pass 535-nm primary dichroic beam splitter and a 565-nm secondary dicroic beam splitter. The CMFDA fluorescent emission was detected through a 540 ± 30-nm band pass filter, and Cy3 fluorescent emission was detected through a 570-nm long pass filter. The digitized images were collected and analyzed using Image Score software (Molecular Dynamics).

Isolation of Cytoskeletal Proteins Associated with E-Selectin

Paramagnetic beads (10^8 Dynabeads® M-450; Dynal, Inc., Lake Success, NY) coated with a polyclonal goat-anti–mouse IgG were incubated with 20 μg of purified H18/7, W6/32 (murine HLA class I mAb; binding control IgG) (Luscinskas et al., 1989), or K16/16 (murine nonbinding IgG) (Luscinskas et al., 1989) in DPBS + 0.25% BSA for 12 h at 4°C. In some experiments, tosyl-activated Dynabeads® M-450 were coated with RGD peptides as previously described (Plopper and Ingber 1993). The beads were then blocked for nonspecific binding by incubation in RPMI + 1% BSA for 3 h at 37°C. HUVEC were cultured in 150-mm tissue culture dishes and activated with IL-1β for 4 h at 37°C. The cells were dispersed with HBSS + 5 mM EDTA, placed in 50-ml polypropylene tubes, suspended (10^6 cells/ml) in RPMI + 1% BSA containing the mAb-coated magnetic beads (5×10^6/ml), and then incubated for 30 min at 37°C with gentle rotation. Magnetic beads and bound cells were collected using a side-pull magnetic separator system (Perspective Diagnostics, Cambridge, MA), and suspended in 10 ml CSK buffer (10 mM Pipes, 50 mM $NaCl_2$, 300 mM sucrose, 3 mM $mgCl_2$, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM AEBSF, pH 6.8) (Plopper and Ingber, 1993). After washing with 10 ml CSK buffer, cells were extracted with 10 ml of 0.1% Triton X-100 in CSK buffer for 1 min on ice. The magnetic bead pellets were sonicated for 15 s and homogenized in a dounce homogenizer. The pellets were washed five times with CSK buffer containing 0.1% Triton X-100. Proteins in the bead complex were dissolved in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, 1 mM PMSF, 200 mM sodium vanadate, pH 7.2). The protein concentration was determined by the Bradford method (BioRad Labs., Richmond, CA). Equal amounts of protein sample were subjected to 8% SDS-PAGE under reducing conditions and Western blot analysis carried out as described above, using mAbs specific for various cytoskeletal proteins.

Biomechanical Analysis of Membrane Protein–Cytoskeletal Linkage

HUVEC, or WT-, and ΔCYTO-E-selectin–transfected COS-7 cells were plated (2×10^4/well) in 0.1% gelatin-coated Immulon 2® Removawell® (Dynatech Laboratories, Inc., Chantilly, VA) and cultured for 24 h. 4 mg of carboxyl ferromagnetic beads (Chromium dioxide, 4.5-μm diameter, Spherotech Inc., Libertyville, IL) were coated either with E-selectin mAb H18/7, a synthetic RGD-containing peptide (Peptite 2000; Telios, San Diego, CA), which is a specific ligand for integrin receptors, or AcLDL, all at 480 μg/ml, following the manufacturer's protocol. Precoated ferromagnetic beads were allowed to bind to the monolayers for 15 min at 37°C. After washing out unbound beads with RPMI + 1% BSA, each well was subjected to a rotational force in a magnetic twisting cytometer, as de-

scribed previously (Wang et al., 1993; Wang and Ingber, 1994). Briefly, a strong magnetic field (1000 Gauss for 10 micros) was applied to magnetize all surface-bound beads, followed by a weaker twisting magnetic field (30 Gauss for 1 min) applied perpendicular to the original field. The latter field was not sufficient to realign the magnetic moment of the beads but served to rotate the beads in place (Wang and Ingber, 1994). The average bead rotation (angular strain) induced by the twisting force was measured by an in-line magnetometer. The applied stress was calibrated in a viscous standard (Wang and Ingber, 1994). For these beads, a 30-Gauss twisting field corresponded to an applied stress of 15.2 dynes/cm^2. The stiffness of the cytoskeleton-membrane protein association was defined as the ratio of stress to strain (in radians) (Wang and Ingber, 1994). To confirm that this resistance to the applied force depended upon an intact cytoskeleton, the twisting force also was applied to the same cells after incubation with 1 μg/ml of cytochalasin D for 30 min, a treatment which is known to disrupt the actin network (Schliwa, 1982).

Results

Leukocyte Adhesion Induces Cytoskeletal Association of E-Selectin in Cytokine-activated HUVEC

To visualize the effect of leukocyte adhesion on the distribution of E-selectin in cultured monolayers of HUVEC, we used laser scanning confocal immunofluorescence microscopy, and H4/18, a nonfunction blocking murine monoclonal antibody to the human E-selectin molecule (Pober et al., 1986; Bevilacqua et al., 1987). HUVEC monolayers were activated with IL-1β (10 U/ml, 4 h) to stimulate maximum expression of E-selectin at the cell surface, and then incubated with either HL-60 cells, a human leukocyte cell line that adheres through E-selectin (Bevilacqua et al., 1985) or JY cells, another human leukocyte cell line that adheres via LFA-1/ICAM-1 (Rothlein and Springer, 1986) (Fig. 1). Before leukocyte adhesion, E-selectin exhibited a diffuse cell surface-staining pattern, as well as intracellular accumulations consistent with Golgi-endoplasmic reticulum localization (data not shown). Adhesion of HL-60 cells (Fig. 1, *a* and *b*), but not of JY cells (Fig. 1 *c*), to activated HUVEC monolayers induced a clustering of E-selectin molecules in the vicinity of the cell–cell attachment sites. To investigate whether this localization of E-selectin during the process of leukocyte adhesion involved interactions with the endothelial cytoskeleton, a detergent-extraction–based protein analysis was performed. HUVEC monolayers were activated with IL-1β (10 U/ml, 4 h) and incubated with sufficient HL-60 or JY cells for 10 min to allow comparable numbers of E-selectin–dependent and E-selectin–independent adhesion events, respectively, to occur. After removal of nonadherent leukocytes by gentle washing, extraction with 0.1% Triton X-100 in PHEM buffer was performed. Both the detergent-soluble and the detergent-insoluble (cytoskeletal) fractions were separated by electrophoresis on an 8% SDS-PAGE under nonreducing conditions, and the separated proteins transferred to a PVDF membrane for Western blotting with the E-selectin mAb H4/18. In the absence of HL-60 adhesion, essentially all immunoreactive E-selectin was found in the soluble fraction at its predicted molecular mass of ~115 kd (Fig. 2). However, after adhesion of HL-60 cells, a significant amount of immunoreactive E-selectin was found in the insoluble fraction (Fig. 2 *a*). In contrast, after adhesion of comparable numbers of JY cells, essentially no E-selectin was detected in the insoluble fraction (Fig. 2 *a*). A small amount of immunoreactive E-selectin also was observed at >200 kD in these gels, which might represent aggregates of E-selectin proteins, or covalent complexes of E-selectin with other proteins which remained intact under nonreducing conditions (Garfin, 1990).

Antibody Clustering of E-Selectin at the Endothelial Surface also Induces Cytoskeletal Association

To stimulate localized clustering of E-selectin as might occur on the endothelial cell surface at sites of leukocyte adhesion, a saturating amount of the function-blocking murine monoclonal antibody H18/7, which recognizes adhesion supporting epitopes in the extracellular portion of the E-selectin molecule, was bound to activated HUVEC and cross-linked by a goat anti–mouse polyclonal IgG to induce cell surface clustering of E-selectin. Epifluorescent microscope analyses confirmed that this cross-linking procedure induced visible clustering of E-selectin mAb on the cell surface, whereas incubation with anti–E-selectin mAb alone, or with the goat anti–mouse polyclonal IgG alone, did not (data not shown). Detergent extraction, performed as described above, revealed a significant redistribution of E-selectin protein from the soluble to insoluble fractions, following antibody-induced E-selectin clustering that was not observed with either H18/7 or the polyclonal IgG alone (Fig. 2 *b*). The fraction of E-selectin that became cytoskeletal-associated in the presence of saturating amounts of bound E-selectin mAb was greater than that observed in HL-60 adhesion (c.f., Fig. 2 *a*). Similar results also were obtained using another E-selectin–specific, but nonfunction-blocking, monoclonal antibody H4/18 (data not shown). However, the cross-linking of mAb W6/32, directed to abundant but functionally unrelated transmembrane cell surface molecules (HLA class I), did not induce any cytoskeletal association of E-selectin (data not shown). In these experiments, higher molecular weight E-selectin immunoreactive bands were again observed.

Cytoplasmic Domain Deletional Mutant of E-Selectin Fails to Show Leukocyte-Adhesion–induced Cytoskeletal Association

To examine whether the cytoplasmic domain of E-selectin is necessary for this leukocyte adhesion–induced cytoskeletal association, a deletion mutant (ΔCYTO) of E-selectin was constructed using site-directed mutagenesis (Fig. 3 *a*). COS-7 cells transfected with both native (wild type, WT) and ΔCYTO-E-selectin showed comparable levels of surface expression of E-selectin proteins as confirmed by FACscan analyses using mAb H18/7 (Fig. 3 *b*). The relative mean channel fluorescence values for WT- and ΔCYTO-E-selectin were 144.9 and 154.03, respectively, and 31% of WT-transfectants and 27% of ΔCYTO-transfectants were positive for E-selectin surface staining. Quantitative monolayer adhesion assays, performed with fluorescently labeled HL-60 cells under nonstatic conditions (see Materials and Methods), documented that both WT- and ΔCYTO-E-selectin–transfected COS-7 cells supported comparable amounts of adhesion (WT; 8196 ± 691, ΔCYTO; 6911 ± 1098, mean ± SD, relative fluorescent intensity units, three replicates, three experiments, $P > 0.16$), which were ~100-fold higher than mock-transfected COS-7 cells (11.1 ± 19.4, $P < 0.005$). COS-7 cells trans-

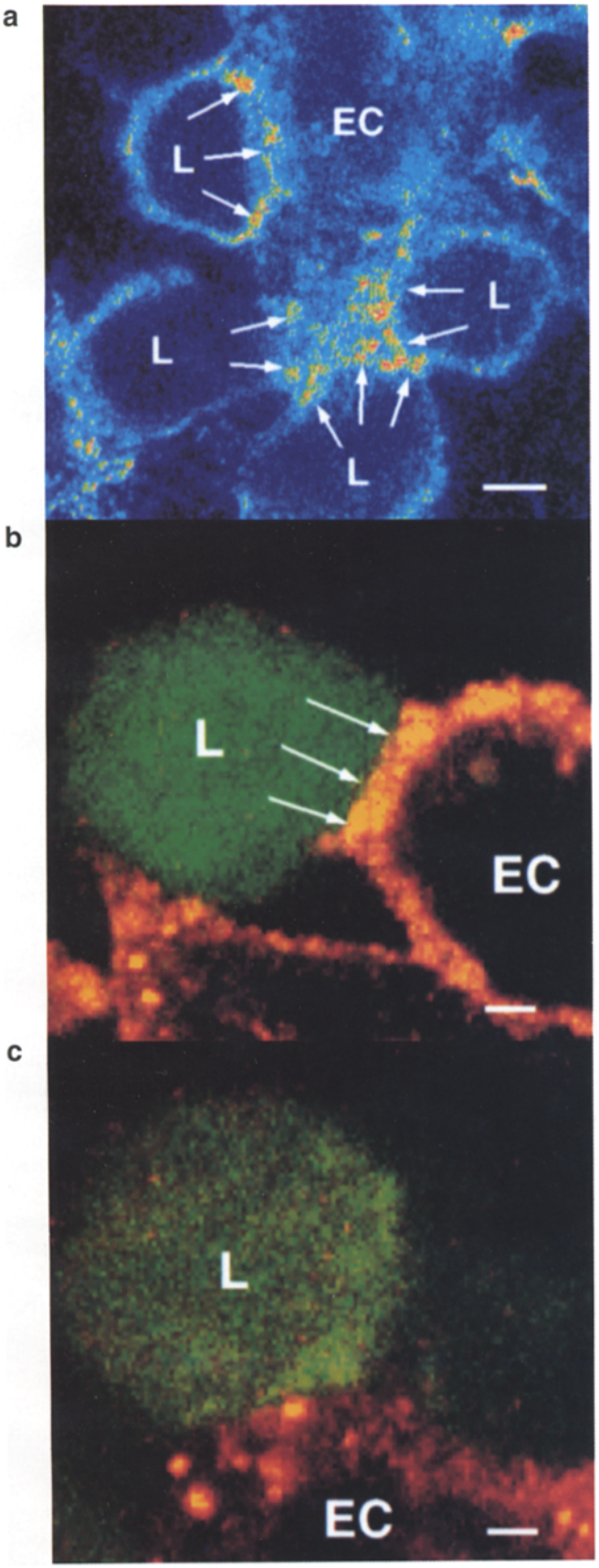

Figure 1. (*a*) Leukocyte adhesion induces clustering of E-selectin in cultured endothelial cells. Fluorescent confocal laser scanning micrograph of an activated HUVEC monolayer to which HL-60 cells (a leukocyte cell line that expresses ligand(s) for E-selectin) have adhered for 10 min at 37°C, stained with a nonblocking anti–E-selectin mAb (H4/18) decorated with a fluorescently conjugated goat-anti–mouse IgG. Pseudocolor image enhancement (blue to yellow/red indicating low to high fluorescence intensity). Clustering of E-selectin was observed at leukocyte attachment sites (*arrows*). *EC*, HUVEC; *L*, HL-60; Bar, 5 μm. (*b* and *c*) Clustering of E-selectin is selectively induced by HL-60, not by JY, cell adhesion. Activated HUVEC were incubated for 10 min at 37°C with either (*b*) CMFDA (*green fluorochrome*)-labeled HL-60 cells, or (*c*) CMFDA-labeled JY cells, another human leukocyte cell line which binds to activated HUVEC primarily by non-E-selectin–dependent mechanisms, and confocal laser scanning microscope in dual color (*red/yellow* for E-selectin and *green* for HL-60 or JY cells) was performed as above. Adhesion of HL-60 cells (*b*), but not of JY cells (*c*), to activated HUVEC monolayers induced a clustering of E-selectin (*b*, *arrows*). *EC*, HUVEC; *L*, HL-60 or JY; Bar, 2 μm.

fected with WT- and ΔCYTO-E-selectin were then incubated with unlabeled HL-60 cells under static assay conditions, and cytoskeletal association was assessed by detergent extraction as described above. Association of E-selectin with cytoskeleton did not occur in COS-7 cells transfected with ΔCYTO-E-selectin in the presence or absence of leukocyte adhesion (Fig. 3 *c*), thus confirming the involvement of the cytoplasmic domain in this interaction. COS-7 cells transfected with WT-E-selectin cDNA showed a pattern of adhesion-induced cytoskeletal association of E-selectin protein (Fig. 3 *c*) similar to that observed with the native E-selectin in cytokine-activated HUVEC (Fig. 2 *a*). However, in COS-7 cells, unlike cytokine-activated HUVEC, association of E-selectin with the cytoskeleton also occurred in the absence of leukocyte adhesion, although to a lesser degree (Fig. 3 *c*). As was observed with activated HUVEC, immunoreactive E-selectin was detected in higher molecular weight complexes (>200 kD) which were more prevalent in the insoluble (cytoskeletal) fraction following leukocyte adhesion.

Identification of Cytoskeletal Proteins Associated with E-Selectin

To directly analyze the nature of the endothelial cytoskeletal association with E-selectin, the protein components associated with E-selectin were physically isolated, using a recently described magnetic bead immunoextraction procedure (Plopper and Ingber, 1993; Plopper et al., 1995). This novel technique, which has been developed for study of the transmembrane association of integrin receptors with focal adhesion complexes, combines specific antibody-coated microbeads with various biochemical and physical extraction procedures to selectively isolate those cytoskeletal components that are complexed with a particular transmembrane protein of interest. Beads coated with appropriate positive and negative control antibodies were used to test the immunoselectivity of the isolation. In preliminary experiments, it was documented that magnetic beads coated with the anti–E-selectin mAb H18/7 bound selectively IL-1β–activated, but not to unactivated HUVEC monolayers, and also that this binding was blocked by preincubation with excess soluble H18/7 mAb (data not shown). We reasoned that the binding of these relatively

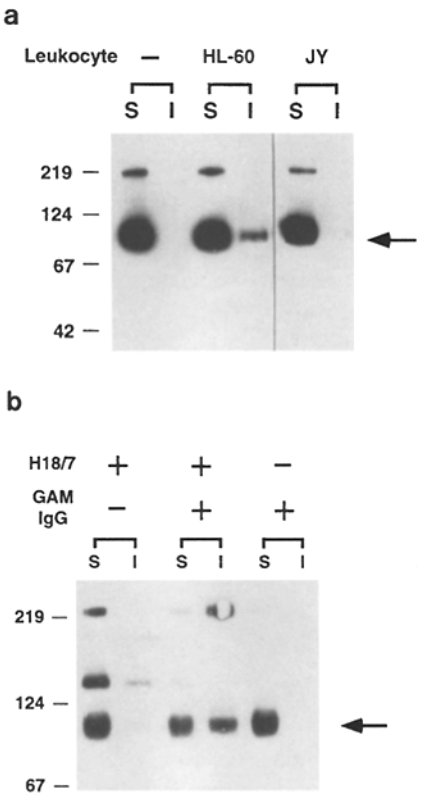

Figure 2. (*a*) Leukocyte adhesion induces cytoskeletal association of E-selectin in activated HUVEC. Activated HUVEC monolayers (IL-1β, 4 h, 37°C) were incubated for 10 min with leukocytes, and then extracted with Triton X-100, as described in Materials and Methods. The samples from the detergent soluble (*S*) and the detergent insoluble (*I*; cytoskeletal) fractions were subjected to 8% SDS-PAGE under nonreducing conditions and Western blotted, using the nonblocking anti–E-selectin mAb H4/18 (10 μg/ml). Note that a significant amount of E-selectin protein (*arrow*) is recovered in the insoluble fraction after HL-60, but not JY, cell adhesion, and that virtually no E-selectin is detectable in the same fraction in the absence of leukocyte adhesion. Comparable patterns were observed in three other experiments. (*b*) Antibody cross-linking of E-selectin can mimic leukocyte adhesion–induced cytoskeletal association of E-selectin. HUVEC were activated with IL-1β for 4 h at 37°C. The cells were incubated with the adhesion-blocking anti–E-selectin mAb, H18/7 (10 μg/ml) for 10 min at 4°C, followed by incubation with cross-linking polyclonal antibody (1:200 dilution) for 10 min at 37°C. The cells then were extracted and subjected to 8% SDS-PAGE and Western blotting with mAb H4/18, as above. Significant amounts of E-selectin (*arrows*) are recovered in the insoluble fraction after the incubation with mAb H18/7 and a goat anti–mouse polyclonal IgG, but not mAb H18/7 or goat anti–mouse IgG alone. These data are representative of three separate experiments.

large (4.5-μm diameter) beads would mimic the adhesion of a leukocyte via an E-selectin–dependent mechanism to the endothelial cell surface (c.f., Fig. 2, *a* and *b*). Magnetic beads were also prepared with mAb W6/32, directed to HLA class I molecules, and a nonbinding murine hybridoma IgG, K16/16 (Luscinskas et al., 1989) as controls for molecular selectivity and antibody specificity. After incubation with activated HUVEC, bead-associated proteins were extracted, and equal amounts subjected to an 8% SDS-PAGE and Western blotting using antibodies to selected cytoskeletal proteins. The binding capacity of both H18/7- and W6/32-coated magnetic beads were confirmed using surface-biotinylated HUVEC in the same condition. Both H18/7- and W6/32-coated magnetic beads were able to extract comparable amounts of E-selectin and HLA class I molecules, respectively, detected by Western blotting using HRP-conjugated streptavidin (data not shown). As seen in Fig. 4 *a*, vinculin, filamin, and α-actinin showed preferential association with the anti–E-selectin antibody-coated beads. In other E-selectin immunoextractions, FAK and one of its substrate proteins, paxillin, also were copurified, whereas another cytoskeletal protein talin was not detected (Fig. 4 *b*). Actin was detectable in both H18/7 and W6/32 bead extracts, although quantitatively less was recovered with W6/32-coated beads (data not shown). This result is similar to that produced using AcLDL-beads which bind transmembrane receptors on the endothelial cell surface (Plopper et al., 1995 and unpublished data). In parallel experiments, RGD-coated magnetic beads were applied to activated HUVEC to extract integrin-associated focal adhesion complexes. As shown in Fig. 4 *b*, FAK, paxillin, and talin were detected as previously reported (Plopper and Ingber, 1993; Plopper et al., 1995).

Direct Measurement of Mechanical Linkage between E-Selectin and the Cytoskeleton in Activated HUVEC

To confirm the physical linkage between cell surface E-selectin molecules and intracellular cytoskeletal elements, H18/7-coated ferromagnetic beads bound to the surface of activated HUVEC were subjected to a mechanical stress (15.2 dyne/cm^2) and the induced bead rotation (angular strain) was simultaneously measured using a magnetic twisting cytometer (Wang et al., 1993; Wang and Ingber, 1994). The mechanical stiffness of the structural linkages between surface E-selectin and internal cytoskeletal structures was determined by calculating the ratio of stress to strain. The results obtained with E-selectin mAb-coated beads were compared first to RGD peptide-coated beads which exhibited a relatively strong transmembrane linkage to cytoskeleton via integrin receptors (Wang et al., 1993), and second, to AcLDL-coated beads, which bind to transmembrane receptors in the endothelial cell that only display minimal resistance to mechanical distortion (Wang et al., 1993). As seen in Fig. 5, beads bound to E-selectin on the surface of the activated HUVEC exhibited resistance to mechanical stress and showed a relative stiffness intermediate between that of integrins and AcLDL receptors. In the presence of cytochalasin D, which specifically disrupts actin microfilaments (Schliwa, 1982; Cooper, 1987), both integrin- and E-selectin–bound beads showed significantly enhanced rotational movement (i.e., reduced stiffness), confirming that the mechanical response measured resulted from structural tethering to filamentous actin cytoskeleton. In contrast, cytochalasin D had no effect on the rotational movement of AcLDL-coated beads, which previously have been shown not to be linked to actin-associated cytoskeletal proteins, such as α-actinin, vinculin, paxillin, or talin, in endothelial cells (Plopper and Ingber, 1993; Wang et al., 1993; Plopper et al., 1995). In parallel experiments (data not shown), beads coated with mAb

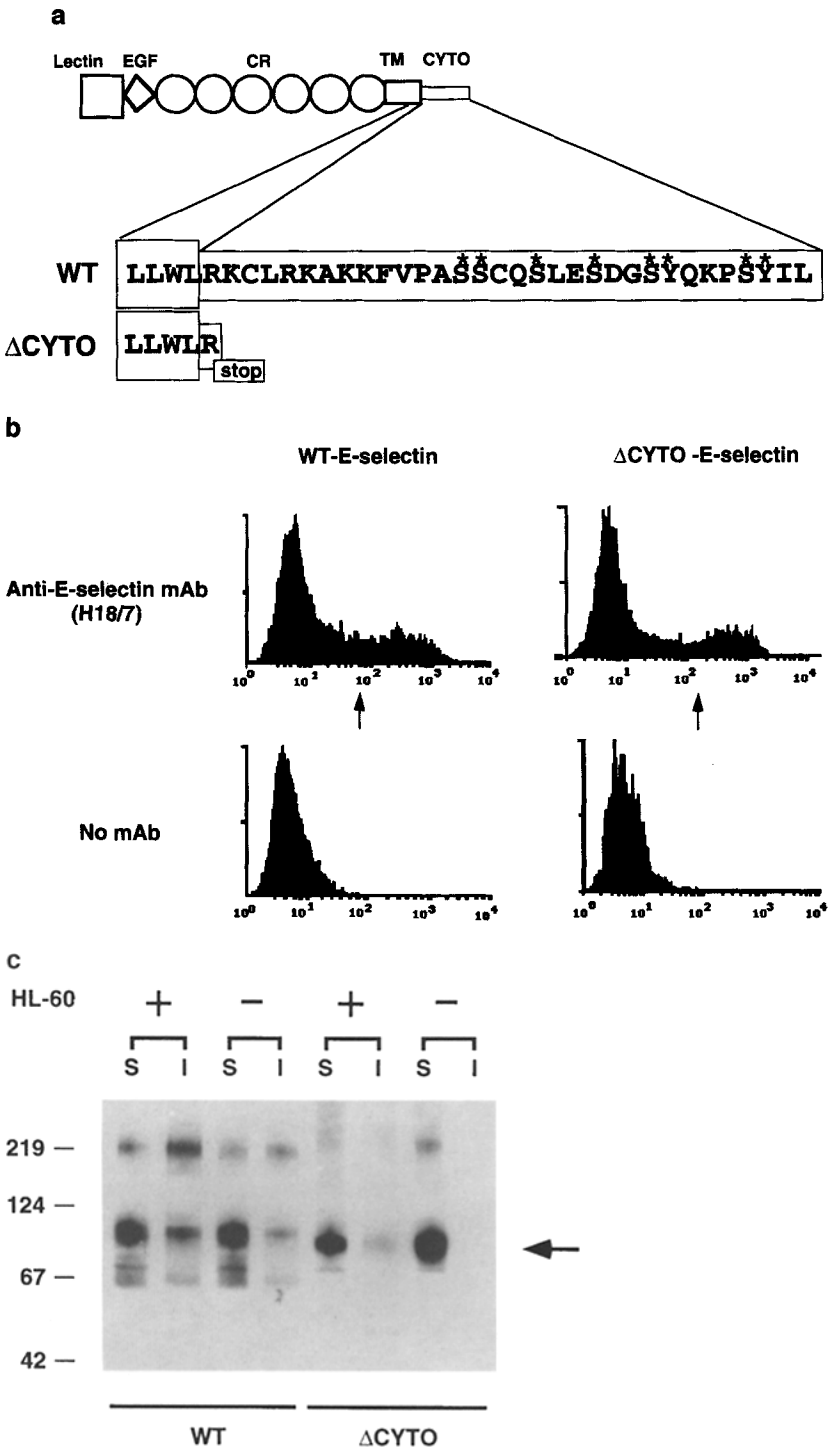

Figure 3. (*a*) Schematic structure of wild-type and cytoplasmic tailless E-selectin. A stop codon was introduced into the second amino acid of the cytoplasmic domain of the intact wild-type (*WT*) E-selectin cDNA, using site-directed in vitro mutagenesis, to generate a "cytoplasmic tailless" mutant cDNA (*ΔCYTO*). Mutated sequence was confirmed by the dideoxy sequencing method. (*Lectin*, lectin-like domain; *EGF*, EGF-like domain; *CR*, complement regulatory protein-like repeats; *TM*, transmembrane domain; *CYTO*, cytoplasmic domain). Asterisks indicate possible phosphorylation sites. (*b*) Surface expression of E-selectin in transfected COS-7 cells. 72 h after transfection, WT- and ΔCYTO-E-selectin–transfected COS-7 cells were incubated with anti-E-selectin mAb (*H18/7*) followed by a FITC-conjugated goat anti-mouse polyclonal IgG, or the secondary antibody alone (*No mAb*). Fluorescence intensity was measured by a FACscan analysis. Approximately 30% of both COS-7 populations are positive for E-selectin surface expression (gate set as indicated by arrow). (*c*) Cytoskeletal association of wild-type but not cytoplasmic tailless E-selectin in transfected COS-7 cells. WT- and ΔCYTO-E-selectin–transfected COS-7 cells were subjected to the detergent-extraction based cytoskeletal analysis described in the text. Note that ΔCYTO-E-selectin (*arrows*) fails to associate with the cytoskeleton during leukocyte adhesion (compare with Fig. 2 *a*). These data are representative of two independent experiments.

W6/32, directed to constitutively expressed transmembrane HLA class I molecules, showed behavior comparable to AcLDL-coated beads (i.e., negligible stiffness which was not influenced by cytochalasin D treatment). This result is more consistent with these receptors being associated with the highly elastic submembranous cytoskeleton, rather than with the more rigid filamentous actin cytoskeleton (Luna and Hitt, 1992).

To further investigate whether the cytoplasmic domain is important in this mechanical linkage, COS-7 cells transfected with WT- and ΔCYTO-E-selectin (expressing comparable number of cell surface immunoreactive E-selectin by FACS analysis) were also examined in the magnetic twisting cytometer. As shown in Fig. 5 *c*, H18/7 beads bound to WT-E-selectin–transfected COS-7 cells exhibited significantly greater resistance to the twisting force than that of AcLDL receptor, whereas ΔCYTO-E-selectin failed to show any significant resistance. Cytochalasin D treatment significantly reduced the stiffness of WT-E-selectin but not ΔCYTO-E-selectin. These results thus

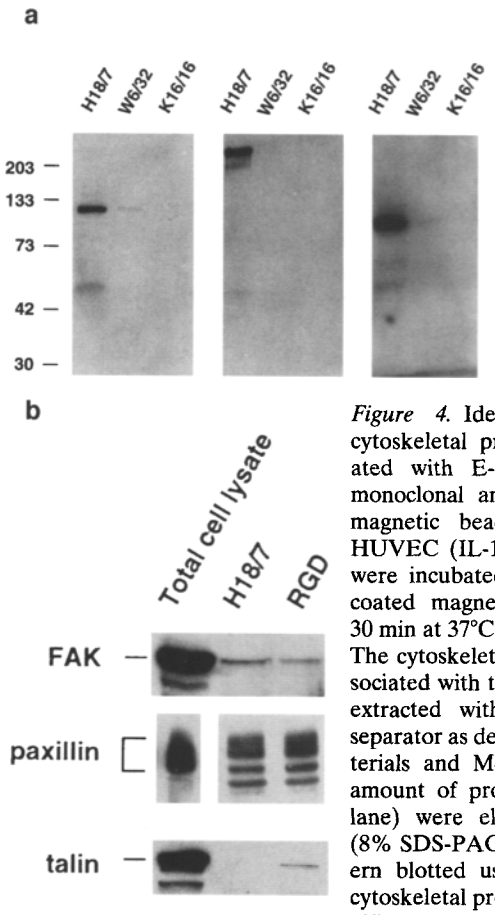

Figure 4. Identification of cytoskeletal proteins associated with E-selectin using monoclonal antibody–coated magnetic beads. Activated HUVEC (IL-1β, 4 h, 37°C) were incubated with H18/7-coated magnetic beads for 30 min at 37°C in suspension. The cytoskeletal proteins associated with the beads were extracted with a magnetic separator as described in Materials and Methods. Equal amount of proteins (10 μg/lane) were electrophoresed (8% SDS-PAGE) and Western blotted using mAbs to cytoskeletal proteins. (*a*) Significant amounts of α-actinin, filamin, and vinculin were associated with H18/7-(anti–E-selectin) coated beads, in contrast to W6/32 (anti-HLA class I molecules) or K16/16 (nonbinding IgG)-coated beads. One of two similar experiments is shown. (*b*) E-selectin–associated cytoskeletal components (by H18/7-coated beads extraction) were compared with integrin-focal adhesion complex by RGD-coated beads extraction. H18/7-coated beads extracted paxillin and FAK, but not talin. RGD-coated beads extracted paxillin and FAK as well as talin as reported previously. Similar data were obtained in two separate experiments.

confirm that the cytoplasmic domain of E-selectin is necessary for mechanical linkage of E-selectin to cytoskeleton.

Discussion

In this report, we demonstrate that E-selectin, an inducible member of the selectin family of adhesion molecules, becomes associated with the endothelial cytoskeleton during the process of leukocyte adhesion. Adhesion of HL-60 cells, which bind via E-selectin to IL-1β–activated HUVEC monolayers (Bevilacqua et al., 1987), induced a clustering of E-selectin molecules at sites of cell–cell contact (Fig. 1, *a* and *b*), and a concomitant association of immunoreactive E-selectin protein with the detergent-insoluble (cytoskeletal) fraction of the endothelial cell (Fig. 2 *a*). These phenomena were not induced by the adhesion of comparable numbers of JY cells (Figs. 1 *c*, 2 *a*), another human leukocyte cell line that adheres to activated HUVEC largely via ICAM-1/LFA-1 interactions (Rothlein and Springer, 1986), thus suggesting a requirement for specific binding to the E-selectin molecule. HL-60 cells adhered comparably to COS-7 cells transfected with either an intact E-selectin or a cytoplasmic tailless E-selectin mutant, but failed to induce cytoskeletal association of the mutant molecule (Fig. 3 *c*), thus demonstrating involvement of the cytoplasmic domain in this process. Cross-linking of E-selectin molecules on the endothelial cell surface induced a pattern of cytoskeletal association similar to that seen with leukocyte adhesion (Fig. 2 *b*). Antibody-coated magnetic beads were used to isolate the cytoskeleton components interacting directly, or indirectly (e.g., as part of a secondary complex), with E-selectin. These included α-actinin, vinculin, filamin, FAK, and paxillin but not talin (Fig. 4). Biophysical measurements, using antibody-coated ferromagnetic beads bound to E-selectin molecules on HUVEC monolayers indicated a cytochalasin-inhibitable resistance to mechanical stress analogous to that exhibited by integrin receptors (Fig. 5). In contrast to E-selectin, two other molecules present on the surface of endothelial cells (AcLDL receptors and HLA class I heterodimers) showed no evidence of any significant biochemical or biophysical transmembrane association with the filamentous actin cytoskeleton. Taken together, we interpret these observations as indicating that E-selectin molecules present at the apical ("luminal") surface of an activated endothelial cell become associated, via their cytoplasmic domains, with certain components of the filamentous actin cytoskeleton, as a consequence of leukocyte attachment to their extracellular domains. This process may have potentially important biochemical and biophysical implications for endothelial function during inflammation.

The clustering of E-selectin on the surface of activated endothelial cells at sites of leukocyte adhesion may represent a previously unrecognized transition step between initial rolling interactions and subsequent stable arrest in the leukocyte-endothelial adhesion cascade (Butcher, 1991; Springer, 1994). The ability of leukocytes that adhere via E-selectin–dependent mechanisms to selectively induce this surface clustering and concomitant cytoskeletal linkage of the E-selectin molecule suggests that a relatively specific outside-in signaling mechanism is involved. Conceivably, a transmembrane signal could be initiated by the binding of complex carbohydrate ligands on the leukocyte surface to the extracellular lectin domains of E-selectin molecules. However, binding of monoclonal antibodies to cell surface E-selectin, alone, failed to induce this cytoskeletal association, whereas cross-linking mediated by a polyclonal anti-murine IgG antiserum was sufficient to mimic the leukocyte adhesion-induced pattern. Recent studies with integrin receptors have revealed a complex relationship between receptor-occupancy dependent and receptor-clustering dependent mechanisms in transmembrane signaling and organization of focal adhesions (Miyamoto et al., 1995). Our observation that the clustering of E-selectin via a nonadhesion blocking mAb (H4/18) also caused cytoskeletal association suggests that receptor-clustering per se is sufficient to induce this phenomenon. However, further studies involving the (complex carbohydrate) natural E-selectin ligands present on the surface of circulating blood leukocytes are indicated.

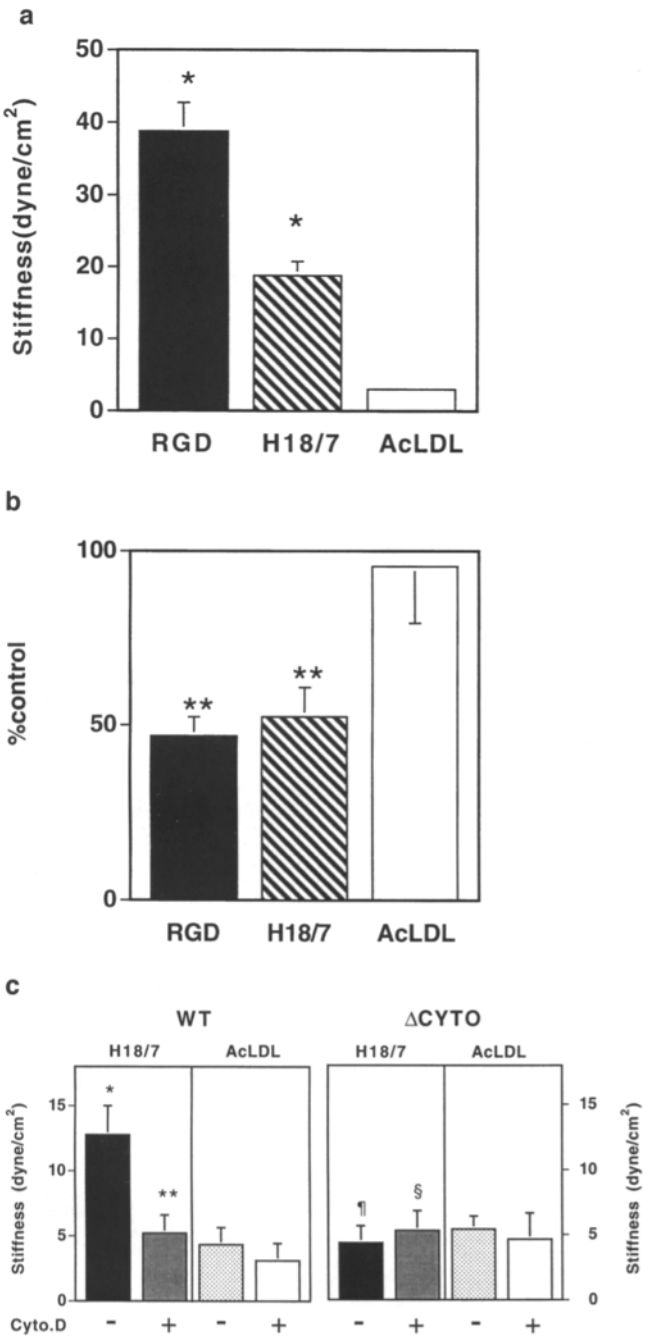

Figure 5. Mechanical linkage between cell surface E-selectin and the actin-cytoskeleton as revealed by a magnetic twisting cytometer. (*a*) Comparison of RGD-, mAb H18/7-, and AcLDL-coated magnetic beads. HUVEC replicate-plated in microtiter wells were activated (IL-1β 10 U/ml, 4 h, 37°C) and 80-μg aliquots of magnetic beads coated with either H18/7, RGD peptides, or AcLDL were added to each microwell. After incubation for 15 min at 37°C, each well was subjected to a magnetic twisting stress (15.2 dyne/cm^2) and the resulting angular strain was measured. The stiffness associated with E-selectin, integrins, or AcLDL receptors was quantitated as the ratio of the applied stress to strain. Note that H18/7-coated beads showed a relative stiffness intermediate between that of RGD- and AcLDL-coated beads. The data are representative of three replicate experiments. * $P <$ 0.0005; RGD- or H18/7-coated beads vs AcLDL-coated beads (*t* tests). (*b*) Effect of cytochalasin D treatment on observed stiffness. Relative stiffness was calculated as the ratio of the resistance to a mechanical deformation with and without cytochalasin D treatment (1 μg/ml for 30 min). Note that addition of cytochalasin D significantly reduced stiffness associated with both H18/7- and RGD-, but not AcLDL-coated beads. The data are representative of three replicate experiments. ** $P < 0.0001$; (+) vs (−) cytochalasin D for RGD or H18/7-coated beads (*t* tests). (*c*) Role of cytoplasmic domain in E-selectin mechanical linkage. WT- and ΔCYTO-E-selectin–transfected COS-7 cells were plated in microtiter wells and 80-μg aliquots of H18/7- or AcLDL-coated magnetic beads were added. After incubation for 15 min at 37°C, a magnetic twisting stress was applied and the "stiffness" of E-selectin or AcLDL receptors, was quantitated as described above. In WT-E-selectin–transfected COS-7 cells, H18/7-coated beads showed significant stiffness (compared to AcLDL-coated beads) that was reduced by the incubation with cytochalasin D (1 μg/ml for 30 min). In contrast, ΔCYTO-E-selectin–transfected COS-7 cells, no significant stiffness (compared to AcLDL-coated beads) was observed before or after cytocholasin D treatment. The data are representative of three replicate experiments. * $P < 0.002$, H18/7-coated vs AcLDL-coated beads; ** $P < 0.05$, before vs after cytochalasin D; ¶ $P > 0.4$; H18/7-coated vs AcLDL-coated beads; § $P > 0.5$ (*t* tests).

The failure of ΔCYTO-mutant E-selectin molecules to associate with the cytoskeleton during leukocyte adhesion establishes a necessary role of the cytoplasmic domain in this process. This result further supports a model of direct transmembrane signaling and/or physical linkage of E-selectin to adjacent structural elements within the endothelial cell. E-selectin's relatively short (32 amino acid) cytoplasmic domain contains consensus sequences required for endocytosis of other receptors via coated pits (Kristakis et al., 1990), as well as several potential phosphorylation sites (six serines and two tyrosines; cf. Fig. 3 *a*). E-selectin molecules present at the surface of cytokine-activated HUVEC, in the absence of adherent leukocytes, appear to be phosphorylated on one or more serine residues (Smeets et al., 1993), but potential regulation of phosphorylation on serines and/or tyrosines in the cytoplasmic domain by ligand binding to the extracellular lectin domain of E-selectin remains to be studied. The cytoplasmic tails of other selectin family members appear to function in the sorting of newly synthesized molecules to storage granules, in the case of P-selectin (Subramaniam et al., 1993), and in leukocyte rolling and attachment to high endothelial venules, in the case of L-selectin (Kansas et al., 1993). A recent study (Waddell et al., 1995) has demonstrated that ligation of L-selectin by monoclonal antibodies or sulfated glycolipids induces tyrosine phosphorylation in human neutrophils. ΔCYTO mutants of L-selectin (lacking the COOH-terminal 11 amino acids of the cytoplasmic domain) fail to support normal adhesive interactions when transfected into a mouse pre-B cell line (Kansas et al., 1993). This is in contrast to our observation that the ΔCYTO mutant of E-selectin expressed in COS-7 cells supports HL-60 cell adhesion to the same extent as WT-E-selectin transfectants. Interestingly, although significant homologies do exist among the corresponding extracellular domains of P, L, and E-selectins, their cytoplasmic domains lack homology (Bevilacqua et al., 1989; Johnston et al., 1989; Ord et al., 1990), suggesting divergent functions.

Adaptation of a recently described magnetic bead extraction procedure (Plopper and Ingber, 1993) allowed

isolation of the cytoskeletal proteins that become associated with E-selectin molecules during their clustering by antibody-coated beads at the endothelial cell surface. Certain of these, e.g., vinculin, filamin, and α-actinin, previously have been shown to be associated with integrins in the focal adhesion complexes (Pavalko and Otey, 1994), such as those that occur on the basal aspect of endothelial cells at points of attachment to the subjacent extracellular matrix. α-Actinin also has been reported to associate with ICAM-1 in transfected COS cells, and an analogous cytoskeletal anchorage has been hypothesized to occur in endothelial cells as a "foothold" for leukocytes undergoing emigration (Carpen et al., 1992). Recent biochemical studies (Pavalko et al., 1995) have demonstrated that the cytoplasmic domain of L-selectin can interact directly with α-actinin which, in turn, forms a complex with vinculin and talin. Interestingly, talin, an actin-binding cytoskeletal component typically present in focal adhesion complexes, which copurifies with integrin receptors using RGD-coated beads (Plopper and Ingber, 1993), was not isolated by our anti–E-selectin–bead extraction procedure (Fig. 4 *b*). E-cadherin, a member of yet another group of adhesion molecules expressed at the lateral cell–cell junctions of epithelial cells and certain vascular endothelial cells, has been reported to colocalize with several cytoskeletal proteins, such as vinculin and α-actinin, but not with talin (Geiger and Ginsberg, 1991). Thus, the cytoskeletal protein complexes which form in association with the cytoplasmic domains of clustered E-selectin molecules at the apical endothelial surface, or those associated with cadherin molecules at the lateral endothelial junctions, appear to have distinct features compared to integrin-containing focal adhesion complexes at the basal endothelial surface. The presence of FAK and its substrate proteins, vinculin and paxillin, in these E-selectin–associated complexes suggest a potential regulatory role for phosphorylation in their formation.

A further analogy to the transmembrane linkages formed by integrins was apparent in the biophysical measurements of the mechanical properties of clustered E-selectin surface molecules (Fig. 5 *a*). The stiffness of their cytoskeletal association, estimated from the resistance of anti–E-selectin–coated beads to an applied magnetic twisting force, in the presence and absence of the cytoskeletal-disrupting agent cytochalasin D (Fig. 5 *b*), resembled that of cell surface integrin receptors probed with RGD-coated beads.

Moreover, the failure of the tailless mutant E-selectin to exhibit any significant resistance to an applied twisting force further confirmed the functional role of the cytoplasmic domain in this phenomenon (Fig. 5 *c*). These observations thus are consistent with a mechanical transmembrane linkage of clustered E-selectin molecules with actin microfilaments in the endothelial cytoskeleton, presumably via their cytoplasmic domains.

Our observations lead us to speculate on several functional implications of leukocyte adhesion–induced E-selectin clustering and cytoskeletal association. First, the transmembrane anchoring of a cluster of E-selectin molecules on the endothelial surface could serve as a physical nidus for countertraction during the spreading and movement of leukocytes to intercellular junctions that follows their stable arrest (Butcher, 1991; Springer, 1994). Second, intracellular signals, both chemical and mechanical, transmitted to the cytoskeleton, during E-selectin clustering, could influence the function of other endothelial adhesion molecules, such as ICAM-1, VCAM-1, and PECAM-1, that participate in subsequent steps in the adhesion cascade (Carpen et al., 1992; Muller et al., 1993; Luscinskas et al., 1994). Third, these processes may target the subpopulation of surface E-selectin receptors that participate in leukocyte interactions for subsequent internalization and degradation (Von Asmuth et al., 1992; Kuijpers et al., 1994). The ability of ΔCYTO-mutant E-selectin transfectants to support comparable numbers of leukocyte adhesion events as wild-type E-selectin transfectants, under nonstatic assay conditions, indicates that the cytoplasmic domain of E-selectin (and the molecular interactions it supports) is not critical for initial attachment. These E-selectin transfectants thus may provide a useful model for further study of the molecular mechanisms and physiologic consequences of the transmembrane signaling and cytoskeletal anchoring mediated via E-selectin during leukocyte–endothelial interactions.

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