

Protein Kinase C–dependent Effects of 12(S)-HETE on Endothelial Cell Vitronectin Receptor and Fibronectin Receptor

Dean G. Tang,* Yong Q. Chen,* Clement A. Diglio,† and Kenneth V. Honn*§||

Departments of *Radiation Oncology, †Pathology, and §Chemistry, Wayne State University, Detroit, Michigan 48202; and ||Gershenson Radiation Oncology Center, Harper Hospital, Detroit, Michigan 48202

Abstract. 12(S)-HETE, a lipoxygenase metabolite of arachidonic acid induced a nondestructive and reversible endothelial cell (EC) retraction. 12(S)-HETE induced EC retraction was inhibited by protein kinase C inhibitors calphostin C and staurosporine but not by the protein kinase A inhibitor H8. The role of EC integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ in 12(S)-HETE induced EC retraction was investigated. In confluent EC cultures, $\alpha v\beta 3$ is localized to focal adhesions at both the cell body and cell-cell borders and is colocalized with vinculin-containing focal adhesions. In contrast, $\alpha 5\beta 1$ is primarily enriched at the cell-cell borders, demonstrating codistribution with cell cortical microfilaments and extracellular fibronectin. Both receptors were functional in mediating cell-cell or cell-matrix interactions based on the observations that specific antibodies inhibited EC adhesion to intact subendothelial matrix and disrupted the monolayer integrity. 12(S)-HETE induced a multistep, temporally defined redistribution of the $\alpha v\beta 3$ -containing focal adhesions, leading to an eventual decrease in $\alpha v\beta 3$ plaques in the retracted ECs. This effect of 12(S)-HETE was inhibited by cal-

phostin C but not by H8. The alterations of $\alpha v\beta 3$ -containing focal adhesions preceded the development of EC retraction. 12(S)-HETE also enhanced EC $\alpha v\beta 3$ surface expression as revealed by immunofluorescence, flow cytometry, and digitized image analysis. 12(S)-HETE-induced $\alpha v\beta 3$ rearrangement (i.e., decreased focal adhesion localization and enhanced surface expression) did not result from altered mRNA transcription (as revealed by semi-quantitative RT-PCR analysis) or protein translation (as revealed by Western blotting). In contrast to its effect on $\alpha v\beta 3$, 12(S)-HETE did not demonstrate a temporally related, well-defined effect on the distribution pattern and the surface expression of $\alpha 5\beta 1$, although the cell-cell border staining pattern of $\alpha 5\beta 1$ was disrupted due to EC retraction. It is concluded that 12(S)-HETE-induced decrease of $\alpha v\beta 3$ localization to focal adhesions may contribute to the development of EC retraction and that 12(S)-HETE induced increase in $\alpha v\beta 3$ surface expression may promote adhesion of inflammatory leukocytes as well as tumor cells to endothelium.

ENDOTHELIAL cells (ECs)¹ express diverse cell surface receptors which mediate cell-cell and cell-matrix interactions. Among these receptors are Ca²⁺-dependent and -independent adhesion molecules involved in intercellular adhesion (Bavisotto et al., 1990; Albelda et al., 1990) and integrins mediating both cell-cell and cell-matrix interactions (Ruoslahti, 1991). All integrins are transmembrane heterodimers composed of α and β subunits and are connected, via the carboxyl-terminal end of either the α chain or the β chain (or both), to the cytoskeleton (primarily microfilament), thus possibly establishing an inductive communication across the membrane. Originally, these glycoprotein complexes were classified into three major subfamilies ($\beta 1$, $\beta 2$, and $\beta 3$) based on a common β subunit in association with

different α subunits (Hynes, 1987). However, other β subunits expressed on epithelial cells, fibroblasts, or some carcinoma cells have been described. To date, at least 20 integrins formed from various combinations of 12 α subunits and 9 β subunits have been reported (Ruoslahti, 1991; Hynes and Lander, 1992; Honn and Tang, 1992).

Knowledge of EC integrins stemmed from the observation that vascular ECs synthesize a membrane protein that is immunologically and biochemically related to the platelet glycoprotein $\alpha IIb/\beta 3$ complex (Fitzgerald et al., 1985). EC $\alpha IIb/\beta 3$ -like receptors were later identified to be the vitronectin receptor ($\alpha v\beta 3$), which shares the $\beta 3$ subunit with $\alpha IIb/\beta 3$ and mediates EC adhesion to fibrinogen, vitronectin, von Willebrand factor (Cheresh, 1987; Dejana et al., 1990; Albelda et al., 1989), thrombospondin (Lawler et al., 1988), laminin (Kramer et al., 1990), fibronectin (Cheng et al., 1991; Charo et al., 1990), and possibly collagen (Agrez et al., 1991). The reason why $\alpha v\beta 3$ is such a promiscuous

1. *Abbreviations used in this paper:* EC, endothelial cell; ECM, extracellular matrix; HETE, hydroxyeicosatetraenoic acid; PKA, protein kinase A; PKC, protein kinase C; RT-PCR, reverse transcription PCR.

and versatile integrin receptor for so many different ligands (in contrast to $\alpha 5\beta 1$, which specifically mediates cell adhesion to fibronectin) may be explained by the fact that the αv subunit, in addition to $\beta 3$, is able to associate with several other β subunits, such as $\beta 1$, $\beta 5$, $\beta 6$, $\beta 8$, and a βs subunit in an osteosarcoma cell line (for review see Honn and Tang, 1992; Hynes and Lander, 1992). Thus far members of two integrin subfamilies have been identified on ECs: the VLA subfamily ($\beta 1$) and the cytoadhesins ($\beta 3$). Of the six α subunits in the $\beta 1$ subfamily, $\alpha 2$, $\alpha 3$, and $\alpha 5$ have been found on both large vessel and microvascular ECs (Albelda et al., 1989; Cheng and Kramer, 1989; Kramer et al., 1990), while $\alpha 1$ and $\alpha 6$ subunits appear to be present only on microvessel ECs (Kramer et al., 1990; Giltay and van Mourik, 1988). Interestingly, both types of ECs appear not to express $\alpha 4$ (Albelda et al., 1989; Kramer et al., 1990). Although ECs apparently lack $\beta 2$ integrins which participate in cell-cell adhesions, EC integrins also appear to play an essential role in maintaining endothelial monolayer integrity (Lampugnani et al., 1991; and this study).

We previously reported that a lipoxygenase metabolite of arachidonic acid 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] induced an extensive retraction of large vessel ECs (Honn et al., 1989). Recently, we observed that 12(S)-HETE produced a similar effect on capillary ECs (Tang et al., 1992). The effect appeared to be mediated via activation of protein kinase C (PKC) and phosphorylation of EC cytoskeletal proteins, subsequently rearranging vinculin-containing adhesion plaques, actin microfilaments, and intermediate filament vimentin (Tang et al., 1992). Integrins play an essential role in linking the cytoskeleton to the extracellular matrix (ECM) and maintaining the integrity of EC monolayer (Lampugnani et al., 1991). Our work has demonstrated that 12(S)-HETE enhances the surface expression and modulates the function of integrin $\alpha IIb/\beta 3$ on tumor cells (Grossi et al., 1989; Chopra et al., 1991). Therefore, in the present study, we investigated the effect of 12(S)-HETE on EC integrins, i.e., $\alpha v\beta 3$ and $\alpha 5\beta 1$. The results demonstrate that 12(S)-HETE rearranges and disrupts $\alpha v\beta 3$ -enriched focal adhesions, but has little defined effect on $\alpha 5\beta 1$. In addition, 12(S)-HETE increases the surface expression of $\alpha v\beta 3$ in microvascular ECs. 12(S)-HETE-induced disruption of $\alpha v\beta 3$ -containing focal adhesions may contribute to EC retraction and 12(S)-HETE-enhanced $\alpha v\beta 3$ surface expression may promote tumor cell and leukocyte adhesion to vascular EC.

Materials and Methods

Cell Culture

Microvascular ECs (CD3) were isolated from mouse pulmonary microvessels, cloned and characterized as described previously (Chopra et al., 1990). Large vessel ECs (rat aortic EC or RAEC) were isolated from Sprague-Dawley rat aortic rings as described previously (Diglio et al., 1982). These ECs were routinely maintained in DME supplemented with 10% FCS (GIBCO BRL, Gaithersburg, MD). Cells were cultured in a humidified atmosphere with 5% CO₂, and the culture medium was changed every 48 h.

Chemicals and Antibodies

12(S)-HETE was purchased from Cayman Chemical (Ann Arbor, MI). Type IV collagen and laminin were isolated from Englebreath-Holm-Swarm murine sarcomas (Chemicon, Segundo, CA). Fibronectin was isolated from

human plasma by affinity chromatography (GIBCO BRL). Vitronectin was affinity purified from bovine serum using monospecific antibodies (GIBCO BRL). The protein kinase inhibitor, staurosporine (the IC₅₀ values for PKC = 0.0027 μ M, for PKA = 0.0082 μ M, for cGMP-dependent protein kinase = 0.031 μ M, and for myosin light chain kinase = 0.0019 μ M), was obtained from BIOMOL Res. Labs. Inc., (Plymouth Meeting, PA). PKC inhibitor, calphostin C (the IC₅₀ values for PKC = 0.05 μ M, for PKA > 50 μ M, for cGMP-dependent protein kinase > 25 μ M, and for myosin light chain kinase > 5 μ M), was isolated from *Cladosporium cladosporioides* (Kamiya Biochem Co., Thousand Oaks, CA). The protein kinase A (PKA) inhibitor, H8 (the IC₅₀ values for PKC = 15 μ M, for PKA = 1.2 μ M, for cGMP-dependent protein kinase = 0.48 μ M, and for myosin light chain kinase = 68 μ M), was purchased from Seikagaku America, Inc. (St. Petersburg, FL). Rhodamine-conjugated phalloidin was purchased from Molecular Probes Inc. (Eugene, OR). Fluorescein (FITC)- or Rhodamine-labeled goat anti-mouse, rabbit anti-goat and goat anti-rabbit IgG were obtained from ICN (Lisle, IL). Monoclonal anti-vinculin antibody was purchased from the Boehringer Mannheim Corp. (Indianapolis, IN). A mAb against human platelet glycoprotein $\alpha IIb/\beta 3$ (7E3) which cross reacts with the EC $\alpha v\beta 3$ but not $\alpha 5\beta 1$ (Fitzgerald and Phillips, 1989; Charo et al., 1987), was kindly provided by Dr. B. S. Coller (Stony Brook, NY). A mAb (OPG-2) recognizing the $\beta 3$ subunit was kindly provided by Dr. T. J. Kunicki (The Blood Center, WI). Polyclonal anti- $\alpha v\beta 3$ and anti- $\alpha 5\beta 1$ were raised in rabbit (Chemicon International, Temecula, CA). Goat antibody to $\alpha 5\beta 1$ and monoclonal anti-human αv (clone VNR 147) were obtained from Telios Pharmaceuticals Inc. (San Diego, CA). The specificity of these antibodies was characterized by immunoblotting and immunoprecipitation. Two polyclonal antibodies to $\alpha 5\beta 1$ stained identical bands on Western blots, i.e., a 110-kD band under nonreducing conditions and a 130-kD band under reducing conditions (data not shown), which represents the $\beta 1$ subunit of the $\alpha 5\beta 1$ receptor. Rabbit anti- $\alpha v\beta 3$ immunoprecipitated a 110- and a 130-kD band under reducing conditions and a 90- and a 150-kD band under nonreducing conditions (data not shown), which corresponded to the $\beta 3$ and αv subunits, respectively. Rabbit anti- $\alpha 5\beta 1$, under reducing conditions, immunoprecipitated a 130- and a 135-kD band, which corresponded to the $\beta 1$ and $\alpha 5$ subunits, respectively (data not shown). This antibody immunoprecipitated a 155-kD ($\alpha 5$) band and a 110-kD ($\beta 1$) band from surface-labeled CD3 cells under nonreducing conditions (data not shown). Monoclonal anti-human $\alpha 5$ was purified from mouse ascites fluid by nondenaturing liquid chromatography (Oncogene Sciences, Manhasset, NY). Nonimmune rabbit IgG (Copper Biochemical, Malvern, PA) and MOPC 21 tumor cells ascites (Sigma Immunochemicals, St. Louis, MO) were used as polyclonal and monoclonal antibody controls, respectively. Goat whole serum (Sigma Immunochemicals) was used to block nonspecific Fc receptors.

Cell Plating

Glass coverslips were cleaned by sonication in 70% ethanol for 30 min and dried. 1×10^5 of the ECs were plated onto treated coverslips (18 mm²) and cultured in DME supplemented with 10% FCS. Higher density of cells (1×10^6) was plated for those experiments using confluent cells and the cells were cultured for 3–4 d to reach full confluence. To determine whether the expression of VnR and FnR in ECs is dependent on the specific substratum, ECs were seeded onto glass coverslips coated with fibronectin (10 μ g/ml), laminin (20 μ g/ml), fibrinogen (10 μ g/ml), type IV collagen (20 μ g/ml), or vitronectin (10 μ g/ml). These substrate proteins were reconstituted to their respective final concentrations in PBS, and 100 μ l of the solution was laid on coverslips (18 mm²) for 2 h at 37°C. After washing, the coated coverslips were further incubated with 2% BSA in PBS for another half an hour to block unoccupied binding sites. Then 1×10^5 of ECs were seeded onto the coverslips and cultured in the absence of serum for 3 h before treatment (see below).

Preparation of Intact ECM

In some experiments (e.g., adhesion study), ECM sheet derived from confluent ECs was used as the adhesion substrate. Confluent ECs were removed by incubation with 25 mM ammonium hydroxide for 20 min at 25°C. The integrity and uniformity of the obtained ECM was characterized as previously described (Menter et al., 1987).

Eicosanoid Treatment

Cells were washed with DME and then treated with 0.1 μ M of 12(S)-HETE in DME for 5 min. The same amount of absolute ethanol (solvent) was used as the control. After the treatment, the cells were washed twice and then cul-

tured in FCS-containing DME for different time periods. For dose study, cells were treated with different concentrations of 12(S)-HETE or corresponding amounts of ethanol for 15 min at 37°C. In some experiments (e.g., flow cytometry studies), cells were continuously exposed to the eicosanoid in 4% BSA (in DME) for different periods of time.

Protein Kinase Inhibitor Studies

To examine the role of different protein kinases in the 12(S)-HETE effect, several general or specific protein kinase inhibitors were used in the EC retraction assay and immunofluorescence, i.e., staurosporine (which inhibits the catalytic domain of PKC and other protein kinases), calphostin C (which preferentially interacts with the regulatory domain of PKC; Tamaoki, 1991), and H8 (which has markedly higher affinities to cyclic nucleotide dependent protein kinases than other kinases; Hidaka et al., 1991). For the dose and time studies, staurosporine (at the doses of 0.005, 0.05, and 0.5 μ M), calphostin C (at the doses of 0.05, 0.5, and 5 μ M) or H8 (at the doses of 0.05, 0.5, 5 and 50 μ M) was added, together with 12(S)-HETE (0.1 μ M) or ethanol, to the culture medium. The EC monolayer was monitored by phase contrast microscopy at timed intervals, i.e., at 15, 30, 45, and 60 min after addition.

EC Retraction Assay

To quantitatively determine the role of different protein kinases in 12(S)-HETE-induced EC retraction, we performed the EC retraction assay in the presence of kinase inhibitors, using our established protocol (Honn et al., 1989). Briefly, confluent CD3 cells grown on collagen IV in 96-well plates were treated with 12(S)-HETE (0.1 μ M) or ethanol, or 12(S)-HETE plus different doses of inhibitors (see above) for 30 min. Then the cells were washed (three times in PBS), fixed, and incubated with 125 I-labeled mAb to collagen type IV (Collaborative Res.; 45 min, 27 μ g total protein; 1.2×10^5 cpm/ μ g protein/well). The contents of the wells were washed ($3 \times$ with PBS) and removed with 1 ml of 0.5% trypsin and 0.5% type IV collagenase and counted using a Minaxi (AUTO-GAMMA 5000 series) gamma counter (Packard Instrument Company, IL). The results were expressed as total cpm/well. Each condition was run in triplicate and the experiment was repeated three times with comparable results.

Flow Cytometric Analysis of EC Surface Expression of Integrin Receptors

ECs were nonenzymatically removed from the culture flasks. Briefly, cells were incubated for 15 min in 5 mM EDTA in PBS containing 0.5% BSA and 10 mM PMSF and then detached by gentle pipetting. Subsequently, cells were washed once with serum-free DME. Cell treatment was performed at 37°C for 15 min with different doses of 12(S)-HETE (0.01, 0.1, 0.5 and 1.0 μ M) or equivalent amounts of ethanol. After washing, cells were fixed with 0.1% sodium azide (in PBS containing 1% BSA) for 20 min on ice. At the end of fixation, cells were washed twice and blocked with normal goat serum (in PBS containing 2% BSA) for 20 min at 4°C. Thereafter, the cells were incubated with rabbit anti- α v β 3 or rabbit (or goat) anti- α 5 β 1 (1:40) for 45 min at 4°C. After washing, the cells were incubated with FITC-conjugated goat anti-rabbit or rabbit anti-goat IgG (1:200 in PBS containing 4% BSA) for 45 min at 4°C. Finally, cells were postfixed with 1% paraformaldehyde for 15 min. Samples were measured with an Epics Profile II flow cytometer (Coulter, Hialeah, FL) for fluorescence intensity. The data were analyzed by comparing the relative fluorescence (Grossi et al., 1989) or mean log fluorescence.

Immunofluorescence

After treatment with 12(S)-HETE or 12(S)-HETE plus kinase inhibitors, ECs grown on various substrates were fixed with 3.7% paraformaldehyde in PBS (containing 1 mM of Ca^{2+} and Mg^{2+} , and 5% sucrose, pH 7.4) for 15 min. After washing with PBS, the residual aldehyde groups were neutralized with 1% NH_4Cl in PBS. For surface integrin labeling, cells were incubated with 20% normal goat whole serum in 4% BSA-PBS at 37°C for 30 min to block the nonspecific binding. For intracellular labeling, cells were first permeabilized with the extraction buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM CaCl_2 and 0.5% Triton X-100) for 3 min, then blocked with the goat whole serum. After extensive rinsing, cells were reacted with various primary antibodies, i.e., monoclonal anti- α v and anti- α 5, 7E3 (at the final concentrations of 10 μ g/ml), or polyclonal anti- α 5 β 1 and anti- α v β 3 (at the final concentrations of 30 μ g/ml). Then cells were incubated with FITC-conjugated goat anti-mouse, goat anti-

rabbit or rabbit anti-goat IgG (1:200) for 45 min at 37°C. To double-label microfilaments and α v β 3 or α 5 β 1, a secondary antibody mixture of FITC-conjugated rabbit anti-goat IgG and Rhodamine-conjugated phalloidin was used. Double labeling of vinculin and α v β 3 or vinculin and α 5 β 1 was performed using a mixture of primary antibodies (i.e., 10 μ g/ml of monoclonal anti-vinculin and rabbit anti- α v β 3 or - α 5 β 1) and a mixture of secondary antibodies (i.e., goat anti-mouse IgG-FITC and goat anti-rabbit IgG-rhodamine). Samples were mounted on clear slides with glycerol-PBS (9:1) containing 0.1% p-phenylenediamine to retard fluorescence fading. Fluorescence microscopy was performed using a Nikon Orthoplan microscope. Micrographs were recorded on Kodak T-Max 400 panchromatic films (Eastman Kodak Co., Rochester, NY).

Quantitation of α v β 3 and α 5 β 1 Surface Expression by Fluorescence Imaging (ACAS Analysis)

To further evaluate the effect of 12(S)-HETE on the surface expression of α v β 3 and α 5 β 1 integrins on ECs and to circumvent the potential influence of cell dissociation using EDTA on the integrin surface labeling, adherent CD3 cells on coverslips stained as described for immunofluorescence were analyzed for fluorescence intensity using the ACAS 470 argon laser fluorescence imaging cytometer (Meridian Instruments, Okemos, MI). This automated digital imaging system determines the subcellular distribution and the in situ expression of interested molecules in single cells or in a population of cells. CD3 cells (either 70% confluent or confluent) grown in 33-mm² petri dishes were treated with 12(S)-HETE and then surface labeled as described for immunofluorescence using either rabbit anti- α v β 3 or rabbit anti- α 5 β 1. The secondary antibody used was goat anti-rabbit IgG conjugated to FITC. Samples were loaded onto the stage of the laser image analyzer and the surface expression of these two integrin receptors determined. The system uses laser irradiation to effect fluorescence excitation. FITC label was excited with the argon laser beam at 488 nm, and the fluorescence emission measured above 530 nm using a 530/30-nm longpass filter. The intensity of each single point emission was recorded and displayed as a color-coded image. Fluorescence intensity is given in arbitrary units per pixel. At least five areas randomly chosen in each sample were analyzed and each condition was run in triplicate. The experiments were repeated with comparable results.

Adhesion Assay

In vitro antibody inhibition studies were performed to determine the relative functions of α v β 3 and α 5 β 1 in mediating EC adhesion to and spreading on intact ECM. CD3 cells were detached with trypsin (0.01%) and EDTA (1 mM) and allowed to recover in serum-containing medium for 30 min at 37°C. After washing, 1×10^5 of suspended cells were put into one of the 24-well plates (Falcon) and the adhesion was conducted in the presence of various dilutions of rabbit anti- α v β 3 and goat anti- α 5 β 1, monoclonal anti- α IIb/ β 3 (7E3), or equivalent amounts of nonimmune rabbit IgG (in DME without serum) for 30 min at 37°C. The assay was stopped by aspirating off nonadherent cells, washed and then fixed with 3.7% paraformaldehyde. Each condition was run in quadruplicate and the experiment was repeated at least three times with comparable results. The number of adherent cells was determined as described previously (Honn et al., 1989).

Antibody Perturbation Study

Specific antibodies to α v β 3 and α 5 β 1 or to the separate subunits (i.e., α v and α 5) were used to determine the roles of these two integrin receptors in maintaining the integrity of EC monolayer. Confluent CD3 cells grown on uncoated coverslips were washed twice with DME containing 2% BSA. Rabbit anti- α v β 3, rabbit anti- α 5 β 1 or nonimmune rabbit IgG (final concentration 30 μ g/ml), or monoclonal anti- α v, monoclonal anti- α 5, monoclonal 7E3 or MOPC ascites (final concentration 10 μ g/ml) were diluted with 1% FCS-containing DME. 200 μ l of the above solution was added onto each coverslip. Then ECs with the various antibodies or control IgG fractions were cultured for different intervals at 25°C, and the integrity of the EC monolayer was monitored by both phase-contrast microscopy and F-actin staining using phalloidin. Cells were fixed at several time points, i.e., 15 min, 30 min, 1, 2, and 24 h after antibody addition.

Immunoblotting

Unlabeled CD3 cells were scraped off the culture flasks into the TNC lysis buffer (0.01 M Tris-acetate, pH 8.0, 0.5% NP-40, and 5 mM Ca^{2+}) containing a cocktail of protease inhibitors (10 mM PMSF, 1 mM leupeptin, 1%

aprotinin, 1 $\mu\text{g/ml}$ of pepstatin and chymostatin) and extracted on ice for 20 min. The whole cell lysates were centrifuged at 14,000 g to obtain the supernatants. Proteins were separated on 7.5% SDS-PAGE under both reducing (5% 2-mercaptoethanol) and nonreducing conditions. Then proteins were transferred to nitrocellulose membrane and the target proteins were detected using the ECL (Enhanced Chemiluminescence) Western blotting detection kit (Amersham, Arlington Heights, IL) as described previously (Tang et al., 1993). To quantitatively determine whether 12(S)-HETE alters the protein level of $\alpha\text{v}\beta\text{3}$ integrin, confluent CD3 cell monolayers grown in six-well culture plates were treated with 0.1 μM 12(S)-HETE or ethanol for 5 min and then transferred to FCS-containing DME and cultured for different time intervals (i.e., 5, 15, 30, 60, and 120 min). Cells were harvested with the TNC lysis buffer and the supernatants obtained as described above. An equal amount of protein (as determined by Bradford) was loaded onto a denaturing 7.5% SDS-PAGE under reducing conditions and proteins transferred to nitrocellulose paper as described above. The amount of $\alpha\text{v}\beta\text{3}$ integrin was probed by a monoclonal antibody (OPG-2) recognizing the β3 subunit and the intensity of the band determined by scanning using an XL Ultrascan densitometer.

Semi-quantitative RT-PCR

To determine whether 12(S)-HETE has any effect on the $\alpha\text{v}\beta\text{3}$ and $\alpha\text{5}\beta\text{1}$ expression at the transcriptional level, the integrin mRNAs were amplified by RT-PCR (Chen et al., 1992) and quantitated by Southern blotting using αv , α5 , β1 , or β3 cDNA as respective probes. Total RNA was extracted from confluent CD3 cells (treated with ethanol or 0.1 μM of 12(S)-HETE for 1, 4, or 24 h) with guanidinium thiocyanate-CsCl method (Chang et al., 1992). One μg of total RNA was reverse transcribed in 20 μl solution containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 10 mM DTT, 0.5 mM dNTPs, 20 U of RNasin (Promega), 1 μM of anti-sense primer, 200 units of M-MLV reverse transcriptase (GIBCO BRL) at room temperature for 15 min then at 37°C for 1 h. To the 20 μl of reverse transcriptase reaction, 1 μM of the sense primer, 80 μl of 1 \times PCR buffer (20 mM Tris pH 8.3, 50 mM KCl, 2.5 mM MgCl_2 , 0.1 mg/ml BSA) and 1 U AmpliTaq DNA polymerase (Perkin Elmer Corp., Norwalk, CT) were added and PCR was run in the GeneAmp PCR system 9600 (Perkin Elmer Corp.) 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C for 30 cycles. The sense primers used in RT-PCR were 5'-CATTTCGAGTCTGGGCCAA-3' (α5), 5'-TGTTCAGTGCAGAGCCTTCA-3' (β1), 5'-GGGGACTGCCTGTGTGACTC-3' (β3). The anti-sense primers were 5'-TGGAGGCTTGAGCTGAGCTT-3' (α5), 5'-CCTCATACTTCGGATTGACC-3' (β1) and 5'-CTTTTCGGTCGTGGATGGTG-3' (β3). For the αv subunit, two pairs of primers were used: 5'-CGGGACCATCTCATCACTAAG-3' (sense) and 5'-TCTTGTCTTCTTGAGGTGGC-3' (anti-sense), and 5'-GTTGGGAGATTAGCAGAGGA-3' (nested sense) and 5'-CAAAACAGCCAGTAGCAACAA-3' (nested anti-sense). 20 μl of PCR-amplified cDNA was migrated on a 2% agarose gel and transferred to Genescreen Plus membrane (NEN, Du pont) using a Posi-Blot Pressure Blotter (Stratagene, La Jolla, CA). DNA was UV-linked to the membrane, hybridized with ^{32}P -labeled cDNA probes and the intensity of the bands determined by scanning with a LKB Ultrascan XL laser densitometer.

Results

12(S)-HETE Induces EC Retraction which Is Inhibited by Calphostin C But Not by H8

Normal CD3 ECs form tightly apposed, "cobblestone"-like monolayer in culture (Fig. 1 *a*). Treatment with 0.1 μM of 12(S)-HETE induced a retracted cell phenotype and formation of cell-cell gaps (Fig. 1 *b*). Calphostin C (Fig. 1 *c*), a selective PKC inhibitor (Tamaoki, 1991), at a dose of 0.05 μM (data not shown) or 0.5 μM , applied together with 12(S)-HETE, significantly inhibited 12(S)-HETE-induced CD3 cell retraction. The effect was more prominent at a higher dose (5 μM) of calphostin C (Fig. 1 *d*). By comparison, H8, a PKA inhibitor (Hidaka et al., 1991), at a dose of 5 μM (Fig. 1 *e*) or even higher (up to 50 μM , data not shown) did not demonstrate any effect. Staurosporine at all doses examined (i.e., 0.005, 0.05, 0.5, and 5 μM) displayed less of an inhibi-

tory effect than calphostin C on 12(S)-HETE-induced EC retraction (Fig. 1 *f*). These inhibitors alone, at the doses tested, did not significantly alter the morphology of EC monolayer, although staurosporine at high doses (e.g., 5 μM) appeared to induce a retractive phenotype of EC by itself (data not shown). Quantitative measurement of EC retraction using labeled anti-collagen IV binding assay (14) was performed to evaluate the effects of these protein kinase inhibitors and the results confirmed the morphological observations (data not shown).

Expression and Functional Characterization of EC $\alpha\text{v}\beta\text{3}$ and $\alpha\text{5}\beta\text{1}$

The subcellular distribution of $\alpha\text{v}\beta\text{3}$ and $\alpha\text{5}\beta\text{1}$ integrins in microvascular CD3 ECs was studied after plating cells on coverslips for various time periods. These two integrin receptors demonstrated different localization patterns (Fig. 2). Subconfluent CD3 cells (4 h after plating) expressed $\alpha\text{v}\beta\text{3}$ prominently at the focal adhesions, colocalizing with actin microfilaments (Fig. 2, *a* and *b*, arrowheads) and vinculin (data not shown; also see Fig. 7). When ECs reached confluency, the $\alpha\text{v}\beta\text{3}$ -containing focal adhesions were distributed at both the cell body and cell-cell borders and continued to colocalize with the stress fibers (Fig. 2, *c* and *d*). In contrast, $\alpha\text{5}\beta\text{1}$ in microvascular ECs appeared to follow a different distribution pattern. In subconfluent CD3 cells (4 h after plating), $\alpha\text{5}\beta\text{1}$ did not demonstrate typical focal adhesion labeling, but a line- or strand-like staining pattern reminiscent of extracellular fibronectin staining (Fig. 2 *f*). In addition, $\alpha\text{5}\beta\text{1}$ demonstrated only occasional and very weak colocalization with stress fiber endings (Fig. 2, *e* and *f*, arrowhead) and vinculin-containing focal adhesions (data not shown; also see Fig. 7). In confluent, and especially in postconfluent CD3 monolayers, $\alpha\text{5}\beta\text{1}$ was enriched primarily and almost exclusively to the cell-cell borders (with very little cell body focal adhesion labeling) and codistributed with the cortical actin filaments (Fig. 2, *h* and *g*, arrowheads), demonstrating a colocalization with extracellular fibronectin (data not shown) but not significantly with vinculin-containing focal adhesions (data not shown; also see Fig. 7). Staining of the large vessel ECs (RAEC) with the same antibodies revealed an identical distribution pattern (data not shown; also see Tang et al., 1993). When CD3 microvessel ECs were plated on individual specific adhesive ligands, the expression of these two integrins in focal adhesions demonstrated a clear substrate dependency. CD3 cells expressed $\alpha\text{v}\beta\text{3}$ to focal adhesions when cells were plated on vitronectin or fibrinogen, but not on fibronectin, collagen IV or laminin (data not shown). Similarly, $\alpha\text{5}\beta\text{1}$ was enriched to focal adhesions only when the cells were plated on fibronectin, but not on other substrates such as laminin, collagen IV, fibrinogen or vitronectin (data not shown).

CD3 cell adhesion to intact ECM was performed in the presence of various concentrations of rabbit anti- $\alpha\text{v}\beta\text{3}$, 7E3 (a monoclonal against the $\alpha\text{IIb}/\beta\text{3}$ complex that cross reacts with $\alpha\text{v}\beta\text{3}$ but not with $\alpha\text{5}\beta\text{1}$; Fitzgerald and Phillips, 1989; Charo et al., 1987; Grossi et al., 1989) and goat anti- $\alpha\text{5}\beta\text{1}$ (Fig. 3). The results indicated that all three antibodies inhibited EC adhesion to ECM in a dose-dependent manner (Fig. 3 *A*). Experiments with rabbit anti- $\alpha\text{5}\beta\text{1}$ also demonstrated an inhibitory effect on CD3 adhesion to ECM (data not shown). When these antibodies or monoclonal anti- αv or

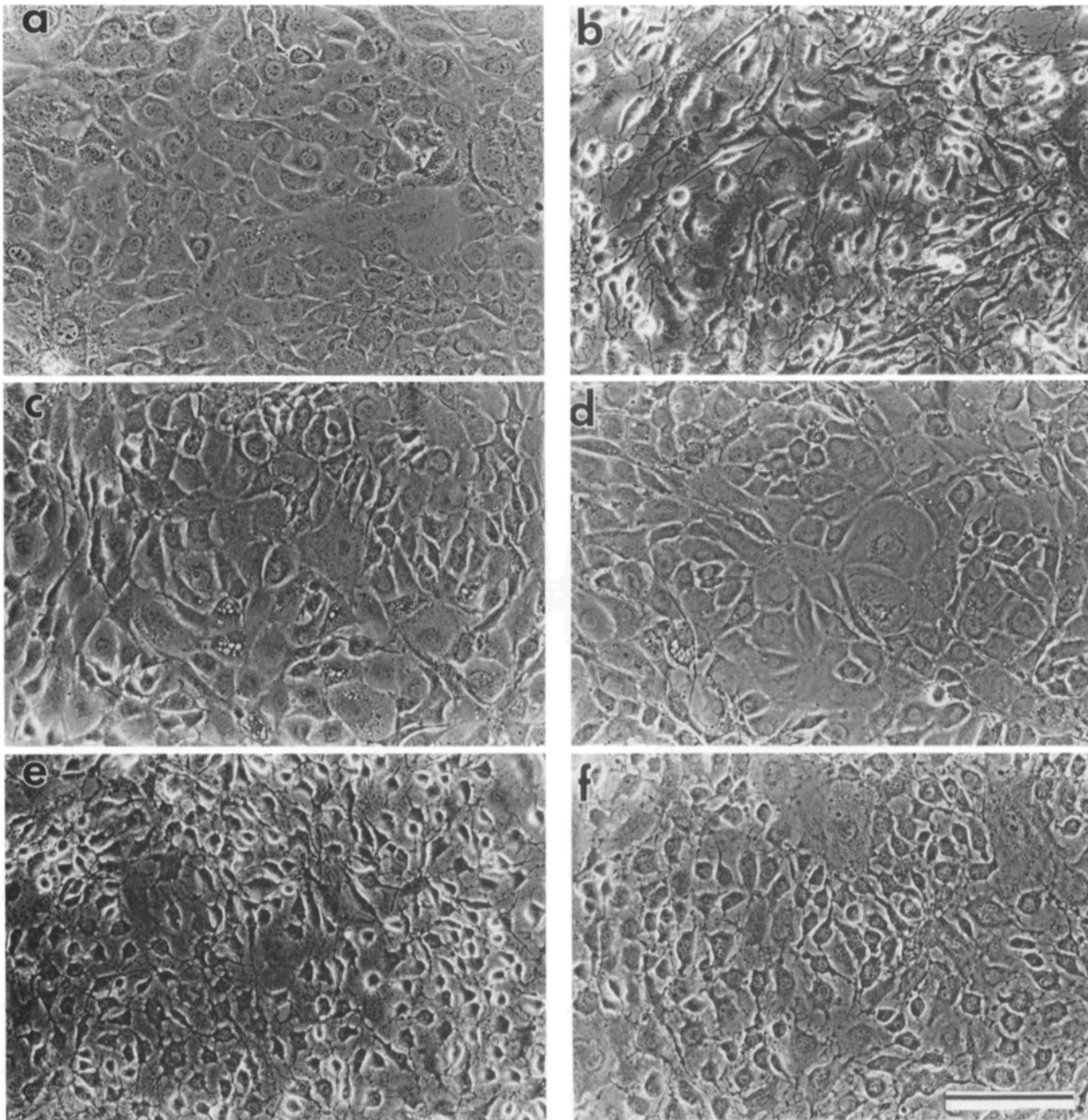


Figure 1. 12(S)-HETE-induced EC retraction is inhibited by PKC inhibitor calphostin C but not by protein kinase A inhibitor H8. Shown are micrographs of confluent CD3 cells 45 min post treatment by ethanol (a), 0.1 μ M 12(S)-HETE alone (b), 12(S)-HETE (0.1 μ M) plus 0.5 μ M (c) or 5 μ M (d) of calphostin C, 12(S)-HETE (0.1 μ M) plus 5 μ M H8 (e), or 12(S)-HETE (0.1 μ M) plus 0.5 μ M staurosporine (f). Bar, 3.5 μ m.

anti- $\alpha 5$ were added to confluent CD3 monolayers, the EC sheet was disrupted as revealed by both phase-contrast microscopy (data not shown) and actin staining (Fig. 3 B). The antibody effect was time dependent. 15 min after adding polyclonal anti- $\alpha v \beta 3$ or monoclonal anti- αv , but not other antibodies (i.e., antibodies to $\alpha 5 \beta 1$ or $\alpha 5$), retracted EC morphology, formation of cell-cell gaps, and decreased staining of stress fibers were observed (data not shown). By 60 min, as shown in Fig. 3 B, these alterations became more prominent with anti- $\alpha v \beta 3$ and anti- αv (Fig. 3 B, b and c). 7E3 demonstrated a disrupting effect comparable to that of

the anti- αv (Fig. 3 B, d). By comparison, both rabbit anti- $\alpha 5 \beta 1$ (Fig. 3 B, e) or goat anti- $\alpha 5 \beta 2$ (data not shown) and monoclonal anti- $\alpha 5$ (Fig. 3 B, f) produced only a slight disruption of the EC monolayer. Rabbit IgG treatment did not result in any alteration of the confluent CD3 monolayer (Fig. 3 B, a).

12(S)-HETE Redistributes EC $\alpha v \beta 3$ -containing Focal Adhesions

Both confluent (Fig. 4 a) and subconfluent (Fig. 4 b) CD3

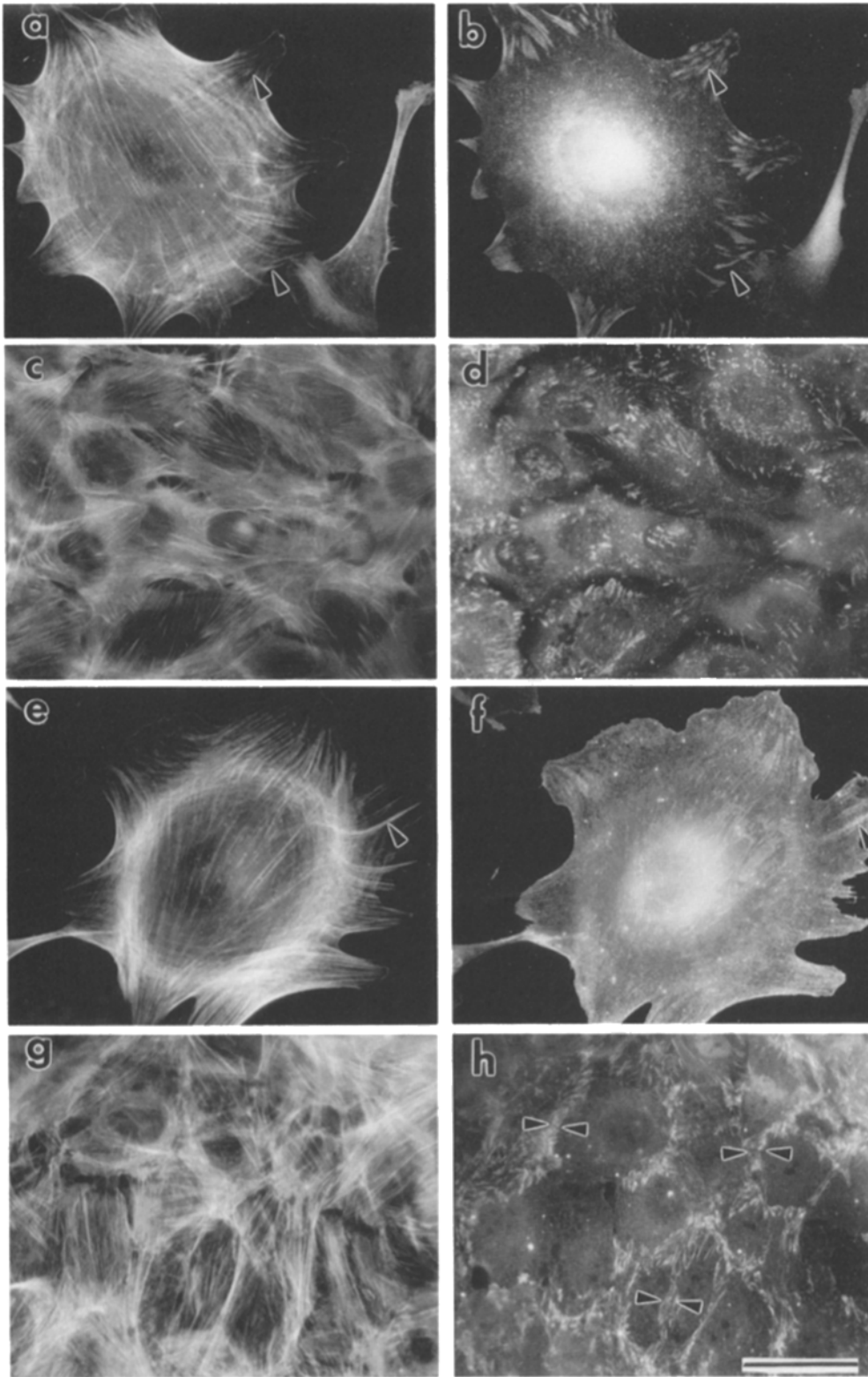


Figure 2. Detection of $\alpha v\beta 3$ and $\alpha 5\beta 1$ in subconfluent (*a*, *b*, *e*, and *f*) and confluent (*c*, *d*, *g*, and *h*) microvascular ECs. Shown are micrographs of cells double labeled for microfilaments with rhodamine-phalloidin (*a*, *c*, *e*, and *g*) and $\alpha v\beta 3$ or $\alpha 5\beta 1$ with rabbit anti-human $\alpha v\beta 3$ (*b* and *d*) or goat anti-human $\alpha 5\beta 1$ (*f* and *h*) followed by fluorescein-conjugated secondary antibody. Bar, 8 μm .

cells expressed $\alpha v\beta 3$ in focal adhesions at the cell body as well as along the cell-cell borders (cell edges) when stained with polyclonal anti- $\alpha v\beta 3$. This distribution pattern is very similar to that of vinculin (see Fig. 7). 12(S)-HETE induced a similar effect on $\alpha v\beta 3$ distribution to its effect on vinculin (unpublished observations). 12(S)-HETE induced a series of

time-dependent alterations in the distribution pattern of $\alpha v\beta 3$ -enriched focal adhesions. 5 min after stimulation, no detectable retraction was observed by light microscopy. However, the typical focal adhesion labeling of $\alpha v\beta 3$ was disrupted in groups of cells in focal areas. In these cells, $\alpha v\beta 3$ -containing focal adhesions appeared to be "cleaved,"

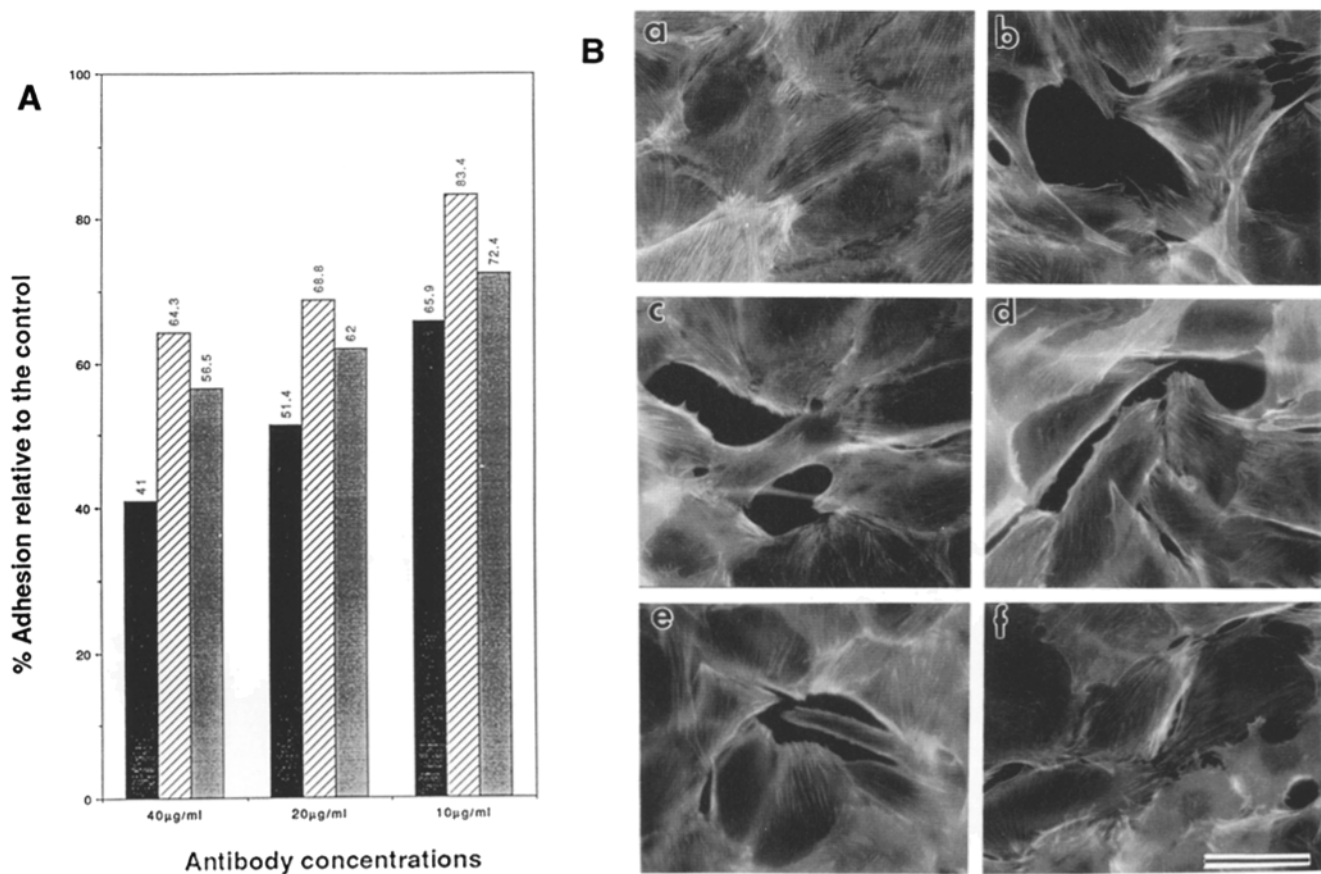


Figure 3. Functional characterization of EC $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$. **A**, adhesion to ECM was performed with resuspended CD3 cells in the presence of rabbit anti- $\alpha\text{v}\beta 3$ (30 $\mu\text{g/ml}$) (■), goat, or rabbit (data not shown), anti- $\alpha 5\beta 1$ (30 $\mu\text{g/ml}$) (▨), monoclonal anti- $\alpha\text{IIb}/\beta 3$ 7E3 (10 $\mu\text{g/ml}$) (▩), or nonimmune rabbit IgG (30 $\mu\text{g/ml}$) for 30 min. Each condition was run in quadruplicate, the number of adhered cells was determined as described (Honn et al., 1989), and the results were expressed as the percentage of the adhesion relative to the control (rabbit IgG treatment). The SD was always < 5% (data not shown). **B**, antibody perturbation study. Confluent CD3 monolayer was incubated with nonimmune rabbit IgG (30 $\mu\text{g/ml}$, a), rabbit anti- $\alpha\text{v}\beta 3$ (30 $\mu\text{g/ml}$, b), monoclonal anti- αv (10 $\mu\text{g/ml}$, c), 7E3 (10 $\mu\text{g/ml}$, d), rabbit anti- $\alpha 5\beta 1$ (30 $\mu\text{g/ml}$, e), goat anti- $\alpha 5\beta 1$ (30 $\mu\text{g/ml}$; data not shown), monoclonal anti- $\alpha 5$ (10 $\mu\text{g/ml}$, f), or MOPC ascites (10 $\mu\text{g/ml}$, not shown) for different time intervals. The integrity of the EC sheet was monitored by actin staining. Shown is 60 min of incubation. Bar, 8 μm .

leading to formation of numerous small $\alpha\text{v}\beta 3$ -enriched focal adhesions (data not shown). As retraction developed in the CD3 monolayer, 15 min after 12(S)-HETE (0.1 μM) treatment, the $\alpha\text{v}\beta 3$ -containing focal adhesions were decreased at both the cell body and periphery (Fig. 4 c, *single arrowheads*; Fig. 4, d, *double arrowheads*), especially for those cells in the retracted area. In some cells in or near the retracted area, large patches of “merged” $\alpha\text{v}\beta 3$ -containing focal adhesions could be observed (Fig. 4 c, *double arrowheads*). Temporally, these patches of $\alpha\text{v}\beta 3$ were observed subsequent to the redistributed, “cleaved” $\alpha\text{v}\beta 3$ -containing focal adhesions, suggesting that the former (i.e., patches) might represent a further rearrangement of $\alpha\text{v}\beta 3$ -enriched focal adhesions. By 30 min, large areas of exposed matrix could be easily discerned in retracted areas (data not shown), and CD3 cells demonstrated a greater decrease in their cell-body $\alpha\text{v}\beta 3$ plaques (Fig. 4 f, *double arrowheads*) and only a few residual “aggregated” $\alpha\text{v}\beta 3$ -containing focal adhesions could be observed at the cell edge (Fig. 4 f, *single arrowheads*). 1 h after treatment, a further decrease in the staining

of $\alpha\text{v}\beta 3$ -enriched focal adhesions was observed both in the cell body (Fig. 4, e and h, *arrowheads*) and the cell margin. 4 h after 12(S)-HETE stimulation, cells still demonstrated decreased overall labeling of $\alpha\text{v}\beta 3$, especially at the cell edges (data not shown). By 24 h, cells recovered their normal “cobblestone” morphology and the complete monolayer was reformed. There was a corresponding increase in $\alpha\text{v}\beta 3$ -containing focal adhesions, both at the cell body and the cell periphery (Fig. 4 g).

12(S)-HETE-induced EC $\alpha\text{v}\beta 3$ Rearrangement Is Inhibited by PKC Inhibitor Calphostin C

As in the retraction study (Fig. 1), 12(S)-HETE treatment (30 min) induced EC retraction and decreased $\alpha\text{v}\beta 3$ staining (Fig. 5 b). Simultaneous treatment of CD3 cells with 0.1 μM of 12(S)-HETE and 0.5 μM of calphostin C totally inhibited both EC retraction and the decrease in $\alpha\text{v}\beta 3$ staining (Fig. 5 c). However, 5 μM or higher doses (up to 50 μM) of H8, a protein kinase A inhibitor, did not demonstrate any effect

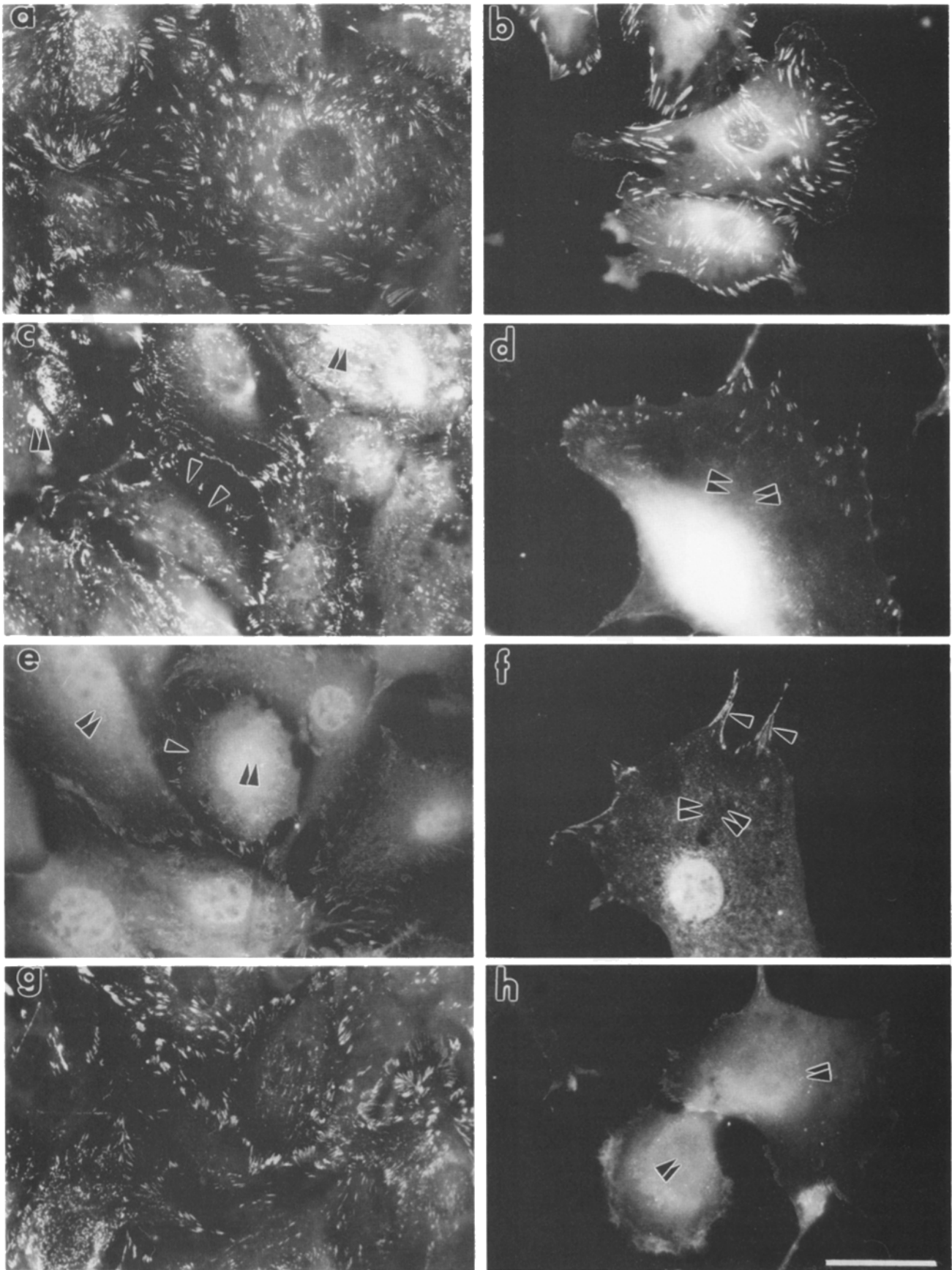


Figure 4. 12(S)-HETE effect on $\alpha 5\beta 3$ of confluent (a, c, e, and g) and subconfluent (b, d, f, and h) CD3 cells. CD3 cells were treated with 0.1 μ M 12(S)-HETE or equivalent amount of ethanol for 5 min and cultured in serum-containing DME for different time points. $\alpha 5\beta 3$ was stained using rabbit anti- $\alpha 5\beta 3$ and FITC-conjugated goat anti-rabbit IgG after fixing and permeabilizing cells. Shown are micrographs of CD3 cells treated by ethanol (1 h after the treatment, a and b) or 12(S)-HETE 15 min (c and d), 30 min (f), 60 min (e and h), or 24 h (g) after treatment. Bar, 10 μ m.

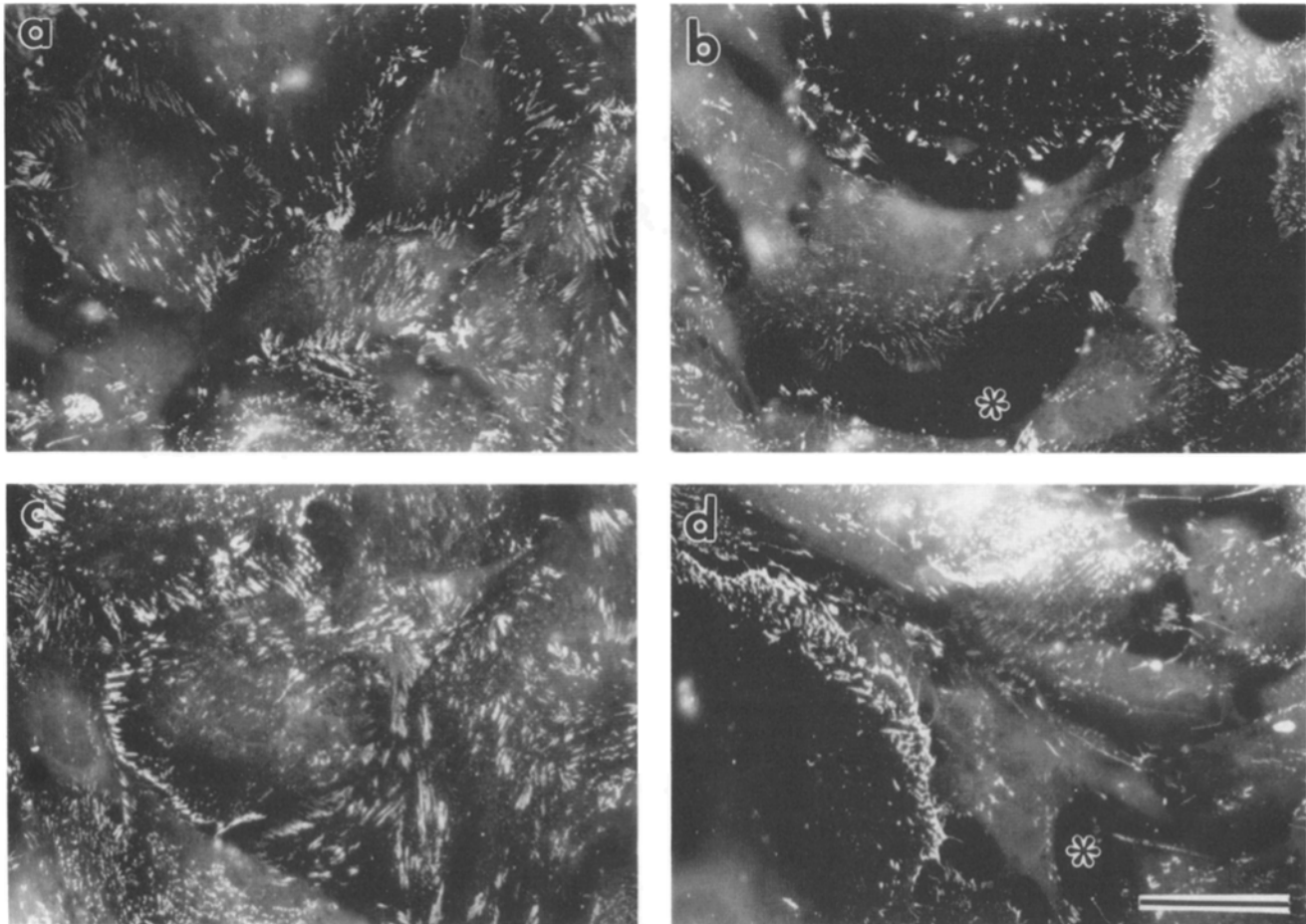


Figure 5. 12(S)-HETE-induced rearrangement of EC $\alpha v\beta 3$ is inhibited by PKC inhibitor calphostin C. Confluent CD3 monolayer was exposed to ethanol (a), 0.1 μM 12(S)-HETE (b), 12(S)-HETE plus 0.5 μM calphostin C (c), 12(S)-HETE plus 5 μM H8 (d), or 12(S)-HETE plus 0.5 μM staurosporine (not shown) for 30 min. Then cells were processed for $\alpha v\beta 3$ staining using rabbit anti- $\alpha v\beta 3$. Note that calphostin C strongly inhibits 12(S)-HETE-induced CD3 cell retraction and $\alpha v\beta 3$ rearrangement (c). H8, at even 50 μM (not shown), is ineffective. Bar, 10 μm .

on 12(S)-HETE-induced EC retraction and $\alpha v\beta 3$ rearrangement (Fig. 5 d). Again, staurosporin demonstrated a less potent effect than calphostin C (data not shown).

12(S)-HETE Does Not Induce a Defined Rearrangement of $\alpha 5\beta 1$ Integrin

As described above, CD3 cell $\alpha 5\beta 1$ demonstrated a different distribution pattern from that of the $\alpha v\beta 3$ integrin. In confluent (Fig. 6 a) and especially in postconfluent (data not shown) CD3 monolayer, $\alpha 5\beta 1$ was almost exclusively enriched to the cell-cell borders (Fig. 6 a, *arrowheads*), colocalizing with cell cortical microfilaments and extracellular fibronectin but not significantly with typical vinculin-containing focal adhesions which are characteristically localized to the cell-matrix contact sites. Interestingly, in contrast to its effect on the redistribution of EC $\alpha v\beta 3$, 12(S)-HETE did not demonstrate any specific effect on the $\alpha 5\beta 1$ receptor in both confluent (Fig. 6, c and e) and subconfluent (Fig. 6, d and f) CD3 cells. No alteration in the distribution pattern of $\alpha 5\beta 1$ was observed preceding EC retraction following 12(S)-HETE stimulation (i.e., 5 min post treatment) (data not shown), in contrast to the rearrangement of $\alpha v\beta 3$ -

containing focal adhesions (see above) at this time point. EC retraction was observed at 15 min (Fig. 6 c, *asterisks*) and became intensified by 60 min (Fig. 6 e, *asterisks*) after treatment. Simultaneously the typical cell-cell border staining pattern of $\alpha 5\beta 1$ was disrupted, however, no clear pattern of alteration was observed. Similarly, no definitive rearrangement or quantitative alteration of $\alpha 5\beta 1$ integrin was observed in subconfluent CD3 cells (Fig. 6, compare b with d and f). By 24 h, EC monolayer reformed and typical cell-cell border staining of $\alpha 5\beta 1$ was again observed (data not shown). Staining with goat anti- $\alpha 5\beta 1$ revealed identical results (data not shown).

Differential Effects of 12(S)-HETE on $\alpha v\beta 3$ and $\alpha 5\beta 1$ May Result from Different Distribution Patterns of These Two Integrins

The mechanism by which 12(S)-HETE exerts a differential effect on these two integrins is at present unclear. One possible explanation may be different subcellular localizations of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins in microvascular ECs. Double immunofluorescent labeling of integrins and vinculin, a protein marker for focal adhesions, was performed to examine this

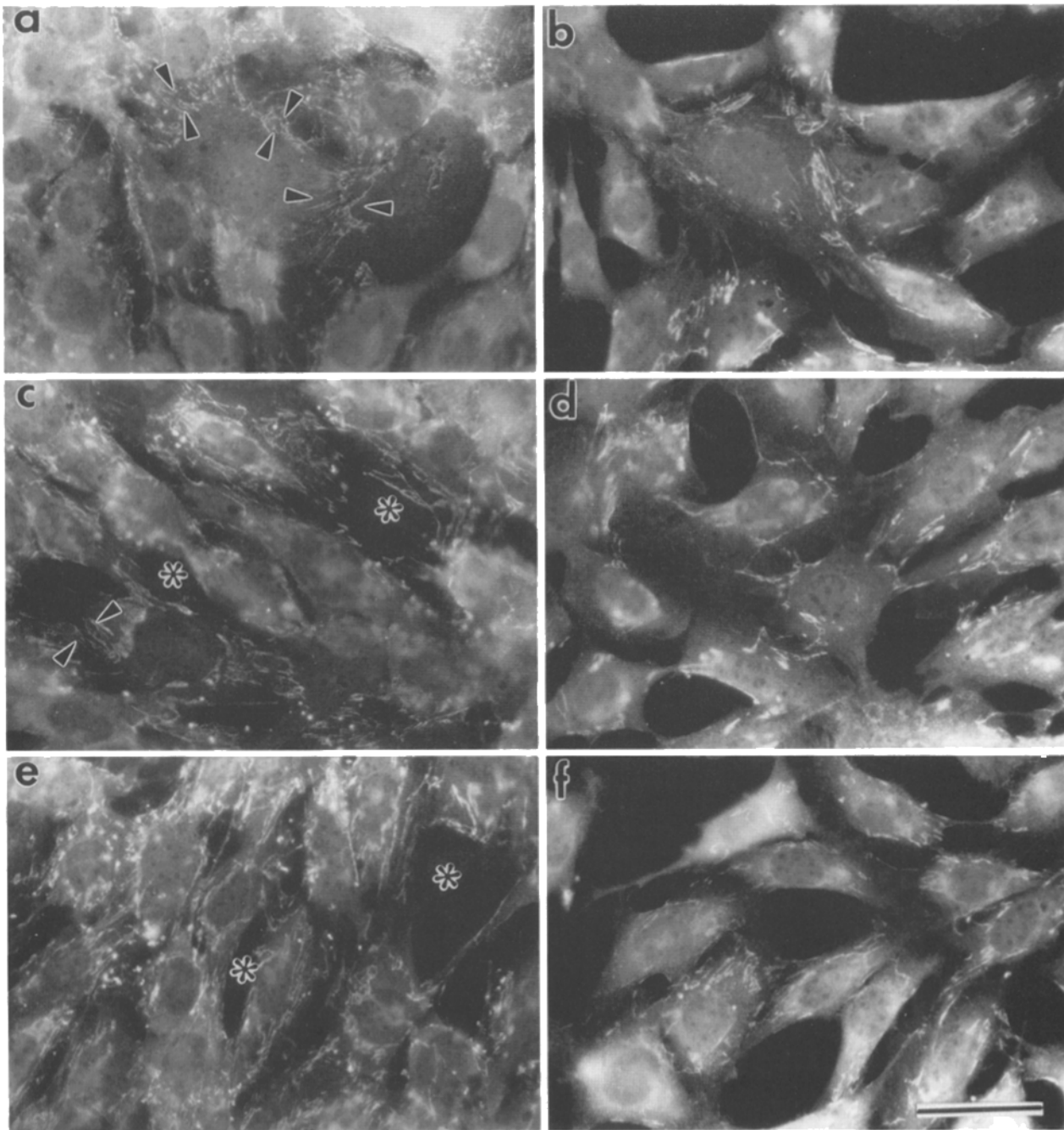


Figure 6. Effect of 12(S)-HETE on $\alpha 5\beta 1$ of confluent (a, c, and e) and subconfluent (b, d, and f) CD3 ECs. CD3 cells grown on coverslips were treated as described in Fig. 5 and stained for $\alpha 5\beta 1$ with rabbit anti- $\alpha 5\beta 1$ and FITC-conjugated goat anti-rabbit IgG. The time points represent 1 h after ethanol treatment (a and b), or 15 min (c and d) or 60 min (e and f) after exposure to 0.1 μ M 12(S)-HETE. Asterisks indicate the retracted areas. Bar, 8 μ m.

possibility. As shown in Fig. 7, $\alpha v\beta 3$ -containing focal adhesions (Fig. 7 a) demonstrated an identical codistribution with vinculin-containing focal adhesions (Fig. 7 b, *double arrowheads*). Colocalization of $\alpha v\beta 3$ and vinculin in focal adhesions also was observed in fully confluent CD3 monolayers (data not shown). In contrast, $\alpha 5\beta 1$ integrin (Fig. 7 c) was present in strand-like structures which were reminiscent of extracellular fibronectin and they did not demonstrate a significant colocalization with vinculin-containing focal adhesions (Fig. 7 d, *arrowheads*). In confluent ECs, $\alpha 5\beta 1$ was primarily localized to the cell-cell borders and did not

demonstrate colocalization with vinculin-containing focal adhesions (data not shown). Previous work demonstrated that 12(S)-HETE also disrupted vinculin-containing focal adhesions in retracted CD3 ECs (Tang et al., submitted). Therefore, it appears that 12(S)-HETE specifically disrupts the integrity of focal adhesions.

12(S)-HETE Enhances the Surface Expression of $\alpha v\beta 3$ But Not $\alpha 5\beta 1$

We recently observed that a 15-min treatment of large vessel EC (i.e., RAEC) with 0.1 μ M of 12(S)-HETE significantly

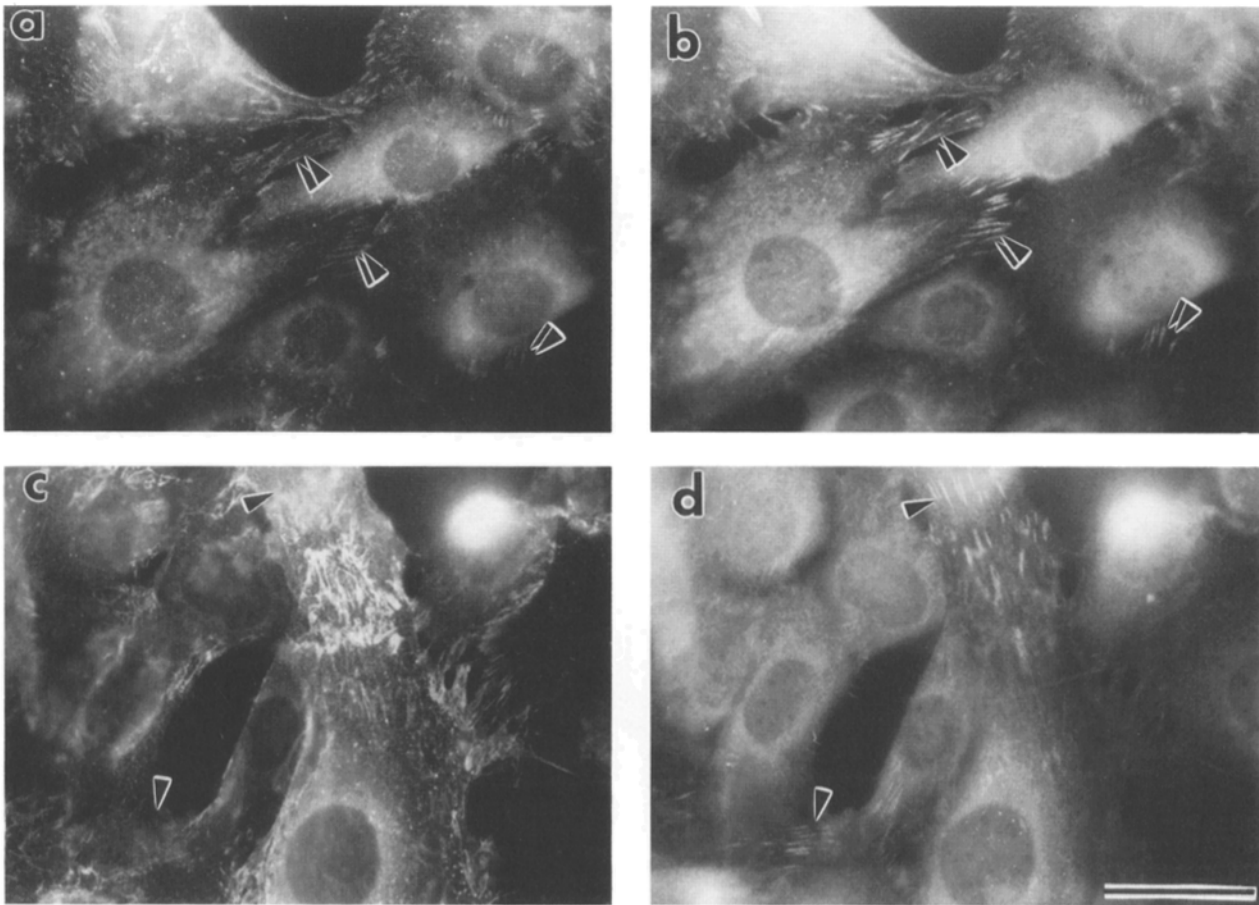


Figure 7. Double labeling of integrins (*a* and *c*) and vinculin (*b* and *d*). Immunofluorescent labeling was processed as detailed in Materials and Methods. Subconfluent CD3 cells were labeled with rabbit anti- $\alpha 5\beta 3$ (*a*) or anti- $\alpha 5\beta 1$ (*c*) and vinculin (*b* and *d*). Bar, 10 μm .

increased the surface labeling for $\alpha 5\beta 3$ using mAb7E3 (Tang et al., 1993). In this study, we found that 12(S)-HETE also stimulated the surface expression of $\alpha 5\beta 3$ on microvessel CD3 cells. Both adherent and resuspended (via nonenzymatic dissociation; see Materials and Methods) CD3 cells were used for surface labeling of $\alpha 5\beta 3$ and $\alpha 5\beta 1$ integrins. Immunofluorescence studies (data not shown) and digitized image analysis (Fig. 8) using adherent ECs indicated that CD3 cells stimulated with 0.1 μM of 12(S)-HETE demonstrated enhanced surface expression of $\alpha 5\beta 3$. This stimulating effect of 12(S)-HETE was found to be both time and dose dependent. 15 min of treatment by 0.1 μM of 12(S)-HETE led to an increase in $\alpha 5\beta 3$ surface labeling. By 1 h, the 12(S)-HETE effect reached peak level (Fig. 8 *c*) and the mean relative fluorescence intensity (represented by the relative color values as shown in Fig. 8) of at least five individual measurements in each condition was increased from 389 ± 59 (arbitrary units) in control (Fig. 8 *a*) to 1286 ± 371 in the 12(S)-HETE-treated (Fig. 8 *c*) group. As shown in the composite histogram in Fig. 8 *e*, there was a dramatic shift (i.e., increase) in the average fluorescence of the 12(S)-HETE-treated samples (*red bars*) compared with the control (*green bars*). After 1 h, the surface expression of $\alpha 5\beta 3$ rapidly decreased to the unstimulated level (data not shown). No effect of 12(S)-HETE on the surface expression of $\alpha 5\beta 1$ was observed (Fig. 8, *b*, *d*, and *f*). At the basal (i.e., unstimulated)

level, CD3 cells appeared to express more $\alpha 5\beta 1$ than $\alpha 5\beta 3$ on their surface (Fig. 8, compare *a* and *b*). 12(S)-HETE treatment did not significantly alter the surface expression level of $\alpha 5\beta 1$ (1049 ± 118 in control vs 1020 ± 171 in 12(S)-HETE-treated group) (Fig. 8, *b* and *d*). No shift in fluorescence intensity of $\alpha 5\beta 1$ surface labeling was observed after 12(S)-HETE stimulation (Fig. 8 *f*). Quantitative flow cytometric measurement of fluorescence intensity revealed a dose-dependent effect on $\alpha 5\beta 3$ surface expression by 12(S)-HETE (15-min stimulation), with a maximum effect on 0.1 μM (increasing the fluorescence intensity by more than two-fold; data not shown). In contrast, 12(S)-HETE did not increase the overall surface expression of $\alpha 5\beta 1$ as revealed by flow cytometry using rabbit anti- $\alpha 5\beta 1$ (data not shown).

12(S)-HETE Effect on $\alpha 5\beta 3$ Is a Posttranscriptional Process

12(S)-HETE demonstrated two seemingly opposite effects on EC $\alpha 5\beta 3$ integrin receptors, i.e., rearranging (decreasing) $\alpha 5\beta 3$ at focal adhesions and increasing $\alpha 5\beta 3$ surface expression. Semi-quantitative RT-PCR followed by Southern hybridization using specific cDNA probes for different integrin subunits was performed to determine whether 12(S)-HETE alters $\alpha 5\beta 3$ expression at the transcriptional level. The same experiment was also run for $\alpha 5\beta 1$. The results in-

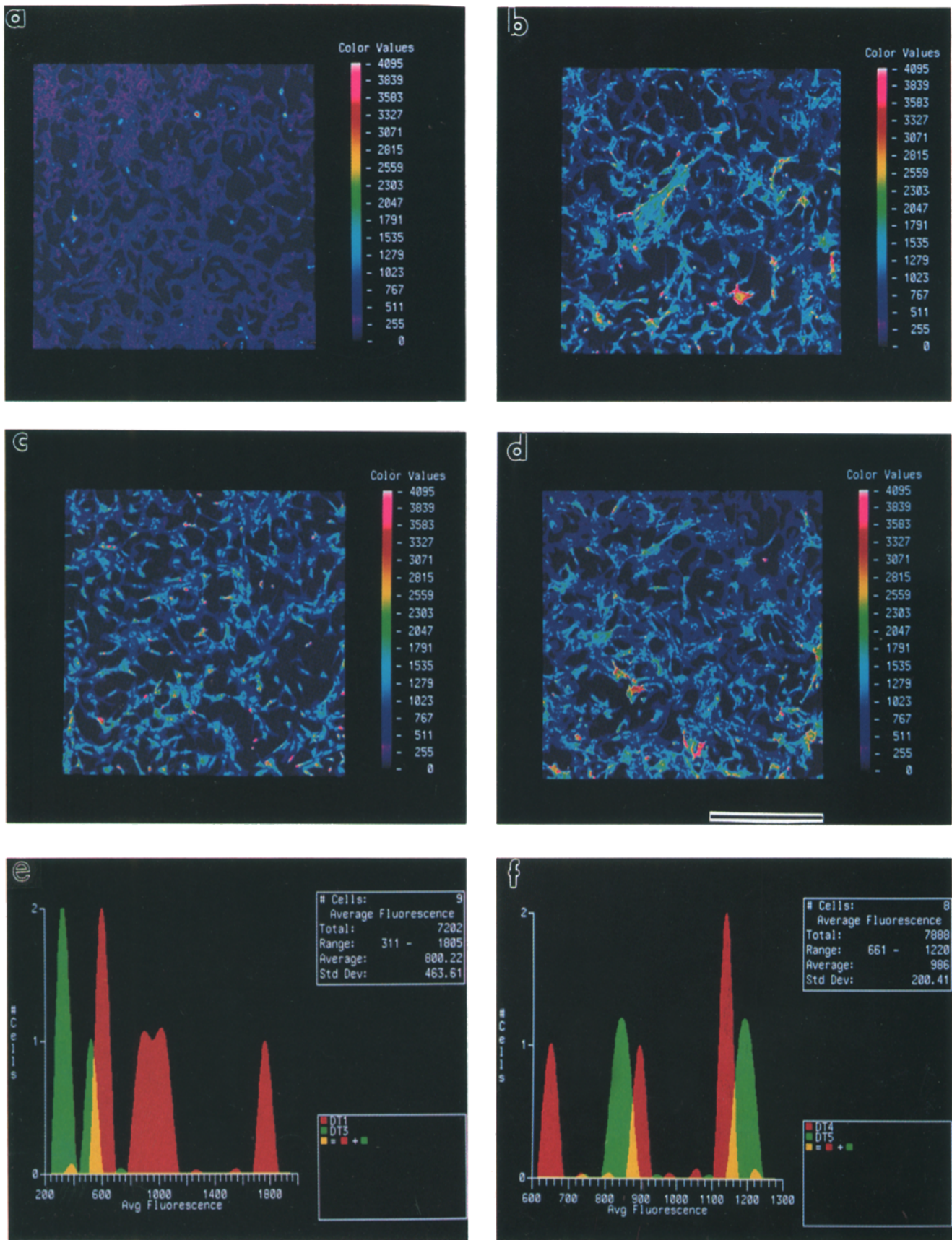


Figure 8. 12(S)-HETE exhibits differential effects on the surface expression of EC $\alpha v \beta 3$ and $\alpha 5 \beta 1$. Adherent subconfluent or confluent (data not shown) CD3 cells were treated with ethanol (*a* and *b*) or 0.1 μM 12(S)-HETE (*c* and *d*) for 5 min, cultured for different time periods (shown here is 60 min after treatment) and then surface stained for $\alpha v \beta 3$ (*a* and *c*) or $\alpha 5 \beta 1$ (*b* and *d*) with rabbit anti- $\alpha v \beta 3$ and

icated that 12(S)-HETE did not significantly alter the mRNA levels of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins. To further support the concept that 12(S)-HETE-induced alterations in $\alpha v\beta 3$ may be posttranscriptional, quantitative western blotting of $\beta 3$ in whole cell lysates using monoclonal anti- $\beta 3$ antibody (OPG-2) was performed. The results indicated that there was no significant alterations in the overall level of $\beta 3$ proteins after 12(S)-HETE (0.1 μM) treatment (data not shown), as determined by densitometric scanning.

Discussion

Physiologically ECs form a continuous and polarized boundary between flowing blood and the underlying tissues. Specific interactions of lymphocytes (Ford et al., 1976), neutrophils (Belloni and Tressler, 1989), and circulating tumor cells (Pauli and Lee, 1988; Weiss et al., 1989), with components of the vascular wall are important for their targeting to different tissues and their exit from circulation. The maintenance of normal hemostasis, the regulation of inflammation, and prevention of blood-borne tumor cells from dissemination, all depend on the physical intactness of the EC monolayer, which in turn is determined by the well-organized cell-cell and cell-matrix adhesion structures. Integrins are the most important transmembrane molecules linking ECM to intracellular cytoskeleton. Cultured large and small vessel ECs express a variety of $\beta 1$ integrin complexes as well as the $\alpha\text{IIb}/\beta 3$ -related $\alpha v\beta 3$ complex (Fitzgerald et al., 1985; Dejana et al., 1990; Albelda et al., 1989; Kramer et al., 1990). Using immunofluorescence, immunoblotting, immunoprecipitation with specific antibodies, as well as adhesion assay and antibody perturbation study, we demonstrated that both large vessel (unpublished observations) and microvessel ECs express $\alpha v\beta 3$ and $\alpha 5\beta 1$, and that both receptors are functional in mediating initial EC adhesion to ECM and maintaining the integrity of the established monolayer. It is well known that the ECM could modulate the organization of EC cytoskeleton and cell shape (Young and Herman, 1985). Distribution of EC integrin receptors to specific cell microdomains, i.e., the cell-cell or cell-matrix contact sites, is also regulated by specific ECM ligands (Cheng and Kramer, 1989; Dejana et al., 1988). In our experiments, we also noted that the distribution of both $\alpha 5\beta 1$ and $\alpha v\beta 3$ in focal adhesions is dependent on specific substratum composition. In confluent microvessel EC monolayer (i.e., EC growing on their own natural ECM), $\alpha 5\beta 1$ and $\alpha v\beta 3$ appear to follow different distribution patterns; the former primarily concentrated at the cell-cell borders colocalizing with cell cortical microfilaments and extracellular fibronectin while the latter codistributed with typical vinculin-containing focal adhesions in both the cell body and cell-cell borders. This different distribution pattern of $\alpha v\beta 3$ and $\alpha 5\beta 1$ implies that these two integrins may play a differential role in mediating EC-matrix interactions and maintaining the EC monolayer integrity and also provides an explanation for the specific effect of 12(S)-HETE on $\alpha v\beta 3$.

12(S)-HETE induced a time-dependent redistribution of $\alpha v\beta 3$ -containing focal adhesions and there exists a cause-and-effect relationship between 12(S)-HETE-induced $\alpha v\beta 3$ rearrangement and EC retraction. 5 min after 12(S)-HETE stimulation when no EC retraction could be detected (both morphologically and quantitatively), redistribution (i.e., cleavage) of $\alpha v\beta 3$ -containing focal adhesions was observed. Subsequently rearrangement of $\alpha v\beta 3$ integrin led to an overall decreased localization of this integrin to focal adhesions, the most important cellular structures linking cells to ECM through the cytoskeleton. The general alterations of $\alpha v\beta 3$ -containing focal adhesions are very similar to those of vinculin plaques (unpublished observations), i.e., an eventual decrease in those retracted cells, implying that 12(S)-HETE has a global impact on the focal adhesions. Focal adhesions are complex cellular structures involved in transducing signals from the outside to the inside, or vice versa, through a chain of proteins, i.e., extracellular adhesive glycoproteins such as fibronectin and vitronectin linked to membrane-spanning integrins which are further connected to cytoskeleton via talin, vinculin, α -actinin, etc (for review, see Burridge et al., 1988). A number of regulatory proteins, such as tyrosine kinases (e.g., pp60^{c-src}, Burridge et al., 1988), proteases (Beckerle et al., 1987), PKC isoforms (Jaken et al., 1989), and so on, are also concentrated at focal adhesions to maintain the dynamic entity. How 12(S)-HETE alters the normal organization of $\alpha v\beta 3$ integrins is currently unknown, but two nonexclusive alternatives could be hypothesized. One possibility is that $\alpha v\beta 3$ redistribution results from the alteration of other components of the focal adhesions, such as microfilament, vinculin or α -actinin. In CD3 cells, $\alpha v\beta 3$ integrin is colocalized with vinculin, stress fiber endings, and α -actinin in focal adhesions (this study and unpublished observations). In fact, we have found that 12(S)-HETE rearranges vinculin-enriched focal adhesions, reorganizes the actin filaments, and mobilizes α -actinin to the membrane "ruffles" (Tang et al., 1992). All of these alterations would undoubtedly disrupt the normal $\alpha v\beta 3$ -containing focal adhesions. Preliminary results obtained from vinculin- $\alpha v\beta 3$ double labeling experiments suggest that alteration (i.e., disruption and decrease) of vinculin-containing focal adhesions appear to occur before that of $\alpha v\beta 3$ -containing focal adhesions. The other possibility is that 12(S)-HETE directly exerts its effect on $\alpha v\beta 3$, for instance, through phosphorylation, since this eicosanoid alters the organization of the cytoskeletal elements by enhancing PKC-dependent phosphorylation (Tang et al., 1992). Several integrin subunits, including $\beta 3$, can be phosphorylated (Hilgery et al., 1991; Haimovich et al., 1991). Interestingly, 12(S)-HETE-induced $\alpha v\beta 3$ rearrangement also appears to be dependent on PKC, since its effect could be totally abolished by the selective PKC inhibitor calphostin C, but not by the protein kinase A inhibitor H8. This finding is in accord with our recent observations that this eicosanoid induces EC cytoskeleton alterations in a PKC-dependent manner (Tang et al., 1992), and that it activates tumor cell PKC by translocating the enzyme from the cytosol to the plasma membrane

rabbit anti- $\alpha 5\beta 1$, respectively. After incubation with secondary antibody, cells were subjected to digitized surface fluorescence analysis on an ACAS 470 laser image analyzer as detailed in Materials and Methods. *e* and *f* represent composite histograms of a distinct population of CD3 cells stained for $\alpha v\beta 3$ (*e*) or $\alpha 5\beta 1$ (*f*). Green bars, control samples; red bars, 12(S)-HETE-treated samples. Bar, 2 μm .

(Liu et al., 1991). It would be interesting to determine which isoform of PKC is responsible for the 12(S)-HETE-induced $\alpha v\beta 3$ rearrangement. Preliminary results indicate that, in addition to the PKC- α , CD3 cells also express two other isoforms. Interestingly, in all of our inhibitor studies, we observed that staurosporine is a less effective inhibitor than calphostin C (based on their relative IC_{50} values; see Materials and Methods) on 12(S)-HETE-induced cytoskeleton phosphorylation and reorganization (Tang et al., 1992), $\alpha v\beta 3$ rearrangement and EC retraction (this study). One possible explanation for these observations is that staurosporine (which has very similar IC_{50} values for most protein kinases) exerts inhibitory effects on, in addition to PKC, a variety of other protein kinases including PKA, myosin light chain kinase and tyrosine kinases. It is known that in many circumstances these protein kinases play differential or antagonizing roles. Therefore, the promiscuous effects of staurosporine may mask its "specific" action on PKC. In addition, many groups have reported that staurosporine possesses some biological activities (such as inducing a dissolution of microfilaments) independent of its effect on PKC (Hedberg et al., 1990). We also observed (both morphologically and quantitatively) that higher doses of staurosporine actually disrupted the EC monolayer integrity.

12(S)-HETE is a monohydroxy fatty acid and a lipoxygenase metabolite of arachidonic acid produced by platelets, leukocytes, and a variety of cell types including many solid tumor cells (for a review, see Spector et al., 1988; Marnett et al., 1990; Liu et al., 1991). During hematogenous spreading of tumor cells 12(S)-HETE may reach a concentration in localized foci of tumor cell-platelet-leukocyte-EC interactions high enough to initiate its biological activity on these interacting cells. Our previous work has demonstrated that this eicosanoid enhances the surface expression of the integrin receptor $\alpha IIb\beta 3$ in cultured B16 amelanotic melanoma (B16a) and Lewis lung carcinoma cells (Grossi et al., 1989; Chopra et al., 1991). More recently, we observed that 12(S)-HETE upregulated $\alpha v\beta 3$ integrin surface expression in large vessel EC, resulting in an enhanced tumor cell adhesion to the stimulated EC monolayer (Tang et al., 1993). In the present study, we observed that 12(S)-HETE increased, in a dose- and time-dependent manner, the surface expression of $\alpha v\beta 3$ in microvessel CD3 cells, as revealed by immunofluorescence studies, digitized imaging analysis and flow cytometry. The expression of individual integrins is strictly regulated during tissue development and cell segregation. Cell- and matrix-adhesion molecules, as well as ECM components are known to respond to and be regulated by signals from growth factors and cellular oncogenes (Damsky and Bernfield, 1990). Typical examples are the up-regulated mRNA expression of integrin receptors (all 3 major subfamilies) by the transforming growth factor- β (Ignatz et al., 1989), the downregulation of $\alpha v\beta 3$ surface expression in ECs by 13-hydroxyoctadecadienoic acid (13-HODE), a lipoxygenase metabolite of linoleic acid (Buchanan et al., 1990), and enhanced surface expression of $\alpha IIb\beta 3$ expression by 12(S)-HETE in tumor cells (Grossi et al., 1989). In this study, we observed two effects of 12(S)-HETE on the EC $\alpha v\beta 3$, i.e., a decreased focal adhesion labeling and an increased surface staining. At present, we are uncertain about the relationship between these two phenomena. However, it is highly possible that increased $\alpha v\beta 3$ surface expression af-

ter 12(S)-HETE stimulation may, at least partly, result from decreased (or mobilized) focal adhesion localization since these two processes appear to be temporally related. Integrins, after their synthesis, are targeted to different microdomains of the cell such as the cell-cell border, focal adhesions and the cell surface. What determines the route of sorting or whether there is any minor difference between the integrin receptors targeted to different cellular microdomains is currently unknown. What is known is that localization to each of these microdomains is not fixed, but under constant exchange depending on the status of the cells (e.g., motile vs resting). Another potential mechanism for 12(S)-HETE-enhanced $\alpha v\beta 3$ surface expression is mobilization of this integrin receptor from an intracellular pool to the plasma membrane. Recently we observed that 12(S)-HETE enhanced the surface expression of the integrin $\alpha IIb\beta 3$, a close relative to $\alpha v\beta 3$, in B16a tumor cells and that this effect was mediated through cytoskeleton-dependent translocation (Chopra et al., 1991). These results suggest that a similar mechanism may exist for $\alpha v\beta 3$ upregulation in EC by 12(S)-HETE. The 12(S)-HETE effect on tumor cell $\alpha IIb\beta 3$ surface expression was found to be time-dependent (within 1 h), independent of transcriptional activation, but dependent on the tumor cell cytoskeleton, i.e., microfilaments and intermediate filaments (Chopra et al., 1991). In this study, the stimulation of $\alpha v\beta 3$ surface expression in CD3 cells by 12(S)-HETE effect is also time-dependent, and proved to be a post-translational process. Semi-quantitative RT-PCR followed by Southern hybridization did not reveal significant alterations in the mRNA levels of both αv and $\beta 3$ after 12(S)-HETE stimulation. Semi-quantitative immunoblotting of the $\beta 3$ subunit also did not reveal any significant alterations in the protein level after 12(S)-HETE treatment. It is possible that the increased cell surface labeling of $\alpha v\beta 3$ results from the decreased focal adhesion enrichment of this integrin receptor as well as from increased protein translocation from a cytosolic pool to the membrane. Theoretically, decreased focal adhesion labeling of $\alpha v\beta 3$ may result from decreased protein synthesis, however, this possibility has been excluded by quantitative Western blotting and RT-PCR. Alternative possibilities include diminished targeting of $\alpha v\beta 3$ to the focal adhesions due to post-translational protein modification (such as phosphorylation), and/or increased proteolytic cleavage due to protease activation. However, at present, substantive evidence for these possibilities is lacking.

In contrast to $\alpha v\beta 3$, $\alpha 5\beta 1$ did not undergo a well-defined and time-dependent rearrangement (e.g., redistribution and decrease of $\alpha 5\beta 1$ -containing focal adhesions), although the typical cell border localization of $\alpha 5\beta 1$ in confluent EC monolayers was disrupted following 12(S)-HETE stimulation. No specific pattern of alteration in $\alpha 5\beta 1$ was observed, nor did 12(S)-HETE alter the surface expression of $\alpha 5\beta 1$ in ECs. Not unexpectedly, 12(S)-HETE did not alter the mRNA level of EC $\alpha 5$ or $\beta 1$. Unlike $\alpha v\beta 3$ -containing focal adhesions whose rearrangement precedes development of EC retraction, the disruption of cell border localization of $\alpha 5\beta 1$ was observed to occur simultaneously with EC retraction. This observation suggests that the disruption of $\alpha 5\beta 1$ localization to the cell-cell borders is not an initiating factor in retracting ECs although it may contribute to the later phase of EC retraction. In microvessel ECs, integrin $\alpha 5\beta 1$, despite cell junction localization, appears to be less impor-

tant than $\alpha v\beta 3$ (which are localized in typical cell matrix contact sites or focal adhesions in both the cell body and cell-cell borders) since anti- $\alpha 5\beta 1$ antibodies are much less potent than anti- $\alpha v\beta 3$ in disrupting the established EC monolayer. Most probably, the differential effect of 12(S)-HETE on $\alpha v\beta 3$ and $\alpha 5\beta 1$ is due to the differential distribution patterns of these two integrins, i.e., $\alpha v\beta 3$, but not $\alpha 5\beta 1$, is codistributed with vinculin-containing focal adhesions. We conclude that 12(S)-HETE appears to affect specifically the protein components, e.g., microfilaments, vinculin, α -actinin and $\alpha v\beta 3$, in focal adhesions.

The significance of $\alpha v\beta 3$ rearrangement after 12(S)-HETE treatment can be multi-factorial. $\alpha v\beta 3$ is organized at focal adhesions and demonstrates colocalization with vinculin. In addition, it connects the intracellular cytoskeleton to the extracellular adhesive glycoproteins, and thus makes the cell and its environment an integrated system. $\alpha v\beta 3$, like vinculin, is also localized to the intercellular junctions. Therefore we believe that 12(S)-HETE-induced disruption of this integrin organization will alter the normal cell-matrix and cell-cell adhesion, break down the EC monolayer integrity, and contribute to the retracted EC phenotype. The increased cell surface expression of $\alpha v\beta 3$, which results from redistribution of focal adhesion-localized $\alpha v\beta 3$ and/or mobilization of $\alpha v\beta 3$ from the cytosolic pool, may increase the adhesivity of blood-borne cells (i.e., leukocytes, tumor cells, etc.) to endothelium (Shaughnessy et al., 1991; Tang et al., 1993). Enhanced tumor cell adhesion and extravasation due to EC retraction may facilitate metastatic dissemination. Similarly, adhesion of leukocytes to EC and enhanced exudation of blood components (both cellular and acellular) may contribute to inflammatory responses.

This work was supported by National Institutes of Health grants CA-47115 and CA-29997.

Received for publication 4 August 1992 and in revised form 19 January 1993.

References

- Agrez, M. V., R. C. Bates, A. W. Boyd, and G. F. Burns. 1991. Arg-Gly-Asp-containing peptides expose novel collagen receptors on fibroblasts: implications for wound healing. *Cell Regul.* 2:1035-1044.
- Albelda, S. M., M. Daise, E. M. Levine, and C. A. Buck. 1989. Identification and characterization of cell-substrate adhesion receptors on cultured human endothelial cells. *J. Clin. Invest.* 83:1990-2002.
- Bavisotto, L. M., S. M. Schwartz, and R. L. Heinmark. 1990. Modulation of Ca^{2+} -dependent intercellular adhesion in bovine aortic and human umbilical vein endothelial cells by heparin-binding growth factors. *J. Cell. Physiol.* 143:39-51.
- Beckerle, M. C., K. Burridge, G. N. DeMartino, and D. E. Croall. 1987. Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell.* 51:569-577.
- Belloni, P. N., and R. J. Treessler. 1989/1990. Microvascular endothelial cell heterogeneity: Interactions with leukocytes and tumor cells. *Cancer Metastasis Rev.* 8:353-389.
- Buchanan, M. R., M. C. Bertomeu, T. A. Hass, S. Gallo, and L. Eltringham-Smith. 1990. Endothelial cell 13-HODE synthesis and tumor cell and endothelial cell adhesion. *Adv. Prostaglandin Thromboxane Leukotriene Res.* 21:909-912.
- Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: Transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487-525.
- Chang, Y. S., Y. Q. Chen, J. Timar, K. K. Nelson, I. M. Grossi, L. A. Fitzgerald, C. A. Diglio, and K. V. Honn. 1992. Increased expression of $\alpha I\text{Ib}\beta 3$ integrin in subpopulations of murine melanoma cells with high lung-colonizing ability. *Int. J. Cancer.* 51:445-451.
- Charo, I. F., L. S. Bekeart, and D. R. Phillips. 1987. Platelet glycoprotein IIb-IIIa-like proteins mediate endothelial cell attachment to adhesive proteins and the extracellular matrix. *J. Biol. Chem.* 262:9935-9938.
- Charo, I. F., L. Nannizzi, J. W. Smith, and D. A. Cheresch. 1990. The vitronectin receptor $\alpha v\beta 3$ binds fibronectin and acts in concert with $\alpha 5\beta 1$ in promoting cellular attachment and spreading on fibronectin. *J. Cell Biol.* 111:2795-2800.
- Chen, Y. Q., X. Gao, J. Timar, D. G. Tang, I. M. Grossi, M. Chelladurai, T. J. Kunicki, S. E. G. Fligiel, J. D. Taylor, and K. V. Honn. 1992. Identification of the $\alpha I\text{Ib}\beta 3$ integrin in murine tumor cells. *J. Biol. Chem.* 267:17314-17320.
- Cheng, Y. F., and R. H. Kramer. 1989. Human microvascular endothelial cells express integrin-related complexes that mediate adhesion to the extracellular matrix. *J. Cell. Physiol.* 139:275-286.
- Cheng, Y. F., R. I. Clyman, J. Enenstein, N. Waleh, R. Pytela, and R. H. Kramer. 1991. The integrin complex $\alpha v\beta 3$ participates in the adhesion of microvascular endothelial cells to fibronectin. *Exp. Cell Res.* 194:69-77.
- Cheresch, D. A. 1987. Human endothelial cells synthesize and express an arg-gly-asp-directed adhesion receptor involved in attachment to fibronogen and von Willebrand factor. *Proc. Natl. Acad. Sci. USA.* 84:6471-6475.
- Chopra, H., S. E. G. Fligiel, J. S. Hatfield, K. K. Nelson, C. A. Diglio, J. D. Taylor, and K. V. Honn. 1990. An in vivo study of the role of the tumor cell cytoskeleton in tumor cell-platelet-endothelial cell interactions. *Cancer Res.* 50:7686-7696.
- Chopra, H., J. Timar, Y. Q. Chen, X. H. Rong, I. M. Grossi, L. A. Fitzgerald, J. D. Taylor, and K. V. Honn. 1991. The lipoxygenase metabolite 12(S)-HETE induces a cytoskeleton-dependent increase in surface expression of integrin $\alpha I\text{Ib}\beta 3$ on melanoma cells. *Int. J. Cancer.* 49:1-13.
- Damsky, C. H., and M. Bernfield. 1990. Cell-to-cell contact and extracellular matrix. *Curr. Opin. Cell Biol.* 2:813-814.
- Dejana, E., S. Colella, G. Conforti, M. Abbadini, M. Gaboli, and P. C. Marchisio. 1988. Fibronectin and vitronectin regulate the organization of their respective arg-gly-asp adhesion receptors in cultured human endothelial cells. *J. Cell Biol.* 107:1215-1223.
- Dejana, E., M. G. Lampugnani, M. Giorgi, M. Gaboli, and P. C. Marchisio. 1990. Fibrinogen induces endothelial cell adhesion and spreading via the release of endogenous matrix proteins and the recruitment of more than one integrin receptor. *Blood.* 75:1509-1517.
- Fitzgerald, L. A., and D. R. Phillips. 1989. Structure and function of platelet membrane glycoproteins. In *Platelet Immunology: Molecular and Clinical Aspects*. T. J. Kunicki and J. N. George, editors. J. B. Lippincott Co., Hagerstown, MD. 9-30.
- Fitzgerald, L. A., I. F. Charo, and D. R. Phillips. 1985. Human and bovine endothelial cells synthesize membrane glycoproteins similar to platelet membrane GpIIb and GpIIIa. *J. Biol. Chem.* 260:10893-10896.
- Ford, W. D., M. Sedgely, and M. E. Smith. 1976. The migration of lymphocytes across specialized endothelium. *Cell Tissue Kinet.* 9:351-361.
- Giltay, J. C., and J. A. van Mourik. 1988. Structure and function of endothelial cell integrins. *Haemostasis.* 18:376-389.
- Grossi, I. M., L. A. Fitzgerald, L. A. Umbarger, K. K. Nelson, C. A. Diglio, J. D. Taylor, and K. V. Honn. 1989. Bidirectional control of membrane expression and/or activation of the tumor cell IRGpIIb/IIIa receptor and tumor cell adhesion by lipoxygenase products of arachidonic acid and linoleic acid. *Cancer Res.* 49:1029-1037.
- Haimovich, B., B. J. Aneskievich, and D. Boettige. 1991. Cellular partitioning of $\beta 1$ integrins and their phosphorylated forms is altered after transformation by Rous sarcoma virus or treatment with cytochalasin D. *Cell Regul.* 2:271-283.
- Hedberg, K. K., G. B. Birrell, T. Hablston, and O. H. Griffith. 1990. Staurosporine induces dissolution of microfilament bundles by a protein kinase C-independent pathway. *Exp. Cell Res.* 188:199-208.
- Hidaka, H., M. Watanabe, and R. Kobayashi. 1991. Properties and use of H-series compounds as protein kinase inhibitors. In *Protein Phosphorylation (part B): Analysis of Protein Phosphorylation, Protein Kinase Inhibitors, and Protein Phosphatases*. T. Hunter and B. M. Sefton, editors. *Methods Enzymol.* 201:328-339.
- Hillery, C. A., S. S. Smyth, and L. V. Parise. 1991. Phosphorylation of human platelet glycoprotein IIIa (GPIIIa): Dissociation from fibrinogen receptor activation and phosphorylation of GPIIIa in vitro. *J. Biol. Chem.* 266:14663-14669.
- Honn, K. V., and D. G. Tang. 1992. Adhesion molecules and cancer cell interaction with endothelium and subendothelial matrix. *Cancer Metastasis Rev.* 11:353-375.
- Honn, K. V., I. M. Grossi, C. A. Diglio, M. Wojtukiewicz, and J. D. Taylor. 1989. Enhanced tumor cell adhesion to the subendothelial cell matrix resulting from 12(S)-HETE-induced endothelial cell retraction. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:2285-2293.
- Hynes, R. O. 1987. Integrins, a family of cell surface receptors. *Cell.* 48:549-555.
- Hynes, R. O., and A. D. Lander. 1992. Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. *Cell.* 68:303-322.
- Ignatz, R. A., J. Heino, and J. Massague. 1989. Regulation of cell adhesion receptors by transforming growth factor- β . Regulation of vitronectin receptor and LFA-1. *J. Biol. Chem.* 264:389-392.
- Jaken, S., K. Leach, and T. Klauk. 1989. Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. *J. Cell Biol.* 109:697-704.
- Kramer, R. H., Y. F. Cheng, and R. Clyman. 1990. Human microvascular endothelial cells use $\beta 1$ and $\beta 3$ integrin receptor complexes to attach to laminin. *J. Cell Biol.* 111:1233-1243.

- Lampugnani, M. G., M. Resnati, E. Dejana, and P. C. Marchisio. 1991. The role of integrins in the maintenance of endothelial monolayer integrity. *J. Cell Biol.* 112:479-490.
- Lawler, J., R. Weinstein, and R. O. Hynes. 1988. Cell attachment to thrombospondin: the role of arg-gly-asp, calcium, and integrin receptors. *J. Cell Biol.* 107:2351-2361.
- Liu, B., J. Timar, J. Howlett, C. A. Diglio, and K. V. Honn. 1991. Lipoxygenase metabolites of arachidonic and linoleic acids modulate the adhesion of tumor cells to endothelium via regulation of protein kinase C. *Cell Regul.* 2:1045-1055.
- Marnett, L. J., M. T. Leithauser, K. M. Richards, I. Blair, K. V. Honn, S. Yamamoto, and T. Yoshimoto. 1990. Arachidonic acid metabolism of cytosolic fractions of Lewis lung carcinoma cells. *Adv. Prostaglandin Thromboxane Leukotriene Res.* 21:895-900.
- Menter, D. G., B. W. Steinert, B. F. Sloane, N. Gundlach, S. M. O'Gara, L. J. Marnett, C. A. Diglio, D. Walz, J. D. Taylor, and K. V. Honn. 1987. Role of platelet membrane in enhancement of tumor cell adhesion to endothelial extracellular matrix. *Cancer Res.* 47:6751-6762.
- Pauli, B. U., and C. L. Lee. 1988. Organ preference of metastasis: the role of organ-specifically modulated endothelial cells. *Lab. Invest.* 58:379-387.
- Ruoslahti, E. 1991. Integrins. *J. Clin. Invest.* 87:1-5.
- Shaughnessy, S. G., R. M. Lafrenie, M. R. Buchanan, T. J. Podor, and F. W. Orr. 1991. Endothelial cell damage by Walker carcinosarcoma cells is dependent on vitronectin receptor-mediated tumor cell adhesion. *Am. J. Pathol.* 138:1535-1543.
- Spector, A. A., J. A. Gordon, and S. A. Moore. 1988. Hydroxyicosatetraenoic acids (HETEs). *Prog. Lipid Res.* 27:271-323.
- Tamaoki, T. 1991. Use and specificity of staurosporin, UCN-01, and calphostin C as protein kinase C inhibitors. In *Protein Phosphorylation (part B): Analysis of Protein Phosphorylation, Protein Kinase Inhibitors, and Protein Phosphatases*. T. Hunter and B. M. Sefton, editors. *Methods Enzymol.* 201:340-347.
- Tang, D. G., I. M. Grossi, C. A. Diglio, J. D. Taylor, and K. V. Honn. 1992. 12(S)-HETE-induced endothelial cell retraction is mediated by PKC-dependent cytoskeleton rearrangement and phosphorylation. *Proc. Am. Assoc. Cancer Res.* 33:88.
- Tang, D. G., I. M. Grossi, Y. Q. Chen, C. A. Diglio, and K. V. Honn. 1993. 12(S)-HETE promotes tumor cell adhesion by increasing surface expression of $\alpha v \beta 3$ integrins on endothelial cells. *Int. J. Cancer*. In press.
- Weiss, L., F. W. Orr, and K. V. Honn. 1989. Interactions between cancer cells and the microvasculature: a rate-regulator for metastasis. *Clin. & Exp. Metastasis.* 7:127-167.
- Young, W. C., and I. M. Herman. 1985. Extracellular matrix modulation of endothelial shape and motility following injury in vitro. *J. Cell Sci.* 73:19-32.