Localization of 13-Hydroxyoctadecadienoic Acid and the Vitronectin Receptor in Human Endothelial Cells and Endothelial Cell/Platelet Interactions In Vitro

By Michael R. Buchanan, Maria C. Bertomeu, Thomas A. Haas, F. William Orr, and Louise L. Eltringham-Smith

Blood/vessel wall cell interactions depend, in part, on the expression of adhesion receptors on cell surfaces, such as expression of the vitronectin receptor (VnR) on the apical surface of endothelial cells (ECs) for platelet/EC adhesion. However, it is unclear how receptor expression is regulated from within cells. In previous studies, we found that ECs metabolize linoleic acid into the lipoxygenase monohydroxy acid, 13-hydroxyoctadecadienoic acid (13-HODE), and that the intracellular level of 13-HODE correlates inversely with VnR expression and platelet adhesion to the EC apical surface. In this study, we determined the physical associations of 13-HODE and VnR in unstimulated and stimulated ECs, i.e., at times when ECs were and were not adhesive for specific ligands and platelets, using double antibody immunofluorescent staining techniques and binding assays. 13-HODE and the VnR were colocalized within unstimulated ECs. When ECs were stimulated, 13-HODE was no longer detectable, either in or outside the ECs, and the VnR was detected on the apical surface of the ECs. These changes were paralleled by increased vitronectin binding and increased platelet adhesion to the ECs. We suggest that colocalization of 13-HODE with VnR reflects a 13-HODE/VnR interaction, confining the VnR in a nonadhesive form inside unstimulated ECs, and, as a result, the ECs are nonadhesive. When the ECs are stimulated, 13-HODE and VnR dissociate, allowing the VnR to relocate on the EC surface, where the VnR undergoes a conformational change resulting in increased EC adhesivity.

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MATERIALS AND METHODS

Materials. A rabbit Ig to human vWF was obtained from Dako (Copenhagen, Denmark), Murine monoclonal antisera to the VnR α chain and the RGD recognizing site of the β chain (LM142 and LM609, respectively) were obtained from Dr D.A. Cheres (Scripps Clinics, La Jolla, CA). The characteristics of these antisera are described in detail elsewhere. Antimouse Ig conjugated to fluorescein was obtained from Dimension Laboratories Inc (Missisauaga, Ontario, Canada). Human fibronectin and vitronectin were obtained from Collaborative Research Inc (Bedford, MA). Human von Willebrand factor (vWF), fibronectin and vitronectin were obtained from Collaborative Research Inc (Bedford, MA).

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Fig 1. Immunofluorescent detection of 13-HODE and VnR in stimulated and unstimulated ECs ± IL-1. 13-HODE and the VnR were colocalized in fixed and permeabilized unstimulated ECs as highlighted by the same areas indicated by the arrows in (A) and (B). There was no immunofluorescence when fixed and permeabilized unstimulated ECs were incubated with PS-13H and authentic 13-HODE (C). When the EC monolayers were incubated with IL-1, immunofluorescence was only detected using the LM609 on fixed ECs, and the pattern of immunofluorescence was concentrated on the EC surface and towards the periphery (D).

obtained from Dr F.A. Ofosu (Canadian Red Cross, Hamilton, Ontario, Canada). Human serum albumin was obtained from Sigma Chemicals (St Louis, MO). Human recombinant IL-1α (IL-1) was obtained from Hoffman-La Roche (Nutley, NJ). The GRGDS and GRGES peptides were obtained from Peninsula Lab Inc (San Francisco, CA). Human umbilical cords were collected by the nursing staff of the Labour and Delivery unit of the McMaster Health Science Centre (Hamilton, Ontario, Canada). All tissue culture plasticware were obtained from Johns Laboratories (Toronto, Ontario, Canada). All cell culture media and supplements were obtained from GIBCO (Grand Island, NY). Linoleic acid and soybean lipoxigenase were obtained from Sigma Chemicals. 125I Iodine (100 μCi/mL, 13 mCi/μg) was obtained from New England Nuclear (Boston, MA).

Preparation of 13-HODE antiserum. 13-HODE was synthesized from linoleic acid using soybean lipoxigenase and purified by high performance liquid chromatography (HPLC) as previously described.22 The purified 13-HODE was coupled to bovine serum albumin, using water soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The 13-HODE/albumin conjugate was purified by exhaustive dialysis against distilled water.

Six male rabbits were injected intramuscularly with the 13-HODE/albumin conjugate (250 μg of 13-HODE, suspended in Freund’s complete adjuvant). Booster injections were administered to each rabbit at 6-week intervals for another 5 months. The rabbits then were anesthetized and exsanguinated via a carotid cannula. The blood was centrifuged at 3,000g for 30 minutes to obtain cell-free serum, which was then passed over a DEAE-CL-6B column at a flow rate of 32 mL/h at a pressure difference of 42 cm. Fractions were collected at 1-minute intervals for 30 minutes. Those fractions collected between 10 and 15 minutes were pooled, as they were identified as those fractions that contained the bulk of the IgG. These fractions were used throughout the study.

To determine the specificity and titre of the pooled antiserum fractions (PS-13H), the following characterization was performed. Known amounts of authentic 13-HODE, 15-hydroxyeicosatetraenoic acid (15-HETE), and 12-HETE (5 to 25 pg of each) were added to aliquots of normal pooled human cell-free plasma. Increasing amounts of PS-13H were then added to each aliquot. All aliquots were subsequently extracted and analyzed by HPLC as previously described.21 In addition, increasing amounts of authentic 13-HODE (0 to 300 pg) or 15- or 12-HETE (0 to 600 pg) were added to some of the EC monolayer preparations used in the immunofluorescence assays (described below) to determine which monohydroxide, if any, blocked the detection of 13-HODE by the PS-13H in the immunofluorescence assay.

Human ECs culture. ECs isolated from human umbilical veins were grown to confluent monolayers according to the method of Jaffe et al.,23 with the following modifications. The ECs were grown to confluence in T-25 flasks containing M199 medium supple-
Fig 2. Immunofluorescent localization of 13-HODE in an unstimulated EC monolayer using confocal laser microscopy with the optics focused intracellularly 0.3 μm below the apical surface of the ECs.

Preparation of nonpermeabilized and permeabilized EC monolayers. The EC monolayers on the discs were first rinsed twice with Hanks’ Balanced Salt Solution (HBSS) and then incubated with 10 ng/mL of IL-1 in 500 μL fresh medium. Four hours later, the monolayers were fixed with either (1) 1% paraformaldehyde in 0.15 mol/L phosphate-buffered saline (PBS), pH 7.3, at 4°C for 18 hours; or (2) fixed with 1% paraformaldehyde in 0.15 mol/L PBS as described above, and then permeabilized by incubating the cells with 2% Triton X-100 (vol/vol) for 3 minutes at 22°C. All EC monolayers were then rinsed four times (5 min/wash) with 0.15 mol/L PBS and washed with 0.1% glycine (vol/vol) in Milloning’s buffer (1.68% NaH₂PO₄ · H₂O, 0.385% NaOH, 0.54% glucose, wt/vol, pH 7.2) for 30 minutes. This method is described in detail elsewhere.²⁴

Localization of 13-HODE, VnR α chain, and VnR β chain. The fixed EC monolayer preparations were initially incubated with 10% normal goat serum for 30 minutes to saturate any nonspecific IgG binding sites, thereby blocking any nonspecific binding of the test antiserum. (Goat IgG was used because it does not crossreact with mouse or rabbit IgG.) The EC monolayers were then rinsed four times with 0.15 mol/L PBS to wash off any unbound goat serum. Next, the EC monolayers were incubated with the test antibody diluted 1/100 at 22°C, ie, the rabbit polyclonal 13-HODE antiserum, PS-13H, or one of the murine monoclonal anti-VnR ascites, LM609 or LM142. A dilution of 1:100 was used for each antiserum,
Platelet/EC adhesion assay. Confluent EC monolayers grown on the fibronectin-coated glass discs were rinsed with PBS containing 2 mmol/L Ca\textsuperscript{2+} and 1 mmol/L Mg\textsuperscript{2+} (pH 7.35) and incubated with IL-1, as described above. Four hours later, the medium was removed. A 1-mL aliquot of \textsuperscript{3}H-platelet suspension was added to each monolayer and incubated for 30 minutes with gentle shaking in a 37°C waterbath. The EC monolayer-covered discs were then removed and gently washed three times in HBSS to remove any nonadherent platelets. The washed monolayers were placed into liquid scintillation vials and their radioactivities were measured to assess the number of platelets adherent/EC monolayer. This adhesion assay is described in detail elsewhere.\textsuperscript{12}

RESULTS

When unstimulated ECs, fixed with paraformaldehyde but not permeabilized, were incubated with PS-13H in PBS, and then viewed through a green filter, no 13-HODE could be detected on the surface of the ECs (data not shown because all photographs were blank, ie, there was no fluorescein fluorescence). However, when fixed and permeabilized ECs were incubated with the PS-13H, there was a distinct punctate pattern of fluorescein fluorescence that appeared to be highlighted in specific vesicles (Fig 1A). These vesicle-like structures were situated immediately below the plasma membrane in the ECs, as determined by depth focusing (Fig 1A) and confirmed by confocal laser microscopy (Fig 2). When 300 pg of authentic 13-HODE (the average amount of 13-HODE produced by each EC monolayer) was added to the permeabilized ECs immediately before adding the PS-13H, all fluorescein fluorescence was blocked (Fig 1C). In contrast, when 150 to 600 pg of 12- or 15-HETE (lipoxygenase metabolites of arachidonic acid) was added, EC fluorescein fluorescence was unchanged (data not shown).

These observations suggested that the PS-13H was specific for 13-HODE and that 13-HODE was localized to specific vesicles within the ECs.

The specificity of PS-13H for 13-HODE was confirmed by HPLC. When known amounts of 13-HODE, 12-HETE, and 15-HETE were added to pooled human plasma and then extracted and assayed by HPLC, an HPLC tracing such as that illustrated in Fig 3, inset A, was obtained. The area under each peak is expressed as 100% recovery of each specific monohydroxide in the larger graph of Fig 3. When increasing amounts of PS-13H were added to the pooled plasma containing the monohydroxides before extraction, there was a concentration-dependent decrease in the area of 13-HODE recovered (Fig 3). This is also illustrated as the decrease in area beneath the curve of peak 1 (see insets B, C, and D, Fig 3). There was no decrease in 12- or 15-HETE recovery, ie, no change in area under the curve (Fig 3). Furthermore, when comparable volumes of an antiserum to vWF or to prostaglandin F\textsubscript{2α} (PGF\textsubscript{2α}) were added, recoveries of all monohydroxides were unaffected (data not shown).

When the same unstimulated EC monolayers were incubated with LM142 (which recognizes the V\textsubscript{\textalpha}R chain) and viewed through a red filter, the pattern of rhodamine red fluorescence was identical to the pattern of fluorescein green

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Inhibition of 13-HODE detection by HPLC by increasing amounts of PS-13H. At 100% recoveries of 25 ng of 13-HODE (m, peak 1), 15-HETE (m, peak 2), and 12-HETE, (c), peak 3), an HPLC tracing such as shown in inset A was obtained. When increasing volumes of PS-13H were added, there was a rapid decrease in 13-HODE recovery only. Insets B, C, and D show HPLC tracings in the presence of 5, 7, and 10 \mu L PS-13H, respectively.}
\end{figure}
fluorescence obtained with the PS-13H antiserum (compare Fig 1A and B). These observations suggest that VnR is colocalized with 13-HODE within the same vesicle-like structures. When LM609 (which recognizes the RGD epitope of the VnR β chain) was used, there was no EC rhodamine red fluorescence, either on the surface (Fig 4a) or inside the unstimulated ECs (Fig 4b). These data indicate that the antibody-binding site of VnR β was not accessible to the LM609, suggesting that the VnR present inside the unstimulated ECs was in a nonadhesive form.

When IL-1-stimulated ECs were fixed and permeabilized, there was no fluorescence of the EC monolayers, using either PS-13H (fluorescein, Fig 4c) or LM142 (rhodamine red [not shown], micrograph identical to Fig 4c). However, when the stimulated ECs were only fixed and not permeabilized (to detect the surface antigens) and then incubated with LM609, there was a marked rhodamine fluorescence on the ECs, concentrated on the periphery of the EC surface (Fig 4d). 13-HODE was not detectable on the surface of these cells (Fig 4d). These data suggest that the VnR was now detectable in an adhesive form because the LM609 antigenic site is related to the RGD-adhesive site of the VnR β subunit.

When increasing concentrations of 125I-labeled vitronectin, -fibronectin, -fibrinogen, or -albumin were added to unstimulated EC monolayers, each ligand bound to the ECs. This binding was rapidly saturated (Fig 5A, B, C, and D), and did not differ among the four proteins. When these 125I-ligands were incubated with IL-1-stimulated ECs, 125I-vitronectin ligand binding increased significantly (P < .002), whereas the binding of the other three proteins remained unchanged at all concentrations tested. When increasing concentrations of unlabeled protein were added to unstimulated EC monolayers to which 5 pg/mL of 125I-vitronectin had been added (ie, to which 2.4 ± 0.1 pg bound/monolayer), the unlabeled vitronectin displaced the 125I-la-
...beled vitronectin in a concentration-related manner (Fig 6). However, when the same concentrations of unlabeled vitronectin were added to IL-1-stimulated ECs (to which 3.9 ± 0.1 pg/monolayer had bound), the unlabeled vitronectin did not displace the \(^{125}\)I-labeled vitronectin (Fig 6). Similar results were seen when 25 pg/mL of \(^{125}\)I-labeled vitronectin was added to the unstimulated and IL-1-stimulated ECs (to which 6.4 ± 0.1 and 8.3 ± 0.1 pg/monolayer bound, respectively, see inset, Fig 6). None of the other three proteins displaced the \(^{125}\)I-labeled vitronectin from unstimulated or IL-1-stimulated ECs (data not shown).

When the basement membranes underlying the EC cultures were incubated with the \(^{125}\)I-labeled ligands, there was no difference in the amounts of vitronectin, fibronectin, fibrinogen, or albumin bound to the basement membrane surfaces (Fig 7). These data indicate that the specific increase in vitronectin binding to IL-1-stimulated EC monolayers was to the apical surface of the ECs and not to any basement membrane components exposed by EC retraction.

When 13-HODE and the \(\alpha\) chain were colocalized inside the ECs (as detected by PS-13H and LM142, respectively, and at a time when the RGD adhesive site of the \(\alpha\) chain was not detectable by LM609), approximately 4 × 10^3 platelets adhered/EC monolayer (Table 1). Adding either 100 μg of the GRGDS or GRGES peptide, or 100 μL of LM142, LM609, or a fibronectin antibody, did not block this "basal" adhesion. However, when 13-HODE was no longer detectable and the adhesive domain of the \(\alpha\) chain was detectable (by LM609) on the apical surface of the ECs, ie, after IL-1 stimulation, platelet adhesion to the ECs increased (\(P < .01\)). This increase in platelet adhesion was only blocked by the GRGDS peptide and LM609, but not by the GRGES peptide or LM142 (Table 1).

**DISCUSSION**

There is a large body of literature indicating that adhesion receptors are expressed on blood and vascular wall cells in response to injury or perturbation, thereby facilitating cell/cell interactions. These receptors include the super family of adhesion receptors called integrins, which are reviewed elsewhere.26-30

However, the mechanism by which the expression of these adhesion receptors on the surface is regulated from within the cells is not well understood. Some studies suggest that the lipoxigenase metabolites of arachidonic and linoleic acids act as secondary messengers, modulating tumor cell/EC interactions via the protein kinase C pathway.31 Al...
though these studies clearly indicate a relationship between protein kinase C activation after phorbol 12-myristate 13-acetate and tumor cell adhesion, they do not provide any direct evidence for a secondary messenger effect of the monohydroxides. On the other hand, a number of other studies provide evidence that suggests that specific fatty acid lipid metabolites have physiochemical effects on integrin adhesivity. First, a number of investigators have shown that specific lipid moieties alter the abilities of various integrins to recognize their ligands in purified systems, i.e., in the absence of phosphorylation or other intracellular messenger processes. Moreover, both endogenous and exogenous monohydroxides have been shown to modulate cell/cell interactions in a number of biologic systems both in vitro and ex vivo. These studies consistently show a significant relationship between the concentrations of the monohydroxides derived from linoleic and arachidonic acids (i.e., 13-HODE and 5-, 12-, and 15-HETE) and blood cell/EC adhesion both in vitro and in vivo in the absence and presence of secondary messenger pathways; namely, 13-HODE is associated with decreased cell adhesivity, whereas 12- and 15-HETE are associated with increased cell adhesivity.

There are similar inverse relationships between the adhesivity of intact and injured vessel walls and vessel wall 13-HODE synthesis, both in animal and human tissues. Furthermore, a number of studies suggest that the adhesivity of the extracellular matrix underlying ECs varies inversely with the amount of 13-HODE associated with it. (It should be noted that another study has suggested that exogenously added 13-HODE has no effect on extracellular matrix adhesivity. However, in that study, the matrix was prepared by lysing the ECs with ammonium hydroxide, which may have inadvertently denatured the matrix proteins, thereby rendering them immune to conformational changes by the 13-HODE.) Thus, there is an abundance of data using both purified and biologic systems that suggest that lipids, in general, and monohydroxides, in particular, can directly alter the adhesivity of integrins. The results of our present study provide further evidence in this regard.

First, the present data support the possibility that the inverse relationship between 13-HODE and vessel wall adhesivity is a result of a direct interaction between 13-HODE
and VnR in ECs. 13-HODE and VnR colocalize in vesicle-like structures in unstimulated ECs, as detected by the PS-13H and LM142 antisera using immunofluorescence, and as confirmed by confocal laser microscopy. (Confirmation that PS-13H is specific for 13-HODE is provided by the observations that [1] PS-13H blocked the detection of 13-HODE by HPLC, [2] PS-13H had no affect on the detection of other monohydroxides by HPLC, and [3] authentic 13-HODE blocked the fluorescein fluorescence of the ECs incubated with the PS-13H, whereas [4] 12- and 15-HETE did not block EC/PS-13H fluorescein fluorescence.)

Second, the observation that the VnR could not be detected inside unstimulated ECs using LM1609, which binds to the RGD-adhesive domain of the VnR β chain,26 suggests that, when VnR is associated with 13-HODE inside ECs, the VnR is not adhesive. (Receptors in their nonadhesive form have also been shown to be located in specific vesicles of other cells, such as the laminin and fibronectin receptors in specific “adhesomes” in leukocytes under resting conditions.37)

Third, when VnR was detected on the surface of IL-1-stimulated ECs, it was only detected by LM609, which recognizes an epitope near or at the RGD adhesive site. This also suggests that VnR underwent some conformational change to allow LM609 access to that site. Moreover, its detection was paralleled by a specific increase in vitronectin binding, an increase in platelet adhesion, and no increase in fibronectin, fibrinogen, or albumin binding.

The observation that there were no differences in vitronectin, fibronectin, fibrinogen, and albumin binding to EC basement membrane preparations excludes the possibility that the difference in vitronectin binding before and after IL-1 stimulation is due to a specific binding of vitronectin to basement membrane components exposed after any EC retraction in response to the IL-1 stimulation. (In addition, EC retraction was not detected by light or scanning microscopy.)

The explanation for why LM142 did not detect VnR on the surface of stimulated ECs is not entirely clear, but is also consistent with the VnR undergoing a structural alteration. For example, if LM142 recognizes a tertiary epitope on the α chain of VnR, any conformational change in VnR after EC stimulation may alter the tertiary structure, thereby rendering VnR foreign to LM142. Alternatively, if the LM142-recognizing epitope of VnR is located in or near the membrane spanning region of the α chain, that site may be masked by cytoskeletal or plasma membrane proteins after VnR relocalization. Neither possibility can be excluded.

The observation that 125I-labeled vitronectin was displaced from unstimulated ECs but not from IL-1-stimulated ECs by unlabeled vitronectin is consistent with recent studies that suggest that some VnR or VnR-like moiety is present on the apical surface of unstimulated ECs.16,18 However, the specific binding of vitronectin to the receptor under these conditions appears to be reversible because vitronectin was easily displaced. (Our immunofluorescent studies suggest, however, that the bulk of VnR in unstimulated ECs is intracellular, because EC permeabilization was required for VnR detection.) The observations that 125I-labeled vitronectin was no longer placeable after EC IL-1 stimulation is consistent with one of two possibilities: (1) either an entirely new receptor is expressed on the EC surface, or (2) the nonadhesive VnR undergoes conformation changes that rendered it more adhesive. The first possibility seems unlikely because the specific antibody (LM609) detected VnR on the EC surface under these stimulated condit-

### Table 1. Effects of IL-1 on Platelet/EC Adhesion ± the GRGDS or GRGES Peptide or ± LM609 or LM142 Antiserum

<table>
<thead>
<tr>
<th></th>
<th>NIL</th>
<th>+GRGDS</th>
<th>&lt;GRGES</th>
<th>&lt;LM609</th>
<th>+LM142</th>
</tr>
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<tbody>
<tr>
<td>-IL-1</td>
<td>4.4 ± 0.3</td>
<td>4.8 ± 0.3</td>
<td>4.2 ± 0.4</td>
<td>3.9 ± 0.6</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td>+IL-1</td>
<td>8.0 ± 0.8</td>
<td>4.3 ± 0.4</td>
<td>7.2 ± 0.5</td>
<td>4.3 ± 0.1</td>
<td>8.4 ± 0.9</td>
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One milliliter of 3H-adenine platelets (2 × 10⁶/μL) was added to each EC monolayer (+10 ng/mL of IL-1) and incubated for 30 minutes. Each EC monolayer was then washed and the number of 3H-platelets adherent was determined, based on EC monolayer radioactivity and 3H-platelet-specific activity. Other EC monolayers with IL-1 were fixed with paraformaldehyde ± Triton X-100 and incubated with one of the murine monoclonal antisera, LM142 (α chain) and LM609 (β chain). Adding 100 μg of GRGDS or 100 μL of LM142 had no effect on platelet/EC adhesion ± IL-1. Data are expressed as mean ± SEM, n = 9.
13-HODE and Endothelial Cell VnR Expression

13-HODE and Endothelial Cell VnR Expression

Abstract

We conclude, therefore, that the lipid environment in which the VnR locates influences its conformational configuration and, hence, its adhesivity. The structural properties of VnR and 13-HODE are compatible with this possibility. The membrane spanning region of both the α and β chains of VnR are lipophilic21,22; therefore, VnR will interact with 13-HODE or any other lipid. In addition, both VnR chains are rich in cysteine and are therefore capable of undergoing rapid conformational changes.21,26 We propose, therefore, that under basal conditions, ie, in unstimulated nonadhesive ECs, the lipophilic region of the VnR α and/or β chains interacts with 13-HODE in vesicles located immediately beneath the EC plasma membrane (Fig 8). Under these conditions, the α chain remains in a conformation such that its N-terminus masks the RGD-recognizing domain of the β chain, ie, masks the adhesive site of the β chain. This possibility is consistent with the observations of Smith and Cherish,26 who found that the α chain of VnR blocks the RGD domain of the β chain in vitro. When ECs are stimulated and 13-HODE synthesis is inhibited, VnR dissociates from any remaining 13-HODE and relocates in the EC phospholipid membrane bilayer. As a consequence, the α chain (which now is associated with another lipid moiety) undergoes a conformational change that unmaskes the adhesive site of the β chain (Fig 8). The latter possibility is consistent with the observations of Conforti et al18 and others,19,20 and is consistent with our observations of a specific increase in ligand binding after EC stimulation. Such a model may also explain how 13-HODE, once released albuminarily from ECs into the basement membrane underlying the ECs, renders other adhesive molecules less adhesive after vessel wall injury.12,13,32,34

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Localization of 13-hydroxyoctadecadienoic acid and the vitronectin receptor in human endothelial cells and endothelial cell/platelet interactions in vitro

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